

Understanding how leaf endophytes are affected by climate change: Examining fungi in grass species with warming

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Abstract

Climate change is impacting ecological systems around the globe with much of current research characterizing direct plant and animal responses. However, there is a gap in our knowledge regarding the direct response of fungal symbionts to climate change. Horizontally transmitted leaf endophytes (type II and III) exist within all plant tissue surveyed to date and potentially play a fundamental role in altering plant response to global change in their ability to alter plant physiology and growth in response to stress. Potential benefits observed in endophyte-positive plants include increased resistance to herbivores, increased competitive ability, increased plant vigor, greater drought-tolerance and changes in nutrient cycling and content of plants (Clay & Rudgers 2005, Rudgers et al. 2009, Kivlin et al. 2013).

Here, we examined three grass species from controlled (ambient temperature) or warming plots (2°C increase in temperature) from Gothic, Colorado and assessed their fungal endophytes. We measured physiological responses such as growth rate and *alpha* and *beta* diversity via changes in species richness and composition in response to temperature increases mimicking climate change. We hypothesized that increased temperature would increase endophytic growth rates, decrease diversity and change composition of the endophyte communities. However, our analysis showed that there was no significant relationship between temperature variation and the three dependent variables in culturable fungal endophytes. Therefore, plants may buffer their endophytes against moderate increases in temperature expected with climate change. Analyses also found that species played a integral role in fungal response to increased temperatures.

Introduction

Climate change is occurring at an unprecedented rate with a projected 2–3° C increase in mean global surface temperatures and shifts in precipitation location and frequency occurring in the 21st century (Stott et al. 2010, Kivlin et al. 2013, IPCC 2014). These changing climatic patterns alter responses of plants and other biota through altitudinal shifts in plant community distribution and change in composition of ecological communities. Plants have ubiquitously been shown to associate with and rely on soil microbe symbionts including mycorrhizal fungi, nitrogen fixing bacteria and fungal endophytes. These microbes are increasingly being recognized for their potential to influence host populations and mitigate abiotic stressors associated with climate change (Cahill et al. 2008, Rudgers et al. 2009, Ranelli et al. 2015). Horizontally transmitted leaf endophytes, a specific type of fungal symbiont existing within the plant tissue, have been found in all plant species surveyed to date and have many documented benefits. These benefits include increased resistance to herbivores, increased competitive ability, increased plant vigor, greater drought-tolerance and changes in nutrient cycling and content of plants (Clay & Rudgers 2005, Rudgers et al. 2009, Kivlin et al. 2013). The relationship between

climate change and leaf endophytes is being explored as observations suggest that endophyte distribution is influenced by climate (Kivlin et al. 2013). Understanding how these organisms will respond to change is crucial as they have the potential to influence feedback loops, dictate how plants thrive in changing climates and are helpful in predicting the ecological dynamics of changing communities (Rudgers et al. 2009).

We currently understand the direct responses of plants and animals to climate change, but lack an understanding of how horizontally transmitted leaf endophytes will respond. One such work cataloguing direct plant physiological response to global change found that N additions mimicking atmospheric deposition increased plant biomass across ecosystems (LeBauer & Treseder 2008). The gap in our knowledge is highlighted by the lack of literature relating direct responses of horizontally transmitted leaf endophytes to global change. Giauque and Hawkes (2013) attempted to examine this relationship, however, the piece dealt entirely with endophytes and their response along a precipitation gradient. In 2013, Kivlin et al. sought to understand the indirect plant-fungal symbiont responses to global change via altered species interactions by performing a meta-analysis of 434 studies. The focus of the research was class 1 leaf endophytes, arbuscular mycorrhizal fungi, ectomycorrhizal fungi and dark septate endophytes. While the study did broaden the scientific community's understanding of plant-fungal symbiosis, it also iterated the need to examine the response of type II and III leaf endophytes.

Here, we explored factors associated with climate change, such as warming, that may directly influence the dependent variables of physiology, diversity and composition of endophytic fungi in grasses. We chose grasses as we estimate that there are 10,000 species of grasses in existence which cover roughly 20% of all land area (Shantz 1954, Barker et al. 2001). By constraining hosts to this single host clade, we reduced confounding effects of hosts' ecological roles (Kivlin et al. 2013). Grasses dominate montane and alpine meadows and, unlike trees, span the full altitudinal range (Shaw 2008). This ability to account for altitudinal variation in alpine areas allowed us to survey diverse communities across which many fungi are able to disperse. We included natural sharp gradients to allow for the investigation of the relative importance of abiotic and biotic drivers of fungal distributions (Ranelli et al. 2015). The treatment included the cultivation of leaf endophytes from three grasses (*Poa pratensis*, *Festuca thurberi* and *Achnatherum lettermanii*) from warming and controlled plots and subsequent observation of change in the three dependent variables. We hypothesized that increased temperature would increase endophytic growth rates, decrease diversity and change composition of the endophyte communities.

Methods

Site Selection:

We sampled three grasses from the warming meadows at the Rocky Mountain Biological Laboratory (RMBL) for their leaf endophytes in Gothic, Colorado (Figure 1). We chose grasses in the Poaceae family due to their practicable abundance, diversity and distribution

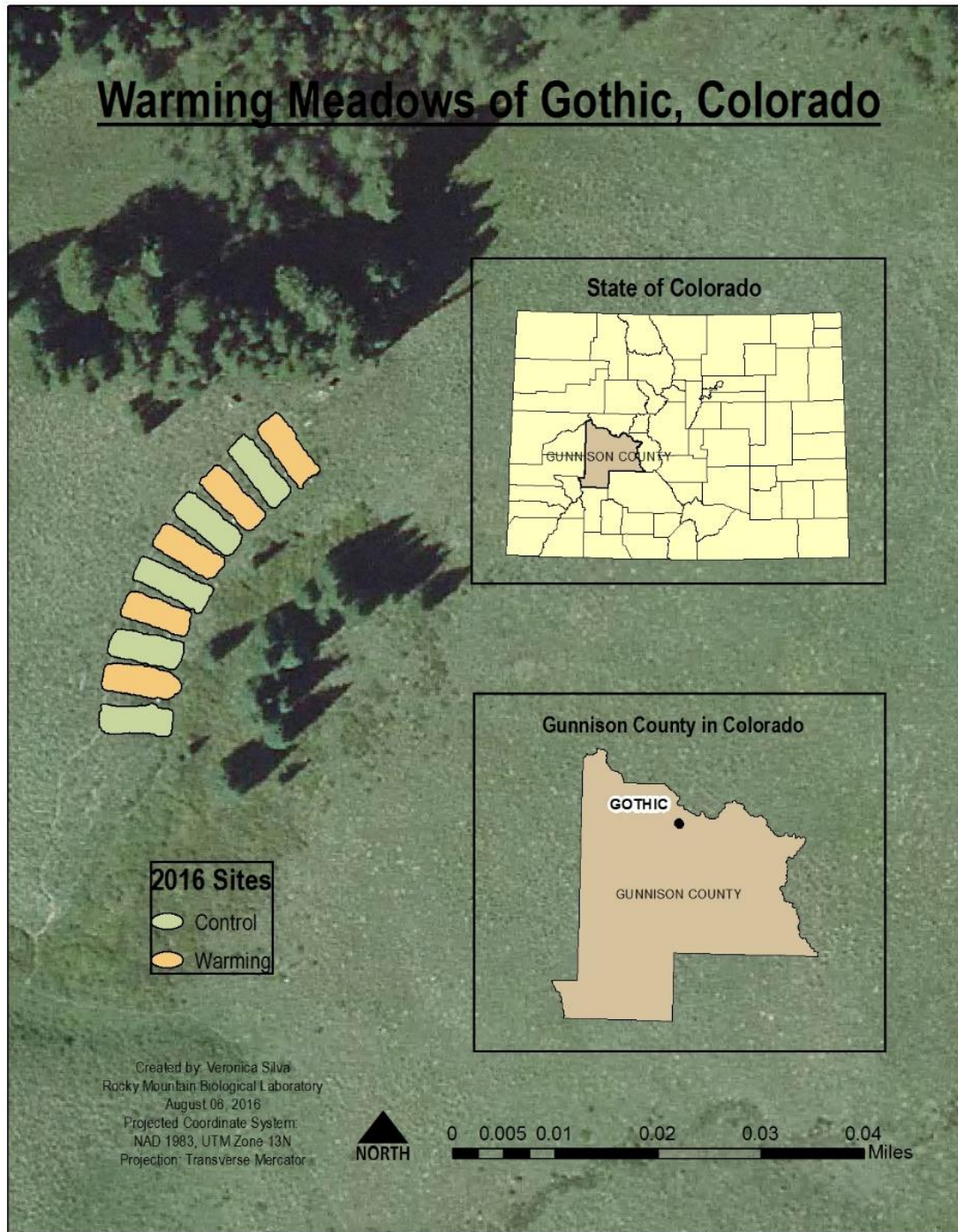


Figure 1. Displays the warming meadows of Gothic, Colorado. The warming meadows exist in the town site of Gothic, where the Rocky Mountain Biological Laboratory has been established since 1928. As shown in the inset maps, Gothic is located in Gunnison County in the West Elk Mountains of Colorado.

The warming meadows are comprised of ten 3×10 m plots located on an east to southeast facing glacial moraine, with their long dimension parallel to the hill slope. Control and experimental plots alternate and are separated by a 3 m gap to ensure that control plots are not affected by the warming experiment (Figure 2). Infrared heaters are suspended 2.5 m above experimental plots and provide uniform heating (22 w/m²) to simulate warming predicted due to doubled atmospheric carbon dioxide levels (Harte et al. 1995). Heaters have been turned on continuously since 1990.

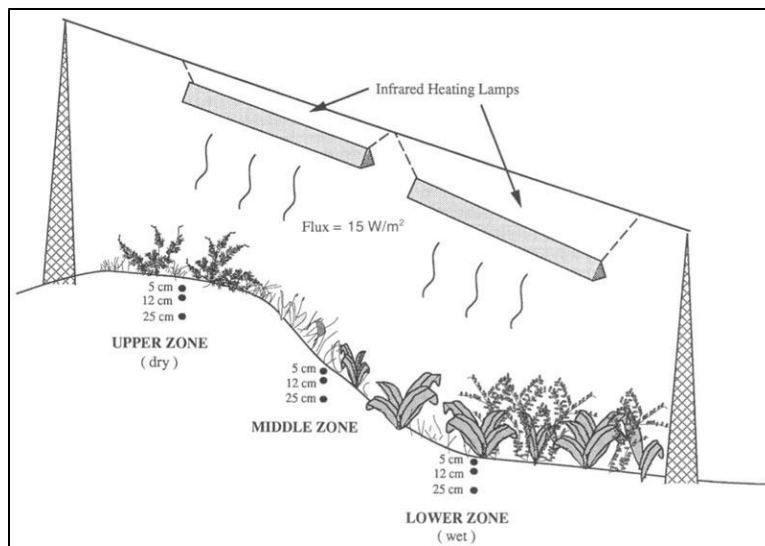


Figure 2. Displays the layout of warming v. controlled plots of RMBL from which samples of the three grasses, *P. pratensis*, *F. thurberi* and *A. lettermanii*, were sampled (adapted from Harte et al. 1995).

Sampling Procedure:

We cultivated leaf endophytes from three different grass species in the Poaceae family: *Festuca thurberi* (FETH), *Poa pratensis* (POPR) and *Achnatherum lettermanii* (ACLE).

3-4 cm long leaf blade segments were obtained from six individuals of each species in each of the ten plots in the warming meadows of RMBL. Prior to being plated, each leaf segment underwent the following surface sterilization procedure: 15 seconds in a 95% ethanol (EtOH) solution, followed by 2 minutes in a 1% sodium hypochlorite (NaOCl) solution then 2 minutes in a 70% ethanol solution. Leaf sections of 1-2cm were plated for each sample across two plates (total of 6 leaf segments per plate).

We subcultured single isolates of culturable fungi from each of those original plates. We then separated each isolate into plates (n=63), separating those into distinct collections: one set was maintained at a temperature of 4° C constantly while the others were kept in warming and variable (20-24° C) incubation temperatures (Figure 3). These temperatures were meant to simulate control and warming treatments in the field to understand if there was any local adaptation of endophytic growth.

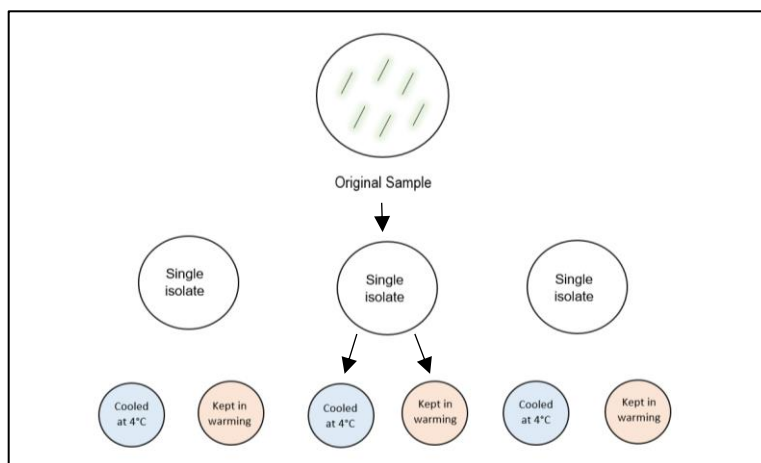


Figure 3. Displays the sampling methodology for this experiment.

Once transferred, digital scans of each secondary petri dish (from both cooled and ambient conditions) were taken at exact 24 hour intervals for 14 days. Once the 14 days elapsed, we analyzed the scans for change in growth area (in cm²). During this time period, we also assessed each isolate by description in order to build an operational taxonomic unit (OTU) matrix to be used in assessing composition and diversity. The term OTU is used to represent each morphospecies of culturable fungi in lieu of species name (as species has not currently been established by means of sequencing).

Data Processing:

Utilizing the program ImageJ and scanned jpegs of each culture, we assessed and recorded fungal area for each culture. Microsoft Excel was then utilized to create graphic representation of growth rates in cultured fungi over the 14-day observation period. We differentiated cultures in terms of treatment (warm versus cold incubation) and species. Using the statistical program R (R Core Team 2016), we then analyzed the data seeking potential effects of increased temperatures on the three dependent variables. Data figures were created using SigmaPlot 12: Systat Software.

Data Analysis:

(Q1) We used a general linear mixed effect model (including the fixed factors of field warming treatment, plant species identity, plate temperature treatment, and all interactions as well as the random effects of plot (in the field experiment, nested in field warming treatment) and fungal isolate identity) to analyze fungal growth rates. Analyses were conducted in the R package lme4 using the lmer function (R Core Team 2016).

(Q2) We recorded fungal OTU composition for each sample as presence/absence of each fungal OTU.. The OTU matrix was analyzed with PERMANOVA including the fixed effects of field warming treatment, plant species identity, and their interaction and the random effect of plot (nested in warming treatment), with 9999 permutations and permutation of residuals under a reduced model. Analyses were conducted in Primer v. 6 (Clark and Gorley 2009). To compare the *beta* diversity of endophyte OTUs across plots, we used PERMDISP analysis with the effect of plant species identity. Finally, we used nonmetric multidimensional scaling analysis with 500 restarts and a Bray-Curtis distance metric to visualize the differences in culturable leaf endophyte composition among warming treatments and plant species.

(Q3) To assess diversity, fungal species richness was analyzed using R (number of fungal OTUs for each plant species X plot combination). We used ANOVA to test for effects of field warming treatment, plant species identity and their interaction on culturable fungal endophyte OTU richness, determined for each plant species X plot combination.

Results

(1) Does long-term field warming alter fungal growth rates under warming (20-24° C) versus cooled (4°C) incubation temperatures?

Fungi consistently grew slower at cooled temperatures versus warmed incubation temperatures ($P < 0.0001$, Figure 4, Table 1). Regardless of laboratory incubation temperature, growth rate also varied among plant hosts ($P = 0.002$). Of the three grasses, fungi isolated from ACLE exhibited the fastest growth rates while FETH exhibited the slowest rates. (Figure 4, Table 1). Finally, the growth response of fungi from different plant species showed a trend toward differences among temperatures ($P = 0.0845$, Table 1). The response change was

most apparent in POPR which generally grew slower at cooled temperatures (much closer to the rate of FETH) than at warm incubation temperatures (when it was almost indiscernible from ACLE) (Figure 4, Table 1).

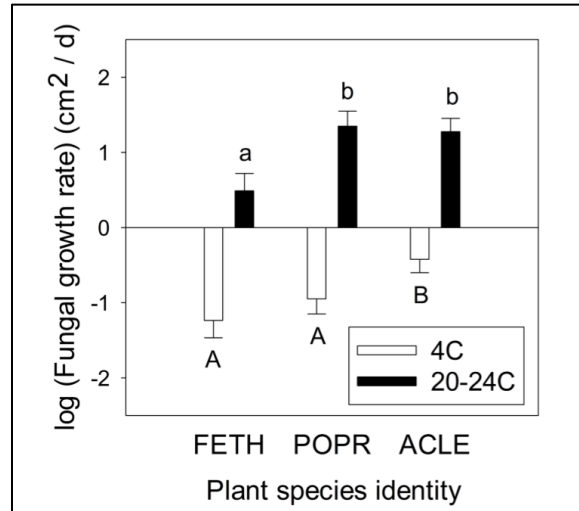


Figure 4. Displays the relationship between temperature, plant species and growth rate in three grasses. Where in warmed incubation temperatures, ACLE and POPR are more similar, however, in cooled incubation temperatures, POPR and FETH are more similar.

Table 1. Statistical results of general linear mixed effect model testing treatment effects on fungal growth rates (log(growth)). Analysis of Deviance Table (Type III Wald chisquare tests).

	Chisq	Df	P
(Intercept)	30.0163	1	4.28E-08
Warming Treatment	0.3392	1	0.560305
Plant Species	12.0866	2	0.002374
Plate Temperature	66.202	1	4.07E-16
Warming Treatment:PlantSpecies	0.3209	2	0.851767
Warming Treatment: Plate Temperature	0.0139	1	0.906055
Plant Species:Plate Temperature	4.9415	2	0.084523
WarmingTreatment:PlantSpecies:Plate Temperature	0.0069	2	0.996557

(2) Does long-term field warming alter culturable fungal endophyte composition for the three plant species?

The composition of cultured fungal endophytes was assessed based on the OTU table of presence/absence of each fungal OTU in each plot X plant species collection. Here, the term OTU is used to represent each morphospecies of culturable fungi in lieu of species name (as species has not currently been established by means of sequencing). The results of a PERMANOVA model show that there was no significant effect of warming ($P=0.69$, Figure 5A) nor any difference among plant species in the effect of warming (warming X plant species, $P=0.47$), but the plant species strongly differed in the composition of leaf endophytes ($P=0.03$, Figure 5B). In addition, *P. pratensis* had higher variability in composition among plots than either of the other grasses. This pattern of higher plot-to-plot *beta* diversity for *P. pratensis* was confirmed by a PERMDISP test (difference from *A. lettermanii*, $P=0.05$, from *F. thurberi*, $P=0.09$).

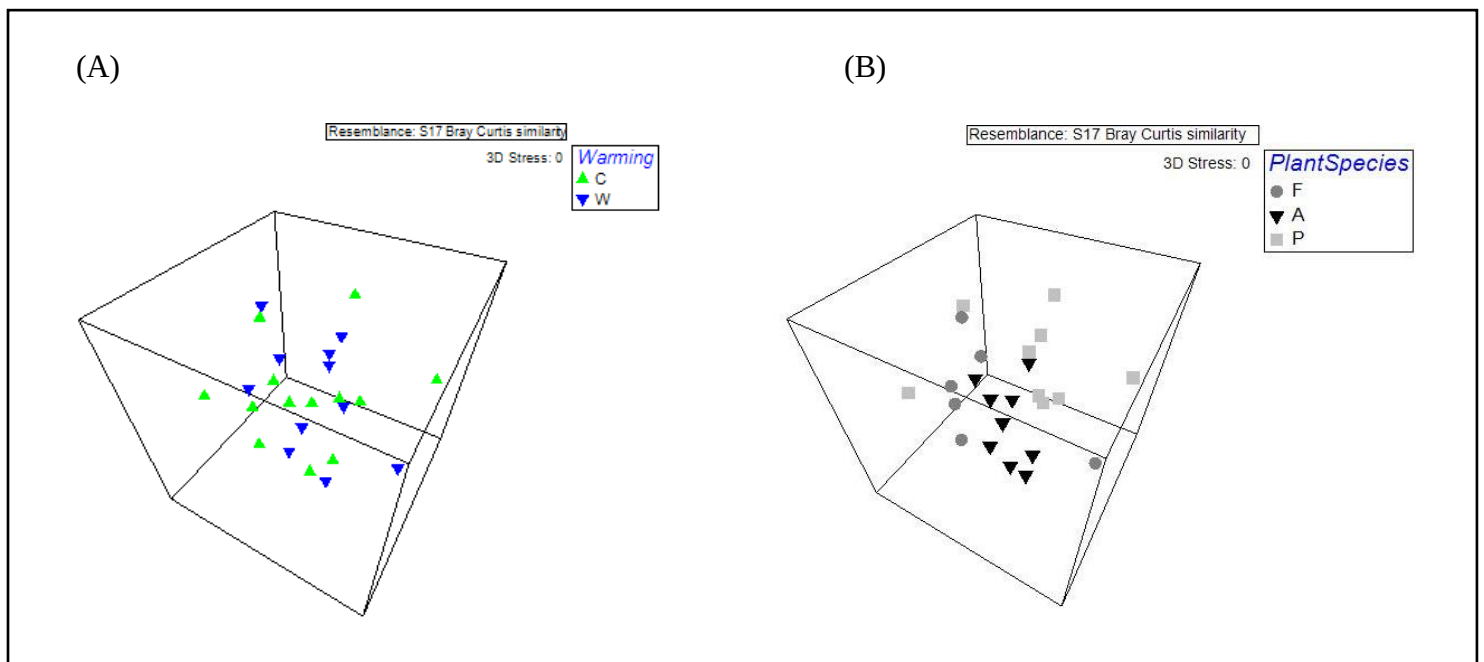


Figure 5. Results of nonmetric multidimensional scaling analysis on leaf endophyte OTU presence/absence (A) showing no effect of long-term experimental warming in the field (C=Control, W=Warmed) and (B) unique fungal communities cultured from three different grass species (F= *Festuca thurberi*, A = *Achnatherum lettermanii*, P = *Poa pratensis*).

(3) Does long-term warming alter diversity in culturable fungal endophyte communities for the three plant species?

The results showed that control plots had 57% higher leaf endophyte richness than the warmed plots, however, the difference was not statistically significant ($P=0.2256$, Figure 6).

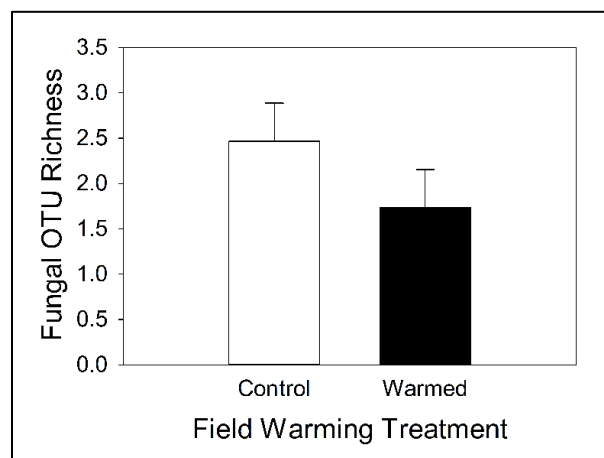


Figure 6. Fungal OTU richness under long-term experimental warming in the field.

Discussion

We hypothesized that increased temperature would increase endophytic growth rates, decrease diversity and change composition of the leaf endophyte communities.

Fungal growth rate was affected more by plant species than the warming treatment. While a trend in decreased fungal growth in response to cooled temperature (simulated control field temperatures with cooled incubation) was concurrent with the literature which predicted a positive correlation between temperature and fungal

metabolism in all fungal symbionts (Kivlin et al. 2013), further study is required to extrapolate the trend. Furthermore, there were no interactions of treatment and current growth temperature. This indicates that fungi are unlikely to shift their physiology in response to future climate change-induced warming and will instead need to move to more suitable habitat.

While neither warming nor plant species had significant effect on composition of fungal endophytes, the trend of shift in composition across species is of interest because this may indicate that fungi will be indirectly affected by climate change if they track their plant hosts.

Lastly, there was no significant effect of warming on diversity within fungal endophyte communities. However, this was based on non-definitive OTU morphospecies and not sequencing.

While this research focused on the response to warming, future research should be broadened to consider the multiple global change factors which will occur simultaneously (such as the interactions between warming temperatures, drying condition, N deposition and CO₂ concentrations) as a result of climate change. By isolating the effect of warming on leaf endophytes, the results may differ from those in actual climatic trends or those ecosystems experiencing gradual temperature changes (Rudgers et al. 2014). And lastly, to truly understand the response of leaf endophytes to climate change, the plant-fungal symbiosis of leaf endophytes should be considered as the species affect one another.

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