Reviewer 1

The article of Adekanmbi and coauthors is aiming to set up an interesting comparison between extracellular and intracellular enzymes and evaluate their temperature sensitivity after being exposed to distinct temperatures for 60 days. However, the authors need to better justify the use of glucose-induced respiration as a proxy for intracellular enzymes. Because the glucose-induced respiration will be the result of various processes and also ultimately depends on the microbial community growth efficiency. While the beta-glucosidase and chitinase activities are capturing only the activity of these enzymes. So, making the comparison between extracellular and "intracellular" enzymes becomes difficult in my understanding. Moreover, it is important to remember that the production of extracellular enzymes will also result in CO2 production. I am concerned that authors' experimental design might not allow to separate between intracellular and extracellular enzymes. Instead of referring to intracellular enzymes authors could refer to "intracellular activity" or "intracellular processes" related to SOM decomposition. This should help to avoid confusion. If authors think that the design allow to make the comparison between extracellular and "intracellular" enzymes they should add an explanation and references to justify their choice.

Nevertheless, I think that the data collected by the authors is valuable and is a good contribution to the field of soil ecology and to the EGU community. It could be interesting to evaluate if the respiration temperature sensitivity and extracellular enzyme temperature sensitivity are coupled or not (are they correlated?). It is also interesting to observe how distinct the two extracellular enzymes responded to the increase in temperatures. I think the authors did a good job in their discussion section.

It is not very clear why authors used a distinct range of temperatures to evaluate the enzyme activation energy for the respiration and extracellular enzymes. Authors could clarify this choice.

Overall, the paper is very well written and is citing the relevant literature in this topic.

We thank Reviewer 1 for their positive and constructive comments on the manuscript which we address below.

Main Comments requiring response:

1. **The authors need to better justify the use of glucose-induced respiration as a proxy for intracellular enzymes. Instead of referring to intracellular enzymes authors could refer to "intracellular activity" or "intracellular processes" related to SOM decomposition.**

We agree with this comment (also made by reviewer 2) about the over-simplification resulting from us referring to 'intracellular enzyme activity' and propose that we revise the manuscript (including the title) so that we refer to 'intracellular metabolic processes' when speaking generally and to 'glucose-induced respiration' when speaking specifically about our results.

2. **It could be interesting to evaluate if the respiration temperature sensitivity and extracellular enzyme temperature sensitivity are coupled or not (are they correlated?).**

Thank you for this suggestion which we have explored using the Q10 data determined over the different temperature ranges. The analysis, however, revealed no relationships between either basal respiration or glucose-induced respiration and the potential extracellular activities at any of the Q10 ranges tested (please see Table). We assume the main interest here is to examine whether the temperature sensitivity of basal respiration is a function of extracellular enzyme temperature sensitivity, given that extracellular activity is usually expected to be rate-limiting to the respiration of monomers. This lack of relationship is likely due to the nature of the extracellular enzyme assays which determine potential activity (rather than in situ activity) and therefore likely do not estimate the actual rate of production of monomeric substrates for subsequent respiration. Because of the reliance on potential activity and that the correlation analysis did not reveal any possible coupling between intracellular and extracellular processes, we would prefer not to include this evaluation in revisions to our manuscript.

Table 1. Pearson correlation coefficients for (a) $Q10_{5\text{-}10}$ (b) $Q10_{15\text{-}26}$ and (c)$Q10_{26\text{-}37}$ between basal respiration (BR), Glucose-induced respiration (GIR), potential beta-glucosidase activity and potential chitinase activity. * denotes significant ($p<0.05$) correlations (n=12).

(a) $Q10_{5\text{-}10}$	BR	GIR	B-Gluc	Chitinase
BR				
GIR	0.758*			
B-Glucosidase	-0.135	-0.310		
Chitinase	-0.135	-0.043	0.609*	
(b) $Q10_{15\text{-}26}$				
BR				
GIR	-0.143			
B-Gluc	0.180	0.013		
Chitinase	-0.084	0.118	-0.133	
(c) $Q10_{26\text{-}37}$				
BR				
GIR	0.190			
B-Gluc	0.068	0.333		
Chitinase	0.034	-0.095	-0.637*	

3. **It is not very clear why authors used a distinct range of temperatures to evaluate the enzyme activation energy for the respiration and extracellular enzymes. Authors could clarify this choice.**

We found that the relationship between assay temperature and reaction rate was non monotonic. However, Arrhenius assumes that reaction rates rise monotonically with temperature and thus does not take in to account the typical unimodal response (discussed in the second paragraph in the discussion) due to declines in activity and thermal denaturation at higher temperatures. We therefore chose to calculate Ea from Arrhenius plots done over the range in which rate increased with increasing temp. We did attempt to fit our data to the Macromolecular Rate Theory (MMRT) model descried by Alster et al., (2016), which better accounts for observed declines in enzyme activity at temperatures below denaturation temperatures and enables the derivation of an optimum temperature. However, our data did not fit this model very well.

Alster, C.J., Baas, P., Wallenstein, M.D., Johnson, N.G. and Von Fischer, J.C., 2016. Temperature sensitivity as a microbial trait using parameters from macromolecular rate theory. Frontiers in microbiology, 7, p.1821.

Reviewer 2

I have read the manuscript titled "Differential Temperature Sensitivity of Intracellular and Extracellular Soil Enzyme Activities" by Adekanmbi et al.

The study has two main objectives. The first objective is to evaluate the thermal sensitivity of the extra- and intracellular steps of soil organic matter decomposition. The second objective is to evaluate the potential of microbial communities to acclimatize/adapt to a temperature treatment over 60 days.

The study is well written and the introduction and discussion sections are well-supported with relevant hypotheses and current literature. I find that the topic and questions raised in this article are of great interest, as there is still a lack of understanding about the thermal sensitivity of soil microorganisms, their potential to adapt to climate change, and the implications on soil carbon decomposition. The study is well-written and has a clear introduction and discussion with well-stated hypotheses and up-to-date bibliography. However, the study has three main limitations that I highlight below.

We also thank Reviewer 2 for their positive appraisal of our work and also respond below to each of the three limitations and outline how we are happy to make revisions to address the minor comments also.

1- I understand the idea of removing substrate limitation by feeding microbes with glucose, but using this as a proxy for intracellular enzyme activity is confusing. Other factors, such as diffusion, active transport, and carbon use efficiency of the microbes, among others, can also impact this step. Additionally, comparing the intracellular decomposition process (which involves multiple enzymes) to an extracellular specific enzyme reaction (such as beta glucosidase or chitinase) seems not appropriate. The authors should rephrase this in their manuscript and consider discussing non-limited respiration or maybe glucose-induced respiration.

We agree with this comment (also made by Reviewer 1) that referring to all intracellular metabolic processes as ‘intracellular enzyme activity’ is overly simplistic. We are therefore happy to revise the manuscript (including the title) so that we refer to ‘intracellular metabolic processes’ when speaking generally and to ‘glucose-induced respiration’ when speaking specifically about our results.

2- I do not understand why the authors are calculating Q10 at different temperatures. It is known that one of the main limitations of Q10 is that it can change depending on the temperature range chosen for calculation. Why did the authors not use linear regression between the natural logarithm of enzyme activity (Vmax) and temperature, and convert to Q10 values based on the relationship: Q10 = exp (10 × slope) (as cited in Zuo et al, 2021, German et al, 2016 and many other articles)? Can you please provide a strong rationale for why this method was not used or present a single Q10 value calculated in this manner.

Refs

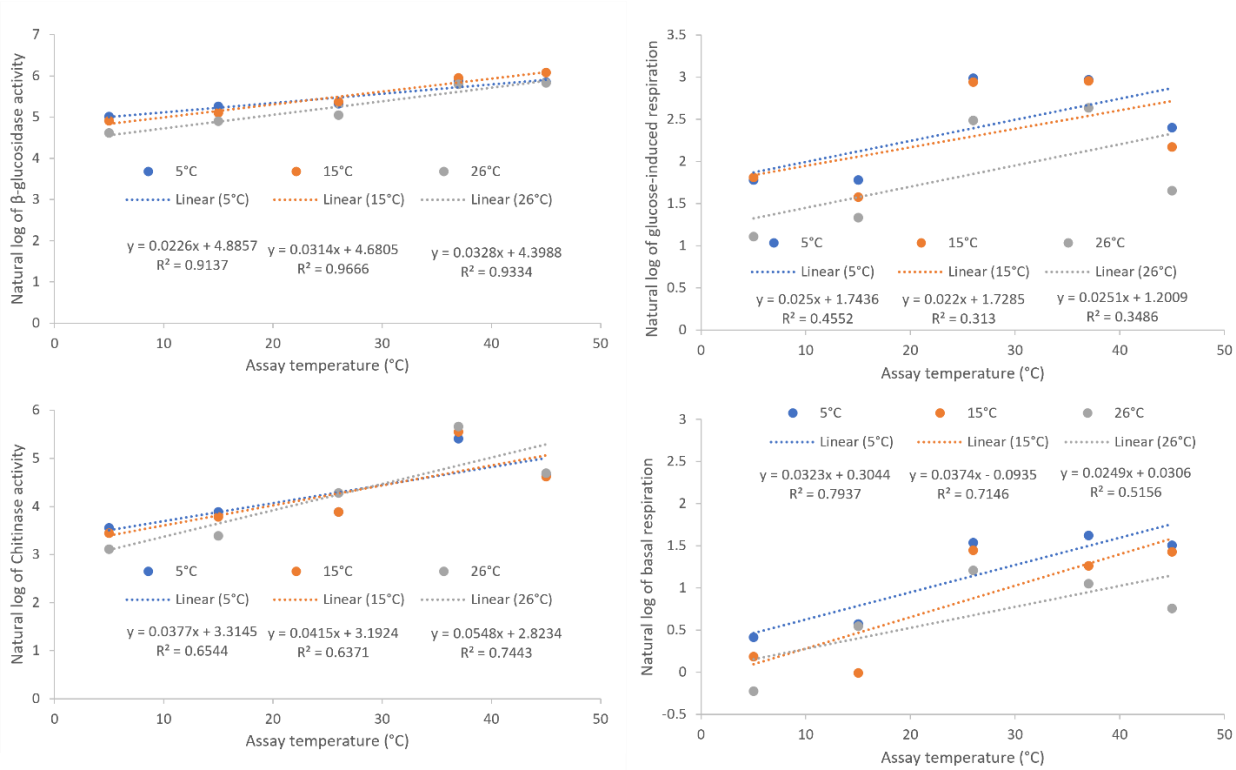
-The effect of soil depth on temperature sensitivity of extracellular enzyme activity decreased with elevation: Evidence from mountain grassland belts. 2021. Yiping Zuo, Hongjin Zhang, Jianping Li, Xiaodong Yao, Xinyue Chen, Hui Zeng, Wei Wang,

-The Michaelis-Menten kinetics of soil extracellular enzymes in response to temperature: A cross-latitudinal study.2016. German, D.P., Marcelo, K.R.B., Stone, M.M., Allison, S.D.

The primary reason why we calculated Q10 at different temperatures and not using the method identified here is because we found that temperature sensitivity was different at different temperature ranges. This meant that there was not a good linear relationship between the natural log of enzyme activity/respiration and temperature apart for β-glucosidase, as demonstrated in the Figure below. We therefore felt that presenting a single Q10 representing temperature sensitivity across the range of temperatures assayed would be misleading and miss some of the nuisances in our findings.

We did attempt to fit our data to the Macromolecular Rate Theory (MMRT) model descried by Alster et al., (2016), which better accounts for non-monotonic relationships between enzyme activity and temperature. However, our data did not fit this model very well either.

Alster, C.J., Baas, P., Wallenstein, M.D., Johnson, N.G. and Von Fischer, J.C., 2016. Temperature sensitivity as a microbial trait using parameters from macromolecular rate theory. Frontiers in microbiology, 7, p.1821.



3- This study only uses one soil and three temperature treatments to explore the relative thermal sensitivity of extra- and intracellular steps of decomposition. I acknowledge that determining thermal sensitivity in the laboratory is a lot of work, but using only one soil and three treatments is still very limited compared to other published studies. The authors should clearly state this limitation in the abstract and main conclusion to avoid extrapolating or overstating the main findings (which are indeed interesting).

We agree that the use of a single soil and only 3 pre-incubation temperatures is a shortcoming that limits the extent to which the results can be generalized. We propose to address this limitation in the revision.

The abstract currently states:

> *"This result implies that depolymerisation of higher molecular weight carbon is more sensitive to temperature changes at higher temperatures (e.g. higher temperatures on extremely warm days) but the respiration of the generated monomers is more sensitive to temperature changes at moderate temperatures (e.g. mean daily maximum soil*

temperature). Therefore, since climate change predictions currently indicate that there will be a greater frequency and severity of hot summers and heatwaves, it is possible that global warming may reduce the importance of extracellular depolymerisation relative to intracellular metabolic processes as the rate limiting step of soil organic matter mineralization."

We propose to add a sentence to the abstract to identify the limitations of our findings so that this passage of text will read:

"This result implies that depolymerisation of higher molecular weight carbon is more sensitive to temperature changes at higher temperatures (e.g. higher temperatures on extremely warm days) but the respiration of the generated monomers is more sensitive to temperature changes at moderate temperatures (e.g. mean daily maximum soil temperature). However, studies using multiple soil types and a greater range of pre-incubation temperatures are required to generalize our results. Nevertheless, since climate change predictions currently indicate that there will be a greater frequency and severity of hot summers and heatwaves, it is possible that global warming may reduce the importance of extracellular depolymerisation relative to intracellular metabolic processes as the rate limiting step of soil organic matter mineralization."

The conclusion previously stated:

"Specifically, for the grassland soil under study, we have demonstrated that potential activities of extracellular depolymerase enzymes (β-glucosidase and chitinase) have greater sensitivity to increases in temperature in the range of temperatures experienced on extremely warm days (between 26 °C and 37 °C) than the potential activity of intracellular enzymes involved in catabolism of monomeric (e.g. glucose) substrates to CO2."

We propose to revise this passage of text in the conclusion so that it now reads:

"Specifically, for our individual grassland soil pre-incubated at just three representative temperatures, we have demonstrated that potential activities of extracellular depolymerase enzymes (β-glucosidase and chitinase) have greater sensitivity to increases in temperature in the range of temperatures experienced on extremely warm days (between 26 °C and 37 °C) than the potential activity of intracellular enzymes involved in catabolism of monomeric (e.g. glucose) substrates to CO2."

We believe these changes would bring the abstract and conclusion into line with the tone of the discussion which includes the following sentence:

"further experiments are required to evaluate the applicability of our finding of a greater temperature sensitivity of extracellular activities at higher (26 °C and 37 °C) temperature ranges to other soil types"

Line to line comments:

Line 100: "measurement of enzyme activity at different temperatures in the lab is not an experimental treatment in itself (compared to the 60 day of temperature treatment). This sentence is misleading. The experiment did not have "60 experimental units," but 12 (3 incubation temperatures x 4 replicates).

We agree that the way this is written is misleading and we propose to revise the section to address this shortcoming. The methodology currently states:

"The experiment was a two factorial experiment involving the 3 pre-incubation temperatures (5 °C, 15 °C, and 26 °C), and 5 assay temperatures (5 °C, 15 °C, 26 °C, 37 °C and 45 °C). This design resulted in 15 treatments replicated 4 times, resulting in 60 experimental units. At the end of the 60-day pre-incubation period, soils were subsampled for determination of basal respiration and substrate induced respiration using glucose as the substrate (Section 2.3), and the potential activity of β-glucosidase (β-1,4-glucosidase) and chitinase (N-acetyl β – D – glycosaminidase) extracellular enzymes (Section 2.4)."

We propose to revise this section so that it reads:

"The experimental design included 3 pre-incubation temperatures (5 °C, 15 °C, and 26 °C), replicated 4 times, resulting in 12 experimental units. At the end of the 60-day pre-incubation period, soils were subsampled for determination of basal respiration and substrate induced respiration using glucose as the substrate (Section 2.3), and the potential activity of β-glucosidase (β-1,4-glucosidase) and chitinase (N-acetyl β – D – glycosaminidase) extracellular enzymes (Section 2.4) all measured at 5 assay temperatures (5 °C, 15 °C, 26 °C, 37 °C and 45 °C)."

Line 97: A space is needed between the two commas.

Here we accidentally included the same reference twice. We propose to correct this in a revision. The current version is:

"by Adekanmbi et al., (2020)(Adekanmbi et al., 2020)."

The revision will be:

"by Adekanmbi et al., (2020)."

Line 128: What does MUB stand for? Was the buffer pre-incubated at different temperatures?

MUB stands for 4-methylumbelliferone and we propose to add this to the sentence to make the methodology clearer. The buffers were pre-incubated at room temperature prior to assays.

We propose to revise this sentence of the methodology so that it reads:

"For each experimental replicate, 1 g of soil was weighed into a 50 ml centrifuge tube and mixed with 4ml pre-incubated 4-methylumbelliferone (MUB) buffer"

Line 326/327: "Accumulation of monomers" needs to be reformulated.

We agree that this phrasing is unclear. The text currently states:

"Such a switch in rate limitation, if applicable generally across all extra- and intracellular reactions, would result in an accumulation of monomers and thus potential for greater losses of C from the soil profile as dissolved organic carbon, an often overlooked component of terrestrial carbon budgets (Evans et al., 2014; Cook et al., 2018)."

We propose to revise this text so that it reads:

"Such a switch in rate limitation, if applicable generally across all extra- and intracellular reactions, would result in a build-up of low molecular weight substrates in the soil and thus potential for greater losses of C from the soil profile as dissolved organic carbon, an often overlooked component of terrestrial carbon budgets (Evans et al., 2014; Cook et al., 2018)."

Line 343: "It is tempting" is not appropriate scientific language. Please rephrase.

The sentence currently reads:

> *"It is tempting to initially suppose that the substrates that are hydrolysed by chitinase and β-glucosidase enzymes in depolymerization reactions might be more recalcitrant than glucose and other lower molecular weight substrates for intracellular respiration and, in consequence, the extracellular-catalysed reactions should have higher temperature sensitivities."*

We propose to revise this sentence in response to the comment so that it reads:

> *"It might be initially supposed that the substrates that are hydrolysed by chitinase and β-glucosidase enzymes in depolymerization reactions might be more recalcitrant than glucose and other lower molecular weight substrates for intracellular respiration and, in consequence, the extracellular-catalysed reactions should have higher temperature sensitivities."*

Line 346: Please remove the hyphen in "trimeric."

The sentence currently reads:

> *"However, it should be recognised that chitinase and β-glucosidase have relatively simple di- or tri-meric substrates in nature and are assayed using artificial and simple substrates that may not be more recalcitrant than those used in intracellular metabolism."*

We propose to revise the sentence so that it reads:

> *"However, it should be recognised that chitinase and β-glucosidase have relatively simple dimeric or trimeric substrates in nature and are assayed using artificial and simple substrates that may not be more recalcitrant than those used in intracellular metabolism."*

Line 349: Please remove the comma.

We think that there is an errant bracket here rather than a comma. The sentence currently reads:

"In addition, the theoretical predictions refer to chemical decomposition reactions and not necessarily those involving enzyme catalysis (Blagodatskaya et al., 2016))."

We propose to revise it so that it reads:

"In addition, the theoretical predictions refer to chemical decomposition reactions and not necessarily those involving enzyme catalysis (Blagodatskaya et al., 2016)."

We thank reviewer 2 for pointing this out.

Line 361: Double space?

We propose to remove the double space here and from several other places between the end of one sentence and the start of another in the revision to the manuscript.

Line 384: Changes in the thermal sensitivity of enzymes could have indicated an adaptation of the enzymes produced by the microbial community.

We agree with this comment but don't want to give the impression that it is the enzymes themselves that are adapting. Rather it is the microbial community that produces the enzymes that is adapting. The sentence currently reads:

"It is likely that such indirect effects of pre-incubation temperature on microbial community composition enzyme pool size masks any direct thermal acclimatation or genetic adaptation of the soil microbial community."

We propose to revise this sentence so that it reads:

"It is likely that such indirect effects of pre-incubation temperature on the microbial enzyme pool size masks any direct thermal acclimatation or genetic adaptation of the soil microbial community and subsequent change in the temperature sensitivity of the enzymes it produces."

We elaborate on this point in the next paragraph.

Line 386: Please use "Vmax" or "apparent Vmax" instead of "concentration," as you did not measure it. Please make sure to use consistent terminology throughout the manuscript.

Throughout the manuscript we refer to potential enzyme activity. However, in these sentences we incorrectly refer to concentration. We did not determine the relationship between rate and substrate concentration in order to estimate Vmax. We therefore propose to revise this sentence and replace 'concentration' with 'potential enzyme activity' rather than Vmax.

The sentence currently reads:

> *"Compared to β-glucosidase, there was less evidence of an effect of pre-incubation temperature on the concentration of chitinase (no significance of pre-incubation as a main effect). The concentration of an enzyme in soil is a function of production versus turnover rate."*

We propose to revise it so that it reads:

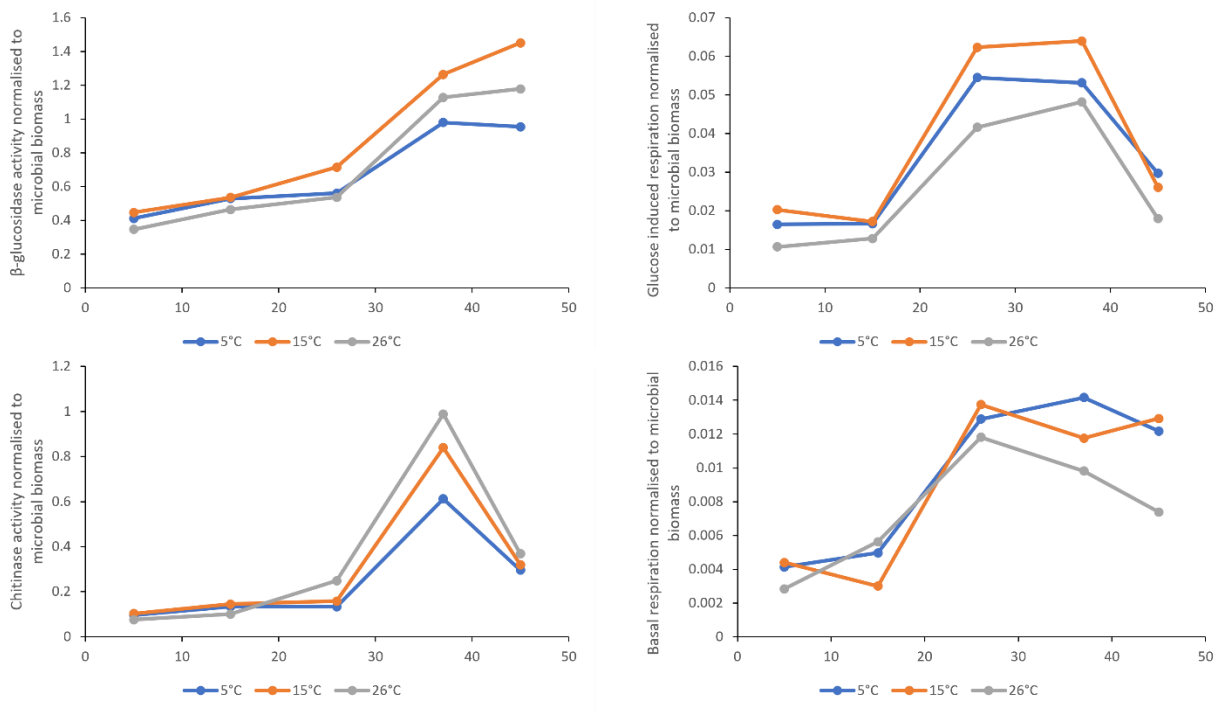
> *"Compared to β-glucosidase, there was less evidence of an effect of pre-incubation temperature on the potential enzyme activity of chitinase (no significance of pre-incubation as a main effect). The potential activity of an enzyme in soil is a function of production versus turnover rate."*

In the supplementary material: Could you please specify if the curves on the graph are the mean of all samples or just one sample for illustrating the reaction? (Figure S-3: β-glucosidase).

Each of the points on the graphs in the Supporting Information represent the mean of three replicates. We propose to revise the Supporting Information to explicitly state this the captions.

Line 391: You could calculate enzyme-specific activity (normalized by microbial biomass) to test if this statement is correct or not?

We normalized all our enzyme assay and respiration measurements to microbial biomass (shown in the figure below) and we can see here that the reason for a lower rate of intracellular metabolic processes in soils incubated at 26 °C is not due to a lower microbial biomass.



We therefore propose to revise the passage of text where this is mentioned as a possibility. The sentence currently reads:

> *"Whilst pre-incubation at 26 °C reduced the rate of intracellular metabolic processes, probably linked to reductions in biomass and relative C availability, it did not lead to an alteration of community intracellular temperature response traits…"*

We propose to revise it so that it reads:

> *"Whilst pre-incubation at 26 °C reduced the rate of intracellular metabolic processes, it did not lead to an alteration of community intracellular temperature response traits"*

Figure 2 caption: Please specify that it is Vmax.

We did not determine the relationship between rate and substrate concentration in order to estimate Vmax. Although we acknowledge this as a limitation, undertaking this task would have increased the number of assays by 5 or 6 fold. Instead we showed that there was a linear relationship between assay product formation and time in our assays (see supplementary information). This indicates that the substrate was supplied at an initial concentration that was sufficiently in excess such that the depletion of substrate

concentration through enzymatic conversion over the assay period did not limit the reaction rate.