

Dear J Grey Monroe and collaborators,

Thank you for submitting your preprint to PCI Evolutionary Biology for recommendation. Two anonymous reviewers, Rodrigo Medel (co-recommender) and I, carefully read your preprint. We found your text was elegantly written. You present an interesting perspective on the way "function-valued traits" influence the response to selection through genetic correlations and plasticity. We think that the main framework needs to be emphasized earlier in the introduction section. In its present form, most of the general context relies on particular issues related with the study system, plant variables, and soil moisture gradients. This is fine, but expanding the context/implication presented in the second paragraph throughout the paper would add substantially to the scope of this contribution.

**Thank you for the great feedback. We are extremely grateful for the thorough and constructive reviews.**

We found the experts' reviews very thorough and thoughtful and agree with their comments. We all agree that your manuscript addresses an important question: considering the continuity and non-linearity of soil moisture effects on plant morpho-physiological traits. We also found this manuscript quite valuable and would be inclined towards recommending it after consideration of comments by the authors. We hope that you will find our feedback useful to revise your manuscript.

Here are additional points that we identified.

1. One motivation of the paper is to assess the benefit of considering the continuous variation of soil moisture when evaluating the environmental response of the phenotype. Your point is that it brings a formally neglected layer of information that brings us one step closer to a more realistic assessment. I fully agree with this but I'm concerned about the presentation of the contextual background and the presentation of the implications of your work in the discussion. My concern comes from the fact that you do not "add up" a layer of detailed information to an otherwise realistic background. You rather proof test whether adding this environment as a continuous variable brings up useful information and you do this in an experimental simplified biological set up in order to properly proof test your hypothesis. Indeed, you use an experiment, a limited number of genetic lineages and summarise the continuous variation of the soil moisture to 6 modalities. This is not independent from another concern: It is sometimes clear, and sometimes less clear throughout the manuscript whether your contribution is specifically about "soil moisture" or whether you address a general issue that concerns all environmental variables. You want to revisit the text of the paper to clarify these aspects throughout the preprint so that no confusion is possible. It will bring value to the paper because your valuable contribution will be more clearly identified by readers as a result (and referred to).

**Thank you for the helpful suggestion. We have made considerable changes to the structure and flow of the manuscript – particularly the introduction – to better frame the research. While we hope that this work will be valuable to researchers studying plant-water relations and responses to environmental variables more generally, we agree with the value of helping the reader by choosing one as the primary focus when presenting the work. We feel that this work will be particularly valuable to researchers in the plant sciences, especially those interested in plant-water relations. Moreover, we understand that plant scientists, especially those studying drought will appreciate the novelty of this work. Large soil-manipulation experiments are notoriously difficult and as we point out in the introduction, therefore almost always limited to one level of drought treatment. We have thus restructured and rephrased some of the writing to emphasize this dimension of the research. Nevertheless, we expect that broader audience will still find value in the combined analytical approaches, function-valued-traits + trait covariances, which are useful for investigations across diverse organisms, traits, and environments.**

2. An important implication of your work is that using environmental variables that are not continuous does not allow to observe nonlinear phenotypic responses. Missing such exponential or logarithmic responses has implications when predicting plant responses, in particular in the current context of climate changes. I totally agree with this and you could actually elaborate a bit more on this aspect and acknowledge more precisely similar work claiming the same message (eg work by Nussey, Wilson, Brommer who you cite, in wild populations, etc.).

**Thank you for the suggestion. We agree that this is an interesting implication of nonlinearities in plain responses to the environment. And have added a paragraph to the discussion about this topic here we have included numerous citations to previous work which has also shared an interest in this topic including work by Nussey, Wilson, and Brommer and others.**

3. You use quantitative genetic approaches and that brings value to this paper. However, the paper lacks contextual background about this aspect. You do not clearly state what are the methods used to assess continuous environmental effects (eg random regression quantitative genetic models) and whether they were used to assess environmental effects on plants. What are the differences and similarities with your approach (the aim is clearly the same)?

We would ultimately accept to recommend this preprint provided that you address these concerns and the reviewers' comments appropriately.

**This is an important point and in the updated manuscript we have more clearly described our approach. We specifically describe our model in relation to the random regression model of Nussey et al (2007) and point out that we elected to treat genotype as a fixed effect rather than random effect due to examining only 5 genotypes per species.**

4. The contrasting patterns of evolutionary constraints between species are quite impressive. I wonder to what extent this contrasting pattern associates to the contrasting life-history strategies as the authors assume, or rather represent non-random phylogenetic signals unrelated to life-history strategies. While this is clearly beyond the focus of the manuscript it would be nice to have additional information on the strategies followed by other populations or sister species to give stronger support to the idea of life-history related genetic constraints.

**We agree that this is an interesting hypothesis. In the revised manuscript we have added a caveat to our discussion about this, pointing out that we have limited power to draw such conclusion but also point out that this will be a potentially interesting hypothesis for future research with the approaches we used here in a broader phylogenetic context.**

Regards,  
Benoit Pujol and Rodrigo Medel

### **Reviews**

*Reviewed by anonymous reviewer, 2020-04-28 12:13*

Review of Monroe, Cai and Des Marais " Trait plasticity and covariance along a continuous soil moisture gradient"- PCI 2020

This manuscript analyses the response (variance and covariance) of 12 traits to continuously varying soil moisture gradient in five genotypes of two *Brachypodium* species (one annual and one perennial). The key findings are that i) most traits exhibit a non-linear response to drought leading to changes in trait correlations, and ii) the shape of the response varied between species, genotype and traits suggesting a context dependent response.

I really enjoyed reading this article. I agreed with the motivation of the article, the design, the code/data provided and the conclusion. Yet, I have to admit I am not a specialist of all the methods the authors used in this manuscript.

I still have some suggestions about the manuscript in its current form.

Introduction

Part of the discussion is about the difference between perennial and annual plants. It would be interesting to bring this subject in the introduction first. I haven't notice that

one sp was annual and the other perennial before the discussion (while it is clearly mentioned by the authors in the material and methods). It would be interesting to get a paragraph in the end of introduction with some hypotheses about difference in traits variance, correlation and evolvability etc. between perennial and annual plants facing drought stress.

**As noted by the recommenders, and in our newly written discussion, we are concerned that our current study lacks power to make formal comparisons between annual and perennial life histories. Thus, while the observed differences between these two annual and perennial species warrants discussion, we are hesitant that including it prominently in the introduction will not properly reflect the cautious interpretation we hope to convey in the discussion. Indeed, rather than being a motivation for the current study, the differences seen between these two species remain an interesting observation that warrants further investigation, but not the primary aim of the study.**

#### Material & Methods

Plasticity through multidimensional trait space. - You specified that the traits are centered, but it is not clear to me if they are also reduced? I guess they are. Please clarify, this information impacts our interpretation of fig3.

**Thank you. Yes the data was reduced such that it has a standard deviation equal to 1. We have added this information to the revised text.**

#### Results

Non-linearity in trait responses to soil moisture. I would be interested to see the R<sup>2</sup> of the best (final) model in Table 1, to have an idea of the part of the variance explained by each model. For each trait, if the R<sup>2</sup> (or AIC) of the best models are very closed I would advise the author to use a model averaging approach (Grueber et al 2011) to get averaged estimates, and then predicted values based on these averaged estimates.

**We have added a table which includes the R<sup>2</sup> and AIC values of the final model.**

I guess the authors plotted in Fig 2 the predicted value for the traits at each soil moisture level, rather than the observed values. Please specify.

**Yes, thank you. We have clarified this in the figure legend.**

In the same line of thought, I think it would be interesting to provide the confident interval around the values (whenever they are predicted or fitted values) plotted in fig 2.

Maybe all the differences observed have overlapping CI, and are not significantly different all along the moisture gradient.

**We appreciate this suggestion. Unfortunately, visualization becomes exceedingly difficult to interpret because each line has 2 confidence interval lines surrounding it. However, the statistical significance of the environmental predictors can be found in the model summary tables we have in the supplemental table S1. We note that it is rare for non-significant predictors to be included in models selected by AIC used here.**

Cohort effect- I would be interested to see some discussion or interpretation of the significance of cohort effect. Indeed in Table1, the harvesting day (H) which is the cohort effect is significant in 18 models on 24. Do you have any explanation? Is it due to a change in trait value with plant age? Or is it an artefact of the experimental design? As you already know, plant traits can shift with plant ontogeny. In one hand, we can think that it is unlikely to see this kind of effect between 5days-difference *Brachypodium* plants. On the other hand, five days differences for a plant of 43 or 56 days is not negligible (9-11% of its lifetime). Please discuss this result.

**We agree this is an important point. First we have added a figure to the paper illustrating the experimental design so that these results might be better understood. Second we have added several comments in the paper regarding this. We have pointed out as you note, the expected effect of harvesting plants of different ages.**

I found the Fig3 b and c complicated to understand, and I am not sure they provide a lot of information to the reader. In my understanding the authors only get one point from them: phenotypes were similar between extreme dry and extreme wet soil moisture contents for *B. sylvaticum*. I have to admit that this result was not particularly obvious for me on fig3b and c. Maybe the authors should consider dropping Fig3 part b and c into appendix to simplify their ms.

**Thank you for the suggestion. After further consideration we agree and have moved parts b and c to the appendix.**

Fig4- So each point is a correlation coefficient calculated across 5 genotypes, right? If yes please specify this in the legend. If No, please add the info about the sample size used to drawn each correlation as different point size in the plot.

**Thank you for the suggestion we have added “Each point is a correlation coefficient calculated across 5 genotypes” to the plot legend.**

#### Discussion

Just a general comment/question to the authors. I agree that the next step would be to search for general patterns of variation and covariation across species (which is a goal of functional ecology) along discrete environmental variables. As you specified, the best approach is to reproduce in an analogous fashion the same experiment on more genotypes or other species. Yet, do you think we could get some insights by compiling individual studies focusing on discrete soil water levels? Like a meta-analysis or a trait database with information on the soil water content (and type of soil)?

**What a cool idea! Presumably this is definitely possible – one could extract data from previous studies where genotypic variation for multiple traits was measured at different soil moisture. It might be difficult to draw explicit comparisons between specific soil moistures since there are so many different methods used to measure this, but one could at least ask some general questions such as, how often and by how much do trait correlations change.**

Minor comments:

- In Abstract please replace “multiple genotypes” by “five genotypes”

**Done**

- In Material & Methods section- Genotypes and species: “For each species, these genotypes represent a range of geographical origin and phenotypic diversity” Please specify into bracket the name of two most distant locations. It will give some clue to the reader without having to go through your previous work.

**Thank you for the suggestion we have added a statement to highlight genotypes of distant geographical origin.**

- In fig 3, please add in legend “*B. sylvaticum* is colored in orange and *B. distachyon* blue”.

**Thank you, done.**

- In section Non-linearity in trait responses to soil moisture is pervasive. “In contrast, SLA, total biomass and shoot mass did not include environmental predictor” please replace “predictor” by “predictor”

**Thank you, fixed.**

- In discussion please check for references in the text. For instance you did not provide the year of publication for Lundgren and Des Marais (current biology).

**Thank you, fixed.**

- In appendix, I would be useful to provide a schema of the experimental design, with information about the number of genotypes, cohort, traits measurement along the five days etc.

**Thank you for the suggestion. We have added a supplemental figure which is a diagram of the experimental design.**

Grueber et al 2011 – Methods in Ecology and Evolution. Multimodel inference in ecology and evolution: challenges and solutions <https://doi.org/10.1111/j.1420-9101.2010.02210.x>

*Reviewed by anonymous reviewer, 2020-05-19 10:36*

The scope of the manuscript is to understand the phenotypic response of genotypes to an environmental gradient of drought, with the goal to better predict the shape and form of phenotypes and response to selection. The authors argue that so far, we have studied phenotypic responses (to abiotic factors) in discrete experimental setups, and it is about time to integrate the complexity of continuous environmental variation in experimental designs so that we can predict phenotypic responses and selection in a more “realistic” way, as opposed to a set of fixed levels. I agree with the authors that experiments under controlled conditions often use a number of fixed levels and measure the response of phenotypes, and we need to understand better the covariation between phenotypes and environmental gradients to estimate selection and the response to selection more accurately. But I disagree with the statement that we have not studied much continuous variation, or that we don't have tools for that. This has been done quite extensively on phenotypic selection studies. The work of Lande & Arnold (1983) *Evolution* 37: 1210-1226 and subsequent methodological papers in the context of phenotypic selection have provided the methods to understand the covariation between traits and fitness to measure selection on phenotypic traits while controlling by genetic correlations (or phenotypic as a proxy of genetic correlations), including non-linear relationships. Furthermore, the approach to model phenotypic traits as a function of environmental traits through model selection has been already used in phenotypic selection studies (e.g. Bolstad et al (2010) *New Phytologist* 188:370-384,

use of model selection to creating a fitness function based on functional traits). Perhaps we have simply failed to quantify environmental factors in natural conditions to better predict the correlation between traits and continuous environmental factors.

**Thank you for the thoughtful comments. We have added citations to Lande & Arnold (1983) and Bolstad et al (2010). Indeed, both are very relevant for the current study.**

But after reading the manuscript, I am left with mixed feelings about the adequacy of their methods. Yes, with their experiment the authors create environmental gradients to measure the reaction norm of genotypes to soil moisture, but this is done in only 5 genotypes per species. And I am not surprised: the experimental design is way too complicated and large when one adds more genotypes. I guess this is why there's been a tendency of using modest and simple experimental approaches where we can increase our sample size of genotypes per species to study the evolution of phenotypes and what constrains a phenotypic response to selection. While the authors used a smart experimental and sophisticated statistical way to investigate the relationship between continuous environmental variation and phenotypes, they are constrained by the scale of the experiment. So in summary, either we make a simple design (fewer levels) and increase the range of genotypes (and potentially phenotypes), or we focused on a subset of genotypes and we get to understand well reaction norms.

**Indeed, this reflects a fundamental trade-off in such experiments – adding genotypes at the cost of environmental resolution or vice versa. We have added comments to the discussion about the limitations of only having 5 genotypes per species.**

I like how the authors approach and recognized the importance of studying specific traits, the structure of genetic correlations and covariation between traits, but also the general multivariate perspective. I admit that the phenotypic plasticity in a multivariate fashion is hard to grasp but necessary. But I was somewhat surprised to see that, while they emphasize the importance of the multivariate approach, little was brought into the discussion, and the focused was on specific traits that somehow “made sense” in the context of life-history traits. So perhaps the authors should think of improving the alignment between the introduction and discussion.

**Thank you for the helpful suggestion. We have made extensive revisions to the introduction and discussion including additional comments about multivariate phenotypes. We hope the result is a more cohesive presentation the study.**

Furthermore, I missed a more ecological and biological context in the introduction to predict responses of the species under study, which is possible since this is a model organism. The life history trait argument and adaptation to specific environments in the context of the geographic range of the species is overlooked in the introduction, and yet it plays an important role to explain plasticity and evolvability in the two species. This is simply discussed in the context of annual and perennial life history strategy and adaptation to drought. But the results are important to predict how will the two species respond to environmental stress through drought and drought tolerance. This is where their experimental manipulation is useful and it could enrich the discussion: what are the implications of their estimates to predict an evolutionary response to an increase in drought? What species will be more constrained and how from a multivariate and single trait approach? What would be the line of least resistance for phenotypic evolution to occur in both species? These seem to me the interesting questions, particularly in the context of their 4th paragraph in the introduction (see below). However, the study includes only 5 genotypes per species and I would assume that this represents only a small fraction of the genetic and phenotypic variation of the two species, and a small fraction of the phenotypic responses if local adaptation and population differentiation is important. This should be, at the very least, acknowledged in the discussion.

**Thank you for raising these important points. We have added to the discussion to distinguish between different sources of constraint – genetic covariances and lack of genetic variation. We have acknowledged the limitations due to the small sample size of genotypes to draw broad conclusions, for example, about differences between annual and perennial life history strategies from the presented results. We have also added a new section in the discussion about how these approaches could be scaled up to include more genotypes, particularly under field conditions.**

The 4th paragraph of the introduction is beautifully presented and argued, and I feel that the authors have put forward an interesting theoretical context as to why is it important to investigate the structure of variance and covariance of traits in environmental gradient to predict phenotypic responses and how traits evolve. I think it is important to return to these ideas in the discussion and use their results to solve the conundrum presented (see above).

**Thank you, we have revised the discussion to better align with the ideas presented in the introduction.**

From a methodological perspective, the study is well executed and the authors displayed a wide collection of statistical tools to investigate phenotypic responses from

a multivariate fashion. However, there are a few flaws in the Methods making necessary much reading between the lines. More detailed is needed to add clarity so that the authors can reach a wider audience, for example:

- In the experiment, the authors stated “For each genotype, 1200 were planted...” but the experimental setting of Figure S2 doesn’t match this. Based on that figure, it looks like the authors have a block design where they used all genotypes x 6 irrigation treatments x 5 replicates, replicating each genotype per block to ensure they can measure all traits. It took a while to figure this out. So there are a total of 10 plants per genotype and treatment, not 100 as it sounded from the initial description. Unless I’m wrong. Please clarify.

**Thank you for pointing this out. Indeed, this diagram was incorrect. We have removed this figure and replaced it with a diagram showing the experimental design which we hope will be more informative.**

- I am confused with the soil moisture estimate, as it appears to be a function of multiple measures (pot weight in day d, weighed dry soil, and field capacity). But if pot weight is used to estimate soil moisture, moisture is somehow influenced by how genotypes used hydric resources right? In other words, they predict traits as a function of how hydric resources are consumed under different drought regimes right?

**This is a good point. To a certain extent yes. The final soil moisture reflects a combination of water input and output through both evaporation from the soil and water use by the plants themselves. Because this is how soil moisture is influenced under natural conditions, this experiment allows us to measure the outcome of the processes. In some sense therefore, the conceptual definition of environment employed in such an experiment is relatable to that put forth by the “niche construction” concept – environmental conditions experienced by an organism are determined in part by what an organism does to its environment. The alternative to the approach we used is to give more water to the plants that use less water, and vice versa, to force soil content to be equal, but this risks “rewarding” water inefficient plants and “penalizing” water efficient plants. While we didn’t want to spend too much time in the paper on this topic, we have added a clarifying statement to the manuscript that addresses this point.**

- Use of model selection to predict traits as a function of genotype, linear, and non-linear soil moisture. It would be good to provide more details on model selection. In general, people usually report AIC values of models to show what’s the best model. It is then confusing to also add p values, as opposed to the coefficients used to build the function to predict the trait, which by the way would be useful for other authors. Those

coefficients are actually more informative than p values. I suppose that it would be impractical in terms of an appendix to add details of model selection for every single trait.

**Thank you this is a good point and we have made changes to the manuscript accordingly. First, we have elaborated the model selection in greater detail. Second, we agree that reporting the p-values adds little value to the manuscript. If anything, they are confusing because we used AIC for model selection rather than model selection based on p-values explicitly. We have thus removed these values and instead simply indicated which predictor variables were included in the final model. We have also removed comments in the results section that spend unnecessary time contrasting p-values between predictor variables.**

- The methods on plasticity through multidimensional trait space are quite obscure, please provide more details as much reading between the lines is necessary to follow this section.

The results are not easy to follow so it would be good to add clarity. I have picked up a few issues:

**Thank you for pointing this out. We have expanded extensively on the methods section describing how plasticity through multidimensional trait space. The revised manuscript provides additional details and equations about how these values were calculated.**

2nd paragraph: Be consistent with the use of acronyms in the text. For example, (...) in leaf relative water content (RWC). This will facilitate your readership to follow the storyline.

**Thank you, we have updated accordingly.**

Figure 2 represents variation in phenotypic responses ranging between 25% to 100%, and the 10% tail is not represented.

**We have added a clarifying statement to the figure legend.**

Also, make sure that you specify the color code in all your figures.

**Thank you. We have now specified the color code for all figures.**

3rd paragraph, description of non-linearity in trait responses: for a few traits, the best model based on AIC does not include the E term, although not all the traits in that situation are included in the description of the results, and it is unclear why do you add the E term in models when it does not seem to be important to predict some traits. I am also confused with the results when looking at Table 1 and Figure 2, e.g. C content in *B. sylvaticum* does not seem to be predicted by any non-linear term, and yet figure 2h shows a non-linear quadratic response, stronger than other traits with a detected quadratic response according to model selection, or even for *B. distachyon* with a model with quadratic E terms. This does not make sense and perhaps the authors should revise their models. This happens for several other traits. It is also unclear how you used model selection. You seem to use both AIC of the model and p values per term in your model, some of which are not statistically significant ( $p > 0.05$ ) and I would imagine that in those cases including or including not those terms would not make any difference (as based on AIC values). So please clarify the logic to create the traits as a function of parameters, because not always you seem to use the best model as defined by model selection.

**Thank you. We have expanded the methods section to describe the logic and implementation of the model selection procedure.**

Clarify 4th paragraph, PCA results. I am somewhat confused here. Looking at figures 3b and 3c it does not look as if *B. sylvaticum* (yellow) displayed similar phenotypes at the extreme of the gradients but in *B. distachyon* (blue). For the species coded in blue, it appears that response presents more variation in PC2 and 3 within genotype. So perhaps the authors need to improve the narrative of the explanation to better reflect the results of the PCA. It's unclear whether they refer to within genotypes or, in general, among genotypes.

**Thank you for the comments. We have taken your suggestion to change the narrative of this section. We have also moved 3b and 3c to the appendix as recommended by another reviewer.**

5th paragraphs, results on significant interaction between genotype and non-linear predictors. It's confusing to use p values and model selection at the same time. In essence, with model selection you look at what model explains best the data collected, and so in some cases, the interaction term is important as it gives the model with the lowest AIC value. But most of the traits did not show that interaction effect term between G x E (be E either linear, quadratic or second degree natural spline parameter. I wonder whether that's a limitation of your experiment: you simply screen 5 genotypes per species.

**Indeed, with only 5 genotypes we unfortunately have limited power to detect G x E.**

I find that the discussion does not really answer the questions of the manuscript. The scope is to study plasticity and covariance along an environmental gradient, but this does not seem to be discussed well. The core of the manuscript is paragraph 4th of the introduction, and I was expecting a discussion around this. So yes, we learn that we can predict a trait as a function of environmental factors (with some limitations as to how many genotypes can we include), and that non-linear relationships are important. But the implications to predict how traits evolve and how phenotypes evolved are poorly discussed. The authors identified really interesting trends on plasticity and evolvability, with the two species showing opposite response to soil moisture, which somewhat seems to reflect life-history traits and the geographic and climatic conditions in which the two species evolved. So while the authors looked at specific traits, the discussion needs to develop more in the context of how phenotypes respond to the environmental gradient, from individual traits (somehow done) but also as complex phenotypes, otherwise the multivariate methods seem pointless.

**Thank you for this insightful assessment. We agree that a better connection between the discussion and goals outlined in the introduction was warranted and have made extensive changes to the discussion accordingly. In particular, we have taken your advice and focused more to discuss the implications species responses to selection might be influenced by the emergent phenomenon identified here – namely changes in trait correlations and multivariate constraints of genetic variation as a function of soil moisture.**

The paragraph on implications for breeding drought adaptation was not expected. I grant you that your work is important for crop breeding, but I don't think it deserves a paragraph. After all, no mention of crop breeding was done in your aims.

**We agree and have rewritten this section. Rather than focusing entirely on breeding, we have changed the focus to a discussion of how these results and experimental methods could be scaled up. We hope that this discussion is more broadly relevant and also addresses another of your concerns about the tractability of scaling these approaches to larger screens of genetic diversity.**

There are a few typos in the text (e.g. it's when it should be its, changes in verb tense) but I guess the authors can fix this in the new version.

**We have gone through the entire manuscript and hopefully fixed all such mistakes.**

A few more comments have been added to the text. I might sound very critical in my review, but I really enjoyed your manuscript and it is inspirational.

End of review

**We are very grateful for the thorough and thoughtful reviews. We hope that the revisions made in response to these helpful comments have substantially improved the manuscript. We have uploaded a revised version of the manuscript to *bioRxiv*.**