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Seasonal disconnects between saprobic and mycorrhizal sporocarp communities in the Southern Appalachian Mountains

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ABSTRACT

The southern Appalachian Mountains in the United States are heavily forested with diverse forest types and vegetation structures and is generally considered a biodiversity hotspot. Fungal sporocarp investigations in this region are not new, but multi-year interseasonal investigations into sporocarp community patterns are rare. Using a 4-year (2014–2017) repeated monthly macrofungal sampling dataset (May–October) across an elevational gradient (associated with vegetation structure), we queried community, seasonal, and guild community ecological patterns. In doing so, we (1) demonstrate a temporal disconnect between saprobic and ectomycorrhizal taxa, (2) describe increased community variability across the growing season, and (3) explore individual taxa occurrences across seasons and examine co-occurrence patterns between taxa and guilds. Further, we explore congruence between sporocarp and metabarcoding fungal datasets and advocate the utility of both sampling schemes.

1. Introduction

The Southern Appalachian mountains are some of the oldest extant montane environments in the United States, and partially due to protection afforded by the lack of glaciation during the last glacial maximum, remain a biodiversity hotspot (Pickering et al., 2002). Forest communities within southern Appalachia are largely structured by elevational gradients (Day and Monk 1974; Lorimer 1980; White et al., 2018) and plant community changes with elevation are often mirrored by patterns in fungal diversity (Veach et al., 2018), lichen diversity (Allen and Lendemer, 2016), and biogeochemical patterns of nitrogen and carbon cycling (Knoepp and Swank 1998; Knoepp et al., 2008, 2018). Fungal communities are generally considered hyperdiverse in these mountains as evidenced by numerous investigations using molecular and morphological datasets (Hesler 1937; Walker et al. 2005, 2008; Baird et al., 2014; Veach et al., 2018; Brown et al., 2019; Barney et al., 2020; Hughes et al., 2020). Despite numerous investigations into fungal diversity in southern Appalachia, basic ecological knowledge is often lacking. There is much we do not know about fungal phenological patterns, drivers of sporocarp occurrence, and interannual community variation within the southern Appalachian Mountains. While several European studies have examined fungal fruiting phenology and variability with climate and other global changes (Andrew et al. 2017, 2018a, 2018b), phenological patterns are not well classified in Southern Appalachia. Southern Appalachia is much older than most of Europe and despite rapid land development, there are very few impacts of land use legacies in more montane regions (Gragson and Bolstad 2006) which results in many virgin forest tracts (Lorimer 1980). Investigations into how phenological patterns manifest themselves in the Southern Appalachian Mountains are crucial as this region is considered particularly vulnerable to predicted climate change scenarios with several important high-elevation tree species expected to either become extirpated or extinct (Potter et al., 2010). Further, with a general trend toward sequence-based ecology, there is a growing call to connect molecular-based studies to traditional fungal species description (Lücking and Hawksworth 2018), global biodiversity and functional databases (Põlme et al., 2020; Větrovský et al., 2020), as well as to sporocarp and/or culture data in the field (Brown et al., 2016) or

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herbaria specimens (Andrew et al., 2019). One of the currently largely unresolved aspects of fungal community ecology is how functional guilds respond to similar ecological factors and this work attempts to bridge this knowledge gap. We also address the differences between fungal seasonality, sporocarp production, and metabarcoding/sporocarp comparability in the Southern Appalachian range.

Knowledge of fungal sporocarp seasonal occurrence patterns in forests is surprisingly sparse outside of common culinary species. In contrast, there is an ever growing body of literature investigating the seasonality of endophytic (Cross et al., 2017; Barge et al., 2019), decomposer microfungal (Ho et al., 2002; Nikolcheva and Bärlocher 2005), animal associated fungal (Anslan et al., 2018), and hypogenous fungal communities (Luoma et al., 1991; Claridge et al., 1993), among others. Of the investigations into fungal sporocarp seasonality, many focus on sporocarp emergence patterns following a disturbance or other successional framework (Jumpponen et al., 2015; Dejene et al., 2017). While most extensive examinations into fungal seasonality and associated changes are from Europe (Heegaard et al., 2017; Andrew et al., 2018a, 2018b; Diez et al., 2020) and Asia (Sato et al., 2012), one of the rare US based studies examined herbaria collection data to elucidate species specific changes in seasonality across Michigan (Diez et al., 2013). Collectively, the evidence strongly suggests that sporocarp (and thus sporulation) seasonal patterns are tightly linked to environmental constraints and are being altered by global change.

As seasons change, so do temperature and precipitation regimes. In the boreal forests of Alaska, it was demonstrated that there was very little interannual variation of fungal communities within a forest type, but there were strong community signals with different forest types (Taylor et al., 2010). Macrofungi sporocarp timing has been demonstrated to be dramatically changing with changing climate (Diez et al., 2013; Gange et al., 2018), but these patterns may be different between forest types (Gange et al., 2007). This phenological shift in sporulation has also been observed for common allergen species (Demain et al., 2021). To complicate things, sporocarp seasonality for mycorrhizal species is tightly linked to host physiological and environmental responses (Gange et al., 2018; Silva-Flores et al., 2019; Silvana et al., 2020). This is further complicated by differential nutrient utilization between mycorrhizal and saprobic taxa that leads to mycorrhizal macrofungi generally having larger sporocarps than saprobes (Bässler et al., 2015), presumably due to carbon availability and uptake efficiency. Further, there is emerging evidence that hyphal associated bacterial interactions, specifically with diazotrophs, can alter sporocarp temporal development (Obase 2020), presumably because diazotrophs may alleviate N-deficiencies. How seasonal and broad phenological patterns will impact fungal guild occurrence patterns in a warming world are not well understood. This work extends the geographic range of multi-year temporal sporocarp studies to include the biodiversity hotspot of the southern Appalachian Mountains to better facilitate an understanding of species patterns in the future.

While there exists several good long-term multi-year fungal sporocarp surveys (Eveling et al., 1990; Straatsma et al., 2001; Büntgen et al., 2012; all from Europe), which generally report peak sporocarp diversity in late summer and throughout the fall, there is a general dearth of information on sporocarp phenological differences between different fungal guilds. A major exception to this is a comprehensive 30-year survey from Japan (Sato et al., 2012) in which a fixed point sampling scheme was undertaken monthly from 1982 to 2011. This provides evidence for a temporal disconnect between ectomycorrhizal (EcM) and saprobic species, but leaf litter decomposing taxa were more similar in diversity patterns to EcM taxa than wood decomposing species. The authors presume these patterns to be due to differential carbohydrate availability where EcM have a ready supply of photosynthates, but saprobic taxa are more constrained by environmental conditions. However, it is not known how representative this temporal disconnection pattern is globally as these long-term surveys are rare in the United States. Here, we expand on this as we examine a multi-year, intra-annual sporocarp sampling dataset collected across different forest community types (which are associated with an elevational gradient) and across two distinct locations in the southern Appalachia region of the United States – Great Smoky Mountains National Park (GRSM - Tennessee) and Coweeta Hydrologic Laboratory (CWT- North Carolina). Field plots at CWT are located in a USDA Forest Service Experimental Forest established in 1934 as part of the NSF-sponsored Long-Term Ecological Research (LTER) program covering different forest types and elevational gradients. These plots, established in 1992 focused on environmental, natural, and human disturbances affecting the ecology of the montane deciduous forests, but fungi at these LTER sites have never been thoroughly examined prior to the current study. The field sites at CWT consist of drier low elevation mixed oak-pine, lower elevation cove hardwood, medium elevation mixed oak, higher elevation mixed oak and high elevation northern hardwood forests (Knoepp et al., 2008). Elevation gradient plots at GRSM were established in 2011 and are located at higher elevations in northern hardwood and spruce-fir habitats. To examine sporocarp community dynamics in a relatively poorly studied biodiversity hotspot, we investigate: (1) the variability of fungal sporocarp communities and individual taxa patterns across seasons and years, and (2) if EcM and saprobic taxa exhibit a temporal disconnect in sporocarp occurrence, as seen in datasets from other regions (Sato et al., 2012). Further, we explore similarities between sporocarp diversity and taxonomic overlap from this study and a previously published, concurrently sampled soil-based metabarcoding analysis (Veach et al., 2018) to assess congruency in genera occurrence between traditional and molecular based investigations. With these data, we expect that there would be a disconnect between EcM and saprobic sporocarp occurrence as has been seen in other studies, but this disconnect would manifest differently in the diverse forest types.

2. Materials and methods

2.1. Sampling locations and dates

Samples were collected across the southern Appalachian Mountains from long-term research plots (LTER) at the Coweeta Hydrologic Laboratory (CWT) in North Carolina and from Great Smoky Mountain National Park (GRSM) in eastern Tennessee. These plots were sampled previously in 2013 (Veach et al., 2018) to investigate elevational impacts of soil-borne fungal communities using a metabarcoding approach. Five plots were selected to represent the dominant vegetation structure and elevations at CWT (Knoepp and Swank 1998; Knoepp et al., 2000; Elliott and Vose 2011) and consist of 80 × 80 m plots (Table S1 for more site location), including: OP (mixed oak-pine, 788 m asl), CH (cove hardwood, 801 m asl), LO (low-elevation mixed oak, 860 m asl), HO (high-elevation mixed oak, 1094 m asl), NH (northern hardwoods, 1389 m asl). Additional plots were established in 2013 within GRSM to extend geographic representation (Veach et al., 2018) and consisted of: HNH (high-elevation northern hardwood, 1539 m asl), NHS (mixed northern hardwood, spruce, fir, 1737 m asl), and SF (spruce fir, 1940 m asl). Within each main plot (6400 m^2), we established 25 16 m^2 subplots. These subplots were randomly selected (five subplots) at each sampling event; sporocarps were collected from individual subplots. Some subplots were located directly adjacent to the LTER plots (rather than within it) to minimize anthropogenic disturbances in the LTER plots. Once a month during the growing season (May–October) for four years (2014–2017), a random subplot (n = 32) was surveyed during each sampling timepoint (n = 24) for a total of 768 total samples. Each subplot was exhaustively sampled (soil, downed wood, etc.) and all visible sporocarps were collected and identified where possible.

2.2. Morphological and molecular identifications

Each sporocarp was identified to species (where possible) using expert knowledge by examining macro-morphology (*similar to* Baird

et al., 2013, 2014) and assigned a morphotype Taxon ID and a Plot x Taxon ID count matrix was generated. Where sporocarp integrity was able to be preserved upon return to the laboratory, a representative sporocarp for each Taxon ID was selected for DNA extraction and barcoding. Genomic DNA from sporocarps was extracted using Qiagen DNeasy Plant Extraction Kit (Germantown, MD, USA) and the ITS regions of the rRNA operon were amplified using the fungal specific primers ITS1f-ITS4 (White et al., 1990; Gardes and Bruns 1993) (PCR parameters following Gardes and Bruns 1993). Amplicons were cleaned using Qiagen Clean Up Kit (Germantown, MD, USA) and Sanger sequences were generated (using the aforementioned primers) by Eurofin Genomics LLC (Louisville, KY, USA). Chromatograms were cleaned, corrected, and contiged using MegAlign Pro (DNASTARl; Madison, WI, USA).

Taxa were identified molecularly to Species Hypothesis (SH) levels where possible by comparing sequences to the UNITE database (Nilsson et al., 2019) using massBLASTer (BLASTn v2.10.0+) as implemented by the PlutoF toolkit (Abarenkov et al., 2010) and best SH or GenBank hit was recorded. If BLASTn identity values were ≥85%, then molecular IDs were used, if <85%, then morphological IDs are used (see Table S2). Where Taxon IDs were determined to be the same species, they were collapsed, and a final Sample x Taxon matrix was compiled and used for additional analyses. Further, fungal functional guilds were determined where possible by querying genus identities and SH epithets against the functional databases Fun^{Fun} (Zanne et al., 2020), FUNGuild (Nguyen et al., 2016), and FungalTraits (Põlme et al., 2020) to identify taxa that are unambiguously ectomycorrhizal (EcM) or saprobic, and the Sample x Taxon matrix was split into separate EcM and saprobic matrices for additional analyses. Downstream analyses were done using the entire fungal community as well as these guild communities.

2.3. Richness, diversity, and community analysis

Data were further processed and analyzed using the programs mothur (v.1.41.3; Schloss et al., 2009) and R (v.3.3.3). Richness (observed) and diversity (1-D, Simpson's Diversity) were calculated and analyzed using separate three-way ANOVA models (whole community, EcM and saprobic communities) after Box-Cox transformations to increase normality (Richness: whole community λ = -0.581, EcM λ = -0.855, saprobic λ = -1.644; 1-D: whole community λ = 1.45, EcM λ = 2.0, saprobic λ = 2.0) and included the model factors Year, Month, and Location. Where a significant factor was observed, *post hoc* analyses (Tukey HSD) were conducted to determine how treatments differed. Further, we investigated whether EcM and saprobic richness differed across sampling months within each subplot using a two-tailed paired Wilcoxon Sign Rank test.

To investigate community dynamics, we generated Canberra community distance matrices (Lance and Williams 1967) for each community type (whole community, EcM, saprobic) and conducted a series of analyses on obtained distance matrices. Canberra distances were chosen as it maximizes the effective distances between samples when highly zero-inflated, a common attribute ii our data as common community dissimilarity metrics (*e.g.* Bray-Curtis) do not perform well with data structure that are highly zero-inflated (Clarke et al., 2006). First, to examine community differences with treatments, we conducted PerMANOVA (Anderson 2001) tests using Canberra distances with the function adonis in the R package *vegan* (Oksanen et al., 2017) with 999 permutations with the following models: Region (CWT or GRSM) to assess if communities were broadly different across the ~70 km distance between locations, and a fully factorial three-way model with Location (plots), Month, Year, and all possible interactions. This was done for the whole community, EcM communities, and saprobic communities separately. Additionally, using mothur implemented tests for homogeneity of molecular variance (HOMOVA; Stewart and Excoffier, 1996) with 1000 iterations, which tests for intrapopulation multivariate heteroscedasticity, we tested if population variances change across months, which may indicate population randomness. These patterns were visualized using non-metric multidimensional scaling (NMDS; as implemented in mothur using 1000 interactions) on Canberra distances and was optimally resolved for 3 dimensions (Stress = 0.18).

Further, to evaluate if EcM and saprobic communities respond similarly across years, months and locations, we utilized a Procrustean Association Metric (PAM) approach (Lisboa et al., 2014; Brown and Jumpponen, 2019). Briefly, individual NMDS loading scores (3 axes) for each guild were used and, using the *vegan* package in R, saprobic NMDS loading scores underwent Procrustes analysis (best matched to EcM loading scores with 999 permutations; SS = 0.9600, correlation = 0.1736, P = 0.005). Then, residual vectors were calculated, and these PAM values were used in a three-way ANOVA model (Year, Month, Location), and where significant, Tukey HSD *post hoc* tests were conducted.

2.4. Individual taxa responses

To elucidate individual taxon changes in abundance over seasons, we used a zero-inflated negative binomial (ZINB) generalized regression framework (Xu et al., 2015; Xia et al., 2018) as our data are highly zero-inflated. ZINB was selected over other zero-inflated models as initial tests demonstrated that ZINB was the best model based on AIC values. For these, each taxon with an absolute count ≥20 (26 taxa for total cumulative relative abundance of 72.07% of the whole fungal community) was tested using the model Year, Month, and Year × Month interactions. Select abundant taxa that differed in abundance across months with ZINB regressions were visualized using simulated fungal occurrences (10,000 simulations using random noise by model [month] based on initial proportions), using kernel smoothing (locally weighted regression) (Cleveland 1979) with the following parameters selected to maximize R^2 (local fit = quadradic, weighted function = cosine, alpha (smoothness) = 0.7) to examine seasonal taxa-specific occurrences. Further, to investigate interannual variation in sporocarp occurrence, we utilized a Kruskal-Wallis one-way analysis of variance approach to identify which taxa change in abundances across years.

2.5. Co-occurrence analysis

To examine if certain taxa co-occur more often than chance, we utilized a SparCC framework (Sparse Correlations for Compositional data; Friedman and Alm 2012), which is robust and well suited for zero-inflated compositional data and minimizes spurious species-species correlations which compositional data can suffer from. Using the SparCC implementation in mothur (samplings = 20, iterations = 10, and permutations = 1000), and limiting our correlations analyses only to taxa with ≥1% relative abundance (globally) we identified taxa that are significantly correlated (with or against) other taxa.

2.6. Statistics and accessions

Statistics were conducted using a combination or JMP Pro (v.15; SAS Institute, Cary, North Carolina, USA), R, and mothur. All obtained ITS sequences are accessioned in GenBank (NCBI) under the accessions MW899379-MW899495.

3. Results

3.1. Taxa distributions

Across our plots over 4-years of repeated seasonal sampling, we had a total of 3015 individual observations and 233 total subplots. We observed and identified 162 unique macrofungal taxa (Table S2). Of our observed taxa, the vast majority belonged to Basidiomycota (95.03%), predominantly Agaricomycetes, with representatives of 14 Orders (of which the Agaricales and the Russulales were the most common at

51.5% and 16.4% respectively), 43 Families (Russulaceae and Cortinariaceae were the most common at 13.3% and 8.9% respectively), and 71 genera (*Cortinarius*, *Russula*, and *Amanita* were the most common at 8.2%, 6.4%, and 6.4% respectively).

3.2. Richness and diversity

Overall, plot species richness (3.06 ± 2.99; Mean ± STD, Max = 16) changed with our ANOVA model ($F_{16,216} = 3.728$, $P < 0.0001$) and was sensitive to month of sampling ($F_{5,216} = 8.551$, $P < 0.001$) and year ($F_{3,216} = 5.061$, $P = 0.002$) but not location ($P = 0.4089$). Tukey HSD *post hoc* analyses indicate richness was higher in August that any other month (Fig. 1) and higher in 2014 than 2015 and 2016. Similarly, richness of EcM taxa were responsive to month ($F_{5,\ 131} = 4.455$, $P = 0.0009$) and year ($F_{3,131} = 4.723$, $P = 0.0037$) but not location ($P = 0.2986$) whereas saprobic richness did not change across our model ($F_{63,131} = 0.7734$, $P = 0.8483$). Richness of EcM and saprobic taxa were similar for most of the months sampled (Fig. 1; May: $W = -12$, $P = 0.6133$; June: $W = 40$, $P = 0.5587$; July: $W = -3.5$, $P = 0.9823$; September: $W = -53$, $P = 0.2436$) but EcM subplot richness was higher than saprobic richness (Fig. 1) for August ($W = -230.5$, $P = 0.032$) and October ($W = -98.5$, $P = 0.044$). In contrast, diversity estimators (1-D) were not different for the entire community ($F_{77,140} = 1.1546$, $P = 0.2787$) or EcM ($F_{80,156} = 0.8257$, $P = 0.8009$) but did differ for saprobic members ($F_{76,147} = 1.600$, $P = 0.233$) for location ($F_{8,147} = 3.166$, $P = 0.0024$), month ($F_{5,147} = 2.695$, $P = 0.0232$), and year ($F_{3,147} = 2.844$, $P = 0.0398$), with early season samples (May) with the highest diversity and August with the lowest.

3.3. Community patterns

The PerMANOVA results suggests that the entire community is similar across plots (vegetation type and elevation) ($F_{7,232} = 0.784$, $P = 0.866$, $R^2 = 0.024$), but interestingly, communities constrained to EcM or saprobes did change with location but with relatively low R^2 values (Table 1). While no factors were significant in the whole community, interactions between location, month, and year explained about 1/3 of the community variability in EcM and saprobic communities (Table 1). In addition, location and year were significant for both, saprobic communities were also responsive to location by month, and month by year interactions.

Analyses of the homogenization of variance (HOMOVA) of Canberra distances across months indicated that multi-dimensional data variability (data spread) was not consistent across months for the entire

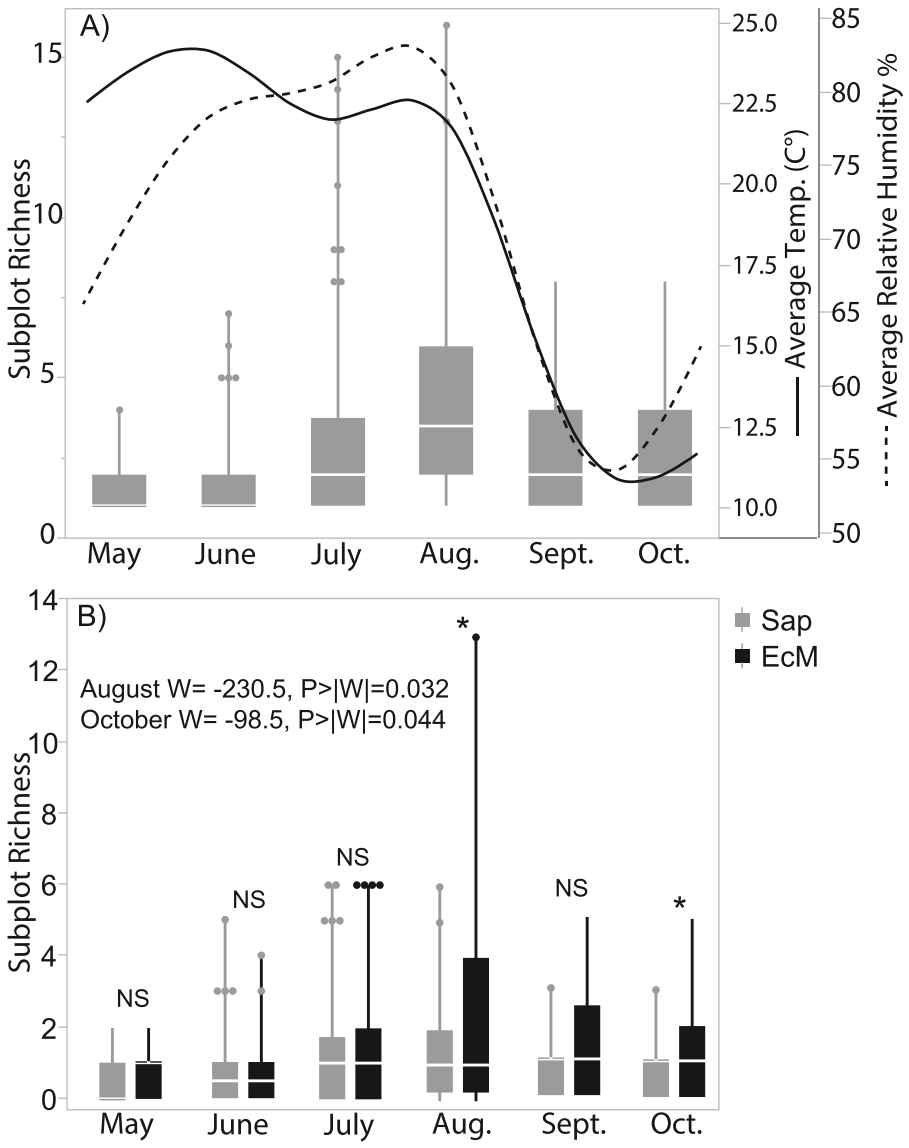

Fig. 1. Fungal richness within each subplot across months presented as quintile (upper and lower bounds are Q3 and Q1 respectively) and horizontal line is median richness for (A) all taxa and (B) saprobic and ectomycorrhizal taxa. Significant differences between guilds for each month based on paired Wilcoxon signed-rank tests are indicated. Also presented are average (30-min window) surface temperature (solid) and relative humidity (dashed) from NEON data at GRSM across sampling years where available and are presented as kernel smoothed averages.

Table 1
Results of PerMANOVA for the entire community, Ectomycorrhizal (EcM) communities, and saprobic communities testing if communities differ across location, month, year, and all possible interactions. Presented are pseudo-F statistics (degrees of freedom in subscript), P-values and R^2 values.

	Entire Community			EcM			Saprobes		
Test	F_{df}	P-value	R^2	F_{df}	P-value	R^2	F_{df}	P-value	R^2
Location	$F_{7,232} = 0.784$	0.866	0.024	**$F_{7,232} = 3.042$**	**0.001**	**0.066**	**$F_{7,232} = 3.251$**	**0.001**	**0.065**
Month	$F_{5,232} = 0.767$	0.834	0.017	$F_{5,232} = 0.900$	0.604	0.014	**$F_{5,232} = 2.044$**	**0.007**	**0.029**
Year	$F_{3,232} = 1.101$	0.352	0.014	**$F_{3,232} = 2.391$**	**0.006**	**0.022**	$F_{3,232} = 1.685$	0.081	0.014
Location x Month	$F_{34,232} = 0.875$	0.734	0.130	$F_{34,232} = 1.385$	0.080	0.146	**$F_{34,232} = 1.605$**	**0.018**	**0.156**
Month x Year	$F_{8,232} = 0.906$	0.648	0.031	$F_{8,232} = 1.572$	0.074	0.039	**$F_{8,232} = 2.490$**	**0.002**	**0.056**
Location x Month x Year	$F_{54,232} = 1.086$	0.300	0.256	**$F_{54,232} = 2.000$**	**0.004**	**0.336**	**$F_{54,232} = 2.159$**	**0.001**	**0.333**
Residuals			0.528			0.376			0.345

fungal community (B = 43.714, P < 0.001), EcM (B = 75.116, P < 0.001) and saprobes (B = 29.578, P = 0.012). *Post hoc* comparisons indicated that for each of these community partitions, samples from August (the height of the fungal growing season in Southern Appalachia) generally were more variable in Canberra-based multi-dimensional space (Fig. 2) than other sampled months.

To investigate whether EcM and saprobic communities are structured similarly and congruently across our sampling design, we used a Procrustes-based analysis. Procrustes association metric vector distances (PAM; across three NMDS axes) differed across our three-way ANOVA model ($F_{12,232} = 2.968$, P = 0.0002), with month ($F_{5,232} = 6.090$, P=<0.0001) and year ($F_{3,232} = 3.029$, P = 0.0279) being different in PAM vector distances but not location (P = 0.156). *Post hoc* analyses and visualized kernel smoothing graphs suggests that the average PAM distance between paired EcM and saprobic data are greatest in August, then in all other months beside September (Fig. 3). This indicates that EcM and saprobic communities behaved similarly in the early season but then diverges in August before converging again in their community responses.

3.4. Individual taxa responses

Of the 26 taxa with greater than 20 observations, ZINB regressions indicated that half of these (50.0%; Table 3, Fig. 4) shifted in occurrence across sampling months, and more (92.3%) were responsive to interactions between month and year, while few (26.9%) differed across years. This suggests that taxa abundance is largely controlled by inter-annual climatic patterns that shift timing of sporocarp development, but taxa are largely, though not exclusively, consistent across years. Further, Kruskal-Wallis tests for differential occurrence across years indicated that 30 taxa were inconsistently found across sampling years (Table S3).

3.5. Co-occurrence analysis

SparCC co-occurrence analysis on abundant taxa demonstrated that 92 of the 420 possible species interactions (21.9%) were significantly correlated with one another (61 were positive associations, and 31 were negative associations; Table 3). Several taxa, where taxon-taxon associations were significant, are monodirectionally significant. They were only positively or negatively associated with other taxa (where a significant correlation can be found), while others had broad positive and

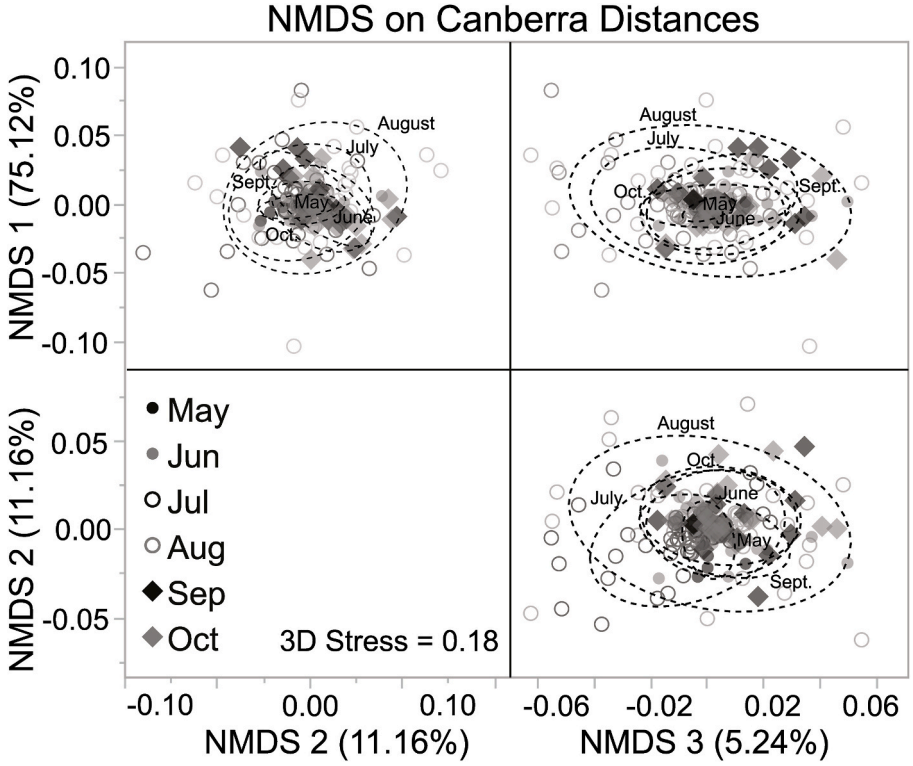

Fig. 2. Non-Metric Multidimensional Scaling (NMDS) plots of sampling plots across months for the entire fungal community with 90% ellipses for the three resolved dimensions. This demonstrates that while communities overlap and have no noticeable differences in community structure (PerMANVOA, Table 1), fungi observed in August are more variable (based on HOMOVA tests) than other months.

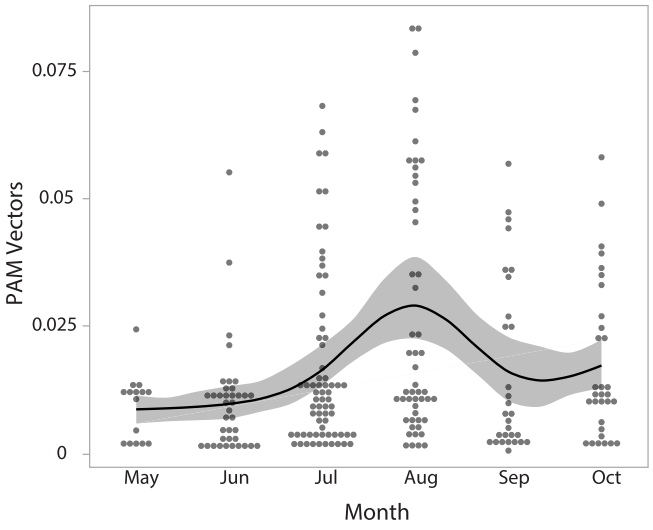

Fig. 3. Procrustes association metric (PAM) vector distances between ectomycorrhizal (EcM) and saprobic communities following Procrustes analyses (Procrustes SS = 0.9699) with rolling average smoothed line with 95% confidence interval. PAM vector distances represent distance on ordination space between EcM and saprobic communities for each sampling plot. EcM and saprobic communities responds similarly in the early months by diverge highly in August suggesting different drivers of these communities in August.

negatives associations. For instance, Taxon 47 (*Gymnopus dichrous*), a small saprobe found on woody material (Hughes et al., 2015) co-occurs more often than chance with other saprobes (Taxon 2, *Stereum ostrea*; Taxon 6, *Rhodocollybia butyraceae*; Taxon 14, *Mycetinis opacus*) and with ectomycorrhizal taxa (Taxon 71, *Russula bicolor*; Taxon 86, *Gyroporus ornatipes*), among others. Similarly, the ectomycorrhizal Taxon 71 (*Russula bicolor*) is only positively or neutrally associated other taxa. Conversely, the saprobic Taxon 126 (*Galerina marginata*) is only negatively or neutrally associated other taxa, and so is the ectomycorrhizal Taxon 143 (*Austroboletus gracilis*).

4. Discussion

This extensive study examining macrofungi populations across the growing season (May–October) for four consecutive years (2014–2017) from repeatedly censused plots provides a unique insight into fungal sporocarp community dynamics in the southern Appalachia Mountains. We demonstrate broad interannual community stability, similar communities from GRSM and CWT and across locations (despite dominant vegetation types and elevation), but known EcM and saprobic communities are different between locations based on PerMANOVA tests (Table 1), which is likely due to shifting vegetation structure and soil chemistry (Veach et al., 2018). Further, similar to Sato et al. (2012), we see a strong temporal disconnect between EcM and saprobic taxa and community structure with maximum disharmony occurring in the month of August and we identify some taxa that seemingly drive this disconnect. It has previously been suggested that seasonal disconnects between EcM and saprobic taxa may be the result of differential carbon utilization (Sato et al., 2012; Bässler et al., 2015), whereby mycorrhizal taxa that get carbon from live plant hosts and may sporulate earlier in the season and saprobic taxa may sporulate later as litter loads increase, which may explain our observed seasonal disconnect. This guild temporal disconnect between EcM and saprobic taxa across months is epitomized by observed differences in guild richness over months (Fig. 1) with mycorrhizal taxa being more species rich than saprobic taxa in August. It should be mentioned that the total fungal richness found here (162 observed taxa) may seem low for a biodiversity hotspot as other large-scale fruiting surveys have found more species, with a European survey of wood decomposing fungi finding 263 species (Bässler et al., 2010) and another finding 668 species (Sato et al., 2012). These exemplar studies either had much larger plot sizes (Bässler et al., 2010), or a much longer timeframe (Sato et al., 2012), which will both impact sporocarp richness. Further, we see extensive variability in fungal communities in July and August (Fig. 2), and this variability is greater than in other months. Additionally, PAM analyses (Fig. 3) demonstrates that differential community dynamics between EcM and saprobic taxa.

Table 2
Results of Zero-inflated Negative Binomial generalized regressions (ZINB) of fungal taxa abundance for taxa with at least a count of 20. Presented are Taxon IDs, relative abundance (RA) across all samples, Generalized R^2 for the model, taxonomic identifies, and tests statistic (Wald χ^2) and P-values for each model component. Significant factors are in bold.

Taxon ID	RA%	R^2	Identity	Month (Wald χ^2, P-value)	Year (Wald χ^2, P-value)	Month x Year (Wald χ^2, P-value)
Taxon 2	3.05	0.144	*Stereum ostrea* †	**51.40, < .0001**	**249.11, < .0001**	**1156.19, < .0001**
Taxon 4	5.57	0.257	Pezizomycetes sp.	**5.57, 0.0182**	**575.66, < .0001**	**1246.34, < .0001**
Taxon 6	2.49	0.126	*Rhodocollybia butyracea* †	0.33, 0.5656	**14.49, 0.0007**	**5183.09, < .0001**
Taxon 7	1.23	0.083	*Cortinarius* sp. ‡	**32.95, < .0001**	6.29, 0.0981	**113.34, < .0001**
Taxon 12	0.86	0.028	*Polyporus varius* †	4.05, 0.1319	0.18, 0.9799	0.72, 0.3931
Taxon 13	0.70	0.061	*Mycorrhaphium adustum* †	3.82, 0.5755	3.60, 0.0575	**25.53, < .0001**
Taxon 14	1.16	0.111	*Mycetinis opacus* †	0.44, 0.5031	1.51, 0.6795	**477.31, < .0001**
Taxon 16	1.33	0.076	*Sebacina candida* ‡	0.26, 0.6062	2.56, 0.1096	**12.28, 0.0065**
Taxon 41	1.16	0.077	*Cortinarius cruentiphyllus* ‡	5.18, 0.3934	1.79, 0.616	**429.57, < .0001**
Taxon 46	3.58	0.168	*Clavulina* sp. ‡	**109.34, < .0001**	5.18, 0.0748	**253.72, < .0001**
Taxon 47	7.99	0.088	*Gymnopus dryophilus* †	**64.15, < .0001**	0.07, 0.9949	**101.81, < .0001**
Taxon 48	9.52	0.283	*Gleoglossum simile* †	10.54, 0.0611	2.36, 0.3071	**1495.26, < .0001**
Taxon 55	1.76	0.169	*Leotia lubrica* †	**7.02, 0.008**	6.03, 0.1098	**447.49, < .0001**
Taxon 71	3.22	0.098	*Russula bicolor* ‡	**21.43, < .0001**	1.50, 0.6802	**42.16, < .0001**
Taxon 81	0.86	0.084	*Xylaria cubensis*	2.91, 0.0875	**152.54, < .0001**	**148.02, < .0001**
Taxon 86	2.75	0.151	*Lactifluus* sp. ‡	**194.10, < .0001**	1.73, 0.1881	11.06, 0.0502
Taxon 117	3.15	0.114	*Hygrocybe chlorophana* †	**16.75, < .0001**	0.45, 0.5002	**23.14, < .0001**
Taxon 126	2.12	0.179	*Lactarius subdulcis* ‡	**13.54, 0.0089**	**22.74, < .0001**	**114.62, < .0001**
Taxon 143	5.37	0.123	*Craterellus* sp. ‡	**36.94, < .0001**	**24.31, < .0001**	**383.21, < .0001**
Taxon 165	1.13	0.061	*Cortinarius tortuosus* ‡	1.68, 0.8902	1.63, 0.6513	**389.58, < .0001**
Taxon 167	5.61	0.205	*Marasmius capillaris*	0.05, 1.00	0.14, 0.9859	**1507.11, < .0001**
Taxon 170	2.26	0.127	*Pestalotiopsis* sp.	**14.81, 0.0001**	**14.82, 0.0001**	**82.84, < .0001**
Taxon 176	2.82	0.082	*Marasmius rotula* †	0.03, 1.00	0.13, 0.9873	**337.45, < .0001**
Taxon 193	0.93	0.040	*Cortinarius* sp. ‡	0.83, 0.3612	0.01, 0.9763	**28.75, < .0001**
Taxon 289	0.80	0.000	*Hygrocybe* sp. †	**11.52×10^{19}, < .0001**	4.60, 0.2033	0.02, 1.00
Taxon 332	0.66	0.063	*Armillaria mellea* †	1.385, 0.9261	6.41, 0.093	**121.78, < .0001**

Table 3
SparCC correlation coefficients (upper triangle) and associated P-values (lower triangle) demonstrating significant (bold) co-occurrence associations between taxa. † signifies saprobic taxa and ‡ signifies mycorrhizal taxa as defined in Table S2.

Taxon ID	2†	4	6†	7‡	14†	16‡	41‡	46‡	47†	48†	55†	71‡	86‡	117†	126‡	143‡	165‡	167	170	176	193‡
2†		−0.01	0.02	0.01	**0.09**	−0.01	0.02	−0.01	**0.09**	0.03	−0.02	0.03	−0.01	**0.10**	−0.02	−0.03	**0.05**	**0.13**	−0.01	**0.20**	0.04
4	0.51		0.04	−0.01	**−0.04**	−0.01	0.04	**−0.06**	−0.01	**−0.08**	**0.11**	0.05	0.06	0.02	−0.02	−0.01	−0.01	0.03	0.01	−0.03	−0.01
6†	0.22	0.07		0.04	0.01	0.03	0.03	−0.03	**0.07**	0.03	0.02	**0.10**	−0.02	0.04	0.01	−0.01	−0.03	0.01	0.02	**0.06**	0.03
7‡	0.24	0.40	0.09		**0.09**	0.02	−0.01	−0.01	−0.01	0.04	−0.02	0.02	0.01	**0.08**	**−0.03**	0.03	**−0.02**	−0.03	−0.02	0.01	0.01
14†	**0.01**	**0.01**	0.58	**0.01**		**0.08**	**−0.03**	−0.01	**0.06**	0.06	−0.01	−0.02	−0.02	0.01	−0.01	0.01	0.01	−0.01	−0.01	0.01	0.01
16‡	0.40	0.30	0.16	0.17	**< 0.01**		**−0.03**	**−0.04**	0.03	−0.02	0.05	0.04	0.04	0.03	−0.04	−0.03	0.09	**−0.03**	−0.01	0.01	−0.01
41‡	0.33	0.11	0.20	0.09	**0.04**	**0.04**		**0.10**	0.05	0.02	0.01	**0.06**	0.01	0.03	−0.02	0.01	0.01	**0.12**	0.03	0.01	**−0.05**
46‡	0.30	**< 0.01**	0.06	0.25	0.36	**0.02**	**< 0.01**		−0.01	0.04	−0.02	0.03	0.04	−0.03	0.01	−0.04	0.03	0.01	−0.01	−0.01	0.03
47†	**0.02**	0.45	**0.04**	0.27	**0.04**	0.23	0.06	0.28		0.04	0.02	**0.11**	**0.12**	0.07	−0.01	−0.04	0.01	**0.08**	**0.09**	0.04	0.04
48†	0.21	**0.03**	0.25	0.12	0.06	0.24	0.29	0.12	0.21		−0.04	−0.04	−0.04	−0.01	**−0.07**	−0.01	0.04	0.07	−0.01	**0.09**	−0.02
55†	0.10	**< 0.01**	0.28	0.10	0.18	0.09	0.66	0.20	0.28	0.08		−0.02	0.01	−0.01	0.03	−0.01	0.01	**−0.03**	0.01	**−0.05**	0.01
71‡	0.20	0.08	**< 0.01**	0.18	0.14	0.07	**0.03**	0.26	**< 0.01**	0.07	0.20		**0.08**	**0.09**	−0.02	0.01	0.04	**0.06**	**0.13**	0.01	0.03
86‡	0.34	0.10	0.19	0.48	0.17	0.23	0.46	0.11	**< 0.01**	0.10	0.47	**0.01**		0.02	0.04	0.02	−0.02	0.05	0.01	−0.01	0.04
117†	**0.01**	0.31	0.12	**< 0.01**	0.54	0.22	0.10	0.10	0.07	0.46	0.44	**0.02**	0.34		−0.01	0.02	−0.01	0.03	0.01	**0.07**	0.03
126‡	0.30	0.27	0.60	**0.04**	0.36	0.06	0.13	0.41	**< 0.01**	0.18	0.12	0.12	0.38			**−0.04**	0.04	0.03	−0.02	−0.01	0.01
143‡	0.13	0.25	0.29	0.18	0.45	0.08	0.35	0.12	0.12	0.34	0.31	0.49	0.35	0.37	**0.02**		−0.02	0.01	**−0.03**	−0.01	0.01
165‡	**0.05**	0.37	0.07	**0.04**	0.37	0.39	0.50	0.22	0.32	0.09	0.34	0.13	0.08	0.35	0.06	0.08		**0.06**	0.03	**0.12**	0.01
167	**0.01**	0.20	0.42	0.07	0.47	**0.04**	**0.01**	0.39	**0.02**	0.06	**0.04**	**0.04**	0.09	0.14	0.24	0.39	**0.04**		−0.01	**0.19**	**−0.04**
170	0.48	0.38	0.25	0.09	0.25	0.18	0.08	0.22	**< 0.01**	0.35	0.52	**< 0.01**	0.38	0.39	0.20	**0.04**	0.01	0.24		−0.01	0.02
176	**< 0.01**	0.11	**0.02**	0.48	0.36	0.49	0.52	0.38	0.16	**0.02**	**< 0.01**	0.57	0.29	**0.02**	0.34	0.42	**0.01**	**< 0.01**	0.21		−0.01
193‡	0.07	0.37	0.14	0.29	0.28	0.37	**< 0.01**	0.14	0.17	0.07	0.50	0.14	0.06	0.12	0.50	0.58	0.42	**0.02**	0.22	0.22	

PAM analysis examines the ordination distances between paired samples after Procrustes analysis.

Taken together, these results strongly suggest that interannual occurrence is more conserved in earlier or later months, but more sporadic in the height of the North American summer. This may be due to biological patterns related to phenology but could also be explained by stochastic patterns that derive from increases in macrofungal richness in July and August. If there are more taxa present, then this noise signal may be amplified; additional investigations are needed to determine the drivers underpinning this pattern. However, it is most likely that this disconnect between EcM and saprobic taxa is a function of carbon availability between guilds as similarly posited by Sato et al. (2012). We see that EcM communities are similar across months based on PerMANOVA tests (Table 1) whereas saprobic communities shift across months. This seasonal stability in EcM communities is suggestive of community stability linked to plant host identity, whereas shifting saprobic communities is indicative of an increase in necromass and leaf litter, which can be affected by local microsite conditions (Bélanger et al., 2019) and plant community structure (Weand 2020). Carbon is available for EcM taxa as long as plants can photosynthesize but increased necromass utilized by saprobic taxa may be temporally controlled, linked to leaf/branch fall, leaf turnover, or cladoptosis events which increase throughout the growing season. Here we expand basic phenological knowledge of sporocarp dynamics across southern Appalachia and expand our understanding of coupled occurrences of sporocarps within and across guilds. This work suggests differential drivers of sporocarp production across taxa, guilds, seasons, and years but this study lacks the ability to define these drivers.

Taxa co-occurrence patterns are generally driven by either similar nutritional requirements or disparate nutrition requirements facilitating co-existence via shared habitat and/or facilitations or niche partitioning respectively (Peršoh et al., 2018; Tu et al., 2020; Ji et al., 2021). However, for sporocarps, which generally are produced during periods of increased resource limitation (Halbwachs et al., 2016), co-occurrence could be a result of ecosystem wide increased resource limitations or could merely be coincidental. This study does not attempt to disentangle these patterns but does provide a framework to investigate which co-occurrence patterns are not coincidental by determining if co-occurrence occurs more often than chance using null models. Here we see numerous taxa have overlapping sporocarp occurrences or have negatively associated sporocarp occurrences, and this pattern seems to be largely (though not exclusively) dependent on guilds. Generally, where SparCC associations are significantly correlated (Table 3), saprobic taxa are positively associated with other saprobic taxa (co-occur in the same plots) whilst ectomycorrhizal taxa tend to be negatively associated with each other, suggesting competitive exclusion of mycorrhizal species. This suggests that some taxa may be beneficial in facilitating occurrence of other taxa across the landscape (positive correlations) or may inhibit or otherwise limit occurrence of some taxa (negative correlations). This mycorrhizal anti-correlative pattern may also be associated with differential vegetation as tree communities vary drastically between many of our plots (Table S1). It appears that at these sites, as long as there is ample organic matter to support broad saprobic action, co-occurrence of saprobes is a common outcome. It should be mentioned, that in even the best co-occurrence and correlation networks, correlated taxa may only co-occur coincidentally and may not be linked biologically, but we see no current reason to doubt the veracity of these observed patterns.

Investigations into the congruency between sporocarp collections and metabarcoding or other molecular approaches are few (Jumpponen et al., 2015) but it has been recommended that the use of both approaches simultaneously can maximize ecological inference (Brown et al., 2016; Frøslev et al., 2019). Here, we have the opportunity to qualitatively interrogate congruency between metabarcoding and sporocarp collection approaches for macrofungi as our plots were previously interrogated using metabarcoding, to answer different questions

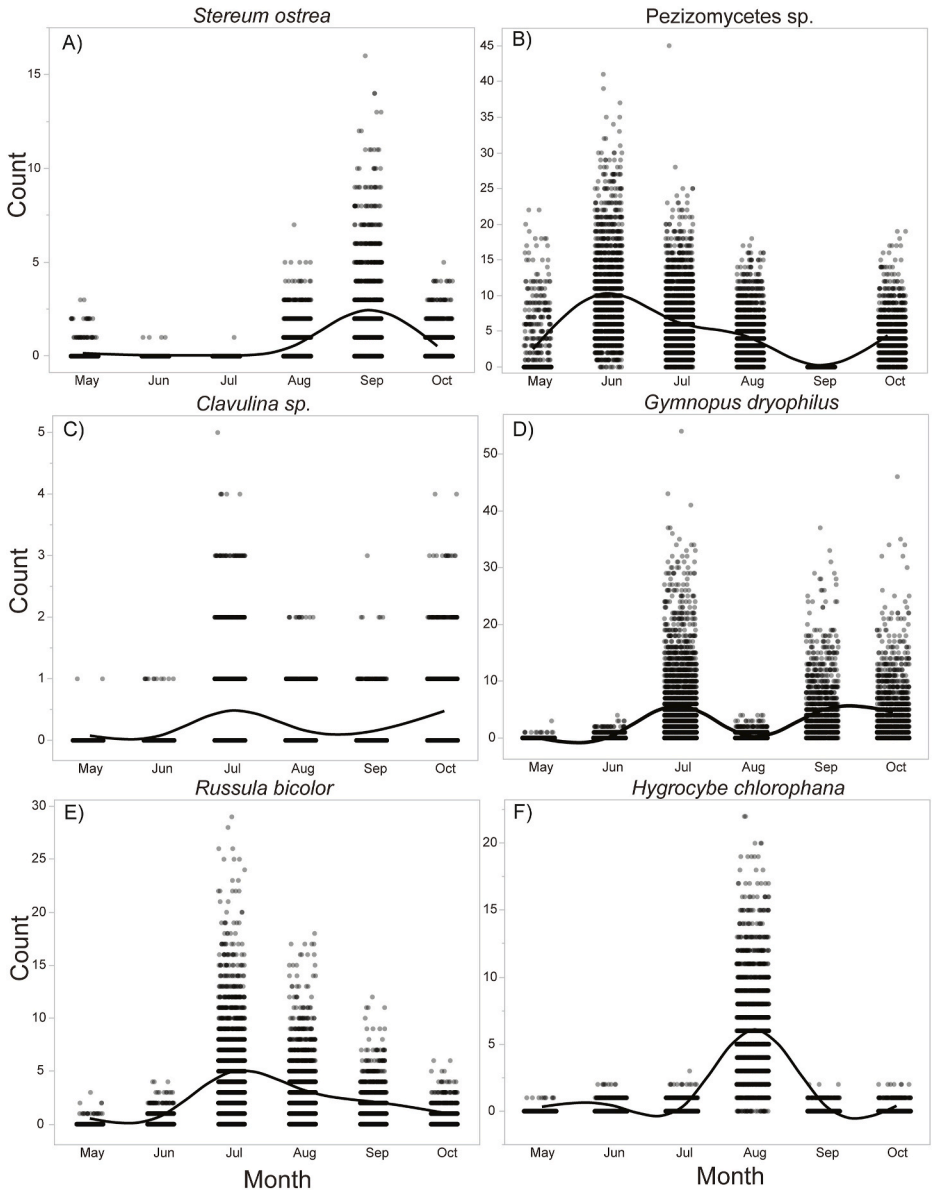

Fig. 4. Simulated fungal sporocarp counts (10,000 simulations) across months and best fit kernel smoothed line of average expected values per plot for (A) Taxon 2, (B) Taxon 4, (C) Taxon 46, (D) Taxon 47, (E) Taxon 71, and (F) Taxon 117 that were significant different using zero-inflated negative binomial regressions (Table 2).

(Veach et al., 2018). It is important to note that samples from Veach et al. (2018) and ours were not collected concurrently. Samples from Veach et al. (2018) were collected in 2013, while our samples were collected between 2014 and 2017). We nevertheless can examine taxa presence congruency as we demonstrated here that interannual community variation is low (total community R^2 across years was 0.014 in our PerMANOVA tests; Table 1). Overall, of the 79 named macrofungal genera of the current study, 62% (49 genera) were also found by Veach et al. (2018) and includes the abundant genera *Clavulina*, *Geoglossum*, *Gymnopus*, *Hydnum*, *Hygrocybe*, and *Inocybe* among others. Further there were numerous genera observed by Veach et al. (2018) but were not observed here. Most of these are not surprising and include microfungi or yeast (or yeast-like) taxa including *Candida*, *Cryptococcus*, *Exophiala*, and *Malassezia*, but also does also include macrofungi that we never observed sporulating during our four-year collection (e.g., *Gymnopilus*). Most interesting are the 30 genera that we observed that were absent from the Veach et al. (2018) dataset and include the commonly found genera *Cantharellus*, *Laccaria*, and *Marasmius*, among others. The absence of common observations from metabarcoding data, which may be the result of limited soil volumes collected per plot, bring up two main points: (1) despite our best efforts, there are still documented primer biases that can occlude true fungal diversity patterns from environmental samples (Mbareche et al., 2021), and (2) to better understand ecological drivers of community dynamics, it is wise to include both metabarcoding and observational data as each will illuminate fungal diversity, but perhaps in different ways. This present work reaffirms that the utility of sporocarp observation data remains strong in an ever molecularly driven analytical world.

In this work, we observed numerous species that occurred across years, but also many whose occurrence patterns differed across years. Using a Kruskal-Wallis framework, we identified 30 taxa whose occurrences significantly differed across years (Table S3). Naturally, several of these taxa were rarities that were only observed once or a few times in a single year (e.g., Taxon 98 *Hebeloma* sp., Taxon 95 *Inocybe* sp., and Taxon 87 *Amanita solaniolens*). Several of these were also highly abundant but had inconsistent interannual fruiting patterns. These include Taxon 167 (*Marasmius capillaris* - 5.6% of all observations), Taxon 4 (morphologically identified as *Galiella rufa* but this could not be

molecularly confirmed so here identified as Pezizomycetes sp. - 5.6% of all observations), Taxon 46 (*Clavulina* sp. [morphologically identified as *C. cristata*] – 3.6% or all observations), Taxon 126 (*Lactarius subdulcis* – 2.1% of all observations), and Taxon 55 (*Leotia lubrica* – 1.8% of all observations). When these data are further interrogated some interesting patterns emerge. A very common taxon, *Marasmius capillaris* is only found during the 2015 sampling year and was conspicuously absent in 2014, 2016, and 2017, and *Lactarius subdulcis, Leotia lubrica, Clavulina* sp. (likely *C. cristata*), Pezizomycetes sp. (likely *Galiella rufa*) exhibit differential abundances across 2014–2016, *Lactarius subdulcis* is most abundant in 2016, *Leotia lubrica* was most abundant in 2014, *Clavulina* sp. had increased in abundance from 2014 to 2016, and Pezizomycetes sp. is most abundance in 2014, but were all absent in 2017. *Marasmius capillaris* is a saprobe often found associated with *Quercus* debris and is generally considered a common sporocarp (Desjardin, 1989), so it is surprising that it was only observed in 2015. The lack of some of these common taxa in 2017 is also an interesting discussion point. While determining why these taxa were found in 2014–2016, but not observed in 2017 is beyond the scope of this work, we can provide some potential reasons. Both sampling locations (GRSM and CWT) were under severe drought conditions from about July 2016–April 2017 (www.drought.gov). This precipitation deficit likely contributed to decreased taxa occurrence, but not all taxa were affected. Additional work needs to be undertaken as to drought responses of these taxa. Further, this drought induced vegetation mortality was a major contributor to one of the worst wildland fire seasons in recent Southern Appalachian Mountain history (winter of 2016–2017; Brown et al., 2019), and while none of our plots were directly burned prior to the 2017 sampling, there may have been significant ashfall which is known to modulate soil chemistry affecting pH and cation exchange capacity (Molina et al., 2007), among others, and might impact fungal and general microbial responses (Noyce et al., 2016).

Here we demonstrate broad-scale effects of location, season, and years on fungal sporocarp communities and, expand our knowledge of sporocarp occurrence patterns in Southern Appalachia, but these patterns are more striking when accounting for fungal guilds. For mycorrhizal and saprobic communities, there are distinct temporal disconnects between occurrence patterns and we document interseasonal differences in community variability. Taken together, this work expands our understanding of interannual, interseasonal, and spatial dynamics of fungal sporocarps and demonstrates the utility of long-term datasets to elucidate community dynamics that may be occluded if only interrogated across a single season or sampling event.

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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.2021.101125.

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