Experimental warming alters potential function of the fungal community in boreal forest

RUNNING HEAD: EXPERIMENTAL WARMING AND FUNGI

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Abstract

Fungal community composition often shifts in response to warmer temperatures, which might influence decomposition of recalcitrant carbon (C). We hypothesized that evolutionary trade-offs would enable recalcitrant C-using taxa to respond more positively to warming than would labile C-using taxa. Accordingly, we performed a warming experiment in an Alaskan boreal forest, and examined changes in prevalence of fungal taxa. In a complementary field trial, we characterized the ability of fungal taxa to use labile C (glucose), intermediate C (hemicellulose or cellulose), or recalcitrant C (lignin). We also assigned taxa to functional groups (e.g., free-living filamentous fungi, ectomycorrhizal fungi, and yeasts) based on taxonomic identity. We found that response to warming varied most among taxa at the order level, compared to other taxonomic ranks. Among orders, ability to use lignin was significantly related to increases in prevalence in response to warming. However the relationship was weak, given that lignin use explained only 9% of the variability in warming responses. Functional groups also differed in warming responses. Specifically, free-living filamentous fungi and ectomycorrhizal fungi responded positively to warming, on average, but yeasts responded negatively. Overall, warming-induced shifts in fungal communities might be accompanied by an increased ability to break down recalcitrant C. This change in potential function may reduce soil C storage under global warming.

Introduction

Global warming is an unprecedented ecological, economic, and global health issue, and it is especially pronounced at high latitudes (IPCC, 2014). Moreover, high latitudes contain large stores of soil organic C—as much as 1024 Pg C (Schuur *et al.*, 2008). The majority of this C is

long-lived in the soil (i.e., recalcitrant), with residence times of a decade or more (Jones *et al.*, 2005). Variation in turnover rates of recalcitrant C compounds can disproportionately influence long-term C storage in soils (Davidson & Janssens, 2006, Parton *et al.*, 1988). If warming increases decomposition rates of recalcitrant C, then the CO_2 released could form a positive feedback on climate change.

Microbes perform decomposition as they break down organic material for energy, C, and nutrients. In particular, fungi are responsible for most of the breakdown of organic C compounds in high latitudes, especially those in more recalcitrant forms (Dighton, 2003, Ponge, 2003, van der Heijden *et al.*, 2008). Previous field studies have reported shifts in fungal community composition under experimental warming (Allison *et al.*, 2010a, Allison & Treseder, 2008, Clemmensen *et al.*, 2006, Deslippe *et al.*, 2011, Deslippe *et al.*, 2012, Penton *et al.*, 2013, Xiong *et al.*, 2014). Fungal responses to increases in soil temperature could have important consequences for global climate. Nevertheless, it is challenging to determine how changes in fungal community composition in response to warming might influence decomposition (McGuire & Treseder, 2010).

Theory based on thermodynamic principles suggests that microbial enzyme activity and respiration should increase rapidly in response to rising temperatures. As a result, emissions of CO_2 to the atmosphere could increase, forming a positive feedback on global warming (Davidson & Janssens, 2006). In fact, increases in soil respiration are common in warming experiments (Rustad *et al.*, 2001). In addition, the Arrhenius function predicts that the breakdown of recalcitrant C compounds should be more sensitive to temperature than should labile C compounds (Davidson & Janssens, 2006). Nevertheless, few field studies have focused on recalcitrant C turnover in response to warming (Conant *et al.*, 2011).

Evolutionary trade-offs, in which one physiological or ecological trait is gained at the cost of another (Darwin, 1859, Stearns, 1989), may constrain the extent to which feedbacks between ecosystems and climate occur. Here, we focus on trade-offs in soil fungi between two ecological traits: (1) the degree to which they proliferate in response to warmer temperatures and (2) their ability to use recalcitrant C compounds. In this case, use of recalcitrant C could provide an advantage to fungi at warmer temperatures, because recalcitrant C is relatively more accessible to enzymatic activity at higher temperatures (Davidson & Janssens, 2006, Waldrop & Firestone, 2004). However, there is a cost to using recalcitrant C compounds—carbon and N is required to construct enzymes for catalysis and uptake of organic compounds, especially large, complex recalcitrant compounds (Allison *et al.*, 2010b, Frankena *et al.*, 1988, Schimel & Bennett, 2004). At colder temperatures, fungal taxa that invest in the requisite enzymes could grow more slowly than those that do not, owing to this cost (Fig. 1). Thus, we hypothesize that fungi that invest in the use of recalcitrant C compounds will receive a better return on these investments when temperatures are warmer, since catabolism of recalcitrant molecules should be relatively more sensitive to temperature (Hypothesis 1). If so, we expect that recalcitrant C users will proliferate under warmer temperatures, potentially leading to greater mobilization of recalcitrant C to the atmosphere.

We also expected functional groups such as ectomycorrhizal fungi, free-living filamentous fungi, and yeasts to vary in their sensitivities to warming. Previous studies have reported that various ectomycorrhizal fungi become more abundant following experimental warming, especially species from the genus *Russula*, *Lactarius* and *Cortinarius* (Allison & Treseder, 2008, Clemmensen *et al.*, 2006, Deslippe *et al.*, 2011, Penton *et al.*, 2013). With respect to other functional groups, free-living filamentous fungi generally possess less genetic capacity for stress

tolerance than do yeasts (Treseder & Lennon, 2015). In fact, yeasts are often frequent in cold and dry environments (Buzzini *et al.*, 2012, Gunde-Cimerman *et al.*, 2003). Accordingly, we hypothesized that warmer temperatures in an otherwise cold boreal forest should shift the community toward free-living filamentous fungi, and away from yeasts (Hypothesis 2). In addition, we predicted that ectomycorrhizal fungi would become more abundant, based on the results from previous studies.

We addressed these questions by combining two approaches: one measuring responses of fungal taxa to warming in a field experiment, and another characterizing the degree to which fungal taxa target labile, intermediate, and recalcitrant C in a field trial adjacent to the warming experiment. Based on Hypothesis 1, we predicted that fungal taxa that use recalcitrant C will be more likely to increase in prevalence under warming than will taxa that use labile or intermediate C. In other words, we expect a positive correlation between indices of warming response and recalcitrant C use across fungal taxa, but weaker (or non-significant) correlations between warming response and labile C use or intermediate C use. For Hypothesis 2, we predicted that taxa representing free-living filamentous fungi and ectomycorrhizal fungi will increase in prevalence in response to warming, but yeasts will decrease.

Materials and Methods

Field site

We tested our predictions in a field experiment established in a boreal forest near Delta Junction, Alaska (63°55'N, 145°44'W). At this site, plant communities were dominated by an overstory of *Picea mariana* (black spruce) and *Populus tremuloides* (quaking aspen) with an understory of grasses, lichens and mosses. Soils were inceptisols, and the local climate was cold

and dry with a mean annual precipitation rate of 303 mm yr^{-1} and a mean annual temperature of –2°C. The plant growing season lasts between May and September.

Warming experiment

Five pairs of 2.5m x 2.5m plots were established in a 1 km^2 area of forest, as reported in Allison and Treseder (2008). One plot from each pair was assigned to the warming treatment while the other served as a control. Soils were warmed passively with closed-top chambers (greenhouses) covered with 6 mm thick greenhouse plastic (Krizek *et al.*, 2005). Gutters and tubing were installed to direct precipitation inside the greenhouses. The warming treatment had been applied during each growing season since 2005; greenhouses were left in place between growing seasons, but the top plastic panel was removed to allow snowfall to reach the soil. The warming treatment increased air temperatures by 1.6 °C and soil temperatures (5 cm depth) by 0.5 ºC, compared to controls (Allison & Treseder, 2008). Soil moisture was reduced 22% in the warming treatment, owing to increased evaporation.

By spring 2013, the warming experiment had been ongoing for eight years. At this time, we used litterbags to bait the active fungi in each control and warmed plot. On May 22, 2013, we collected senescent needles from the canopy of living black spruce trees near the experimental plots. We immediately filled each litterbag with 2 g of senescent needles. The next day, we placed two litterbags on the forest floor in each plot. Both bags from each plot were collected on July 6, 2013; their contents were composited within each plot. Altogether, there were 5 samples (one from each plot) for each treatment (control and warming), for 10 samples total. The samples were stored at –80 °C for DNA sequencing.

Substrate enrichment

In a separate trial, we characterized carbon use of fungi by performing a substrate enrichment trial *in situ* (following McGuire *et al.*, 2010). We added a selection of organic C compounds directly to the soil surface. Fungi that consistently became more prevalent in response to a given compound were likely to use that compound for growth or activity (McGuire *et al.*, 2010). We selected lignin to represent recalcitrant C, cellulose and hemicellulose for intermediate C, and glucose for labile C. In July 2013—the same time we collected the litterbags from the warming experiment—we set up five substrate enrichment plots. Each plot was located between each pair of warming and control plots from the warming experiment.

Lignin, cellulose, xylan (a hemicellulose), and glucose were obtained from Sigma Aldrich (St. Louis, MO). We mixed 200 mg of each organic compound into 10 ml of distilled water, and then immediately sprinkled it onto the surface of the litter layer of each plot in the afternoon of July 4, 2013. An additional set of applications served as controls; they received 10 ml water but no organic C compound. There was one application per organic C compound (or control) per plot. Applications were spaced 10 cm apart, and each application covered a 15 x 15 cm area.

Forty-eight hours after we applied the compounds, we returned to collect the litter layer (dead moss and fragmented litter) from each application site in the afternoon of July 6, 2013. Samples were immediately placed on ice and then transferred to a –80 °C freezer within 18 hours.

DNA sequencing

Needles from warming experiment bags (“warming samples”) and litter samples from carbon enrichment experiment (“enrichment samples”) were thawed at room temperature after removal from the –80°C freezer. All warming and enrichment samples were homogenized by hand. We

extracted DNA with the PowerSoil DNA Isolation Kit (MoBio, catalog #12888) following manufacturer guidelines. In brief, 0.15 g samples were prepared in duplicate (0.3 g total) for cell lysing, DNA binding to spin filter, washing, and eluting DNA. The DNA was assessed with a molecular mass standard on an agarose gel stained with ethidium bromide and imaged using a fluorescent imager. The DNA concentration and quality was also quantified using a NanoDrop. Duplicates were combined, re-assessed, and stored at –20°C until PCR reactions were conducted.

We modified primers targeting the 5.8S encoding gene to amplify the ITS2 region of fungal ribosomal encoding genes. These primers produce a shorter amplicon than do primers targeting the entire ITS region. Thus, these primers reduce species bias and PCR chimeras, but capture a similar level of fungal diversity (Ihrmark *et al.*, 2012). We amplified a ~339 bp region of the fungal ITS2 gene using a forward primer (fITS9; AATGATACGGCGACCACCGAGATCTACAC TCTTTCCCTACA CGACGCTCTTCCGATCT NNNNNGAACGCAGCRAAIIGYGA) plus barcoded, reverse primers with the reverse complement of the 3' Illumina adapter (CAAGCAGAAGACGGCATACGAGAT), a unique 12 base barcode, a pad (AGTCAGTCAG), a linker sequence (CC), and the ITS4 primer (TCCTCCGCTTATTGATATGC). We used a staggered primer design (Tremblay *et al.*, 2015) that included 0, 1, 2 or 3 bases preceding the ITS4 primer (e.g., CC-ITS4, CC-G-ITS4, CC-AG-ITS4, or CC-CAG-ITS4), in order to increase the diversity of amplicon sequences across the Illumina Miseq flowcell early in the read. This approach allows greater accuracy of amplicon cluster detection and resolution, and improves sequence quality in general.

We included the following in each PCR reaction: 21.5 μL Platinum PCR Supermix (Invitrogen, Carlsbad, CA), 0.75 μL of each primer (10 μM), 1 μL BSA (10 mg mL^{-1}), and 1 μL

(10 ng) of DNA. Reactions ran with a hot start at 94 °C for 5 min, 35 cycles of 95 °C for 45 s, 50 °C for 1 min, 72 °C for 90 s, and a final extension step of 72 °C for 10 min. We ran PCR reactions from each sample in triplicate, pooled them, and used Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA) for purification. We quantified purified samples by using the Qubit dsDNA High Sensitivity Assay Kit (Life Technologies, Grand Island, NY), and then pooled samples in equimolar concentrations. The pooled sample was sequenced as 2 x 301 reads with the v3 600 cycle kit on an Illumina MiSeq sequencer at the Genomics core in the Institute for the Integrative Genome Biology at the University of California, Riverside. A spike of 12.5 pM PhiX at 30% was included in the amplicon library to help with cluster diversity visibility for added sample heterogeneity.

Sequences were de-multiplexed and sequence data were processed and analyzed using Quantitative Insights Into Microbial Ecology (QIIME) pipeline (version 1.8.0) (Caporaso *et al.*, 2010). Read 1 and Read 2 paired ends were assembled with SeqPrep (1.1) and ea-utils (r811). We filtered amplicon sequences for quality control by retaining sequences characterized by a minimum Phred score sequence cutoff threshold of 34 and higher. Paired reads were discarded if they contained either less than 75% consecutive high quality reads, or contained more than three consecutive low quality base calls. Sequences were further filtered for chimera detection and removal using USEARCH 6.1 (version 6.1.544), and all global singleton reads were removed. After quality control, our dataset contained 1,128,600 high-quality sequences. Sequences were clustered into operational taxonomic units (OTUs) using UCLUST. Across all samples, we identified 4,912 OTUs. Representative sequences (one per OTU) were aligned with BLAST comparison in the GenBank database. Based on nomenclature classification in the UNITE (version 7) database, a taxonomic assignment was made for each. The analysis used the

'dynamic' release to account for distinct taxonomic assignments between 97–99% nucleotide similarity for particular fungal lineages, assigned manually by experts. Groups of sequences were clustered at 97% nucleotide similarity to be inclusive of taxa at fine levels of resolution for phylogenetic and statistical analyses that otherwise may have been overlooked at lower identity thresholds. All non-fungal OTUs were discarded prior to statistical analysis. To avoid biases due to different library sizes, samples were normalized to 3,928 fungal sequences per sample.

Finally, we categorized OTUs as functional groups based on taxonomy. We used the same assignments as Tedersoo et al. (2014) and Nguyen et al. (2015), although we grouped pathogenic and parasitic OTUs into one category, "pathogens". In addition, we split saprotrophic OTUs into "free-living filamentous fungi" versus "yeasts" based on morphology. Yeasts included facultative and obligate yeasts. The subset of free-living filamentous fungi that were known wood decay fungi were also identified. We were able to classify 48% of OTUs to functional group.

Traits of individual OTUs

We estimated ecological traits of individual fungal taxa. The first ecological trait was "warming response", which we defined as the change in prevalence of the OTU in response to warming (Fig. S1a):

$$Warming\ Response = \left[\left(\frac{W_p}{5}\right) - \left(\frac{C_p}{5}\right)\right] \times 100\%$$

where W_p is the number of warmed plots in which a given OTU is present, and C_p is the number of unwarmed control plots occupied by the same OTU. There were 5 warmed and 5 unwarmed plots in the experiment. Warming response ranged from +100% to –100%. Positive warming responses indicated an increase in prevalence in response to warming; negative values, a

decrease. A separate warming response was calculated for each fungal OTU present in at least one warmed plot or unwarmed control plot. A total of 1,734 OTUs met this criterion, so 1,734 warming responses were recorded (Table S1).

The next set of ecological traits were indices of substrate use, taken from the substrate enrichment trial. Again, substrates were glucose, hemicellulose, cellulose, and lignin. We calculated use of each substrate for individual OTUs (Fig. S1b). For example, lignin use was represented as:

$$Lignin\ Use = \left[\left(\frac{L_p}{5}\right) - \left(\frac{N_p}{5}\right)\right] \times 100\%$$

Where L_p was the number of lignin-enriched plots in which a given OTU was present, and N_p was the number of no-substrate-added plots occupied by that same OTU. Larger positive lignin use for a given OTU indicated that the OTU had become more prevalent following lignin enrichment, likely because it was using lignin (sensu McGuire *et al.*, 2010). Lignin use values were calculated for all OTUs that were present in at least one no-substrate-added plot or lignin-enriched plot (2291 OTUs total, Table S1). We repeated this set of calculations for glucose, hemicellulose, and cellulose, obtaining substrate use values for 1971, 2004, and 2091 OTUs, respectively.

Note that to calculate prevalence of a given OTU, we used presence/absence of that OTU in each plot, rather than number of sequences of that OTU in each plot. We took this approach because the numbers of sequences derived from Illumina sequencing do not necessarily reflect actual abundance of a given OTU, owing to potential primer and PCR biases (Tedersoo *et al.*, 2015).

Statistics

Once we had determined warming response and substrate use for individual OTUs, we could then examine how these traits were phylogenetically distributed, whether they differed among functional groups, and if warming response was linked to substrate use. We performed all statistical tests on traits of individual fungal taxa. In every case, statistical replicates consisted of either fungal OTUs or fungal orders (see Fig. S1 for an example analysis). Note that the experimental plots were used as units for measuring prevalence of OTUs, so we could calculate warming response and substrate use. They were not used as statistical replicates.

Phylogenetic signal of warming response

We examined the phylogenetic signal of the warming response by determining variance in that trait at each taxonomic rank. Variance was calculated using a mixed effects model (lme4 package for R, as detailed in Lennon *et al.*, 2012). Specifically, we calculated the observed variance in warming response at the phylum, subphylum, class, order, family, and genus level. Warming response was the dependent variable. The taxonomic ranks were included as six hierarchical independent variables. The statistical replicates were OTUs. We then generated 10,000 random permutations of observed values among all OTUs, and recalculated the variance explained at each taxonomic level. If the observed level of variance in warming response for a given taxonomic rank was higher than the 95% confidence interval from the randomizations, then there had been more diversification of warming response at that level than expected by chance.

Warming responses of fungal orders

In the current study, variance in warming response was highest (and significant) at the order level (see Results, below). To confirm that orders responded differently to warming, we

conducted an analysis of variance (ANOVA) with warming response as the dependent variable, and order as the independent variable (SPSS, 2009). OTUs were statistical replicates. In addition, to characterize warming responses of each order, we performed a series of one-sample t-tests (SPSS, 2009). For each order, we compared the mean warming response of all OTUs in that order to a mean of 0. Orders with mean warming responses significantly greater than zero became more prevalent with warming; those with responses significantly less than zero became less prevalent. (To describe patterns of warming responses across the fungal phylogeny, we performed a similar series of one-sample t-tests for each taxonomic rank.)

Relationships between warming response and substrate use across orders

Next, we tested whether warming responses were significantly related to use of lignin, cellulose, hemicellulose, or glucose across taxa. Since phylogeny influenced warming responses, we used two analytical approaches to account for phylogenetic structure when examining trait relationships. First, we binned OTUs into orders (sensu Treseder & Lennon, 2015) and performed linear regressions with order as the statistical replicate. Order was chosen because it was the taxonomic rank with the highest variation in warming response. Second, we performed a hierarchical linear mixed model with order as a random effect and OTUs as the statistical replicates ("Functional group, lignin use, and taxonomy", below). Note that we did not perform phylogenetic independent contrasts between warming response and substrate use, because there was too much variation in the ITS region to construct a phylogeny that reliably represented earlier divergences (Schoch *et al.*, 2012). Instead, hierarchical or nested models are an alternate approach, as detailed in Ricklefs and Starck (1996).

To bin traits of OTUs into orders, we calculated the mean warming response of all OTUs within each order. Likewise, we calculated each order's mean lignin use, cellulose use, etc. We

then performed a series of linear regressions with lignin use, cellulose use, hemicellulose use, or glucose use as the independent variable, and warming response as the dependent variable (SPSS, 2009). For this analysis, order was the statistical replicate. Hypothesis 1 would be supported if (1) lignin use and warming response were significantly and positively related to one another across orders, and (2) warming response were not significantly related to cellulose use, hemicellulose use, or glucose use.

Warming responses of functional groups

For Hypothesis 2, we used an ANOVA to test for differences between functional groups in warming response (SPSS, 2009). Functional group was the independent variable, and warming response was the dependent variable. OTUs were statistical replicates. In addition, we used a one-sample t-test on each functional group to determine whether its mean warming response departed significantly from zero. Hypothesis 2 would be supported if functional groups differed significantly in warming responses, with significantly positive responses for ectomycorrhizal fungi and free-living filamentous fungi, and significantly negative responses for yeasts. (To be comprehensive, we also performed a series of one-sample t-tests on substrate use values.)

Functional group, lignin use, and taxonomy

Finally, to simultaneously test for effects of functional group, lignin use, and order on warming response, we applied a hierarchical linear mixed model with functional group and lignin use as fixed effects, order as a random effect, and warming response as the dependent variable (SPSS, 2009). OTUs were the statistical replicates. We expected that effects of functional group and lignin use on warming response should each be significant, independent of order.

Results

Phylogenetic signal of warming response

Warming responses of fungal OTUs were related to their phylogeny— there was significant divergence in warming response at all taxonomic ranks (Fig. 2, mixed effects model, $P < 0.05$). Variation in warming response was greatest at the order level. Indeed, orders differed significantly from one another in warming response (ANOVA, $F_{58,817} = 4.156$, $P < 0.001$).

Warming responses of fungal orders

Altogether, 10 orders responded significantly to warming (Fig. 3 and Table S2, one-sample t-test, $P < 0.05$ for all). For example, the yeast-dominated order Cystofilobasidiales was negatively affected by warming ($t = –5.888$, $df = 12$, $P < 0.001$). The Tremellales, which includes facultative yeasts, also declined significantly ($t = –4.471$, $df = 34$, $P < 0.001$). In contrast, the chytrid order Spizellomycetales became more prevalent under warming ($t = 4.583$, $df = 6$, $P = 0.004$).

Nine orders significantly increased in prevalence in response to lignin additions, which suggested that they were capable of breaking down this compound (Table S3). These orders included the Polyporales and Hymenochaetales.

Relationships between warming response and substrate use across orders

Warming response was significantly positively related to lignin use across orders, although the relationship was weak (Fig. 4, linear regression, $r^2 = 0.089$, $F_{1,48} = 4.662$, $P = 0.036$). In comparison, warming response was not significantly related to cellulose use (Fig. S2, $r^2 = 0.011$, $F_{1,44} = 0.473$, $P = 0.495$), hemicellulose use ($r^2 = 0.003$, $F_{1,47} = 0.151$, $P = 0.700$), or glucose use ($r^2 = 0.021$, $F_{1,47} = 0.996$, $P = 0.323$). These results supported Hypothesis 1.

Warming responses of functional groups

Hypothesis 2 was also supported. Functional groups varied significantly in warming response (Fig. 5, ANOVA, $F_{5,528} = 9.669$, $P < 0.001$). In particular, free-living filamentous fungi increased significantly in response to warming (one-sample t-test, $t = 1.983$, $df = 152$, $P = 0.025$), as did ectomycorrhizal fungi ($t = 3.857$, $df = 9$, $P = 0.002$). In contrast, yeasts declined significantly ($t = –0.5627$, $df = 95$, $P < 0.001$). The endophyte ($t = 1.500$, $df = 4$, $P = 0.104$), pathogen ($t = –0.838$, $df = 184$, $P = 0.798$), and lichen-forming ($t = –0.210$, $df = 84$, $P = 0.583$) groups did not respond significantly to warming.

Functional groups also displayed different substrate use profiles (Fig. S3). For example, free-living filamentous fungi responded significantly positively to lignin enrichment (one-sample t-test, $t = 1.852$, $df = 292$, $P = 0.033$). In contrast, yeasts used glucose ($t = 2.029$, $df = 50$, $P = 0.024$) and hemicellulose ($t = 3.030$, $df = 57$, $P = 0.002$). Lichen-forming fungi targeted glucose ($t = 4.498$, $df = 234$, $P < 0.001$), hemicellulose ($t = 2.329$, $df = 238$, $P = 0.010$), and cellulose ($t = 2.662$, $df = 221$, $P = 0.004$).

Functional group, lignin use, and taxonomy

Finally, functional group, lignin use, and order collectively influenced warming response, as indicated by a hierarchical linear mixed model with functional group and lignin use as independent variables, and order as a co-variate. Specifically, effects of functional group ($F_{5,72} = 2.377$, $P = 0.047$) and lignin use ($F_{1,72} = 5.292$, $P = 0.024$) were significant.

Discussion

We found support for the hypothesis that evolutionary trade-offs should favor recalcitrant C users as temperatures increase (Fig. 1). Fungal orders varied in their responses to warming (Figs.

2 & 3), and those that responded most positively to warming were more likely to use lignin (Fig. 4). In contrast, use of labile C (glucose) or intermediate C (hemicellulose and cellulose) were not significantly related to warming response across fungal orders (Fig. S2), which was also consistent with the hypothesized trade-off. Thus, warming-induced changes in prevalence of fungal orders may be accompanied by an increase in recalcitrant C users. In turn, breakdown of recalcitrant C may be augmented.

A number of studies have observed disproportional increases in recalcitrant C turnover as temperatures rise, compared to more labile C (reviewed in Conant *et al.*, 2011). For example, when tropical soils were incubated in a range of temperatures in the laboratory, respiration of older C was more sensitive to temperature than was younger C (Waldrop & Firestone, 2004). Nevertheless, the majority of these studies were performed in the laboratory, which might constrain recalcitrant C dynamics (Conant *et al.*, 2011). Field warming experiments that focus on recalcitrant C cycling are rarer; some infer that recalcitrant C is more temperature-sensitive than labile C (Conant *et al.*, 2008), but not always (Melillo *et al.*, 2002). Our results suggest that *in situ* shifts in fungal taxa may contribute to increases in recalcitrant C turnover in boreal forest.

Previously, McGuire et al. (2010) documented a positive relationship between ability to target recalcitrant C and proliferation under warming in our field site. However, their analysis was limited to five fungal OTUs, which constrained their ability to interpret the relationship. By contrast, in the current study, we compared these traits across 50 orders representing 849 OTUs. Essentially, advances in high-throughput sequencing allowed us to more extensively characterize fungal taxa in our site. Our findings are consistent with those of McGuire et al. (2010), and are more readily generalizable to the broader fungal community. Moreover, the current study

extends our understanding of fungal warming response by documenting phylogenetic distributions of this trait and incorporating functional groups.

Functional groups of fungi also varied in their responses to warming (Fig. 5). In general, free-living filamentous fungi and ectomycorrhizal fungi became more prevalent under higher temperatures, but yeasts declined. The yeasts' responses are consistent with their genetic profiles. Specifically, yeasts possess more genes encoding stress tolerance traits than do free-living filamentous fungi (Treseder & Lennon, 2015). Others have noted increases in taxonomic groups dominated by free-living filamentous fungi (Deslippe *et al.*, 2012, Semenova *et al.*, 2015, Xiong *et al.*, 2014) and ectomycorrhizal fungi (Allison & Treseder, 2008, Clemmensen *et al.*, 2006, Deslippe *et al.*, 2011, Penton *et al.*, 2013) in warming manipulations. Warming may have alleviated cold stress for free-living filamentous fungi, allowing them to proliferate. On the other hand, yeasts may not have been limited by temperature under ambient conditions.

These changes in functional groups might also alter C cycling. In general, free-living filamentous fungi tend to possess a relatively large complement of genes for recalcitrant C breakdown (Treseder & Lennon, 2015). Moreover, the free-living filamentous fungi responded positively to lignin enrichment in the current study (Fig. S3), which also suggests they can use recalcitrant C. An increase in this functional group could augment recalcitrant C breakdown. In contrast, yeasts tend not to possess as many recalcitrant C-targeting genes (Treseder & Lennon, 2015). Instead, they targeted labile C (glucose) and intermediate C (hemicellulose) in the substrate enrichment trial (Fig. S3). Thus, the yeasts' decline may not directly influence recalcitrant C breakdown. Overall, the shift from free-living filamentous fungi to yeasts may contribute to faster recalcitrant C turnover in response to warming.

Warming responses varied most among fungal orders (Fig. 2). For example, the Cystofilobasidiales, an order of Basidiomycetous yeasts, significantly decreased with warming (Fig. 3). With respect to the other orders, the increases in the Pezizales, Hypocreales, Eurotiales, and Sordariales in the current study (Fig. 3) have likewise been documented in other warming experiments (Deslippe et al., 2012, Semenova et al., 2015, Xiong et al., 2014). Variance in warming response was also significant—but less marked—at the other taxonomic ranks.

Regardless of the statistical approach, lignin use accounted for only a portion of warming responses across taxa. For example, in the linear regression on fungal orders, about 9% of the variation in warming response was explained by lignin use (Fig. 4). Functional group identity may have contributed to some of the remaining variation, given that functional group and lignin use were each significantly related to warming response when both were included in the hierarchical mixed model on fungal OTUs. Other fungal traits may also coincide with temperature responses, such as melanin production to reduce damage from extreme heat and cold (Bell & Wheeler, 1986, Butler & Day, 1998), incorporation of β1,3-glucan in cell walls to deter freeze-thaw damage (Bowman & Free, 2006, Latgé, 2007), and production of cold-induced RNA helicases to unwind cold-stabilized RNA (Ellison *et al.*, 2011, Schade *et al.*, 2004). We do not know how these other traits were distributed among the fungal taxa in this study. As our knowledge of the phylogenetic distributions of these and other fungal traits develops, so will opportunities to identify potential causes and effects of shift in fungal communities in response to warming.

Our measurements were conducted over a relatively brief timeframe. Although warming had been manipulated for eight years in the field site, the black spruce needles we used as “bait” to detect active fungi remained in those plots for just six weeks. Thus, we only captured taxa that

were active in the first half of the growing season. Since fungal communities shift seasonally in cold environments (e.g., Schadt *et al.*, 2003, Schmidt *et al.*, 2007), this may represent only a subset of soil fungi in the study site. Nevertheless, fungal growth is highest in May and June in this site (Treseder *et al.*, 2004), so we had timed the litterbag incubation to cover this period. A total of 1374 fungal OTUs colonized the needles during those six weeks (Table S1). For comparison, 1338 fungal OTUs were recovered from litter layer in the no-substrate-addition plots (Table S1), which was primarily composed of dead moss and fragmented litter from previous seasons. The fungal community in the black spruce needles was not noticeably depauperate, despite the short decomposition time.

The substrate addition trial was also short-term—it lasted 48 hours. Previous studies have indicated that longer incubation times allow more cross-feeding among fungi (e.g., DeRito *et al.*, 2005, Hanson *et al.*, 2008, Morris *et al.*, 2002). In other words, fungal taxa might proliferate because they are using products released by the substrate users, not because they are using the substrate themselves. We used a short incubation time to avoid this artifact. Inevitably, we sacrificed the capacity to detect substrate users that responded more slowly. Nevertheless, even lignin can be catabolized in soil within 24 hours by fungi (Hanson *et al.*, 2008). Moreover, the known wood decay OTUs in our samples responded positively to lignin additions within the incubation time (Fig. S1c), as did orders dominated by wood saprotrophs (Table S3). In addition, McGuire et al. (2010) used nucleotide analog labeling to identify arginine, glutamate, lignocellulose, and tannin-protein users in the same study site, and they also documented responses by taxa within 48 hours. In other ecosystems as well, active microbial communities can shift within 2–4 days after substrate enrichment (Goldfarb *et al.*, 2011, Hanson *et al.*, 2008, Murase *et al.*, 2012, Yin *et al.*, 2000, Zumsteg *et al.*, 2013).

In this study site, soils in the warmed plots are 22% drier than the unwarmed controls (Allison & Treseder, 2008). The greenhouses are equipped with gutters that collect rainwater from the roofs and direct it to the soil inside, so water inputs are the same in both treatments. Rather, drier conditions are a consequence of increased evaporation owing to the higher temperatures (Allison & Treseder, 2008). This climate change scenario might become common in boreal forests (Allison & Treseder, 2011, Chapin *et al.*, 2000, Potter *et al.*, 2001). Thus, drying can serve as a proximal mechanism influencing shifts of fungal taxa under warming. Accordingly, the changes in prevalence of fungal OTUs could have been influenced by drying as well as warming.

In conclusion, we examined potential evolutionary trade-offs between ability to use recalcitrant C and proliferation in response to warming by fungal taxa. Specifically, we characterized responses of fungal taxa to warming in a field manipulation in boreal forest. We compared these warming responses to profiles of organic C use that were derived from a complementary substrate enrichment trial in the same ecosystem. The results were consistent with an evolutionary trade-off: warming response was positively (albeit weakly) related to use of lignin, but not to use of labile or intermediate C. In addition, free-living filamentous fungi and ectomycorrhizal fungi became more abundant under warming, while yeasts declined. Moreover, phylogenetic relationships influenced warming responses, with fungal orders exhibiting the most variation in this trait. Overall, recalcitrant C use, functional group, and taxonomy (especially order) were each related to the responses of fungal taxa to warming. Thus, shifts in fungal taxa under global warming could augment the potential for breakdown of recalcitrant C. This response might contribute to a decline in soil C storage in boreal forests at high latitudes.

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Supporting Information captions

Table S1. OTU data for the warming experiment and the substrate enrichment trials.

Table S2. Warming responses of taxonomic groups of fungi, as mean ±1SE (n OTUs) change in % plots present.

Table S3. Taxonomic groups of fungi that displayed significantly positive responses to lignin, as mean ±1SE (n OTUs) of change in % plots present.

Table S4. Warming responses and substrate use profiles of fungal orders, as mean change in % plots present with warming or substrate addition.

Figure S1. Sample calculations of warming response and lignin use for individual OTUs, and analysis of lignin use by wood decay fungi. **(A)** OTU0054 was identified as a *Friedmanniomyces* species and assigned to the "free-living filamentous fungi" functional group (Table S1). It was detected in two (40%) of the unwarmed plots and five (100%) of the warmed plots. Its warming response was calculated as the change in percentage of plots occupied in response to warming (+60%). **(B)** OTU4578 was classified as a species of *Trechispora*, which are known wood saprotrophs (Nguyen *et al.*, 2015). Its lignin use was calculated as +40%, which was the percentage of lignin enriched plots (40%) minus no-substrate-added plots (0%) occupied by the OTU. **(C)** We obtained lignin use values for eight additional OTUs that matched known wood decay fungi (Table S1). Their lignin use ranged from +20% to +40%. The mean lignin

use of these nine OTUs was significantly greater than zero ($t = 8.315$, $df = 8$, $P < 0.001$), suggesting that this functional group can break down lignin.

Figure S2. Relationships between warming response and substrate use across fungal taxa. Each symbol represents a fungal order. Positive values indicate an increase in prevalence in response to warming or substrate addition; negative values, a decrease. For each relationship, the analysis was restricted to fungal orders that were detected in both the warming experiment and that substrate's enrichment trial. Thus, $n = 46$ orders for cellulose use, $n = 49$ orders for glucose use, and $n = 49$ orders for hemicellulose use. None of these relationships were significant ($P > 0.05$). Order-level means for warming responses and substrate use are reported in Table S4.

Figure S3. Substrate use profiles of fungal functional groups. Bars are means ±1SE of the change in prevalence in response to substrate additions for all OTUs within each functional group. Positive values indicate an increase in prevalence in response to substrate additions; negative values, a decrease. Colors represent significant increases (red) or decreases (blue) in prevalence (*$P < 0.05$).

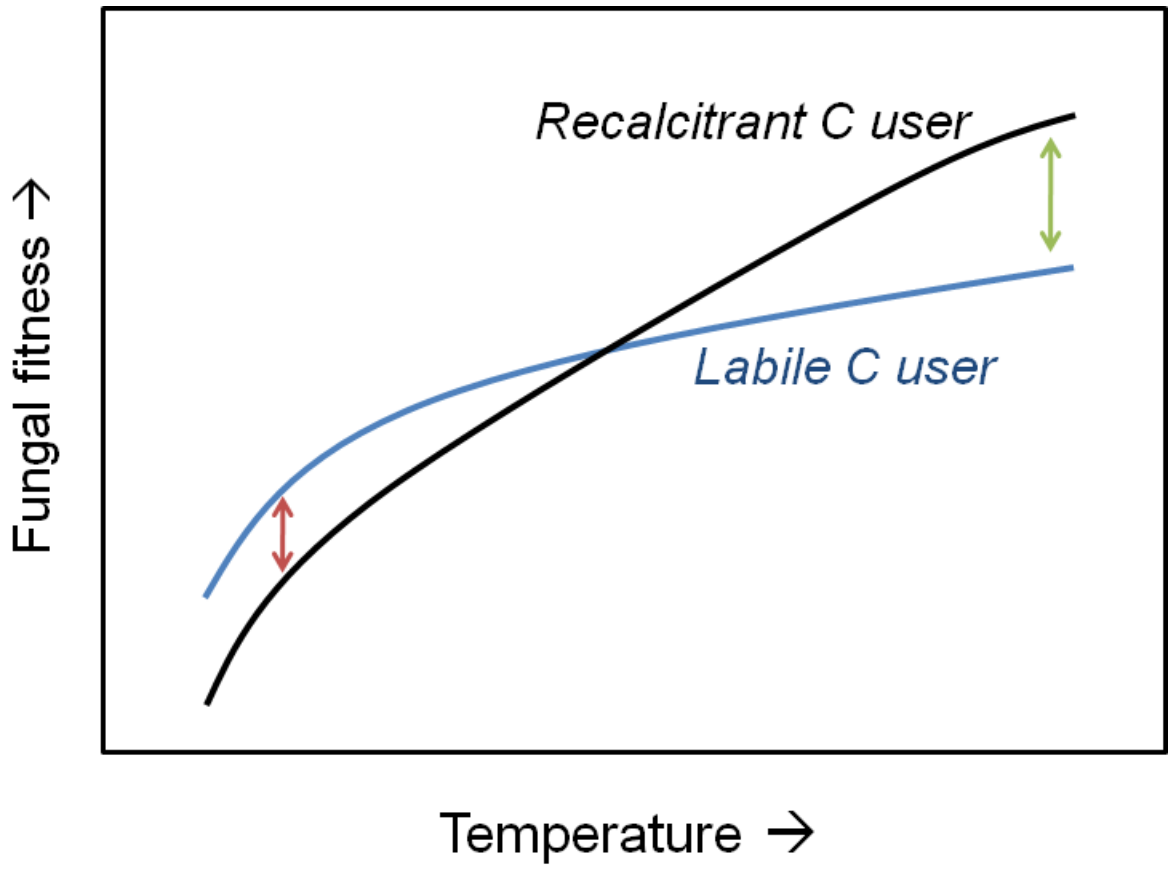

Figure 1. Hypothesized trade-offs between recalcitrant C use and warming responses. Green rightmost arrow: At higher temperatures, recalcitrant C users proliferate faster than do labile C users, because recalcitrant C users can access an additional source of energy. Red leftmost arrow: At lower temperatures, recalcitrant C is less available, and the cost of investing resources in enzymes is disadvantageous for recalcitrant C users.

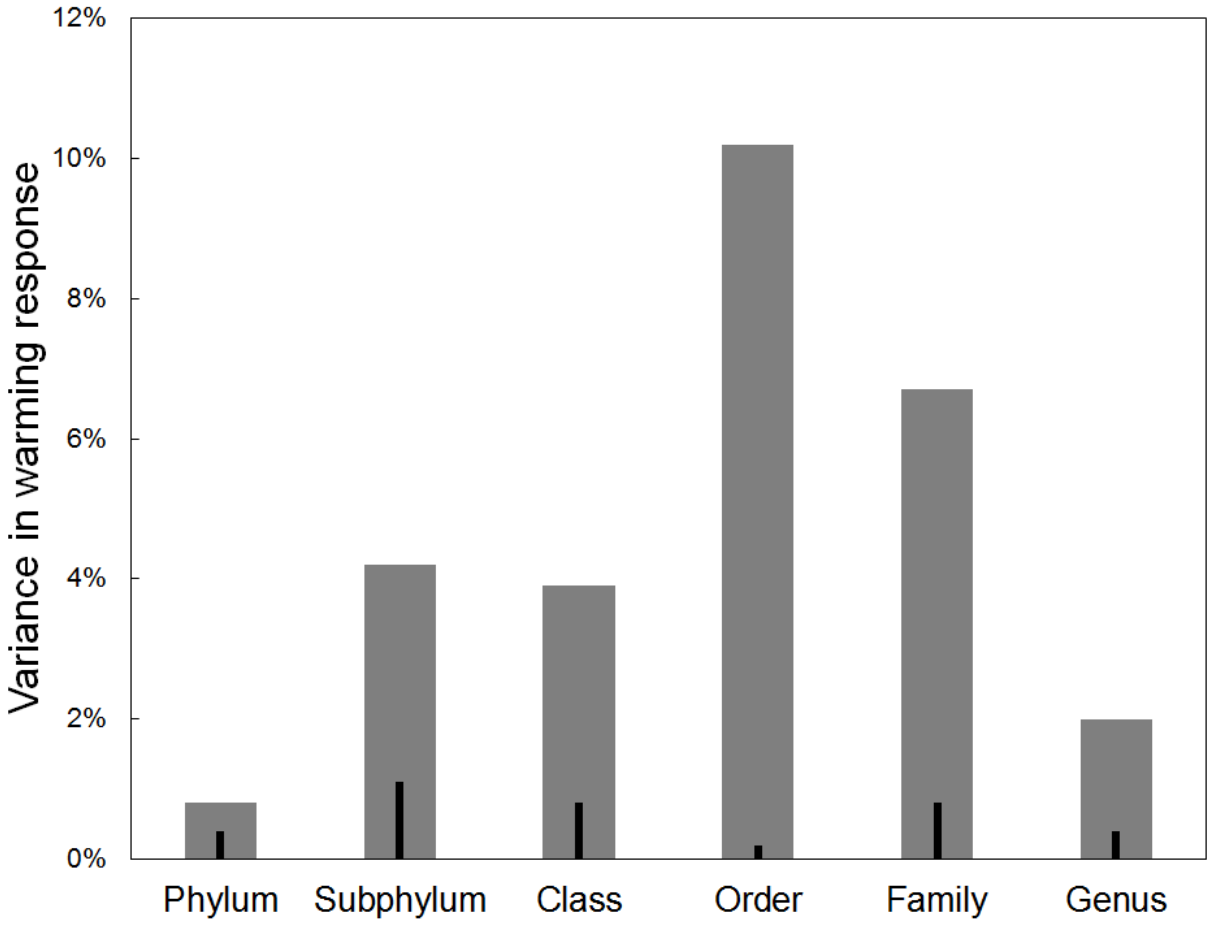

Figure 2. Variance in warming response of fungal OTUs at each taxonomic rank. Warming response for each OTU was calculated as change in percentage of plots occupied by that OTU in the warmed versus control treatments. Grey bars are observed variance. Black bars are 95% confidence intervals of expected variance based on randomization of warming response across OTUs. At all taxonomic ranks, observed variance was greater than expected variance. Thus, variation in warming response was significant at each taxonomic rank ($P < 0.05$).

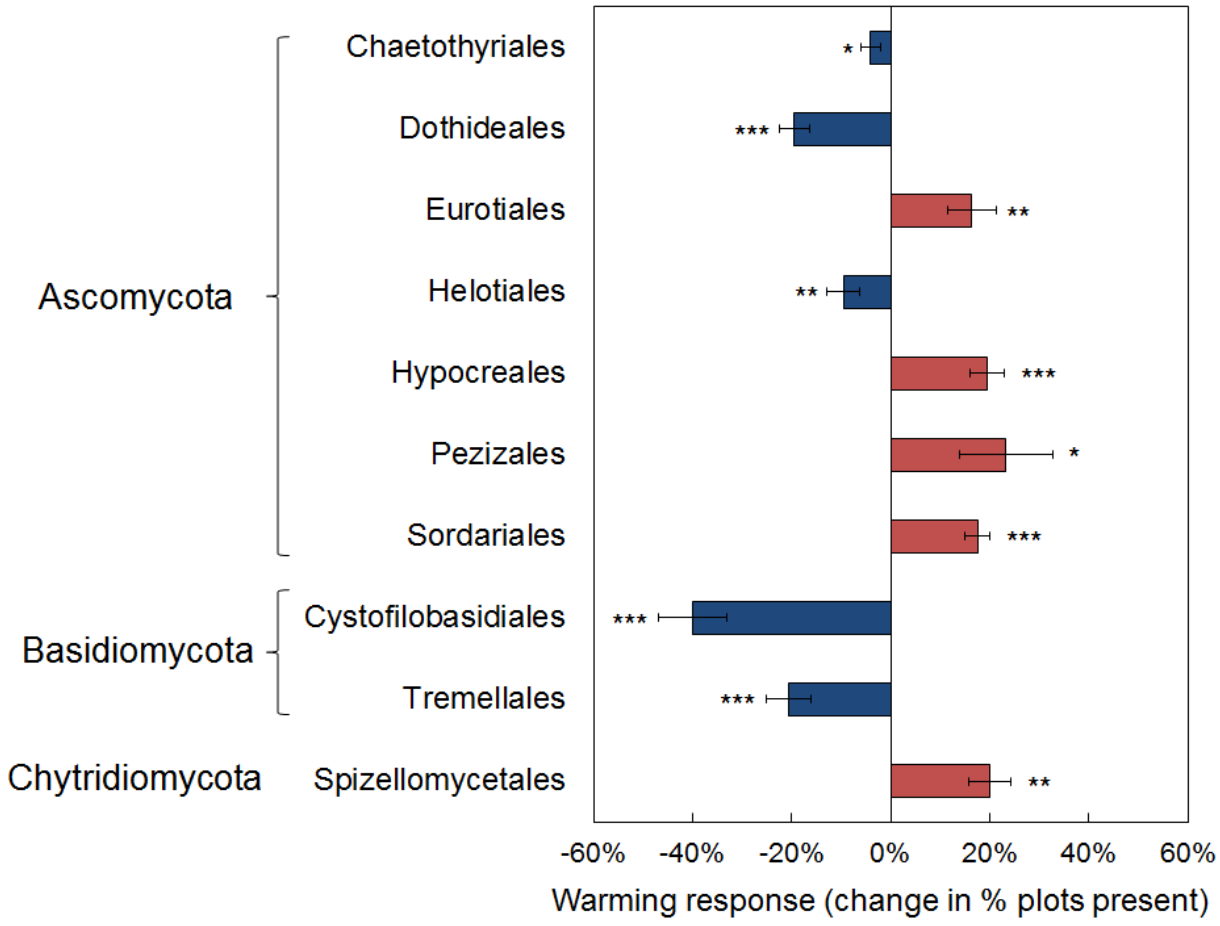

Figure 3. Fungal orders that displayed significant responses to warming. Bars are means ±1SE of 8 (Sordariales) to 141 (Chaetothyriales) OTUs within each order. Positive values indicate an increase in prevalence in response to warming; negative values, a decrease. Red = increased prevalence, blue = decreased prevalence. *P < 0.05, **P < 0.01, ***P < 0.001. Changes in prevalence for all fungal orders (and other taxonomic ranks) in the study are reported in Table S2.

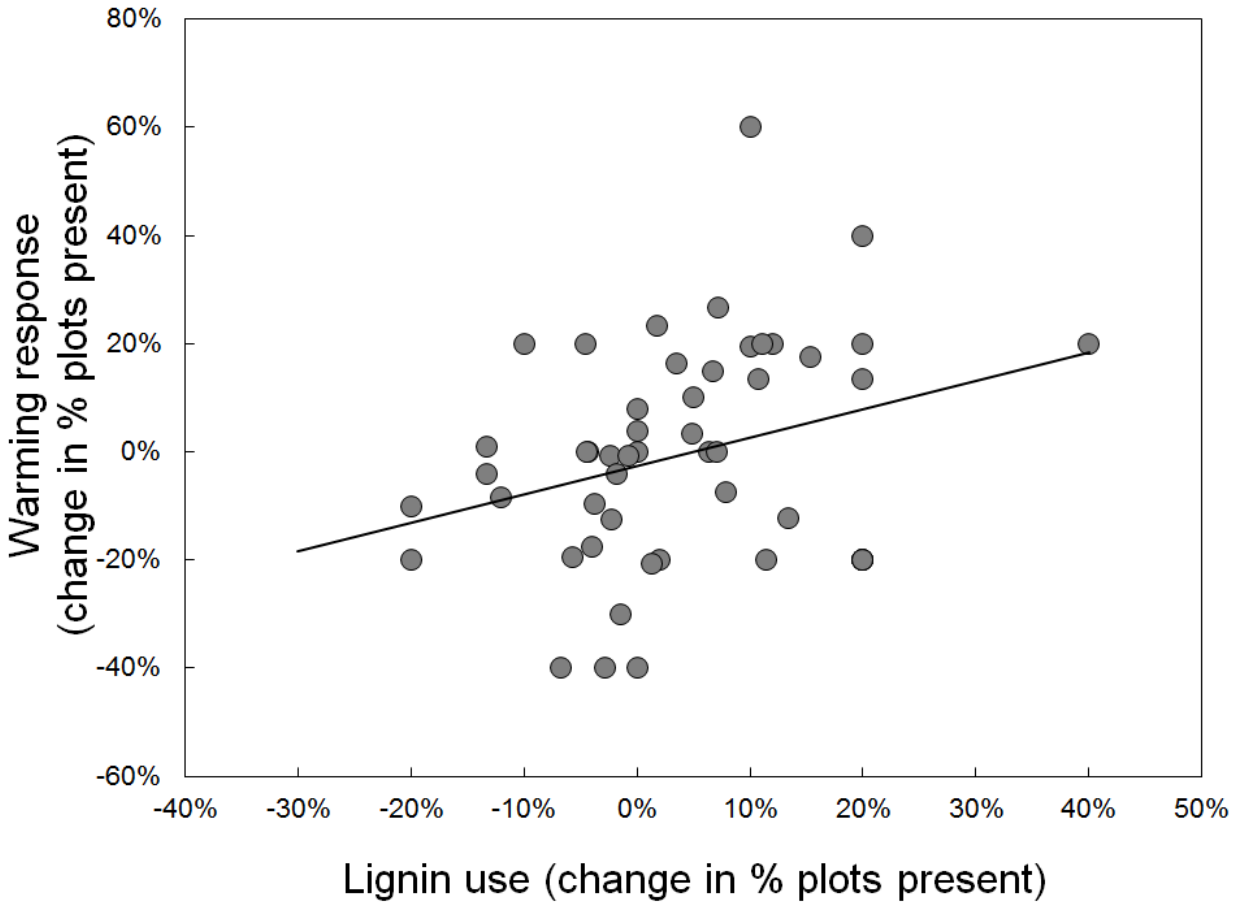

Figure 4. Relationship between lignin use and warming response across fungal orders. Each symbol represents a fungal order. Positive values indicate an increase in prevalence in response to warming or substrate addition; negative values, a decrease. Fifty orders were detected in the warming experiment as well as the lignin enrichment trial. These orders represented a total of 849 OTUs. Lignin use and warming response were significantly related (linear regression, n = 50, $r^2 = 0.089$, P = 0.036). Line is best fit. Order-level means for warming responses and substrate use are reported in Table S4.

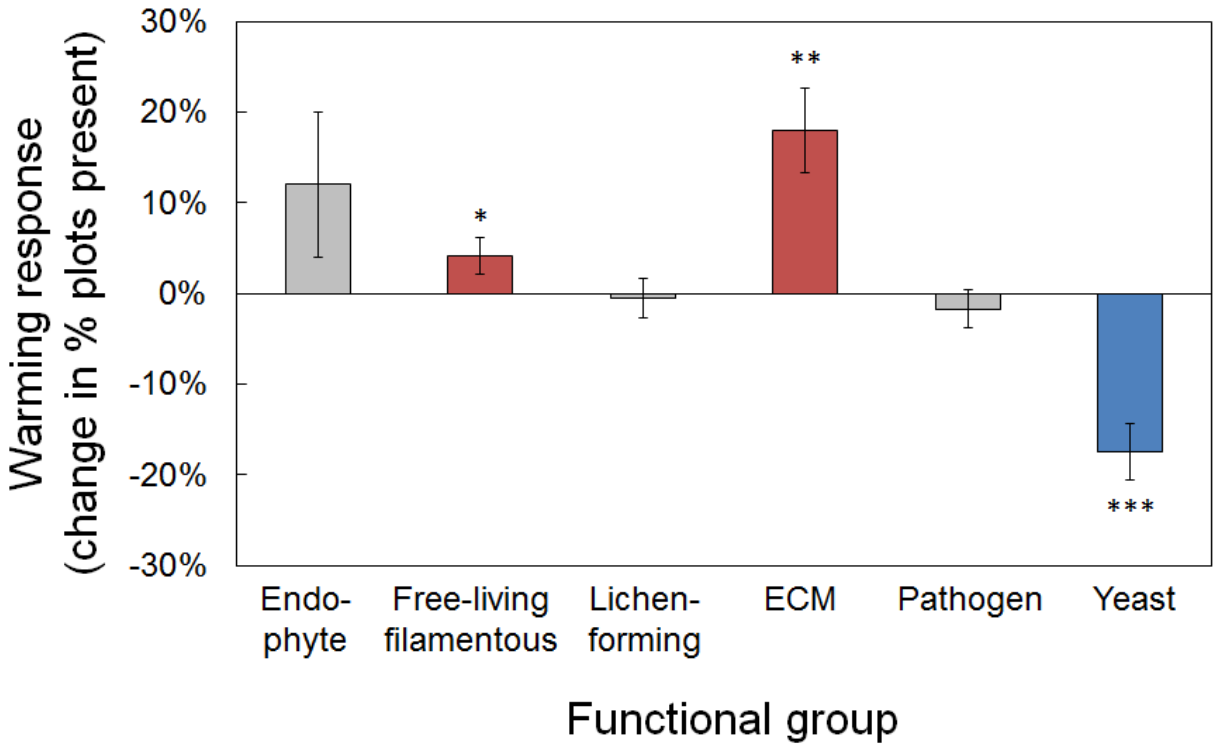

Figure 5. Warming responses of fungal functional groups. Bars are means ±1SE of the change in prevalence in response to warming for 3 (endophyte) to 185 (pathogen) OTUs within each functional group. Positive values indicate an increase in prevalence in response to warming; negative values, a decrease (*P < 0.05, **P < 0.01, ***P < 0.001). Colors represent significant increases (red) or decreases (blue) in prevalence. ECM = ectomycorrhizal.