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Nitrogen and phosphorus additions affect fruiting of ectomycorrhizal fungi in a temperate hardwood forest

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ABSTRACT

The functioning of mycorrhizal symbioses is tied to soil nutrient status, suggesting that nutrient availability should influence the reproduction of mycorrhizal fungi. To quantify the effects of nitrogen (N) and phosphorus (P) availability on ectomycorrhizal fungal fruiting, we collected >4000 epigeous sporocarps representing 19 families during the course of a season in a full factorial NxP addition experiment in six replicate forest stands. Nutrient effects on fruiting shifted as the season progressed, with early fruiting species responding more to P and late-fruiting species responding more to N. The composition of species fruiting in young successional forests differed more with nutrient addition than in mature forests. Sporocarp abundance and species richness were suppressed by N addition. This work shows that N and P availability affect ectomycorrhizal fungal fruiting, with these effects taking place within a context defined by stand age and the progression of fruiting across the season.

1. Introduction

Earth is losing biodiversity at an alarming rate (Kim and Byrne 2006). Due to the ephemeral nature of fungal sporocarps and the difficulty of identifying species from vegetative structures, fungal species are underrepresented in biodiversity inventories. Ectomycorrhizal (EM) fungi form mutualistic symbioses with dominant tree species in many temperate and boreal forests, where the composition and diversity of these fungi help structure plant communities and drive ecosystem processes (van der Heijden et al., 2015; Tedersoo et al., 2020). These fungi are sensitive to changes in nutrient availability (eg. Arnolds, 1991; Lilleskov et al., 2001; Avis et al., 2003; Janssens et al., 2010) but the responses of many species to specific disturbances and changes remain unclear. The community composition of EM fungi has consequences for tree nutrient acquisition and growth (Anthony et al., 2022). Without an understanding of the environmental pressures that affect fungi at the population and community level, we have little power to predict changes in fungal species biodiversity or to foresee how these changes could impact ecosystems locally and globally.

Nitrogen (N) pollution is one of the drivers of global ecological change, with potential to alter EM interactions and fungal communities (Lilleskov et al., 2019). As a factor limiting plant growth in many terrestrial ecosystems (Vitousek et al., 2010), elevated N can alleviate nutrient limitation, reducing the benefit of EM fungi to trees. This mechanism can be induced experimentally; adding N to potted plants reduces the benefit of mycorrhizal inoculation (Hoeksema et al., 2010). Elevated N availability may also shift the structure of EM fungal communities (e.g., Lilleskov et al., 2001, 2002; Avis et al., 2003; van der Linde et al., 2018; Yang et al., 2020). Reduced belowground allocation of carbon to EM fungi following N additions may select for nitrophilic fungi adapted to environments with high N availability, especially inorganic N sources. In contrast, nitrophobic species (e.g., many *Tricholoma*, *Cortinarius*, and *Piloderma* spp.) display reductions in abundance and species richness following N addition (Agerer 2001, 2006; Hobbie and Agerer 2010), possibly because these taxa are adapted to mine for organic N under N-limited conditions (Lilleskov et al., 2011). The length and exploration characteristics of extraradical hyphae, as well as their production of N mobilizing enzymes, seem to be the most important factors predicting whether a species will respond to N deposition (Hobbie and Agerer, 2010; Lilleskov et al., 2011).

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EM symbioses are also involved in uptake of P (Baxter and Dighton, 2001; Köhler et al., 2018), which, like N, commonly limits or co-limits primary productivity (Vitousek et al., 2010). Phosphorus limitation may select for different EM fungi than N limitation due to differences in competitive fungal functional traits under different soil nutrient conditions. Few studies have investigated the sensitivity of EM fungal communities to P addition, and results differ among these studies. In a study on P addition in a P-limited spruce forest, soil EM fungal community composition changed in response to P addition, but there was no change in soil fungal biomass (Almeida et al., 2018). This result contradicts those from a mixed beech-maple forest, in which P fertilization had no effect on the composition of EM fungal communities isolated from root mats, bulk soil, or root tips (Carrino-Kyker et al., 2016; Burke et al., 2021). Other biotic and abiotic factors (including N availability) could impact EM fungal responses to changes in P limitation. With further investigation we may begin to understand which groups of EM fungi are 'phosphophilic', persisting following increased P availability and which are 'phosphophobic', as well as how environmental variables impact EM responses to changes in P availability.

EMF primarily live and function belowground in host roots and soil substrates. While the abundance and composition of sporocarps may differ from that of hyphae and mycorrhizae belowground, fruiting can be an important indicator of fungal responses to environmental change such as nutrient enrichment (Boddy 2016), which has broad consequences for forest ecosystems. Sporocarps are food sources for a diversity of organisms including invertebrates, birds, and small and large mammals including humans (Hernández-Santiago et al., 2020; Elliott et al., 2022; Ferreira et al., 2023), and sporocarps are critical to the fitness and dispersal of many EM fungi. Thus, changes in sporocarp production have implications for the population dynamics of EM fungal species, community assembly (e.g. Peay et al., 2007) and trophic interactions. Carbon allocated to fruiting originates as photosynthate from a tree acquired by EM fungal symbionts in exchange for nutrients, so shifts in sporocarp production driven by nutrient deposition may be an indicator of broader changes in tree-fungus source-sink dynamics that have extended consequences for belowground carbon pools (e.g., Frey 2019). Starting with the benchmark paper of Arnolds (1991) it has been firmly established that the fruiting of many EM species is negatively influenced by elevated N (e.g. Peter et al., 2001; Lilleskov et al., 2001; Avis et al., 2003; Janssens et al., 2010), while there is limited information regarding the influence of elevated P or N and P together on EM fungal fruiting (but see Ohenoja 1978). Importantly, fungal species can differ in the timing of their fruiting over a season (Sato et al., 2012) and the age or successional stage of their habitat (Vogt et al., 1981; Smith et al., 2002; Nara et al., 2003), and these factors may also affect fruiting responses to nutrient additions.

The long-term study of Multiple Element Limitation in Northern Hardwood Ecosystems (MELNHE) represents an ideal setting to test the individual and synergistic effect of N and P on EM fungal fruiting. Initiated in 2011, MELNHE is a full factorial N x P addition to a temperate mixed hardwood forest in the northeastern United States. Successional stands were dominated by Betula alleghaniensis, B. papyrifera and Acer rubrum, and mature stands dominated by Fagus grandifolia, B. alleghaniensis and A. sacchrum. Findings to date include increased soil N and P availability (Fisk et al., 2014); increased foliar N and P (Hong et al., 2022; Gonzales and Yanai 2019; Gonzales et al., 2023); increased tree diameter growth following P addition, on average (Goswami et al., 2018), or N addition, in larger trees (Hong et al., 2022); and increased root growth in response to N addition (Shan et al., 2022) or N + P addition (Li et al., 2023). These results suggest that communities of EM fungi, as the mediators of soil nutrient uptake in trees, will likely also be affected by N and P addition.

We censused sporocarps in the MELNHE experiment to examine how EM fungal communities respond to nutrient availability. Because trees support mycorrhizae to improve soil nutrient uptake, we hypothesized that overall EM fungal productivity, indicated by total fruiting abundance and aboveground biomass, would decrease with N and P additions. EM fungal species are functionally divergent in their nutrient uptake and exchange capacities with trees, so we also expected that nutrient addition would shift overall sporocarp species composition and impact species diversity, potentially disfavoring fungal taxa specialized in nutrient uptake from organic matter. The fruiting of EM fungal species can vary in their seasonal phenology and with forest stand age, therefore we sought to understand how these factors set the context for sporocarp community responses to N and P addition. We also characterized how nutrient impacts on EM fungal fruiting differed with stand age and shifted as sporocarp composition changed throughout the season.

2. Materials and methods

2.1. Site Description

Bartlett Experimental Forest (latitude = 44.05°, longitude = −71.3°) is in the White Mountains of New Hampshire, USA. The region experiences warm summers with high temperatures often above 32 °C and average July temperatures of 19 °C. Winter temperatures fall below 0 °C with average January temperatures of -9 °C. Average annual precipitation is 1270 mm distributed throughout the year (USFS Northern Research Station). Soils are Spodosols developed in glacial drift derived from granite and gneiss (USFS Northern Research Station).

The MELNHE project has added N and P to 13 stands in the White Mountains since 2011 (Yanai et al., 2024). This study makes use of six of the MELNHE stands: three successional (clearcut between 1975 and 1978) and three mature (last harvested between 1883 and 1890) stands dispersed across the Bartlett Experimental Forest, with tree composition varying with stand age (Fig. 1). Each stand includes four 50 m × 50 m plots receiving annual additions of low levels of N (30 kg N ha-1 yr-1 as granular NH4NO3) and P (10 kg P ha-1 yr-1 as granular or powdered NaH2PO4) in a factorial design (+N, +P, +N and P, and control). Measurements were conducted in a 30 m × 30 m area in each plot, to allow a 10 m buffer.

2.2. Sampling Methods

Over the 2018 fungal growing season, epigeous sporocarps in these six stands were sampled five times between late July and mid-October (Bashian-Victoroff et al., 2024). Sampling occurred on July 27–29 (average temperature 21.31 °C, antecedent weekly precipitation = 54.1 mm), August 13–15 (21.37 °C, 25.7 mm), August 30-September 2 (21.34 °C, 0.00 mm), September 24–27 (13.62 °C, 51.8 mm) and October 12–15 (8.37 °C, 15.8 mm). The 30 m × 30 m sampling area was divided into nine 10 m × 10 m subplots and each subplot was sampled using a 3.5 minute timed wander to ensure consistent sampling across the plot. Only visible epigeous sporocarps were included in the sampling effort. Sporocarps were enumerated, recorded and photographed. Sporocarps from the center 10 × 10 m subplot of each plot were collected, dried and weighed to provide data on aboveground fungal biomass.

2.3. Sporocarp Identification

Sporocarps were initially sorted into morphological species (morphospecies) based on macroscopic and microscopic characters of fresh specimens, and molecular techniques were used to adjust morphospecies assignments. DNA was extracted from sporocarp tissue of 1–5 representatives from each morphospecies (depending on sporocarp availability for each morphospecies) using a CTAB method following Gardes and Bruns (1993). The nuclear ribosomal internal transcribed spacer (nrITS) region was amplified by PCR using forward primer ITS1-F and reverse primers ITS4 or ITS4-B following Gardes and Bruns (1993) and White et al. (1990). PCR products were visualized with gel electrophoresis. Successfully amplified samples were digested using restriction enzymes *Hinf*I and *Dpn*II to produce RFLP (restriction fragment length

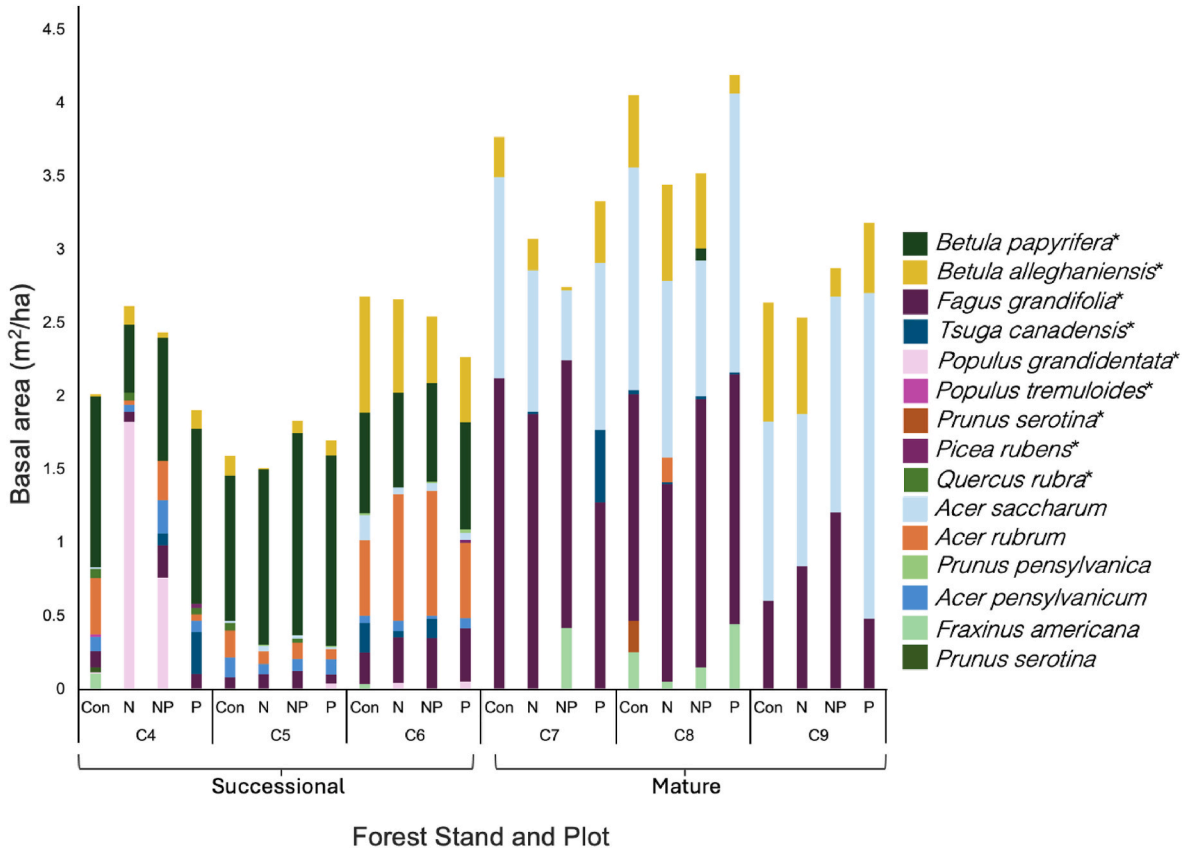

Fig. 1. Basal area of trees in the sampled plots of six stands (C4-C9) Bartlett Experimental Forest NH, based on 2015 inventory of trees ≥10 cm diameter at breast height (Fisk et al., 2022). C = control, N = nitrogen addition, P = phosphorus addition, NP = nitrogen and phosphorus addition. * = Ectomycorrhizal host species.

polymorphism) patterns (Horton 2002). Representatives of each unique combination of morphospecies and RFLP pattern were reamplified for sequencing using ITS1-F and ITS 4 or ITS4-B. Gel electrophoresis was used to confirm successful reamplification and PCR products were cleaned using QIAquick PCR purification kit columns (Qiagen, Valencia, CA, USA). DNA concentration was quantified using a ND-1000 NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA concentrations were adjusted to 20–40 ng/μl for sequencing and sent to Eurofins (Louisville, KY) for DNA sequencing using the primer ITS1-F.

The following methods were used to process sequences and assign taxonomy. Sequences were visually analyzed and edited using FinchTV version 1.4.0 (Geospiza, 2009) and BioEdit version 7.2.1. (Hall, 1999). Cutadapt 1.8 (Martin 2011) was used to trim sequences. Sequences were clustered into denovo OTUs with the QIIME 2 VSEARCH plug-in (Bolyen et al., 2019), by first clustering at 98.5%, then clustering the resulting representative sequences for 98.5% OTUs at 97% similarity. The QIIME 2 feature-classifier plug-in (Bokulich et al., 2018) was used to assign taxonomy to representative sequences of 97% clusters using the naive Bayes classifier (Pedregosa et al., 2011) in conjunction with the QIIME formatted UNITE dynamic species hypothesis dataset (version 8.0, released 2019; Kõljalg et al., 2013; Bolyen et al., 2019). Taxonomy was also assigned individually to all sequences in the dataset to examine consistency with assignments to sequences that fell within the same 97% similarity cluster. Representative sequences of each cluster were also manually compared to database sequences in GenBank (NCBI) and UNITE (Kõljalg et al., 2013) using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). To rule out genetically similar species names from outside of our study region, taxonomic assignments were compared to species occurrence reports from the Global Biodiversity Information Facility (GBIF.org). Results were also compared to known associations with host trees, and sporocarp morphology. If taxonomic assignments conflicted with sporocarp morphology, taxonomic assignments were based on our best hypotheses based on all available data including molecular, ecological, occurrence data, and morphological information. The FungalTraits database (Põlme et al., 2020) was used to verify trophic status of fungal species. We assigned EM soil exploration types to each species we identified using assignments made in Tedersoo and Smith (2013) and references therein. All sequences were accessioned into GenBank following taxonomic assignment.

2.4. Statistical Methods

The first set of analyses aimed to provide a picture of the overall shift in sporocarp composition between the three successional and three mature stands. Non-metric multidimensional scaling (NMDS) was used to ordinate the matrix of sporocarp abundance data, using Bray-Curtis dissimilarity after removing singletons. The Wisconsin double relativization (Bray and Curtis, 1957) was applied prior to ordination, to emphasize within-sample relative abundances after down-weighting the influence of the dominant species. To examine the influence of seasonality on sporocarp fruiting, indicator species analysis (De Cáceres and Legendre, 2009) was conducted separately for each stand age class to identify indicators of individual collection trips, as well as pairs and triplets of consecutive trips, using absolute abundances of sporocarps. Ordination and indicator species analyses were run in R 3.6.3 (R Core Team, 2020) using *vegan* (Oksanen et al., 2019) and *indicspecies* (De Cáceres and Legendre, 2009).

The influence of nutrient treatments on sporocarp composition over the course of the season was tested with PERMANOVA (Anderson 2001) and visualized with constrained analysis of principal coordinates (CAP; Anderson & Willis, 2003). The PERMANOVA model included the fixed

effects of N, P, stand age-class, collection trip, and all possible interactions. PERMANOVA models also included stand nested within age class and plot nested within stand as random effects to account for each plot and stand being sampled multiple times during the season. PERMANOVA were also conducted individually for each collection date, using fixed effects of N, P, age class, and all possible interactions and the random effect of stand nested within age class. CAP was used to visualize responses of sporocarp composition in the different age classes at each sampling date. PERMANOVA and CAP utilized Bray-Curtis dissimilarity and Wisconsin double relativization, after removing singleton species. PERMANOVAs was run in PERMANOVA+ 1.0.5 in Primer 6.1.15 (PRIMER-E, Plymouth, UK), while CAP was conducted in R using *vegan*.

Individual responses of common species (occurring within at least 6 plots) to N and P addition were examined with the *mvabund* package in R (Wang et al., 2012), using generalized linear models (GLMs) with a binomial distribution for sporocarp counts. These analyses were run with a species matrix created by summing sporocarp counts from each collection trip by plot to test the overall effect of the treatments for the season. Models were run separately for data from each stand age class, and included the fixed effect of N addition, P addition, and their interaction, with stand as a blocking variable for permutations. However, to avoid excessive numbers of individual tests, we focused interpretation on the main effects of N and P.

To further test our hypotheses, univariate community variables were examined, including total sporocarp abundance, total sporocarp biomass, Pielou's species evenness, species richness and species richness standardized by total sporocarp abundance. Species richness standardized by total sporocarp abundance was calculated by plot to examine patterns in richness independent of those associated with the total number of sporocarps. Each variable was individually tested using linear mixed effects models that included the fixed effects of N, P, stand age-class, collection trip, and all possible interactions among fixed effects, as well as the random effects of stand and plot. Linear mixed models were run in R using the packages *lmerTest* (Kuznetsova et al., 2017) and *emmeans* (Russell 2020).

3. Results

3.1. Seasonal and stand age effects on fungal sporocarp communities

We observed a total of 4,043 epigeous EM sporocarps (111 species, 19 families, seven orders) in the six stands, with dominance changing over the five collection dates (Fig. 2A, Fig. S1; Bashian-Victoroff et al., 2024). All sequences are available in GenBank (accessions MT345178-MT345282). The most abundant genus was *Lactarius* (26.2 % of sporocarps, seven species), followed by *Russula* (25.4% of sporocarps, 20 species), *Cortinarius* (16.9 % of sporocarps, 24 species), and *Amanita* (16.8% of sporocarps, 13 species). *Russula* and *Amanita* species were dominant in the early and mid-season collections (late July-early September), with some *Amanita* species also fruiting into autumn while *Russula* mostly stopped fruiting by that point. Boletaceae and *Scleroderma citrinum* became prominent members of the sporocarp community during early September to mid October. *Lactarius* species became important fruiters mid-season and remained so into autumn, when they were joined by an abundance of late-season *Cortinarius*.

Sporocarp composition in the two stand age classes diverged as the season progressed (significant collection date x stand age interaction with full season's dataset; Table 1, Fig. 2A). In the last two collection trips (late September, mid October) there was a clear distinction in composition between the two stand age classes that was absent earlier in the season (significant stand age effects only in late September and mid October for PERMANOVA restricted to individual collection dates; Table 2; Fig. 2A). The late-season divergence between the two stand age classes was driven in large part by *Cortinarius* species, which were indicators of late season collection trips in the successional stands (Table S1; Fig. 2A). Species from a variety of lineages were indicators of the two late-season collections in only one of the two age classes (Table S1; Fig. 2A).

3.2. Effects of N and P addition on fungal species composition

We saw significant variation in sporocarp community composition between nutrient treatments. Interestingly, the response of sporocarp composition to N and P addition changed as the season progressed. In the full dataset, there were significant main effects of N and P on composition (Table 1), but the influence of N and P changed with

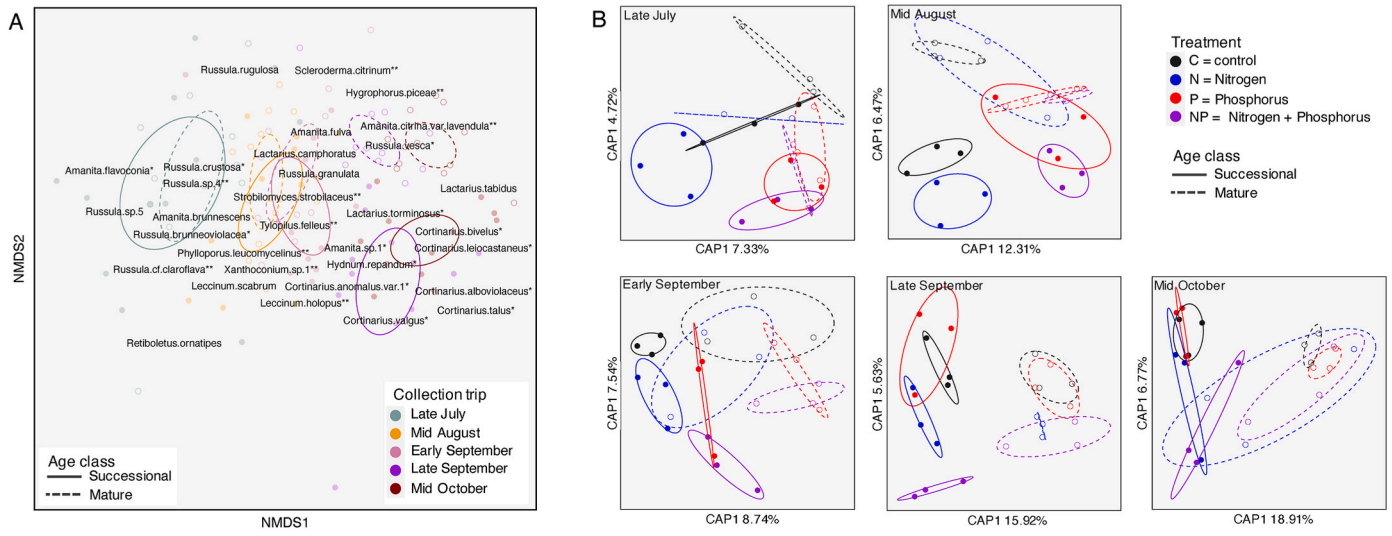

Fig. 2. NMDS ordination of sporocarp communities across the fruiting season, with indicator species of collection trips (or consecutive pairs or triplets of trips) for successional and mature stands also ordinated (A), and CAP ordinations of sporocarp communities for individual collection trips showing nutrient treatments by stand age class (B). C = control, N = nitrogen addition, P = phosphorus addition, NP = nitrogen and phosphorus addition. For A, * = indicators of only successional, ** = indicators of mature stands only, and no asterisk indicates indicators in both age classes. To interpret CAP ordinations in B, focus on following treatment effects in a single stand age-class across the season at a time.

Table 1
PERMANOVA and linear mixed model results for sporocarp community responses to nitrogen (N), phosphorus (P), stand age (successionald or mature) and collection trip (bold = 0.10 ≤ α > 0.05, bold italics = α ≤ 0.05).

Model Factor[a]	Composition[b]	Total sporocarp abundance	Total sporocarp mass	Species evenness	Species richness	Richness per total sporocarps
	F (df) P	*F (df) P*	*F (df) P*	*F (df) P*	*F (df) P*	*F (df) P*
N	1.92 (1, 12)	14.83 (1, 12.0)	6.45 (1, 12.0)	0.00 (1, 12.1)	15.46 (1, 12.0)	1.40 (1, 12.0)
	0.021	***0.002***	***0.026***	0.994	***0.002***	0.259
P	2.19 (1, 12)	1.65 (1, 12.0)	0.24 (1, 12.0)	0.98 (1, 12.0)	1.42 (1, 12.0)	7.85 (1, 12.0)
	0.008	0.223	0.633	0.343	0.256	***0.016***
Age	2.36 (1, 4)	0.50 (1, 4.0)	0.81 (1, 4.0)	1.26 (1, 4.1)	0.29 (1, 4.0)	0.25 (1, 4.0)
	0.032	0.518	0.418	0.324	0.620	0.642
Trip	6.91 (4, 63)	36.96 (4, 64.0)	11.51 (4, 64.0)	2.40 (4, 59.8)	19.95(4, 64.0)	9.57 (4, 63.3)
	<0.001	***<0.001***	***<0.001***	**0.060**	***<0.001***	***<0.001***
N x P	0.82 (1, 12.03)	0.11 (1, 12.0)	0.32 (1, 12.0)	0.08 (1, 12.1)	0.02 (1, 12.0)	0.06 (1, 12.0)
	0.654	0.747	0.582	0.778	0.879	0.808
N x Age	1.24 (1, 12)	0.00 (1, 12.0)	0.67 (1, 12.0)	0.17 (1, 12.1)	0.89 (1, 12.0)	0.80 (1, 12.0)
	0.235	0.961	0.430	0.691	0.364	0.388
P x Age	1.48 (1, 12)	0.38 (1, 12.0)	0.54 (1, 12.0)	0.07 (1, 12.0)	0.08 (1, 12.0)	0.42 (1, 12.0)
	0.142	0.547	0.478	0.794	0.782	0.527
N x Trip	1.41 (4, 63)	1.44 (4, 64.0)	0.31 (4, 64.0)	0.44 (4, 59.9)	0.60 (4, 64.0)	0.38 (4, 63.3)
	0.008	0.231	0.868	0.776	0.662	0.824
P x Trip	1.64 (4, 63)	1.69 (4, 64.0)	0.75 (4, 64.0)	2.43 (4, 59.8)	0.76 (4, 64.0)	1.03 (4, 63.3)
	0.001	0.162	0.563	**0.057**	0.554	0.339
Trip x Age	2.41 (4, 63)	11.51 (4, 64.0)	3.4 (4, 64.0)	1.31 (4, 59.8)	4.70 (4, 64.0)	3.96 (4, 63.3)
	<0.001	***<0.001***	***0.014***	0.278	***0.002***	***0.006***
N x P x Age	0.88 (1, 12)	0.00 (1, 12.0)	2.66 (1, 12.0)	1.25 (1, 12.1)	0.06 (1, 12.0)	0.10 (1, 12.0)
	0.541	0.962	0.129	0.285	0.808	0.754
N x P x Trip	0.97 (4, 63)	0.56 (4, 64.0)	0.12 (4, 64.0)	1.14 (4, 59.9)	0.39 (4, 64.0)	0.35 (4, 63.3)
	0.563	0.692	0.974	0.348	0.814	0.842
N x Trip x Age	1.26 (4, 63)	2.88 (4, 64.0)	0.42 (4, 64.0)	0.445 (4, 59.9)	0.97 (4, 64.0)	1.33 (4, 63.3)
	0.043	***0.029***	0.792	0.775	0.427	0.267
P x Trip x Age	1.25 (4, 63)	1.12 (4, 64.0)	0.14 (4, 64.0)	0.63 (4, 59.8)	2.11 (4, 64.0)	0.44 (4, 63.3)
	0.049	0.355	0.969	0.642	**0.090**	0.782
N x P x Trip x Age	0.1.65 (4, 63)	0.95 (4, 64.0)	1.29 (4, 64.0)	0.17 (4, 59.9)	0.24 (4, 64.0)	0.89 (4, 63.3)
	0.888	0.444	0.283	0.953	0.912	0.475

[a] All models also included individual forest *stand* and *plot* as random effects, but no hypothesis test was applied to these variables.
[b] One plot in Mid-July had zero mushrooms so was excluded from composition analyses.

Table 2
PERMANOVA results for sporocarp composition responses to nitrogen (N), phosphorus (P), and stand age (successionald or mature) for each individual collection trip (bold = 0.10 ≤ α > 0.05, bold italics = α ≤ 0.05).

Model Factor[a]	Mid-July[b] composition	Mid-August composition	Mid-September composition	Late-September composition	Mid-October composition
	F (df) P	*F (df) P*	*F (df) P*	*F (df) P*	*F (df) P*
N	1.22 (1, 11.0)	1.47 (1, 12.0)	2.01 (1, 12.0)	2.04 (1, 12.0)	1.81 (1, 12.0)
	0.285	0.148	***0.027***	***0.034***	***0.051***
P	2.22 (1, 11.0)	3.04 (1, 12.0)	2.12 (1, 12.0)	2.04 (1, 12.0)	1.30 (1, 12.0)
	0.051	***0.002***	***0.018***	***0.031***	0.224
Age[3]	0.524	0.167	0.170	***0.016***	***0.027***
N x P	0.90 (1, 11.0)	0.81 (1, 12.0)	1.06 (1, 12.1)	1.31 (1, 12.0)	0.71 (1, 12.0)
	0.493	0.634	0.398	0.234	0.741
N x Age	1.97 (1, 11.0)	1.02 (1, 12.0)	0.899 (1, 12.1)	1.41 (1, 12.0)	1.25 (1, 12.0)
	0.079	0.433	0.565	0.188	0.266
P x Age	1.38 (1, 11.0)	1.54 (1, 12.0)	1.40 (1, 12.0)	2.18 (1, 12.0)	1.25 (1, 12.0)
	0.216	0.129	0.177	***0.022***	0.245
N x P x Age	1.05 (1, 11.0)	0.54 (1, 12.0)	1.06 (1, 12.1)	1.45 (1, 12.0)	0.413 (1, 12.0)
	0.388	0.862	0.405	0.163	0.946

3 The effect of stand age class was tested with a Monte Carlo procedure because the model structure did not allow for enough permutations to obtain an accurate pseudo *F*-statistic. Therefore, only *P*-values are reported.
[a] All models also included individual forest *stand* as a random effect, but no hypothesis test was applied to this variable.
[b] One plot in Mid-July had zero mushrooms so was excluded from analyses.

sampling date (significant trip x N and trip × P interactions; Table 1). Analyses of composition within each collection trip showed that the influence of P addition started in the beginning of the season, was present through September, and disappeared in October (Table 2; Fig. 2B). In contrast, the effect of N addition on composition was not statistically detectable in the beginning of the season, but emerged as significant in the last three collection trips (Table 2; Fig. 2B). The addition of N and P had a largely additive effect on composition as a whole (i.e., non-significant N × P interaction; Fig. 2B). However, on most collection trips sporocarp composition in successional stands exhibited a more consistent response to N and P than in mature stands, which drove the significant three-way interactions of trip and stand age with both N and P addition (both at $P < 0.05$; Table 1, Fig. 2B).

We observed variable nutrient effects on the sporocarp abundances of individual taxa. Of the common species (present in six or more plots; 33 species total), 16 exhibited statistically significant (α = 0.05) or marginally significant (α = 0.1) responses, to the addition of one or both nutrients (Table S2; Fig. 3). Interestingly, fruiting patterns of individual

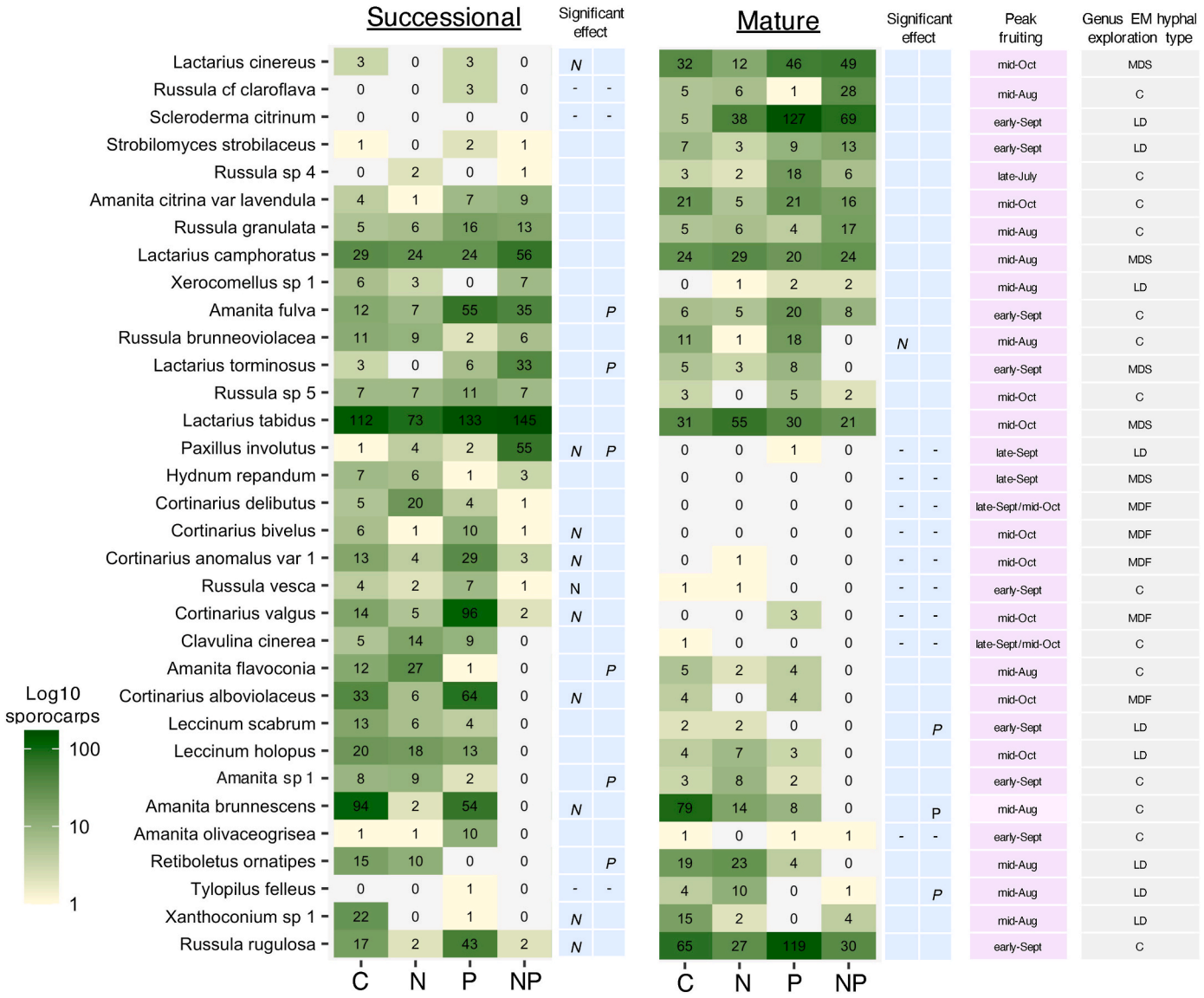

Fig. 3. Heat map of total sporocarp counts summed for the season for treatments within an age class, for species present in six or more plots during the season. C = control, N = nitrogen addition, P = phosphorus addition, NP = nitrogen and phosphorus addition. Significant effects from individual tests run with mvABUND (bold = $0.10 \leq \alpha > 0.05$, bold italics = $\alpha \leq 0.05$) are indicated in the *significant effect* columns to the right of each season's data; a dash indicates a species not tested within an age class because fewer than four sporocarps were found. The collection trip from which the most sporocarps were observed for a species is indicated under the column labeled *peak fruiting*, and the hyphal exploration type of the ectomycorrhiza (EM) for each genus is provided under *Genus EM hyphal exploration type* (C = contact, SD = short distance, MDS = medium distance smooth, MDF = medium distance fringe, LD = long distance).

species exhibited a range of nuanced responses to N and P additions that were dependent on stand age class (Table S2; Fig. 3). In the successional stands, a number of species were affected by the addition of one or both nutrients (Fig. 3). Notably, abundances of four *Cortinarius* species were depressed by N addition and two *Amanita* species were depressed by P addition (Fig. 3). In the successional stands, three species increased in abundance under nutrient addition, with *Paxillus involutus* responding most strongly when both nutrients were added together (Fig. 3). In contrast, in the mature stands, few species exhibited consistent responses to nutrient addition (Fig. 3). Importantly, many of the species that were responsive to N or P addition in the successional stands (e.g., *Cortinarius*) were entirely absent from the mature stands, which drove the difference in response between the two stand age classes (Fig. 3). Additionally, fruiting responses of specific fungi to nutrient additions occurred at different points throughout the season. Species that were common early in mid-July through early September responded more often to added P, whereas species that were common in late-September through mid-October responded more often to added N (Fig. 3).

3.3. Seasonal, stand age and nutrient effects on sporocarp abundance and biomass

The addition of N, but not P, had marked effects on total sporocarp abundance and mass (Table 1; Fig. 4A and B). Nitrogen addition reduced sporocarp abundance by ~30–50% in both stand age classes across the season (Fig. 4A). However, the effects of N and P addition varied within the context of stand age and over the course of the season. The seasonal pattern of fruiting differed between the two stand age classes and further modulated the effects of N addition in unique ways, reflected in multiple interactions of trip, N addition, and stand age (Table 1; Fig. 4A). Specifically, total sporocarp abundance in the successional stands started out very low early in the season and peaked late, with N addition suppressing fruit body counts on most collection trips (Fig. 4A). Sporocarps in the mature stands were more abundant than the successional stands

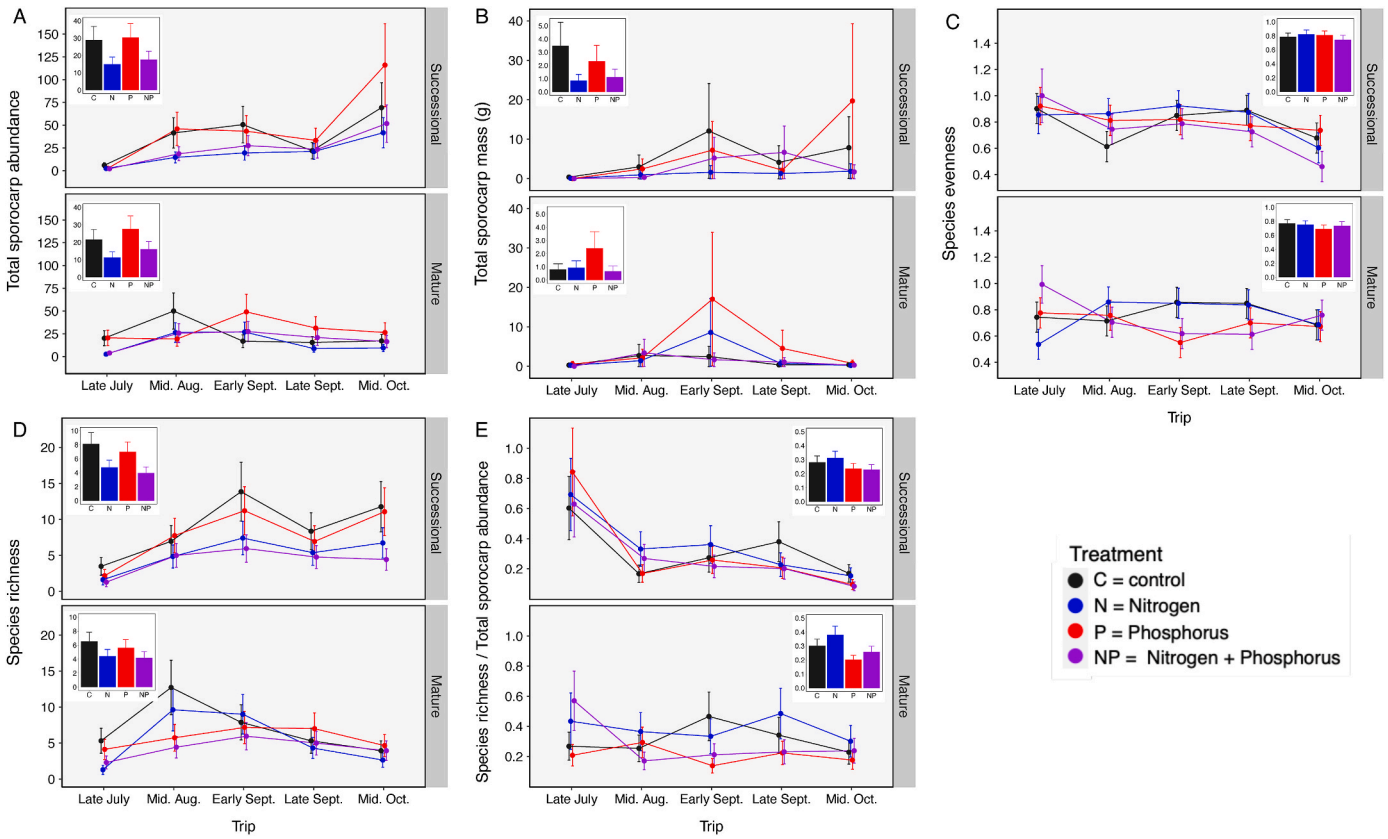

Fig. 4. Total sporocarp abundance (A), total sporocarp dry biomass (B), species evenness (C), species richness (D) and species richness per total sporocarps (E) for each collection trip and averaged for treatments over the season (inset bar graphs). All values are marginal means and standard errors from linear mixed models. C = control, N = nitrogen addition, P = phosphorus addition, NP = nitrogen and phosphorus addition.

early on and remained relatively uniform through the end of the season, with N effects generally being less consistent between dates (Fig. 4A). Total sporocarp mass, like abundance, was reduced by N addition in the successional stands; in contrast, P addition elevated total sporocarp mass in the mature stands, but not consistently enough to attain statistical significance (Table 1, Fig. 4B). The seasonal influence on total sporocarp mass differed by age class (significant trip x stand age effect; Table 1); in the successional stands, mass peaked late in the season, whereas in mature stands, total sporocarp mass was low overall but with a mid-season (early September) peak (Fig. 4B). In contrast with total sporocarp abundance, the effect of nutrient addition on total sporocarp mass was not modulated by the time in the season when data were collected or stand age (i.e., no interactions of trip or stand age with nutrient additions; Table 1).

3.4. Seasonal, stand age and nutrient effects on species evenness and richness

Consistent with our hypotheses, nutrient addition strongly influenced species diversity; these effects were more distinct for richness than evenness (Table 1; Fig. 4C, D, 4E). Species evenness changed little over the season, except under P addition, which caused a modest depression during the mid season (marginal trip and trip × P interaction; Table 1; Fig. 4C). Nitrogen addition suppressed species richness (significant main effect of N; Table 1; Fig. 4D). Although early season species richness was low and similar between the two stand age classes, it rose and then declined by the end of the season in the mature stands but not in the successional stands (significant trip x stand age interaction; Table 1; Fig. 4D). Interestingly, there was a tendency that P addition tended to suppress sporocarp richness, but only in the older stands early in the season (marginal P x Trip × age interaction; Table 1; Fig. 4D). Species richness standardized by total sporocarp abundance tended to flip the seasonal patterns in species richness exhibited before relativizing, most clearly in the successional stands (significant trip and trip x stand age effects; Table 1; Fig. 4E vs Fig. 4D). Furthermore, the negative effects of nitrogen on species richness disappeared after controlling for total abundance, and P addition emerged as an overall negative influence on species richness (Table 1; Fig. 4E).

4. Discussion

We found that EM fungal fruiting responded to N and P addition in a temperate hardwood forest, suggesting that these EM communities are locally sensitive to changes in biogeochemical conditions. Importantly, these changes occurred within the contexts of seasonal timing and forest stand age. To anyone who has foraged for mushrooms, it is not surprising that the community structure of fruiting bodies shifted seasonally and varied between mature and successional forests. However, the influence of nutrient enrichment, particularly N, is typically examined after pooling fruiting records across a season (e.g., Lilleskov et al., 2001, Avis et al., 2003), which provides a robust picture of net effects but may overlook important aspects of how the nutrient impacts unfold in a community context. Stand age effects on fruiting responses to nutrients are also important to consider, as forest harvesting alters environmental conditions and initiates forest succession with consequences for forest composition.

In temperate ecosystems, seasonal changes in temperature and day length stimulate plant phenological events including root growth, the quality and quantity of root exudates, bud break, and leaf senescence. The timing of these events may be influenced by nutrient availability (Yang et al., 2016), and may be consequential for tree nutrient demands (Nord and Lynch 2009), and carbon allocation to roots (Endrulat et al.,

2016). These host phenological events have consequences for mycorrhizal fungi, including fruiting, because the functioning of mycorrhizal symbioses is tied to the source sink-dynamics of plant-fungal carbon and nutrient exchange (Sato et al., 2012; Nahberger et al., 2021). Fungal sporocarp production is stimulated by environmental factors including temperature and rainfall (Gange et al., 2007; Boddy et al., 2014) that change through a growing season, although not all fungal species respond equivalently to these environmental factors (Pinna et al., 2010; Sato et al., 2012). This leads to a situation where different fungal species preferentially fruit when forest trees are at different phenological stages, in part because fungi are responding differentially to abiotic environmental cues as well as to factors more directly associated with host physiology. In our study the seasonal timing of nutrient effects may be driven by the intersection of EM fungal and tree phenology. Interestingly, the effects of P on fungal composition were most significant early in the season and dropped off in late September, while those of N addition began in mid-September and continued to the end of the season. This seasonal offset in nutrient effects may be driven by a seasonal shift in host nutrient needs associated with their phenology or by the timing of microbially driven soil processes that drive differential nutrient availability; these possibilities warrant further investigation. Mycorrhizal fungi, rather than roots, are the chief organs of soil nutrient acquisition (van der Heijden et al., 2008; Köhler et al., 2018), so the seasonal effect of nutrient additions on EM fungal fruiting suggests that further examination of processes that vary with mycorrhizal phenology (as described in Chaudhary et al., 2022) could be fruitful.

Mycorrhizal fungi vary in their morphological, physiological, and phenological traits, which affects their responses to environmental changes. With some exceptions, nutrient responses within taxonomic groups represented in our dataset varied even at the species level. Sensitivity to N or P addition may relate to an organism's functional role in acquiring that nutrient, and decreases in fruiting body production may be an indirect response to decreased C allocation by host trees to roots colonized by nutrient-mining EM taxa. Alternatively, trees may upregulate defense against fungal species providing less nutrient benefit (Hortal et al., 2017), ultimately leading to decreased fruiting of those fungi. Fungi with high biomass exploration type hyphae that produce N-mobilizing enzymes are likely well adapted to mine soil nutrients and are more likely to be suppressed in high-N environments (Hobbie and Agerer, 2010). Consistent with our hypothesis, we found that N addition reduced overall sporocarp abundance, richness, and aboveground fungal biomass, consistent with earlier studies (Arnolds, 1991; Lilleskov et al., 2001; Peter et al., 2001). Decreased fruiting abundance was particularly evident in species of Cortinarius and during late season collections.

There has historically been a paucity of data on EMF responses to elevated P and contradictory responses have been observed across study systems and research methods. For example, in a beech-maple forest, P addition had no detectable effect on arbuscular mycorrhizal (AM) or general fungal community composition on root mats, in bulk soils, on fine root fragments (Carrino-Kyker et al., 2016; Burke et al., 2021), or on EM or AM total colonization of roots (Lydia V. Jahn, personal communication). In contrast, in a P-limited boreal spruce forest, belowground fungal biomass increased when P was added (Almeida et al., 2018), suggesting that P addition may stimulate fungal biomass of some species in P-limited environments (Hagerberg et al., 2002). Interestingly, Almeida et al. (2023) found that changes in EMF belowground biomass production following P fertilization depended on ambient P availability, suggesting that P addition enhances EMF in P-limited forests, but not when P is not limiting. In our study, the addition of P increased species richness relativized by abundance, but not overall sporocarp abundance or aboveground biomass.

Our analyses highlighted 12 EMF species with fruiting responses to added P or interactive effects of N and P addition. To our knowledge, this is the first report of species-level EMF fruiting response to elevated P. Of the species that responded to P, most were in the genus *Amanita* or the family Boletales. The functional traits leading to P sensitivity in EMF remain underexplored relative to N. Long distance exploration type hyphae and as well as the production of P mobilizing enzymes may be traits associated with P acquisition in EMF (Lilleskov et al., 2019). In our study, species of Boletales (e.g., *Leccinum scabrum, Retiboletus ornatipes,* and *Tylopilus felleus*), which have long distance exploration type hyphae, were suppressed with added P, suggesting that these fungi may be adapted to mine for P beyond P-depletion zones and in P-limited ecosystems. Only one taxa, Paxillus involutus, was significantly more abundant when both N and P were added together.

This study was limited to one growing season in one geographic location. To determine whether the fruiting response of EMF to N vs P addition depends on the nutrient status of the site at the time of sampling (Kuyper and Suz, 2023) will depend on similar tests being conducted at multiple sites with varied ambient nutrient availability. Future research endeavors should include sampling soil to determine if composition of soil fungi below ground varies with nutrient treatment.We found that different species of EM fungi responded to soil nutrient additions differently, suggesting that these fungi are functionally diverse in their ability to absorb and transport soil nutrients. Therefore, the sustained diversity of mycorrhizal fungi is important to nutrient uptake and the resiliency of forests to ecosystem change. The formation of sporocarps is an important mechanism for reproduction of most EMF, so changes in fruiting and species richness likely affect species fitness. Our results support the importance of P, in addition to N, in shaping EM fungal communities. Notably, in this temperate hardwood forest, P shaped fungal community composition early in the growing season but N was more important in the later part of the season, suggesting that seasonal changes affect host tree nutrient demands and the functions of mycorrhizal associations.

CRediT authorship contribution statement

Claudia Bashian-Victoroff: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Ruth D. Yanai:** Writing – review & editing, Funding acquisition, Conceptualization. **Thomas R. Horton:** Writing – review & editing, Funding acquisition, Conceptualization. **Louis J. Lamit:** Writing – review & editing, Visualization, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2024.101388.

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