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Thermal adaptation of heterotrophic soil respiration in laboratory microcosms

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Abstract

Respiration of heterotrophic microorganisms decomposing soil organic carbon releases carbon dioxide from soils to the atmosphere. In the short term, soil microbial respiration is strongly dependent on temperature. In the long term, the response of heterotrophic soil respiration to temperature is uncertain. However, following established evolutionary trade-offs, mass-specific respiration (R_{mass}) rates of heterotrophic soil microbes should decrease in response to sustained increases in temperature (and vice-versa). Using a laboratory microcosm approach, we tested the potential for the R_{mass} of the microbial biomass in six different soils to adapt to three, experimentally imposed, thermal regimes (constant 10, 20 or 30 °C). To determine R_{mass} rates of the heterotrophic soil microbial biomass across the temperature range of the imposed thermal regimes, we periodically assayed soil subsamples using similar approaches to those used in plant, animal and microbial thermal adaptation studies. As would be expected given trade-offs between maximum catalytic rates and the stability of the binding structure of enzymes, after 77 days of incubation R_{mass} rates across the range of assay temperatures were greatest for the 10 °C experimentally incubated soils and lowest for the 30 °C soils, with the 20 °C incubated soils intermediate. The relative magnitude of the difference in R_{mass} rates between the different incubation temperature treatments was unaffected by assay temperature, suggesting that maximum activities and not Q_{10} were the characteristics involved in thermal adaptation. The time taken for changes in R_{mass} to manifest (77 days) suggests they likely resulted from population or species shifts during the experimental incubations; we discuss alternate mechanistic explanations for those results we observed. A future research priority is to evaluate the role that thermal adaptation plays in regulating heterotrophic respiration rates from field soils in response to changing temperature, whether seasonally or through climate change.

Keywords: acclimation, adaptation, carbon cycling, climate change, climate warming, CO_2, microbial community, soil respiration, temperature, thermal biology

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Introduction

The respiration of heterotrophic microorganisms decomposing soil organic carbon (SOC) releases carbon dioxide (CO_2) from soils to the atmosphere. In the short term, rates of SOC decomposition and associated soil microbial respiration are strongly dependent on temperature (Kirschbaum, 2006). In the long term, however, the response of heterotrophic soil respiration to temperature is uncertain (Denman *et al.*, 2007). For example, despite expectations of sustained increases in soil respiration in response to increased temperatures (Denman *et al.*, 2007), in field studies elevated soil respiration rates under experimental warming are relatively short-lived (Jarvis & Linder, 2000; Oechel *et al.*, 2000; Luo *et al.*, 2001; Rustad *et al.*, 2001; Melillo *et al.*, 2002; Eliasson *et al.*, 2005). The explanations proposed for this ephemeral response include the depletion of fast-cycling, SOC pools and thermal acclimation of microbial respiration (Kirschbaum, 2004; Eliasson *et al.*, 2005; Knorr *et al.*, 2005). Theoretical models (Kirschbaum, 2004; Eliasson *et al.*, 2005; Knorr *et al.*, 2005) attribute the response of soil respiration to sustained warming to the first mechanism (i.e. 'substrate depletion'), for which there is indirect experimental support (Hartley *et al.*, 2007). When both substrate depletion and thermal acclimation have been directly tested for in experimentally warmed,

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field soils, there is evidence that both phenomena occur (Bradford *et al*., 2008, 2009).

The mechanism of substrate depletion, and not thermal acclimation, to explain soil respiration responses to experimental warming has been argued to be consistent with our understanding of SOC dynamics (Kirschbaum, 2004). Similarly, it has been argued that it is unclear as to what advantage microbes would gain from acclimation to higher temperature regimes (Hartley *et al*., 2008). These arguments appear in conflict with the established evolutionary trade-offs that occur during biochemical adaptation to higher and lower temperature regimes (for a review see Hochachka & Somero, 2002). For example, enzymes adapted to higher temperatures typically have reduced conformational flexibility as a result of their greater rigidity, which is required to maintain their binding structure. This loss of flexibility is associated with a decrease in their catalytic rates (i.e. k_{cat}; the rate at which substrate is converted to product per active site per unit time). In cooler environments the selective force of temperature is not on maintaining binding structure but rather k_{cat} directly (this is the common observation that lower temperatures limit reaction rates) and so there is selection for greater conformational flexibility. This means that if cold- and warm-adapted enzymes are compared at a temperature intermediate to which they are adapted then k_{cat} will be higher for the cold-adapted enzymes (Hochachka & Somero, 2002). Much of the fundamental research on biochemical adaptation to temperature has focused on enzymes of respiratory pathways. Given the evolutionary trade-offs between k_{cat} and enzyme conformational stability (Hochachka & Somero, 2002; Clarke, 2004), physiological adjustment of mass specific respiration (R_{mass}) rates to the ambient thermal environment is a common property of organisms across all three kingdoms of life (Hochachka & Somero, 2002; Atkin & Tjoelker, 2003; Körner, 2003; Lange & Green, 2005; Heinemeyer *et al*., 2006; López-Gutiérrez *et al*., 2008; Malcolm *et al*., 2008; Tjoelker *et al*., 2008). Commonly, organisms can express isoenzymes with different temperature sensitivities under different thermal conditions (Hochachka & Somero, 2002) to physiologically adjust their R_{mass} rates (i.e. respiration rates per unit biomass) under sustained temperature change.

The trade-offs between maintaining binding structure vs. conformational flexibility set up the expectation that the R_{mass} rates of soil microbes decomposing SOC will adapt to the ambient temperature regime. Evidence suggests that the R_{mass} rates of the heterotrophic, soil microbial biomass in field soils do decrease under experimental warming and also track seasonal temperature changes (Bradford *et al*., 2008, 2009). What is not known is whether temperature is the direct driver of these changes or whether other factors which vary seasonally, and in response to experimental warming, function as proximal drivers (Bradford *et al*., 2008, 2009).

Physiological research on thermal adaptation is commonly conducted on whole organisms, organelles, or enzymes (Hochachka & Somero, 2002; Atkin & Tjoelker, 2003; Clarke, 2004). Given that heterotrophic soil respiration is the product of the activities of multiple individuals and species, adjustment of R_{mass} rates of the soil microbial biomass to different ambient temperatures could arise through physiological adjustment of individuals, evolutionary adaptation of populations or species turnover. Given these multiple mechanisms, use of the term 'acclimation' in the debate about soil respiration responses to elevated temperatures has recently been criticised (Hartley *et al*., 2008). Acclimation usually refers to physiological adjustments of individuals (or organs) in response to a single environmental factor under laboratory conditions. The term 'acclimatization' is approximately equivalent but is used in field studies, where causation is often difficult to ascribe to a single variable (Hochachka & Somero, 2002; Körner, 2003). Henceforth, we refer to the adjustments of R_{mass} rates to temperature, of soil microbes decomposing SOC, as 'thermal adaptation'. Such a broad use of this term has been recommended in the physiological literature (Hochachka & Somero, 2002) to encompass the suite of responses organisms exhibit to altered thermal regimes. We suggest that the term can also encompass shifts from cold- to warm-adapted microbial populations (see Bradford *et al*., 2008), as observed in aquatic systems in response to seasonal temperature change (Hall & Cotner, 2007; Hall *et al*., 2008). Notably, other changes in microbial communities in warmed soils that may or not occur in direct response to temperature change could also affect R_{mass} rates. For example, a shift from bacterial to fungal-dominated communities would decrease R_{mass} given the expectation that fungi utilize C more efficiently (Hendrix *et al*., 1986). Also, it is possible that enhanced substrate-limitation under warming might lead to declines in C use efficiency (and hence increased R_{mass}), although Steinweg *et al*. (2008) found no evidence for this.

Here, we use a laboratory microcosm approach to experimentally impose three different thermal regimes, on six different soils. These thermal regimes are imposed by incubating the soils for 77 days at 10, 20 or 30 °C. Periodically we assay the soils across the same temperature range to determine R_{mass} rates of the heterotrophic soil microbial biomass. For these assays we use a published method (Bradford *et al*., 2008) based on those approaches used in plant, animal and microbial thermal adaptation studies (e.g. Hochachka &

Somero, 2002; Atkin & Tjoelker, 2003; Malcolm *et al*., 2008; Tjoelker *et al*., 2008). We amend the soils weekly with glucose across the 77 day, experimental incubations to maintain a source of labile C to the soil microbes. Given that direct linkages between microbial community composition and ecosystem processes are unresolved (Fierer *et al*., 2007; Allison & Martiny, 2008; Green *et al*., 2008), we focus on the measurement of process rates and do not determine if treatment effects result from physiological adjustment of individuals, population or species turnover and/or selection of *de novo* mutations. Our overall objective is to test for the potential for the R_{mass} of heterotrophic soil microbes decomposing SOC to adapt to different thermal environments. We test for the time that adaptation of R_{mass} takes and whether temperature is likely a direct driver. Specifically, using periodic assays we test whether adjustments in R_{mass} rates manifest within days to a couple of weeks, as observed for plants, animals and cultured microbes (Hochachka & Somero, 2002; Atkin & Tjoelker, 2003; Malcolm *et al*., 2008; Tjoelker *et al*., 2008), or whether it occurs more on a seasonal timescale, as field observations of R_{mass} rates of the heterotrophic soil microbial biomass suggest (Bradford *et al*., 2008). The controlled, laboratory approach is designed to test whether temperature is likely the direct driver, because the imposed thermal regimes can be disassociated from many of the other effects (e.g. changing soil moisture) of experimental warming of field soils and/or seasonal temperature change. We do, however, acknowledge that the soil environment and resident communities are highly complex, even in a simplified laboratory design, and so evaluate alternate explanations for our results in the 'Discussion'.

Materials and methods

Study soils

Six soils were used in the study and their general characteristics and the sites from which they were sampled are reported in Table 1. All soils are mineral-based but three are more organic-rich (CH, HOC, HOH) and three more organic-poor (CL, HMC, HMH; see Table 1). For the two soils sampled from the Coweeta Long-Term Ecological Research (LTER) site there was little, humified organic material overlying the mineral soil. Specifically, a deep humified organic layer does not develop at the two sites sampled and so the O horizon was scraped away before sampling the 0–10 cm depth of the surface mineral soils. For the four soils from the Harvard Forest LTER, the litter layer was brushed away and the organic rich surface horizons were sampled, and then the 0–10 cm of the surface mineral horizons below them (Table 1). Soils were passed through a 2 mm sieve to remove roots and stones and immediately transported on ice to the University of Georgia. Note that we selected our soils based on two criteria. First, we have observed (Bradford *et al*., 2008) decreases in R_{mass} rates in the four Harvard Forest LTER soils in response to either seasonal or experimental warming; yet these responses occurred in concert with other changes in the soils (e.g. labile C pool size). A laboratory approach therefore permits a degree of experimental control not afforded in field studies, which aids in discerning causation. Second, given our expectation that the evolutionary trade-offs underlying adjustment of R_{mass} rates to the ambient thermal regime are overarching, we included two additional soils from Coweeta LTER to introduce distinct soil microbial communities. Harvard Forest LTER is in the northeastern United States and Coweeta LTER in the southeastern United States. We did not replicate each soil but rather used each soil as an experimental replicate (giving $n = 6$). This ensured the study design remained feasible (see below for analysis numbers) but provided a conservative test of our expectations. That is, if the mechanisms were truly overarching then only then would we detect a significant effect (i.e. all replicates responding similarly) of the recent thermal regime on R_{mass} rates.

Experimental incubations

In the laboratory, each of the six soils were divided into three 200 g dry weight equivalent aliquots and placed in 'moisture-retention', plastic food containers. This gave 18 containers of soil (i.e. 6 soils $\times$ 3 aliquots). One aliquot of each soil was placed at 10, 20 or 30 °C. Before starting the experimental incubations, soil moisture was adjusted to 50% water-holding capacity (WHC), either by air-drying to the desired WHC or through addition of deionized H_2O. WHC was determined by saturating a subsample of soil in a Whatman #1 filter paper placed in a glass funnel, and then permitting the water to drain for 2 h before determining the gravimetric soil moisture content (for 100% WHC) by drying for 24 h at 105 °C. Determination of the gravimetric moisture contents of the sieved soils, without the water saturation step, permitted calculation of the WHC of the sampled soils and hence the amount of drying or wetting required to adjust the soils to 50% WHC. This WHC is considered to fall within the optimum (or unstressed) range (e.g. Fierer & Schimel, 2002) of 50–70% for laboratory incubations of soils (Paul *et al*., 2001).

To maintain the moisture content of each soil at 50% WHC, and to provide a source of labile C substrate across the 77 day incubations, glucose was added in solution at an amount of 0.84 mg C g dry wt. soil^{-1} every

Table 1 Description of soils and the sites they were sampled from

Site name	CH	CL	HOC	HOH	HMC	HMH
Lat., Long.	35°01′N, 83°27′W	35°02′N, 83°26′W			42°30′N, 72°10′W	
Elevation (m)	1347	795			270–420	
Mean monthly air temp. – high (°C)	≈ 20	≈ 20			≈ 20	
Mean monthly air temp. – low (°C)	≈ 5	≈ 5			≈ −6	
MAP (mm)	≈ 1900	≈ 1900			≈ 1080	
Vegetation type	Northern hardwoods	Cove hardwoods			Northern hardwoods	
Dominant plant spp.	*Betula alleghaniensis*, *Liriodendron tulipifera*, *Quercus rubra*	*L. tulipifera*, *Q. rubra*, *Tsuga canadensis*, *Carya* spp.			*Quercus veluntina*, *Acer rubrum*, *Betula papyrifera*, *Acer pensylvanicum*	
Soil type	Coarse-loamy, mixed, mesic Typic Haplumbrepts	Fine-loamy, mixed, mesic Typic Haplumbrepts			Fine-loamy, mixed, mesic Typic Dystrochrept	
Moisture regime	Mesic	Mesic	Mesic			
Soil total %C	9.9	5.5	17.2	16.8	5.5	5.3
Soil total %N	0.7	0.3	0.8	0.8	0.3	0.3

Two soils were sampled from the Coweeta Long-Term Ecological Research (LTER) site at high (CH) and low (CL) elevation. The other four soils were sampled from the Harvard Forest LTER site in control (HOC, HMC) and heated plots (HOH, HMH), from organic-rich (HOC, HOH) and organic-poor (HMC, HMH) horizons, of the Long-Term Soil Warming Experiment (Peterjohn *et al*., 1994; Melillo *et al*., 2002).
Lat., latitude; Long., longitude; temp., temperature; MAP, mean annual precipitation.

7 days. Preliminary work showed that this glucose amendment rate prevented soil respiration rates from exponentially declining (as observed when soils are incubated without labile C amendment; Paul *et al*., 2001) but was low enough to ensure that labile C did not substantially accumulate across the incubations (see 'Discussion'). We used glucose because it is a common constituent of fast-cycling, SOC pools, the turnover of which fuel much of heterotrophic soil respiration (Gu *et al*., 2004; van Hees *et al*., 2005, Bengtson & Bengtsson, 2007).

Respiration and microbial assays

We tested for thermal adaptation by conducting short-term assays, following similar approaches as in plant, animal and cultured microbe studies (e.g. Hochachka & Somero, 2002; Atkin & Tjoelker, 2003; Malcolm *et al*., 2008; Tjoelker *et al*., 2008). These short-term assays permitted measurement of R_{mass} before the individuals (or in our case communities) could adapt to the assay measurement temperatures. The assays involved placing soils with water or glucose for 24 h simultaneously at 10, 20 and 30 °C before determination of headspace CO_2 concentrations. These measurement temperatures spanned the range experienced by the soils across the 77 day incubation period of our study. We assayed the soils following 1, 7, 21, 50 and 77 days of incubation. At 77 days, we included 15 and 25 °C in the range of assay measurement temperatures.

For each assay we used 50 mL centrifuge tubes, with caps modified for gas analysis. Each tube contained 2 g dry wt. equivalent soil, subsampled from each of the six soils (Table 1), maintained at each of the three, experimental incubation temperatures. After glucose or water addition, assay soils were mixed with the solutions using dissecting needles. Tubes were then capped and flushed with CO_2-free air to remove CO_2 from the headspace before placement at the measurement temperatures. Glucose solution was added to soils at a dose of 42.5 mg C g dry soil^{-1}. Dose–response experiments confirmed that the dose amount was in excess of demand across the assays, which avoided the confounding effect of substrate-limitation when assessing the response of enzyme-catalysed reactions to temperature (Davidson *et al*., 2006). Glucose was used as a representative monosaccharide and is a dominant constituent of rhizodeposited C (van Hees *et al*., 2005), which itself supports much of heterotrophic soil respiration (van Hees *et al*., 2005).

Addition of water or glucose solution raised the WHC from 50% (at which the soils were maintained during the 77 day incubations) to 65%. Both values are within the range deemed favourable for microbial activity (Paul *et al*., 2001). Headspaces were sampled (5 mL overpressurization followed by a 5 mL sample) using a gas-tight syringe (SGE, Vic., Australia) and CO_2 concentrations determined using infrared gas analysis (IRGA). The IRGA (LI-7000, LI-COR, Lincoln, NE, USA) was connected to a system designed to permit injection onto a 1 mL sample loop, the contents of which was transferred to the IRGA using a gas sample valve (VICI Valco Instruments Co Inc., Houston, TX, USA) connected to a CO_2-free air stream. Concentrations of headspace samples were calculated from peak areas by comparison with a CO_2 in air reference standard (1990 μL L^{-1}, Air Liquide America Speciality Gases LLC, Plumsteadville, PA, USA). All assays were performed in duplicate, giving 216 tubes for the measurements on Days 1, 7, 21 and 50 of the incubations, and 360 tubes on Day 77. That is, 6 soils × 3 incubation temperatures × 3 measurement temperatures × 2 solution types × 2 repeats = 216. At 77 days the additional 144 tubes came from inclusion of the two additional measurement temperatures (15 and 25 °C).

Our approach permitted us to estimate potential soil respiration rates (water only assays), potential respiration rates without glucose substrate-limitation (glucose addition assays) and also R_{mass} rates (by dividing respiration rates from the glucose addition assays by microbial biomass values) (see Bradford *et al*., 2008). Henceforth, we refer to these different expressions of soil respiration as Soil *R*, Substrate *R* and Substrate R_{mass}, respectively. Note that potential caveats introduced by relationships between the microbial biomass method and Substrate *R* were not encountered in this experiment. Specifically, relationships between these two variables were weak (r^2 values between 0.07 and 0.27 for soils assayed at 20 °C) (see Bradford *et al*., 2009; Hartley *et al*., 2009).

To estimate microbial biomass we used a modified substrate-induced respiration (SIR) technique (Fierer *et al*., 2003), which uses autolysed yeast as the substrate. Using the 50 mL centrifuge tube and IRGA method described above, 2 g dry wt. equivalent soil per tube was incubated overnight at 20 °C, before addition of 2 mL of yeast solution (12 g yeast to 1 L H_2O). Soils were then incubated uncapped for 1 h, then capped and flushed with CO_2-free air, and then finally incubated at 20 °C for 5 h. We report SIR biomass as the maximum CO_2 production rates (soil + substrate-derived); no conversion factors are used. The short incubation time in the modified SIR method we used may overcome some of the limitations associated with the method if microbes are actively growing (see Blagodatsky *et al*., 2000). All SIR assays were performed in duplicate.

Statistical analyses

To test for incubation and measurement temperature effects on respiration rates (i.e. Soil *R*, Substrate *R* and Substrate R_{mass}) we used linear mixed-effects modelling. Fixed effects were Incubation Temperature (10, 20 and 30 °C), Measurement Temperature (10, 20 and 30 °C) and Experimental Day (1, 7, 21, 50 and 77). To identify the unit of repeated measurement across time and incubation temperature, Soil Identity (CH, CL, HOC, HOH, HMC, HMH) was used in the random effects structure. Given that Substrate R_{mass} is essentially a ratio, we used a covariate approach (Jasienski & Bazzaz, 1999) to evaluate treatment effects where microbial biomass was used as the covariate and Substrate *R* (not Substrate R_{mass}) as the dependent variable. The more complex covariate model (multiple slopes, multiple intercepts) was a significant improvement on simpler covariate structures and so significance values reported for Substrate R_{mass} are derived from the complex model structure. Given that interactions with Experimental Day were observed for all three respiration rate expressions, we used the described model structure to analyse each experimental day individually.

To evaluate further the significant Incubation Temperature effects on Substrate R_{mass} after 77 days of incubation, we recoded the data file to test whether Substrate R_{mass} rates differed at measurement temperatures that were intermediate between two incubation temperatures. Soils experimentally incubated at 10 °C were coded 'Low' when measured at 15 °C with soils incubated at 20 °C (coded 'High'). We did the same for soils incubated at 10 and 30 °C when measured at 20 °C, and for soils incubated at 20 and 30 °C when measured at 25 °C. This gave us two levels (Low or High) of a new variable termed Incubation2, which was assessed at three levels (15, 20 and 25 °C) of a new categorical variable termed Intermediate. We used the covariate approach described above, with a linear mixed effects model where the fixed effects Incubation2 and Intermediate were permitted to interact. Again, Soil Identity was included in the random effects to identify the spatial unit of repeat. We also used linear regression to evaluate the relationship between experimental incubation temperature and Substrate R_{mass} rates at the measurement temperature (20 °C) intermediate across our range (10 to 30 °C) of experimental incubation temperatures.

For statistical significance we assumed an α-level of 0.05. All statistical analyses were performed in S-PLUS 8.0 (Insightful Corporation, Seattle, WA, USA). Data were tested for assumptions of normality and homogeneity of variance; all data were $\log_e$-transformed to meet these assumptions.

Results

The design of our measurement assays permitted us to assess three different response variables. First, the 'water only' assays permitted estimation of potential soil respiration rates (Soil *R*). For this variable, treatment effects might be caused by glucose substrate-limitation, differences in microbial biomass and/or in R_{mass}. We recognized that glucose substrate-limitation would likely occur because our weekly amendments of glucose were not designed to remove glucose substrate-limitation across the experimental incubations but just to maintain labile C supply (see later). We observed a significant Incubation Temperature effect at Days 21, 50 and 77 (Fig. 1, top five plates). Specifically, across the range of assay temperatures, Soil *R* rates were greatest for the 10 °C incubated soils and lowest for the 30 °C soils. The differences in Soil *R* rates between soils incubated at different temperatures appeared to become more pronounced as the experimental incubations proceeded (Fig. 1). There were no interactions between Incubation Temperature and Measurement Temperature (Incubation × Measurement × Day and Incubation × Measurement: $F_{8,235} = 0.34$, $P = 0.95$ and $F_{2,235} = 0.80$, $P = 0.45$, respectively).

The second response variable (Substrate *R*) is calculated following alleviation of glucose substrate-limitation and therefore treatment effects of incubation temperature can only be caused by differences in microbial biomass and/or in R_{mass}. We observed a statistically significant Incubation Temperature effect at Day 77 only (Fig. 1, middle five plates). At this measurement point, across the range of assay temperatures, Substrate *R* rates were greatest for the 10 °C experimentally incubated soils and lowest for the 30 °C soils. However, although we could resolve a statistically significant effect, the differences in Substrate *R* rates were small (Fig. 1) and so may not have been biologically significant. The relative magnitude of the difference in Substrate *R* rates between the different incubation temperature treatments was unaffected by assay temperature (for Day 77, Incubation × Measurement interaction: $F_{2,43} = 0.57$, $P = 0.57$). Similarly, for the whole dataset there were no interactions between Incubation Temperature and Measurement Temperature (Incubation × Measurement × Day and Incubation × Measurement: $F_{8,235} = 0.24$, $P = 0.98$ and $F_{2,235} = 0.11$, $P = 0.90$, respectively).

For the third response variable (Substrate R_{mass}) treatment effects of incubation temperature can only be caused by adjustments in R_{mass} (either through direct or indirect response to incubation temperature). As with the previous two response variables, at every measurement period the Measurement Temperature

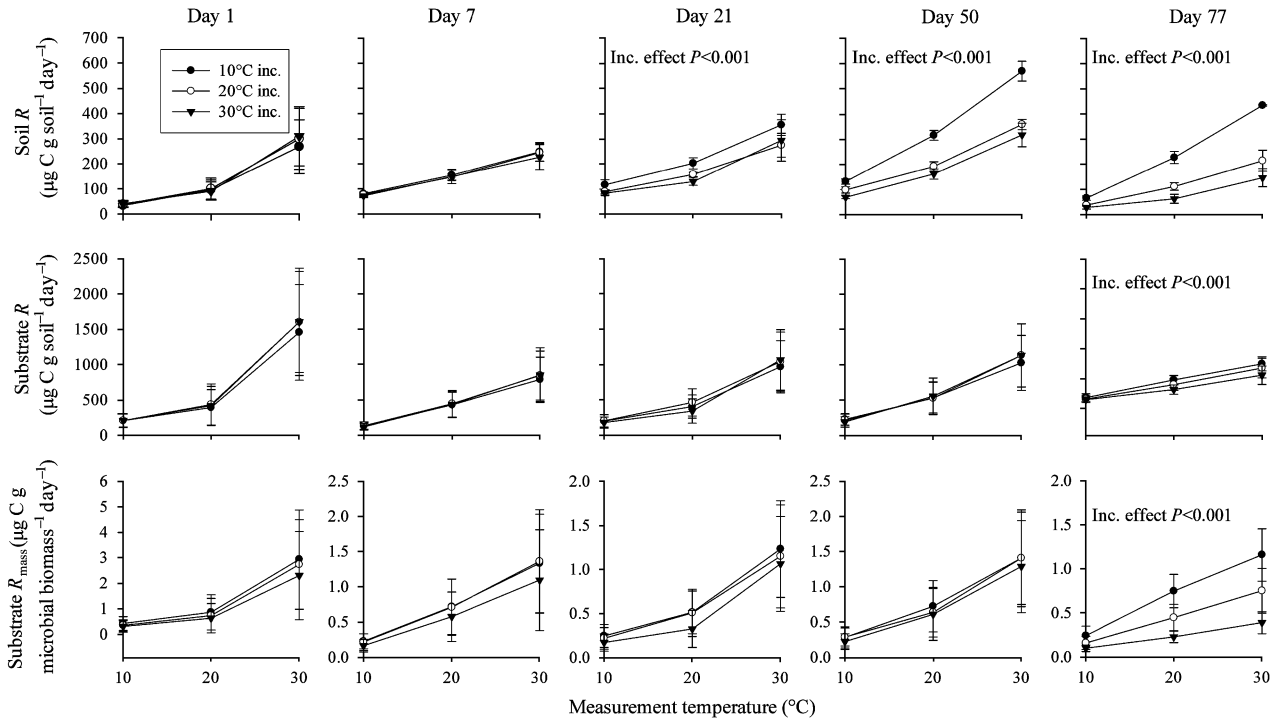

Fig. 1 Soil respiration rates (top five plates), with added glucose substrate (middle five plates) and with added glucose substrate expressed per unit microbial biomass (bottom five plates). Soils were maintained at 10, 20 and 30 °C for 1, 7, 21, 50 and 77 days and at the end of each of these periods assayed across this range of temperatures (i.e. measurement temperature on the *x*-axis). The different mass specific respiration rates (R_{mass}) after 77 days of incubation are consistent with thermal adaptation to lower and higher temperatures. Values are means ± 1 SEM, $n = 6$. Given that Substrate R_{mass} is essentially a ratio, for these values SE were propagated from the errors in the Substrate *R* and substrate-induced respiration microbial biomass data. For each plate, where a significant ($P < 0.05$) incubation effect was observed the significance of this effect is shown. The interactive effects of incubation and measurement temperature were always nonsignificant ($P > 0.05$). Note the changing *y*-axis scales for the Substrate R_{mass} plots.

effect was significant ($P < 0.001$), reflecting the increase in respiration rates with the assay measurement temperature (Fig. 1, lower five plates). We observed a significant Incubation Temperature effect at Day 77 only (Fig. 1, lower five plates). At this measurement point, across the range of assay temperatures Substrate R_{mass} rates were greatest for the 10 °C experimentally incubated soils and lowest for the 30 °C soils, with the 20 °C incubated soils intermediate. The relative magnitude of the difference in Substrate R_{mass} rates between the different incubation temperature treatments was unaffected by assay temperature (for Day 77, Incubation × Measurement interaction: $F_{2,42} = 1.00$, $P = 0.38$). Similarly, for the whole dataset there were no interactions between Incubation Temperature and Measurement Temperature (Incubation × Measurement × Day and Incubation × Measurement: $F_{8,234} = 0.26$, $P = 0.98$ and $F_{2,234} = 0.12$, $P = 0.89$, respectively).

The effect of Incubation Temperature at Day 77 on Substrate R_{mass} rates, shown in Fig. 1 (lower five plates), represents a mean effect for soils that differ markedly in some of their chemical and source site characteristics (see Table 1), as well as microbial biomass (see later). The large error bars on the Substrate R_{mass} means reflect these differences, especially because the errors were propagated from the errors associated with the microbial biomass and Substrate *R* values (see 'Materials and methods'). However, despite the large errors across the mean Substrate R_{mass} values, the same pattern of response (i.e. lower Substrate R_{mass} values for soils experimentally incubated at higher temperatures) was observed for each of the six soils individually. These data are shown in Fig. 2 and are Substrate R_{mass} values for each soil at Day 77 only, and thus correspond with the mean effects shown in the last plate of Fig. 1. Between different soils there were qualitative differences in the Substrate R_{mass} treatment effects, with some soils (e.g. CH) intercepting the *y*-axis at the same point for at least two of the experimental incubation temperatures, and others (e.g. HOC) having distinct intercepts across experimental incubation temperatures (Fig. 2). These differences are indicative of thermal adaptation

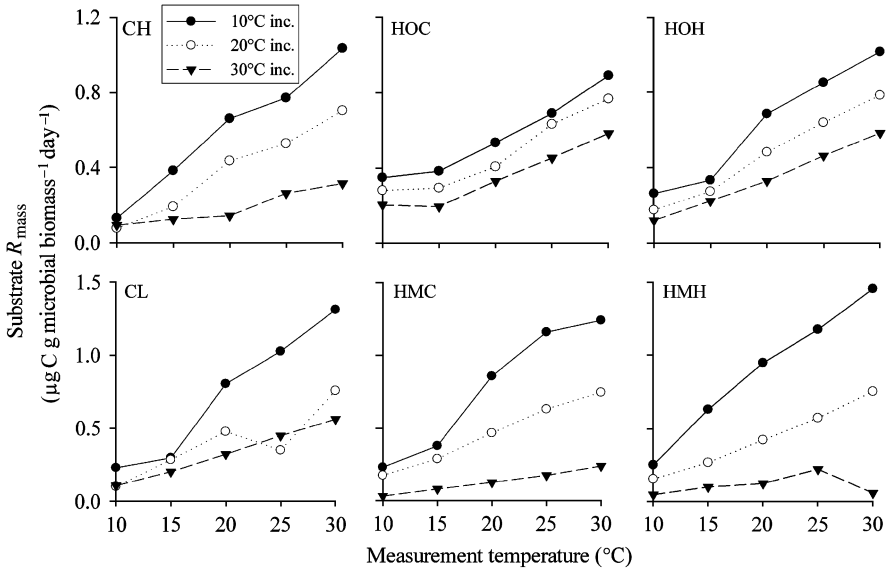

Fig. 2 Soil respiration rates at Day 77 with glucose in excess, expressed as mass specific (per unit microbial biomass) respiration rates (R_{mass}). Each plate shows rates of Substrate R_{mass} for one of six soils, at five measurement temperatures, after incubation at 10, 20 or 30 °C for 77 days. The lower Substrate R_{mass} values for soils incubated at higher temperatures (and vice-versa) are consistent with thermal adaptation. Note the different scales of the *y*-axes for the organic-rich soils (top three plates) compared with the organic-poor soils (bottom three plates). Values are means of two analytical repeats.

phenomena described as type I (i.e. change in temperature sensitivity described by Q_{10}) and II (change in respiration rates with no measurable change in Q_{10}) responses, respectively. See 'Discussion' for further explanation about type I and II adaptation responses.

An 'acid test' of thermal adaptation is to measure metabolic rates of organisms maintained at high and low temperature regimes at an intermediate temperature (e.g. Atkin & Tjoelker, 2003). We used this approach by conducting measurement assays at Day 77 at temperatures intermediate between two of the experimental incubation treatments. For example, soils experimentally incubated at 10 and 20 °C were assayed at a measurement temperature of 15 °C. When the Substrate R_{mass} rates were compared at an intermediate temperature, we observed that the Substrate R_{mass} rates of soils incubated at a 'high' temperature regime were lower, on average, than for soils incubated at a 'low' regime (Fig. 3a). The significant interaction between the low vs. high effect and the intermediate temperature comparison ($F_{2,24}$, $P = 0.037$) probably arose because the relative difference in Substrate R_{mass} was much greater for the soils compared at the measurement temperature of 20 °C than at 15 or 25 °C (Fig. 3a). The low vs. high effect at 25 °C was probably only marginally significant because, while five of the six soils had lower Substrate R_{mass} values when experimentally incubated at 30 as opposed to 20 °C, soil 'CL' did not (Fig. 2). A second 'acid test' for thermal adaptation, commonly used when investigating thermal acclimatization of dark respiration in plant tissues (e.g. Tjoelker *et al.*, 2008), is to regress Substrate R_{mass} rates at an intermediate temperature for individuals maintained at different temperature regimes. Thermal adaptation is associated with a negative relationship between the environmental temperature and Substrate R_{mass}. We observed a negative, linear relationship between Substrate R_{mass} measured at 20 °C and the experimental incubation temperatures (Fig. 3b). The relationship explained 82% of the variation in our Substrate R_{mass} measurements (Fig. 3b).

As would be expected, microbial biomass was greater in the organic-rich than -poor soils and in the majority of the six soils it was greater at Day 1 than at Day 21 (Fig. 4). After this time the microbial biomass remained relatively stable, except for the CH, HMC and HMH soils incubated at 30 °C, which exhibited a marked increase in biomass at Day 77.

Discussion

Our objective was to test the potential for the R_{mass} of heterotrophic soil microbes decomposing SOC to adapt to different thermal environments and to evaluate, if R_{mass} did adapt, the timescales involved. Thermal adaptation of R_{mass} would be expected based on established evolutionary trade-offs associated with biochemical adaptation to higher and lower temperature

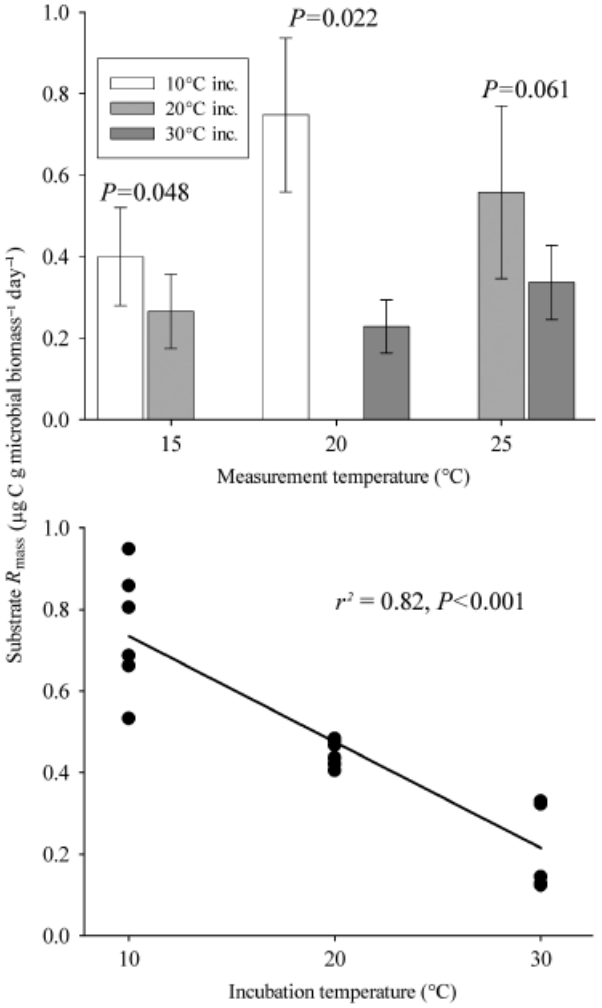

Fig. 3 Soil respiration rates at Day 77 with glucose in excess, expressed as mass specific (per unit microbial biomass) respiration rates (R_{mass}), at intermediate measurement temperatures. (a) Substrate R_{mass} rates at an intermediate measurement temperature (15, 20 or 25 °C) for soils maintained for 77 days at 10 and 20 °C (first pair of bars), 10 and 30 °C (second pair of bars), and 20 and 30 °C (third pair of bars), respectively. Above each pair of bars the significance of the experimental incubation treatment effect is shown. The lower Substrate R_{mass} rates, at the intermediate measurement temperature, for soils maintained at the higher of the two incubation temperatures, is consistent with thermal adaptation. Values are means ± 1 SEM, $n = 6$. Given that Substrate R_{mass} is essentially a ratio, errors were propagated from the errors in the Substrate R and substrate-induced respiration microbial biomass data. (b) Substrate R_{mass} rates at a measurement temperature of 20 °C for soils incubated at 10, 20 or 30 °C for 77 days. Values are means of two analytical repeats for each of six soils from each experimental incubation temperature. The negative relationship between Substrate R_{mass} and experimental incubation temperature, at an intermediate measurement temperature (20 °C), is consistent with thermal adaptation.

regimes (Hochachka & Somero, 2002; Clarke, 2004). For respiratory enzymes, the trade-offs manifest as a reduction in R_{mass} rates for organisms adapted to warmer thermal environments and an increase in R_{mass} rates for organisms adapted to cooler thermal environments (Hochachka & Somero, 2002). After 77 days of incubation at 10, 20 and 30 °C we observed Substrate R_{mass}, at a common temperature, to be highest for soils experimentally incubated at 10 °C and lowest for soils incubated at 30 °C (Fig. 1). This effect was consistent across the six soils (Fig. 2), as would be expected based on fundamental evolutionary trade-offs. Comparisons of Substrate R_{mass} rates at measurement temperatures intermediate between the experimental incubation temperatures, confirmed that soils maintained at higher incubation temperatures had lower Substrate R_{mass} rates (Fig. 3a). Such comparisons at intermediate temperatures, with lower R_{mass} values for warm-adapted individuals, are commonly considered to be a definitive test for thermal adaptation (Hochachka & Somero, 2002; Atkin & Tjoelker, 2003; Körner, 2003). Furthermore, negative relationships between R_{mass} rates of organisms adapted to different thermal regimes, when compared at a common and intermediate measurement temperature, is also often used as evidence of thermal adaptation (Tjoelker *et al*., 2008). The negative relationship we observed for soils assayed at 20 °C, after 77 days of incubation at our three experimental temperatures (Fig. 3b), is therefore further evidence that Substrate R_{mass} adapted to the imposed temperature regimes in our study. Potential indirect effects of substrate availability, and other mechanisms, in the adjustments of R_{mass} across incubation temperatures are evaluated in the ensuing 'Discussion'.

Thermal adaptation of the R_{mass} rates of individuals maintained at different thermal regimes often takes from days to a few weeks (Hochachka & Somero, 2002). However, we did not observe thermal adaptation until after 77 days of incubation (Fig. 1). This may suggest that the changes in Substrate R_{mass} we observed resulted from population, ecotype or species shifts. Seasonal shifts between cold- and warm-adapted microbial communities have been observed in lake ecosystems (Hall & Cotner, 2007; Hall *et al*., 2008). In addition, Bradford *et al*. (2008) observed that daily mean temperature across the previous 9–11 weeks explained the most variation in Substrate R_{mass} rates of field soils assayed using similar approaches to those described here (but see Bradford *et al*., 2009). Alternatively, indirect effects of substrate availability might have played a role (see later). Research on linkages between microbial community structure and the response of R_{mass} of the soil microbial biomass to both temperature and substrate availability may help evaluate the mechanisms

underpinning the time taken for changes in R_{mass} to be observed in our study. However, whether this will be successful is uncertain because direct links between microbial community structure and function are unresolved (Fierer *et al*., 2007; Allison & Martiny, 2008; Green *et al*., 2008).

Heterotrophic soil microorganisms typically experience conditions of C substrate-limitation (Schimel & Weintraub, 2003). Our experiment was designed to replicate these conditions and, at the same time, to try to limit exacerbating the degree of C substrate-limitation in the soils incubated at higher temperatures. It appears that we were able to maintain C substrate-limitation throughout the 77 day incubation. That is, rates of Substrate *R* (assayed with glucose in excess of demand) were markedly greater than Soil *R* rates (assayed with water only) at the same experimental day (Fig. 1). However, there was evidence that the degree of C substrate-limitation differed between soils incubated at different temperatures at Days 21, 50 and likely also Day 77. A 'back-of-the-envelope' calculation, taking the average Soil *R* rate across Days 7 to 77 (i.e. after the first weekly glucose amendment), suggests that across the experiment the soils incubated at 10 °C had a small, net gain of labile C, those at 20 °C a small, net loss of labile C and those at 30 °C a net loss of about twice as much C as was added as glucose. That is, the 11 weekly additions of 0.84 mg C g^{-1} soil were equivalent to 9240 μg C g $soil^{-1}$ across the 77 day incubation and the mean Soil *R* rates for soils incubated at 10, 20 and 30 °C were 98, 152 and 246 μg C g $soil^{-1}$ day^{-1}, respectively. Total respiration over 77 days is then 7521, 11 692 and 18 910 μg C g $soil^{-1}$, respectively. Decreases in readily available C pools have been shown theoretically (Kirschbaum, 2004; Eliasson *et al*., 2005; Knorr *et al*., 2005) and experimentally (Bradford *et al*., 2008) to occur in warmed soils. Although it seems that it is the turnover of these pools, and not their concentrations, that are most relevant to soil respiration dynamics (Bengtson & Bengtsson, 2007), depletion of the pool sizes could be inferred to influence the degree of substrate limitation for microbes. This does seem likely from Day 21 in our incubations and we consider below how this might affect our mechanistic interpretation of the impacts of the thermal environment on R_{mass}.

Given that from Day 21 glucose substrate-limitation appeared to be greater for soils incubated at higher incubation temperatures, we cannot definitively identify whether glucose substrate-limitation and/or higher temperature was the proximal cause of the Incubation Temperature effect on Substrate R_{mass} rates at 77 days. This is especially true for the soils incubated at 30 °C, which had a substantial net loss of C compared with soils incubated at lower temperatures. Certainly, there is the potential for C substrate-limitation to decrease R_{mass} rates (see Steinweg *et al*., 2008) and so the comparison between those soils incubated at 30 °C and the other incubation temperatures might be influenced by the different 'substrate environments'. There is a clear need for studies that factorially modify temperature and substrate regimes to evaluate the potential role of these factors in the adjustment of R_{mass} rates.

The possibility of different substrate availability may be less of an issue for comparisons between soils incubated at 10 and 20 °C, given that their net change in C was much less than for the 30 °C incubated soils. Notably, Substrate *R* rates were more or less biologically equivalent (Fig. 1, middle five panels) and there were no marked declines in microbial biomass (Fig. 4). As the Substrate *R* assays were performed with glucose in excess of demand, this suggests that other forms of C, as well as nutrients such as nitrogen, were not differentially available between soils incubated at different incubation temperatures to an extent that they might have influenced R_{mass} rates. That is, differential limitation of C (other than glucose) and/or nutrients does not appear to be a plausible explanation for the changes we observed in Substrate R_{mass} rates. In addition, the slight accumulation of labile C in the soils incubated at 10 °C does not appear to have changed the soil environment (e.g. through solute potentials) to an extent that it directly affected the potential activity of the total microbial community (i.e. Substrate *R*; Fig. 1) or its biomass (see Fig. 4). What is puzzling is the apparent spike in microbial biomass at Day 77 for three soils incubated at 30 °C (Fig. 4). In experimentally warmed field soils, decreases in microbial biomass accompanied C substrate depletion, as well as decreases in Substrate R_{mass} rates in mineral soils (Bradford *et al*., 2008). In contrast, we observed lower R_{mass} rates to be associated with higher microbial biomass, at least for soils incubated at 30 °C (compare Figs 1 and 4). Soils incubated at 20 °C did not appear to have markedly different biomass values at Day 77 (Fig. 4), although their Substrate R_{mass} rates did differ to soils incubated at 10 °C (Fig. 1). Together, the results of Bradford *et al*. (2008) and those presented here suggest that changes in R_{mass} rates are not necessarily linked to changes in microbial biomass. Future research, however, is required to disentangle conclusively the direct roles of temperature and C substrate availability in affecting the R_{mass} rates of the heterotrophic soil microbial biomass.

Atkin & Tjoelker (2003) defined two functional forms of thermal adaptation for plant dark R_{mass}. Type I adaptation involves changes in Q_{10} values. These changes manifest as similar R_{mass} rates at measurement temperatures near the low end of the thermal niche of cold- and warm-adapted individuals and gradual

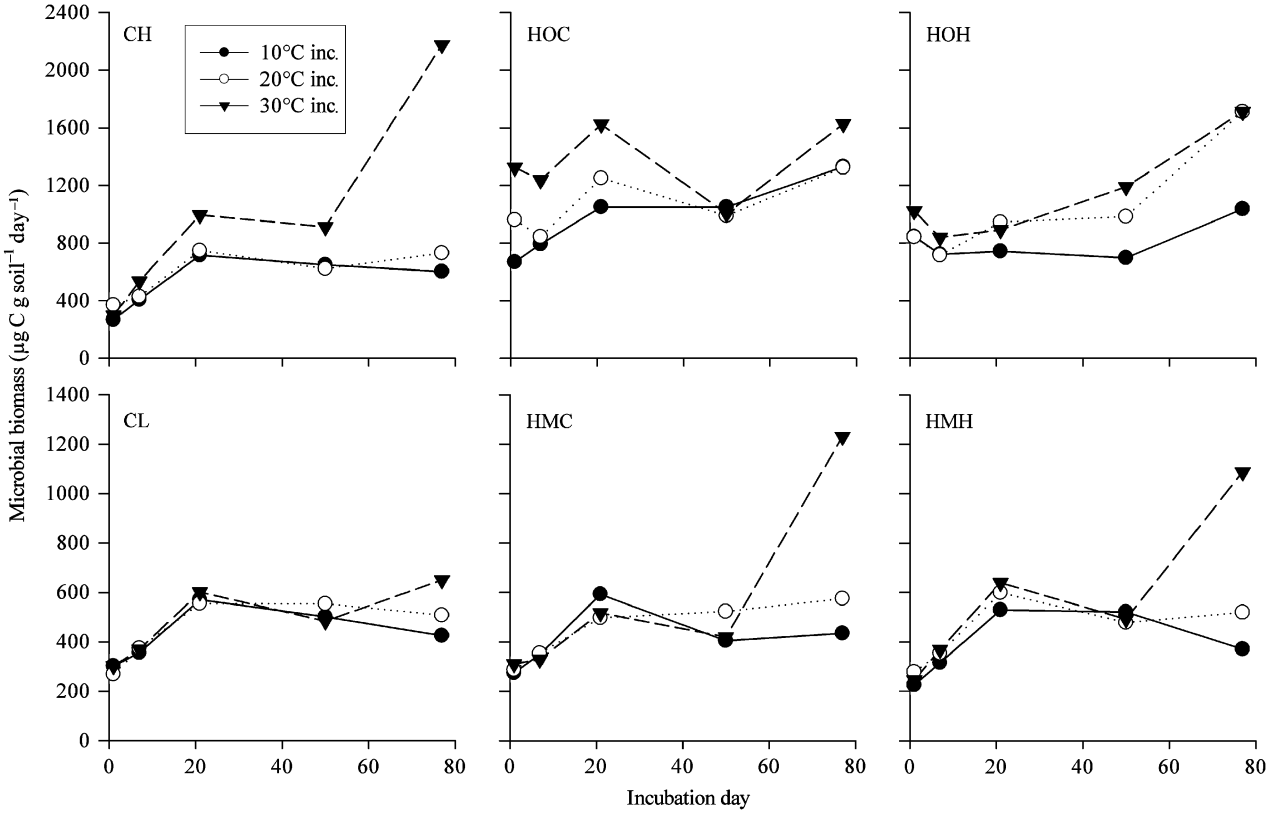

Fig. 4 Soil microbial biomass across the 77 day incubation period. Each plate shows microbial biomass for one of six soils after incubation at 10, 20 or 30 °C for 1, 7, 21, 50 and 77 days. Microbial biomass was measured using substrate-induced respiration. Values are expressed as raw respiration rates; no microbial biomass conversion factors have been applied. Note the different scales of the *y*-axes for the organic-rich soils (top three plates) compared with the organic-poor soils (bottom three plates). Values are means of two analytical repeats.

separation of R_{mass} rates as measurement temperature increases (higher values for cold-adapted individuals). Type II adaptation manifests as lower R_{mass} rates across the entire thermal regime for warm- vs. cold-adapted individuals; no change in Q_{10} is necessary. Visualized graphically (measurement temperature vs. R_{mass}), type I results in the same intercept, different slopes and type II as same slope, different intercepts. Although we observed patterns consistent with both types I and II adaptation across the different soils (Fig. 2), the mean effect across the soils was of different intercepts and similar slopes (i.e. type II; Fig. 1). That is, the nonsignificant interactions between incubation and measurement temperature (see 'Results') suggested that the slopes for soils incubated at the different temperatures were not statistically different. The significant main effect of incubation temperature was indicative of significantly different intercepts (Fig. 1). Davidson *et al.* (2006) argued that research on the temperature sensitivity of soil respiration needs to move beyond discussions of Q_{10}; our findings of statistically different intercepts but similar slopes adds empirical support to this recommendation. Future work should, however, assess whether the different forms of thermal adaptation (type I or II) we observed for different soils is replicable for each soil type. If it is then Q_{10} may be an important factor to consider for at least some soil types when thinking about their R_{mass} rates under warmer thermal regimes.

There are questions as to the extent our results can be extrapolated from the laboratory to SOC dynamics in the field. First, heterotrophic soil microorganisms typically experience conditions of C substrate-limitation (Schimel & Weintraub, 2003). Our Substrate R_{mass} assays necessitated removing glucose limitation given the confounding effects of substrate-limitation on the apparent temperature sensitivity of respiration (Davidson *et al.*, 2006). Without including these confounding effects, we cannot conceive how to measure R_{mass} rates of the soil microbial biomass under conditions of C substrate-limitation. Nevertheless, to extrapolate our findings to the field may require such methodologies to be developed. Second, our experimental design involved substantial soil disturbance, and as opposed to observing

Substrate R_{mass} rates either increase or decline compared with starting conditions, we first observed them decline overall and then separate out experimentally based on incubation temperature. Given that Bradford *et al*. (2008) observed lower Substrate R_{mass} rates with experimental warming and higher seasonal temperatures, the disturbance associated with the current study probably did not significantly influence the qualitative outcome of our test of thermal adaptation. Third, weekly amendment of glucose likely selected for microbial communities that differed from the soils at the start of the experiment (e.g. Hanson *et al*., 2008) and future work should use both different substrates and quantify changes in microbial community composition. This may be especially important for determining those processes that contributed to the change in R_{mass} we observed. For example, we cannot elucidate whether it was through a physiological response of the original community, or whether there were other processes such as turnover in species composition that might result in altered R_{mass} rates (e.g. such as switch between bacterial and fungal-dominance; see Hendrix *et al*., 1986). Fourth, pronounced changes in R_{mass} were only detected at Day 77 and future work should evaluate whether this finding is maintained across longer time-scales. Lastly, our work demonstrates only that intracellular, respiratory activities of soil microbes display thermal adaptation (or at least that Substrate R_{mass} adapts to different temperatures across medium-term incubations). The fundamental evolutionary trade-offs between temperature sensitivity and enzyme stability do suggest that extracellular enzymes decomposing more recalcitrant SOC should also thermally adapt. This remains to be empirically tested, although there is some evidence the activities of these enzymes do track ambient temperature (e.g. Fenner *et al*., 2005). What is certain is that it is the response of these extracellular enzymes to temperature, and not respiration per se, that will be critical in determining solubilization rates of the large pools of SOC with intermediate turnover times, and hence potential feedbacks between climate warming and atmospheric CO_2 concentrations.

The question as to whether heterotrophic soil microbial respiration adapts to ambient temperature challenges the core of our understanding of linkages between microbial and ecosystem ecology. Despite arguments to the contrary (Hartley *et al*., 2008), well-established evolutionary trade-offs in controls on metabolic rates suggest that R_{mass} rates should be lower for organisms adapted to higher temperature regimes and vice-versa (Hochachka & Somero, 2002). Our work demonstrates that, for soils sampled from hardwood forests in the north- and southeastern United States, Substrate R_{mass} rates of the microbial biomass when measured under conditions of excess glucose substrate availability, follow the expectations of established biochemical trade-offs. An important research priority is to evaluate the role that thermal adaptation plays in regulating heterotrophic respiration rates from field soils in response to changing temperature, whether seasonally or through climate change.

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