Temperature acclimation and adaptation of enzyme physiology in *Neurospora discreta*

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Abstract

Fungal metabolic rates could increase under climate warming but may be counteracted by mechanisms of physiological acclimation and evolutionary adaptation. We hypothesized that Vmax and Km parameters of *Neurospora discreta* extracellular enzymes would acclimate to warmer temperatures through compensatory mechanisms. We also predicted that evolution under warmer temperatures would alter enzyme parameters and fungal respiration through adaptive mechanisms. In contrast to these predictions, growth at higher temperature (22ºC versus 16ºC) increased the temperature-corrected Vmax of three enzymes. The carbon substrate used for fungal growth (lignin versus sucrose) had a much greater impact on enzyme Vmax than temperature. Following experimental evolution, the enzymatic parameters of *Neurospora* strains did not adapt to higher temperatures as hypothesized; rather, enzyme Vmax values were unaffected, and respiration rates increased. Together, these results suggest that physiological and evolutionary mechanisms are unlikely to counteract soil carbon losses driven by saprotrophic fungi under climate warming.

Keywords: climate change; experimental evolution; extracellular enzyme; Km; *Neurospora discreta*; physiological acclimation; respiration; soil carbon; thermal adaptation; Vmax

Introduction

Organisms may respond to climate change through multiple mechanisms with implications for ecosystem processes (Parmesan 2006). These mechanisms may include physiological acclimation, evolutionary adaptation, or population change. Organisms that migrate could also respond to climate change by moving along with their thermal niches. The climate responses of soil fungi are particularly important because fungi play essential roles in biogeochemical processes (Treseder et al. 2016; Treseder et al. 2014).

In soils, fungi produce many enzymes such as glycoside hydrolases, oxidases, peptidases, and phosphatases that convert complex organic matter into simple molecules that can be taken up and used for cellular metabolism (Talbot et al. 2014). Fungal metabolism and enzyme kinetics are known to be temperature sensitive (Wallenstein et al. 2011)—as temperature increases, biochemical theory predicts that enzymes and substrates with higher kinetic energy will react more rapidly to form metabolic products (Davidson & Janssens 2006). At the cellular level, these reactions could sum up to increase respiration under higher temperatures. At the ecosystem level, respiration represents a carbon loss pathway, and therefore warming temperatures are expected to accelerate carbon losses from soils and other ecosystem components (Carey et al. 2016; Crowther et al. 2016). Still, questions remain about fungal thermal physiology and consequent impacts on carbon and nutrient cycling under climate change (Allison & Treseder 2011).

One major uncertainty is the potential for fungi (and other organisms) to alter their physiology under sustained climate change (Crowther & Bradford 2013). In addition to altering temperatures, climate change will have indirect effects on other ecological factors. For example, shifts in plant communities and tissue chemistry could alter the organic substrates available to fungi (Suseela & Tharayil 2018; Kardol et al. 2010).

How will fungal physiology respond to these shifts? Responses could involve changes in biomass growth, gene expression, and isozyme production for organisms with multiple copies of the same enzyme gene (Bradford 2013). We define such changes as physiological acclimation because they do not require any genetic changes. Over longer time periods, changing selective pressures of temperature and substrate chemistry could alter fungal genes through evolutionary processes (Romero-Olivares et al. 2015). Strains with genes conferring greater relative fitness under the new conditions should increase in frequency (Tenaillon et al. 2012). Collectively, we refer to these physiological and evolutionary responses as thermal adaptation.

The physiological literature makes clear predictions about thermal adaptation and compensatory mechanisms that maintain homeostasis under temperature variation (Hochachka & Somero 2002; Somero 1978). Most biochemical reaction rates increase at higher temperatures, but changes in organism physiology may partially counteract these rate changes. For example, cold-acclimated (or adapted) organisms should express enzymes with higher catalytic rates, partially offseting the biochemical effects of low temperatures (Lonhienne et al. 2000; Georlette et al. 2004; Siddiqui & Cavicchioli 2006). This compensation response could be achieved by expressing isozymes with higher catalytic turnover rates (k_{cat}) or by increasing the total amount of enzyme expressed. Both of these mechanisms would increase enzyme Vmax—the measured maximum reaction velocity—when enzymes are assayed at a common temperature. By the same logic, thermal compensation for warmer temperatures would reduce enzyme Vmax when measured at a common temperature. If these compensatory mechanisms involve genetic change, then evolutionary adaptation would be required.

Biochemical theory also makes predictions about enzyme-substrate binding under climate warming (Tsuruta & Aizono 2003; Snider et al. 2000; Siddiqui & Cavicchioli 2006). As

temperatures increase, the half-saturation constant for enzymatic reactions (Km) may change. Although Km may decline with increasing temperature for some enzymes, more often Km increases, which weakens enzyme-substrate binding (Allison et al. 2018; Hochachka & Somero 2002). Fungi could compensate for this negative effect of temperature on binding capacity by expressing enzymes with lower Km and/or lower sensitivity of Km to temperature (Hochachka & Somero 2002). Still, it remains unclear if the strength of this compensatory mechanism varies under different thermal regimes, particularly for microbes. Most of the prior work on Km compensation has focused on enzymes from animals (Somero 2004; but see Allison et al. 2018).

Whereas the evolutionary response to thermal environment has been extensively studied in bacteria (Bennett & Lenski 2007), there are very few studies with fungi. Moreover, most bacterial studies have focused on model organisms that do not provide much insight into biogeochemical processes. New studies are needed to address the potential for evolutionary change in fungal taxa and physiological traits that can influence carbon and nutrient cycling under climate change. Furthermore, climate change could have indirect effects on plant litter chemistry and substrate availability (Ilmén et al. 1997) with consequences for enzyme properties.

Neurospora discreta is an ideal fungus for addressing questions about thermal adaptation and substrate use. The *Neurospora* genus is well-characterized genetically, with *Neurospora* serving as a longstanding model system (Gladieux et al. 2015; Ellison et al. 2011). *Neurospora* species are present in soils around the globe where they function as saprotrophs that produce a broad array of extracellular enzymes. *Neurospora crassa* has exhibited thermal adaptation following heat shock (Mohsenzadeh et al. 1998), and *Neurospora discreta* showed relative increases in spore production following experimental evolution at elevated temperature (Romero-Olivares et al. 2015). However, a previous study with 27 strains of *Neurospora discreta*

isolated from sites along a temperature gradient showed no evidence of thermal adaptation of enzyme Vmax or Km (Allison et al. 2018). Therefore the mechanisms of physiological and evolutionary response to increasing temperature remain unclear in *Neurospora*.

To address these mechanisms, we analyzed the respiration and enzyme kinetic properties of *Neurospora discreta* strains before and after experimental evolution at different temperatures. This design enabled us to separate physiological acclimation from evolutionary adaptation. By conducting growth assays at two temperatures and on two substrates, we could contrast the effects of temperature versus substrate change on *Neurospora* physiology. Based on prior theory, we hypothesized that warm-acclimated or warm-adapted strains would express enzymes with lower Vmax at a common temperature but a higher Vmax temperature sensitivity (i.e. the change in Vmax with a change in assay temperature). Such enzymes should also have lower Km and Km temperature sensitivity values. In parallel with Vmax, we hypothesized lower respiration at a common temperature for warm-adapted strains. Finally, we hypothesized that *Neurospora* strains would change their enzyme expression patterns when acclimating to different growth substrates.

Methods

Neurospora strains

Neurospora discreta strains were obtained from a culture collection in J. Taylor's laboratory (Table 1). A subset of five strains from Alaska was used in the evolution experiment. We focused on *Neurospora* taxa because they are distributed globally and produce a wide array of extracellular enzymes involved in the degradation of plant biomass (Gladieux et al. 2015; Shrestha et al. 2011; Allison et al. 2018; Jacobson et al. 2004).

Growth conditions

We prepared an inoculum by growing strains for 5 d on Vogel's minimal medium agar (VMM; 1x Vogel's salts, 1.5% sucrose, 1.5% agar) (Vogel 1956), washing the culture with 20 ml of 1 M sorbitol, filtering the liquid, and harvesting the conidia by centrifugation for 5 min at 1260 × *g*. The conidia were then washed in fresh sorbitol, centrifuged (3x), and stored at −20 °C. Strains were inoculated at a rate of 1 x 10^6 conidia ml^{-1} liquid into septum-capped and sterilized 40 ml glass vials containing 5 ml 1x Vogel's salts and 200 mg sucrose or 200 mg lignin (Sigma-Aldrich 370959). We studied lignin because it is a complex substrate that fungi may degrade to access cellulose or use as a carbon source if other substrates are unavailable (Kirk & Farrell 1987). Vials were incubated overnight (12 h) at room temperature (22ºC) and then transferred to incubators maintained at 16ºC or 22ºC. At 1 d (24 h), 7 d, 14 d, and 21 d after transfer, vials were analyzed for respiration and destructively sampled for enzyme assays. Each strain was grown in triplicate for a total of 48 vials (2 substrates x 2 growth temperatures x 4 time points x 3 replicates). We used a batch culture approach to approximate growth conditions in the field where fungi colonize and consume substrates in a successional process (Voříšková & Baldrian 2013).

Respiration measurements

Each vial was opened under sterile conditions, equilibrated with ambient air for 15 min, closed, and incubated at the growth temperature (i.e. 16ºC or 22ºC) for 2 h. Before incubation, 10 ml headspace gas was removed from the vial through the septum using a needle and syringe. The vial then equilibrated with ambient air pressure through the needle hole and was sampled again after incubation. Headspace samples were injected into an infrared gas analyzer (EGM-4, PP

Systems) to determine CO_2 concentration in ppm. The concentration difference before and after incubation was converted into a respiration flux using the volume of the headspace (35 ml) and the ideal gas law. Cumulative respiration was determined over the 21-d growth period using the trapezoidal rule for integration under a curve based on linear interpolation. Raw respiration data are available in Supplemental Table 1 in units of mmol d^{-1} $vial^{-1}$.

Enzyme assays

We assayed potential enzyme activities using fluorimetric and colorimetric procedures for 96-well plates as reported previously (Allison et al. 2018; German et al. 2011). Fungal mycelium was removed from the growth medium by filtration. The filtrates from the 3 replicates were combined in a 15 ml tube and stored at -80ºC prior to enzyme analysis. *Neurospora* culture filtrate was added to microplate wells and brought to a volume of 125 µl with distilled water; the dilution factor was recorded. To the assay wells, we added 125 µl substrate dissolved in 50 mM maleate buffer, pH 6.0. Substrates were serially diluted by two-fold from the maximum concentrations shown in Table 2 to yield eight substrate concentrations. The OX assay also received 10 µl 0.3% H_2O_2 in all substrate wells. All assays included culture filtrate blanks and substrate controls. For the fluorimetric assays, we included 7-amino-4-methylcoumarin (AMC) standards for leucine aminopeptidase (LAP) and 4-methyumbelliferone (MUB) standards for the other hydrolytic enzymes. Standard wells contained 125 µl of 25 µM MUB or AMC plus 125 µl 1x Vogel's salts; quench controls contained 125 µl culture filtrate instead of Vogel's salts.

We incubated assay plates for 4 h (hydrolytic enzymes) or 24 h (oxidases) at 4, 10, 16, 22, 28, and 34ºC. For hydrolytic enzymes, fluorescence was read at 365 nm/450 nm excitation/emission on a BioTek Synergy H4 microplate reader. For oxidative enzymes,

absorbance was read at 410 nm. Enzyme activities were expressed as nmol h^{-1} ml^{-1} culture filtrate according to German et al. (2011, 2012). We used an extinction coefficient of 3.9 μM^{-1} for pyrogallol and calculated PER activity as OX – PPO activity for each sample. Raw enzyme data are available in Supplemental Table 2 in units of nmol h^{-1} ml^{-1} for Vmax and μM for Km.

Evolution experiment

We initiated the evolution experiment by adding ~5 x 10^6 spores to 30 cm race tubes containing 25 ml media (1x Vogel's salts, 2 g ml^{-1} spruce needles, and 1.2% agar). Upon reaching the end of the race tube, strains were transferred to a new tube by collecting mycelium and conidia from the last 2 cm of the tube and inoculating into the new tube. The experiment was continued for 15 transfers corresponding to ~1500 mitotic generations, assuming 100 cell cycles per 30 cm growth (Dettman et al. 2008). This assumption is very conservative, and the actual number of generations could be higher if based on hyphal branch intervals (Watters et al. 2000). Each of the five strains was evolved in incubators at 16ºC and 22ºC. At 500, 1000, and 1500 generations, mycelium and conidia from the end of the race tube were grown up in liquid VMM in a 50 ml tube, and the resulting conidia were harvested for inoculation of vials for respiration and enzyme measurements. Enzyme activities were analyzed after 1500 generations only.

Statistical analyses

Enzyme data were analyzed after 14 and 21 d of growth. By this time, the strains had achieved peak respiration under most temperature and substrate combinations. Enzyme activity data were quality controlled by removing values associated with pipetting error, bubbles in microplate wells, and other analytical errors. Activity values below detection limits were set to a detection

limit of 1 x 10^{-5}. This step removed negative activities from the dataset that are not biologically meaningful while avoiding bias from under-sampling values near zero. After quality control, activities were fit to the Michaelis-Menten equation using the non-linear least squares (nls) function in *base* R (R Development Core Team 2017). Vmax and Km parameters were extracted from the model fit, and parameters from poor fits were discarded. Model fits were considered unreliable if parameters were negative or had 95% confidence intervals greater than the parameter magnitude for Vmax or twice the parameter magnitude for Km. The data were also visually checked for spurious values by plotting Vmax and Km versus laboratory incubation temperature and removing suspect data points. This quality filtering is somewhat subjective but did not appear to bias the overall measured responses to temperature and substrate based on comparison with unfiltered data. For samples with Vmax values too low to detect, we substituted the minimum detectable Vmax (computed at 16ºC for each enzyme) to avoid under-sampling values near zero.

Extracted parameters were log-transformed and analyzed with linear regression using plate incubation temperature as the independent variable. Regression slopes were extracted as a metric of Vmax or Km temperature sensitivity in terms of change in log(Vmax) or log(Km) per °C. Regression parameters were used to compute enzyme Vmax or Km at 16ºC, hereafter referred to as Vmax or Km for simplicity. We chose 16ºC because it corresponds to the lower temperature used in our growth assays and evolution experiment.

We analyzed respiration and enzyme data with mixed model analysis of variance (ANOVA) and the *lme* function in the R software environment. Strain was included in all models as a random effect, and respiration data were log-transformed to achieve normality. To test for physiological acclimation of respiration, we ran an ANOVA on cumulative respiration from the

20 ancestral strains with substrate and growth temperature (*growth T*) as main effects. To test for evolutionary changes in respiration, we ran an ANOVA on cumulative respiration from the five evolution strains with substrate, growth T, and generation as main effects. Because there was no variance in evolution temperature (*evolution T*) for the ancestral strains (i.e. at generation = 0), we ran another ANOVA with only the evolved strains (i.e. generation ≥ 500) to test for effects of evolution T; this model also included substrate, growth T, and generation as main effects.

To test for physiological acclimation of enzyme properties, we analyzed Vmax, Km, and temperature sensitivity values from 21 d of the growth period for the 20 ancestral strains. The mixed-model ANOVA included substrate and growth T as main effects. We tested for evolutionary change in enzyme parameters over time using data from the five strains evolved at 16ºC (generation ≥ 0) and a model with substrate, growth T, generation, and day as main effects. We tested for effects of evolution T on enzyme parameters using data from the evolved strains at generation = 1500 and a model with substrate, growth T, evolution T, and day as main effects. This designed enabled statistical analysis of evolution T effects independent of growth T. All enzyme data were rank-transformed prior to ANOVA to improve normality. Data and R code are available at github.com (DOI: 10.5281/zenodo.1308189).

Results

Response to substrate

Average cumulative CO_2 produced by the ancestral strains was 16 times greater on sucrose than on lignin (2.7±0.1 vs. 0.17±0.01 mmol CO_2, mean±SEM, $P < 0.001$). Vmax for most enzymes depended strongly on substrate (Fig. 1). AG, AP, BG, LAP, and NAG Vmax were significantly higher on sucrose than on lignin ($P < 0.001$). Although average BG Vmax was greater on

sucrose, several individual strains showed greater BG Vmax on lignin. PER was mainly expressed on sucrose ($P < 0.001$), whereas PPO was mainly expressed on lignin and very low on sucrose ($P = 0.002$). On average, BX Vmax was similar across substrates ($P > 0.05$), but a few strains showed higher values on lignin. CBH Vmax was significantly greater on lignin ($P < 0.001$), especially at 22ºC ($P = 0.051$ for interaction). Because most enzymes were not expressed consistently on one of the two substrates, we did not compare Km or temperature sensitivity parameters across substrates.

Response to growth temperature

There was no effect of growth T on average cumulative respiration for either substrate (averaged across all 20 ancestral strains). However, the maximum rate of CO_2 production on sucrose was generally one week earlier at a growth T of 22ºC compared to 16ºC (Fig. 2). Respiration rates declined by day 21 and were usually higher at 16ºC on that day, likely due to greater substrate remaining (Fig. S1). The temporal patterns on lignin were less clear, but some strains showed an increase in respiration rate peaking at 21 days, but only at 16ºC growth T (Fig. S1).

There were some significant effects of growth T on Vmax and Km, but not Vmax temperature sensitivity or Km temperature sensitivity (Fig. 3, Fig. S2). In contrast to our acclimation hypothesis, growth at 22ºC significantly increased Vmax of AG ($P = 0.007$), BX ($P < 0.001$), and CBH ($P < 0.001$). Consistent with the hypothesis, LAP Vmax declined significantly ($P = 0.015$) driven by changes on sucrose (there was little LAP activity on lignin). For Km, there were some weak but significant interactions between growth temperature and substrate for BG ($P = 0.044$) and OX ($P = 0.011$) (Fig. S2).

Response to evolution temperature

Adaptation to laboratory conditions over hundreds of generations led to changes in respiration (Fig. 4). Cumulative respiration declined significantly by 500 generations ($P < 0.001$ for generation effect; ANOVA including the five ancestral strains and averaging across evolution T). There was also a significant interaction between generation and substrate ($P = 0.003$) indicating that the decline was steeper when respiration was measured on lignin.

To analyze the effect of evolution T on cumulative respiration, we ran an ANOVA with the evolved strains only. We found a significant ($P = 0.003$) effect of evolution T, but also interactions with substrate ($P = 0.011$) and generation ($P = 0.005$). In contrast to our hypothesis, warmer evolution T increased respiration overall and more so when respiration was measured on sucrose compared to lignin. The change in respiration over generations was greater on lignin.

It was difficult to detect the evolutionary response of enzymes to temperature due to a reduction in some enzyme activities over hundreds of generations (Fig. 5). AG Vmax declined when assayed after growth on sucrose ($P < 0.001$, post-hoc ANOVA) but increased after growth on lignin ($P < 0.001$). AP was lower in evolved strains after 14 d but not 21 d of growth ($P = 0.018$ for generation by day interaction). BX declined in evolved strains ($P = 0.016$), as did PER ($P = 0.010$). CBH declined to undetectable levels when grown on sucrose ($P < 0.001$, post-hoc ANOVA) but increased on lignin ($P < 0.001$). For NAG, generation showed a significant interaction with growth T on sucrose ($P = 0.040$, post-hoc ANOVA) and with day on lignin ($P = 0.031$) such that there was no clear response overall. PPO and OX could only be measured reliably on lignin where they showed significant declines following evolution ($P < 0.001$).

Despite low activities of many enzymes after 1500 generations, we used ANOVA to test for effects of evolution T on Vmax and Vmax temperature sensitivity. The Vmax of most

enzymes was not significantly affected by evolution T (Fig. 5). However, for AP, there was an interaction between evolution T, day, and substrate ($P = 0.038$). For CBH, which was only expressed on lignin after laboratory evolution, Vmax was significantly lower for the strains evolved at 22ºC ($P = 0.007$, post-hoc ANOVA). Vmax temperature sensitivity for NAG showed a significant three-way interaction involving evolution T, growth T, and day ($P = 0.023$; Fig. 6), indicating that temperature sensitivity was higher for warm-evolved strains but only at 22ºC growth T and only on day 14 with the pattern disappearing or even reversing by 21 d. We were able to assess Vmax temperature sensitivity on lignin for AG, BG, and CBH, but there were no significant effects of evolution T. Other enzymes could not be analyzed due to low activities. Km and Km temperature sensitivity responses to evolution T were not analyzed due to low sample sizes resulting from enzyme activities below detection limits.

Discussion

We hypothesized that increases in temperature and shifts in substrate chemistry, as expected under climate change, would alter enzyme properties and cellular respiration. Such changes could occur due to physiological acclimation in the short term or evolutionary adaptation in the long term. If enzymatic reaction rates were to increase less steeply under warming due to mechanisms of thermal compensation, positive feedbacks between warming and soil carbon loss could weaken. Under substrate-limited conditions, the intrinsic temperature sensitivity of Km could also limit these positive feedbacks (German et al. 2012a). In contrast, hypothesized increases in Vmax temperature sensitivity and declines in Km and Km temperature sensitivity would sustain carbon losses under warming.

Acclimation

To test for physiological acclimation, we grew *Neurospora* strains at two different temperatures on two different carbon substrates. In contrast to our acclimation hypothesis, there was little evidence that higher growth T reduced enzyme Vmax at a common temperature. In fact, the measured Vmax of several enzymes increased, which may have resulted from a general increase in metabolic rates at 22ºC. If the expression of different isozymes had changed, we might have expected differences in Vmax temperature sensitivity, Km, and Km temperature sensitivity. However, these parameters were largely unaffected by growth T. Likewise, although the timing of respiration differed, cumulative respiration was unaffected by growth T over a 21-day period.

There are several potential explanations for a lack of Vmax thermal compensation. It is possible that our *Neurospora* strains lack the genetic potential to express different isozymes, either due to a limited complement of enzyme functional genes or relatively simple regulatory pathways. However, this explanation seems unlikely given that some *Neurospora* are capable of lignocellulose degradation and show complex regulation of enzyme expression (Shrestha et al. 2011; Hanson & Marzluf 1975). Another possibility is that 16ºC versus 22ºC is not a large enough temperature difference to elicit a strong physiological response. These fungi experience substantial diurnal and seasonal temperature fluctuations in surface soils (Allison & Treseder 2008), so the growth temperatures we used may be well within their thermal niches.

Our ability to disentangle thermal compensation mechanisms was also constrained by our growth assay approach. In our batch cultures, measured Vmax is a function of enzyme k_{cat} values and expressed enzyme concentrations that depend on total fungal biomass as well as biomass-specific expression rates. Biomass in turn depends on substrate availability which declined over the 21-d experiment, especially for sucrose. Therefore compensatory changes in enzyme-level

properties could have been obscured by increases in fungal biomass at 22ºC relative to 16ºC. Unfortunately we did not measure fungal biomass due to difficulties in separating biomass from the growth medium (particularly the lignin component). Still, if there were any changes in fungal biomass, they did not affect cumulative respiration which was unaffected by growth T.

In contrast to the weak temperature response, we found a substantial physiological response to carbon substrate. Respiration rates were much lower on lignin than on sucrose, which is consistent with the chemical differences among the two substrates. Sucrose degradation requires only one hydrolytic reaction prior to entry into central metabolic pathways. Lignin metabolism involves multiple steps and therefore yields much less energy (Kirk & Farrell 1987). Our respiration results suggest that *Neurospora* growth rates were much lower on lignin.

Despite minimal growth, CBH and PPO were preferentially expressed on lignin. Some strains also had greater BG and BX Vmax on lignin compared to sucrose. These results suggest that the presence of lignin in the growth medium triggered the up-regulation of enzymes involved in lignocellulose metabolism, similar to responses observed in the presence of cellulose metabolites (Znameroski et al. 2012). Although we used purified lignin as a growth substrate, a coordinated response to decompose all lignocellulose components would make sense because many *Neurospora* species spend at least part of their life cycles growing on wood and may sporulate following forest fires (Jacobson et al. 2004). Also, some *Neurospora* may produce lignocelluloytic enzymes as they switch from endophytic to saprotrophic life strategies and break through cell walls following plant host death (Kuo et al. 2014). It is also possible that enzyme production by our strains was affected by residual cellulose and hemicellulose in the kraft lignin preparation that we used. Taken together, these results imply that *Neurospora* strains may

acclimate readily to changes in substrate availability driven by vegetation shifts associated with climate change (Suseela & Tharayil 2018).

Evolutionary adaptation

In our evolution experiment, the large decline in respiration rates by 500 generations was probably an adaptive response to the laboratory environment. Adaptation to culture conditions is commonly observed in bacterial evolution experiments (Elena & Lenski 2003), though the rate of adaptation declines over thousands of generations (Lenski & Travisano 1994). Although both 16ºC and 22ºC are greater than the mean annual temperatures experienced by *Neurospora* strains in their native Alaskan environment, higher average temperatures in the laboratory were probably not the key driver of adaptation, otherwise we should have observed a smaller change in respiration over 500 generations for the strains evolved at 16ºC as opposed to 22ºC. Other factors such as growth substrate or reduced temperature variation may have caused the observed declines in respiration rate through selection on physiological traits such as growth efficiency (Manzoni et al. 2012). Another possibility is that race tubes selected for fungi with more rapid linear growth and less hyphal branching, resulting in lower biomass in the respiration assay.

Although it was opposite our prediction, we did observe a significant effect of evolution T. Strains evolved at 22ºC showed a smaller decline in respiration compared to those evolved at 16ºC in the laboratory. This result suggests that evolutionary adaptation is not likely to reduce fungal respiration or feed back negatively on soil carbon loss under warming. If fungi evolve higher respiration rates under warming, more soil carbon would be released to the atmosphere as CO_2. At the same time, evolution of greater respiration rates under warmer conditions may increase fungal fitness. A previous study found an association between increased respiration and

evolution of greater spore production (Romero-Olivares et al. 2015). Assuming that growth substrates are available in the field, evolution of higher respiration rates and greater spore production with increased temperatures is likely to increase fungal activity and soil carbon loss, thereby generating a positive feedback to climate warming.

Enzyme responses to laboratory evolution paralleled the respiration response. Over generations, the potential activities of several enzymes declined significantly. This result was somewhat surprising because the growth medium contained complex carbon and nutrients that require enzymatic action for degradation. However, the fungi could have grown primarily on available monomeric substrates in the litter medium, thereby alleviating selection to maintain extracellular enzymes. Also, selection for rapid linear growth might have caused a tradeoff with investment in enzymatic capacity; evolutionary tradeoffs are apparent across fungal taxa and could also arise within taxa (Treseder & Lennon 2015; Crowther et al. 2014).

Unlike respiration, enzyme parameters showed little response to evolution T under our experimental conditions. There were no clear positive effects of the warmer evolution T on Vmax, suggesting that enzyme production and catalytic rate remained the same. In addition, there was little evidence for evolutionary adaptation of Km or Vmax and Km temperature sensitivities. Therefore, the relatively higher respiration we observed after evolution at 22ºC was probably due to increased uptake of available substrates and/or lower growth efficiency and not changes in enzyme properties (Manzoni et al. 2012).

There are some important caveats with our experimental design. Due to logistical constraints, we only evolved five strains of *Neurospora* on one substrate type at two different temperatures for 1500 generations. If we had used evolution temperatures that differed by more than 6ºC or run the experiment for longer than 1500 generations, enzyme parameters might have

shown stronger adaptive responses to temperature. Still, such a design would have likely required a growth substrate with greater polymer content to maintain selective pressure on enzyme properties. We also recognize that future studies should analyze additional fungal taxa to account for taxonomic variation in the evolutionary response to thermal environment. Finally, the outcomes of evolution may differ under field conditions where biotic and abiotic interactions are much more complex than in the laboratory.

Conclusions

Despite some limitations, our study indicates that *Neurospora* strains are less sensitive to a 6ºC increase in average temperature than a shift in substrate chemistry from lignin to sucrose. The *Neurospora* strains we analyzed do not show evidence of thermal compensation upon growth or evolution at elevated temperature. In fact, these strains showed moderately higher respiration rates when evolved at higher temperature. This response, especially if coupled to higher fungal spore production and fitness, could exacerbate losses of soil carbon under warming. Our findings should be useful for parameterizing new microbial models that incorporate physiological and evolutionary responses of microbes to changes in climate and substrate availability (Wieder et al. 2015a; Wieder et al. 2015b).

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Figure legends

Fig. 1. Enzyme log(Vmax) (computed at 16ºC) compared across growth substrates. Strains were grown on lignin or sucrose for 21 d at 16ºC or 22ºC. Asterisks denote significant substrate effect ($P < 0.05$). Boxplots include median (central line), interquartile range (box), and data range without outliers (whiskers). AG = *α*-glucosidase; AP = acid phosphatase; BG = *β*-glucosidase; BX = *β*-xylosidase; CBH = cellobiohydrolase; LAP = leucine-aminopeptidase; NAG = *N*-acetyl-*β*-D-glucosaminidase; OX = total oxidase; PER = peroxidase; PPO = polyphenol oxidase.

Fig. 2. Histograms showing the day of maximum respiration rate at each growth temperature for ancestral and evolved strains on sucrose and lignin.

Fig. 3. (A) Enzyme log(Vmax) (computed at 16ºC) and (B) Vmax temperature sensitivity. Strains were grown on lignin or sucrose for 21 d at 16ºC or 22ºC. Asterisks denote significant growth temperature effect ($P < 0.05$). Boxplots include median (central line), interquartile range (box), and data range without outliers (whiskers). AG = *α*-glucosidase; AP = acid phosphatase; BG = *β*-glucosidase; BX = *β*-xylosidase; CBH = cellobiohydrolase; LAP = leucine-aminopeptidase; NAG = *N*-acetyl-*β*-D-glucosaminidase; OX = total oxidase; PER = peroxidase; PPO = polyphenol oxidase.

Fig. 4. Cumulative (21-d) respiration over 1500 generations. Strains were evolved at 16ºC or 22ºC and grown on lignin or sucrose at 16ºC or 22ºC. Boxplots include median (central line) and interquartile range (box).

Fig. 5. Mean±SEM log(Vmax) (computed at 16ºC) for enzymes from strains evolved at 16ºC or 22ºC. Strains were grown on lignin or sucrose for 14 or 21 d (data are averaged across growth temperatures). AG = *α*-glucosidase; AP = acid phosphatase; BG = *β*-glucosidase; BX = *β*-xylosidase; CBH = cellobiohydrolase; LAP = leucine-aminopeptidase; NAG = *N*-acetyl-*β*-D-glucosaminidase; OX = total oxidase; PER = peroxidase; PPO = polyphenol oxidase.

Fig. 6. Vmax temperature sensitivity for *N*-acetyl-*β*-D-glucosaminidase enzyme. Data from evolved strains grown on lignin or sucrose and grown at 16ºC or 22ºC for 14 or 21 d.

Table 1. *Neurospora* strain isolation sites and site characteristics. Mean annual temperature (MAT) and mean annual precipitation (MAP) over the period 1981-2010 were obtained from PRISM (http://www.prism.oregonstate.edu/) for sites inside the continental United States. MAT and MAP for other sites were obtained with Climate Reanalyzer (http://cci-reanalyzer.org), Climate Change Institute, University of Maine, USA, using the University of Delaware Air Temperature and Precipitation dataset. Strains with asterisk (*) were used in the evolution experiment.

ID	Genotype	Site	Latitude	Longitude	MAT	MAP
MM10	*Neurospora discreta* from USA	Fairbanks, AK	64.83	-147.72	-1.9	276
MM18*	*Neurospora discreta* from USA	Fairbanks, AK	64.83	-147.72	-1.9	276
MM2*	*Neurospora discreta* from USA	Fairbanks, AK	64.83	-147.72	-1.9	276
MM20*	*Neurospora discreta* from USA	Fairbanks, AK	64.83	-147.72	-1.9	276
MM23	*Neurospora discreta* from USA	Fairbanks, AK	64.83	-147.72	-1.9	276
MM24*	*Neurospora discreta* from USA	Fairbanks, AK	64.83	-147.72	-1.9	276
MM26	*Neurospora discreta* from USA	Fairbanks, AK	64.83	-147.72	-1.9	276
MM30	*Neurospora discreta* from USA	Fairbanks, AK	64.83	-147.72	-1.9	276
MM31*	*Neurospora discreta* from USA	Fairbanks, AK	64.83	-147.72	-1.9	276
MM6	*Neurospora discreta* from USA	Fairbanks, AK	64.83	-147.72	-1.9	276
W1099	*Neurospora discreta* from USA	Morgan Hill, CA	37.11	-121.65	15.7	560
W1101	*Neurospora discreta* from USA	Morgan Hill, CA	37.11	-121.65	15.7	560
W1103	*Neurospora discreta* from USA	Morgan Hill, CA	37.11	-121.65	15.7	560
W1111	*Neurospora discreta* from USA	Morgan Hill, CA	37.11	-121.65	15.7	560
W1289	*Neurospora discreta* from Spain	Macanet de la Selva, Spain	41.78	2.73	13.7	701

W1303	*Neurospora discreta* from Switzerland	Leuk, Switzerland	46.32	7.63	0.4	798
W792	*Neurospora discreta* from USA	Bernalillo, NM	35.3	-106.55	13.0	268
W793	*Neurospora discreta* from USA	Bernalillo, NM	35.3	-106.55	13.0	268
W794	*Neurospora discreta* from USA	Bernalillo, NM	35.3	-106.55	13.0	268
W795	*Neurospora discreta* from USA	Bernalillo, NM	35.3	-106.55	13.0	268

Table 2. Enzymes and substrates analyzed in the current study.

Enzyme and abbreviation		Substrate target	Synthetic substrate and maximum concentration (μM)	
α-glucosidase	AG	Starch degradation products	4-MUB-*α*-D-glucopyranoside	1000
Acid phosphatase	AP	Organic phosphorus	4-MUB Phosphate	4000
β-glucosidase	BG	Cellulose degradation products	4-MUB-*β*-D-glucopyranoside	2000
β-xylosidase	BX	Hemicellulose degradation products	4-MUB-*β*-D-xylopyranoside	2000
Cellobiohydrolase	CBH	Cellulose degradation products	4-MUB-*β*-D-cellobioside	1000
Leucine-aminopeptidase	LAP	Polypeptides	L-leucine-7-amido-4-methylcoumarin hydrochloride	1000
N-acetyl-*β*-D-glucosaminidase	NAG	Chitin degradation products	4-MUB-*N*-acetyl-*β*-D-glucosaminide	2000
Total oxidase	OX	Lignin and phenolics	Pyrogallol + H_2O_2	1000
Polyphenol oxidase	PPO	Lignin and phenolics	Pyrogallol	1000
Peroxidase	PER	Lignin and phenolics	(equal to OX – PPO)	

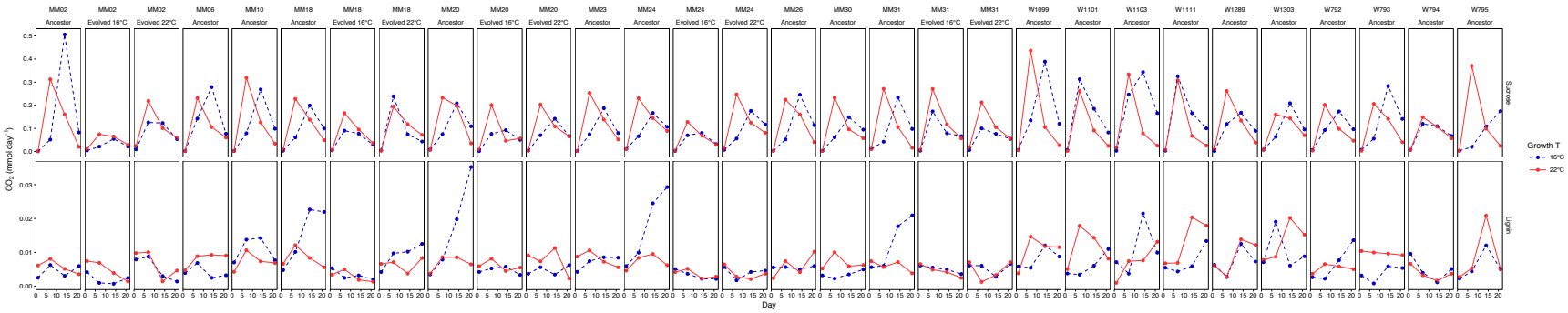

Fig. S1. Strain respiration over time. Respiration rate is shown at each growth temperature for ancestral and evolved strains on sucrose and lignin.

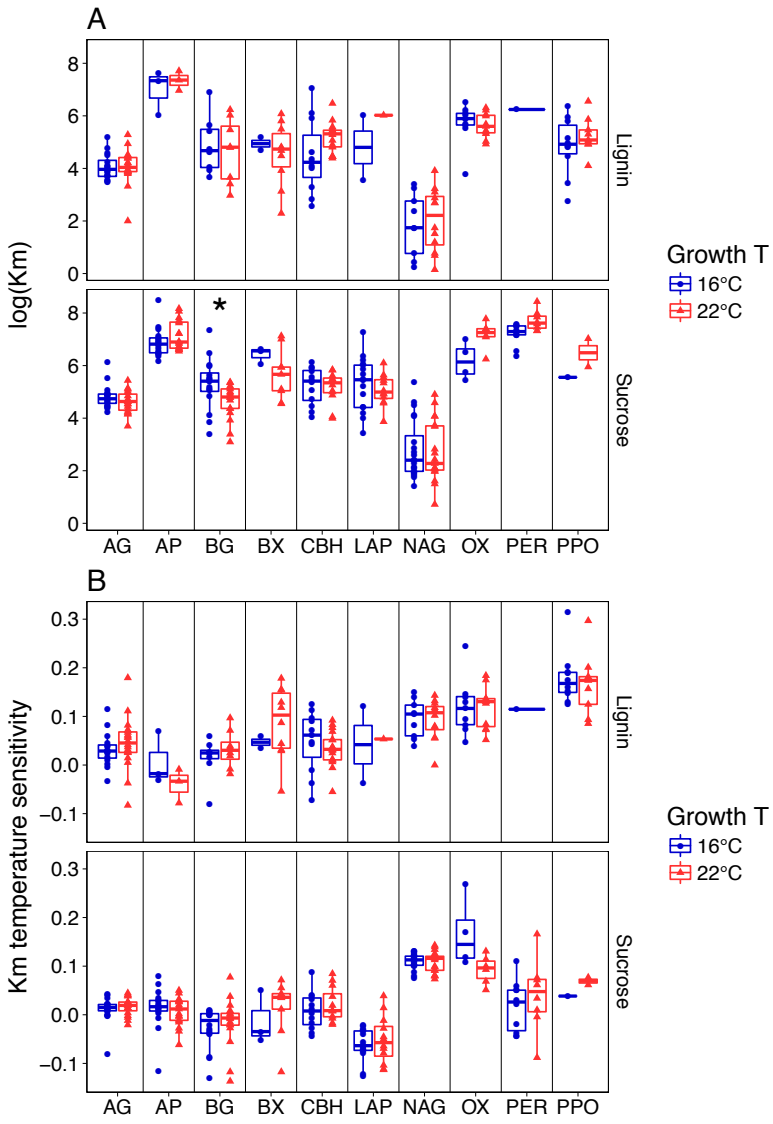

Fig. S2. (A) Enzyme log(Km) (computed at 16ºC) and (B) Km temperature sensitivity. Strains were grown on lignin or sucrose for 21 d at 16ºC versus 22ºC. Asterisks denote significant growth temperature effect ($P < 0.05$). Boxplots include median (central line), interquartile range (box), and data range without outliers (whiskers). AG = α-glucosidase; AP = acid phosphatase; BG = β-glucosidase; BX = β-xylosidase; CBH = cellobiohydrolase; LAP = leucine-aminopeptidase; NAG = *N*-acetyl-β-D-glucosaminidase; OX = total oxidase; PER = peroxidase; PPO = polyphenol oxidase.