Editor and Reviewers comments:

Our responses to the comments provided by the editor and both reviewers are shown in blue.

Comments to the Author:  
  
Dear Authors,

Two leading experts have reviewed your manuscript, and they found it a beneficial contribution to understanding better how base water potential for seed germination can be utilised as potential traits for understanding the seed functional ecology of a species. They found novelty in your approach to studying this unexplored aspect of plant regeneration ecology and appreciated your efforts in conducting detailed experimental studies. I agree with the reviewers that your study is novel and will become a reference for future research. However, some sections, especially the methodological and result sections at several places, are hard to understand quickly, and reviewers need to dig deeper to get a better understanding, thus needing revision (see detailed comments of reviewers). It is, therefore, suggested that a significant revision be made and the critical comments of both reviewers addressed.

Dear Editor,

Thank you for your positive evaluation of our manuscript. The comments and suggestions of the reviewers have helped us to identify key points of the methods and the results that needed revision to improve clarity. We have considered all comments and we have incorporated them into a revised version. Please find below our point-by-point responses to the comments made by the reviewers. We hope that you find this revised manuscript acceptable for publication in Functional Ecology. All authors contributed to and approved this revised submission.

Yours sincerely,

The authors

REVIEWERS' COMMENTS TO THE AUTHORS  
Reviewer: 1  
  
Comments to the Corresponding author

This is a very interesting study that brings new attention to an overlooked functional trait (base water potential for germination) that could have consequences for how species respond to a changing climate. Base water potential for germination is a challenging trait to estimate, both from an experimental and a modeling perspective, and I appreciate that this study is accessible – the writing is generally very clear, and they take advantage of an R package that simplifies the estimation process for base water potential. Moreover, they test a question that I’ve never really seen evaluated before by asking whether seed germination response traits can vary over small spatial scales (10m) in correspondence to microsite differences in a heterogeneous environment. For a seed, 10m can be a very large difference in terms of the experienced microenvironment, yet we have very little understanding in the field of ecology of whether and how microsite variation leads to different eco- and evolutionary processes during regeneration, or scales up to influence community structure and response to larger-scale climatic changes.  
  
I would like to see this paper be published and am hopeful that it will generate increasing interest in the functional significance of germination responses. However, I have a few questions about the analysis and think that the manuscript would benefit from a bit more detail about the hydrotime metrics and more nuanced, comprehensive interpretation of what the observed patterns might mean, ecologically. I note these questions and suggestions below and hope they will be helpful in strengthening an important manuscript.

We were very pleased to see that Reviewer 1 had a positive view of our research and valued the novelty of the question and the approach we used. We deeply appreciate the additional time dedicated to improving this manuscript. Please see below our response to your comments.

Comments in the PDF:

L21-22 “These results support that intraspecific variation in the ψb for germination has functional significance” --  I think this wording is a little strong. Demonstrating that trait variation exists, and is observationally tied to the environment, is important but still not a direct test of functional significance, in my mind. To demonstrate that, I think we would need a subsequent empirical tests of germination and recruitment outcomes in the field to demonstrate that differences in base water potential lead to differences in performance or fitness in the environment. Studies from other semi-arid systems have suggested that variation in base water potential may exist, but is not necessarily a strong driver of differences in germination (relative to temperature) – and that predictive models assuming a common base water potential for all species (i.e. a ‘wet-thermal model’, assuming no variation in base WP) capture most of the expected variation in germination generated by fully hydrothermal models that take variable base water potential into account (e.g., introduction of Hardegree it al. 2018: <https://doi.org/10.2135/cropsci2017.11.0666)> This is not to argue that variation in base water potential doesn’t matter – on the contrary, I think this paper is very timely because we have not fully examined this question and utilized field experiments to understand how it does matter. Rather, I think the results presented here are a compelling first step and suggest that this trait could be functionally significant in this system, but that additional work is still needed to demonstrate that and understand how (And this future work would be very valuable to the field!).

Thanks for this comment, with which we fully agree. We have rephrased the specific sentence (L 20-21) that Reviewer 1 highlighted but also made small changes along the manuscript to remark that this is a potential trait that need further inspection. See changes in L 376-377, 403, 432, 446 and 494. Furthermore, we read the paper by Hardegree et al., which was suggested by the reviewer, and include it as another discussion point in L 470-475.

L24 Again, I think these patterns suggest that this trait may have consequences for fitness, but the results don’t demonstrate that outright... especially when we consider that we also do not know if this variation is heritable and adaptive from the current study.  These data only demonstrate an observational correlation between trait and microclimate, which is a very interesting place to start. So I suggest being more careful about language and interpretation throughout the manuscript.

We agree, and made changes accordingly across the manuscript, see specific lines above.  
  
L79 I might replace ‘largely’ with ‘likely’, as regenerative responses may depend on many different processes, not just germination response

Thank you for this observation. We made the change.  
  
L214 Replace ‘has’ with ‘have’

Thank you for this observation. We made the change.

L212-220 I’m a little confused about the soil moisture data. In the methods above, it sounded like you had two gypsum sensors per each of four sites (8 total), but one of them was damaged (I imagine this is why there are only 7 datapoints). But in Figure 3B there are no data from Cañada but four datapoints from Penouta. Is this just an error in the coloring of the figure?

We provide more clarification below, and in the text, and fixed the wrong color palette. Each Microlog SP3 datalogger has two gypsum sensors only separated by 5 cm. When checking the data, both values were very similar, thus we decided to calculate the mean value of both sensors per year, i.e. at the end we have 1 water potential value per each Microlog SP3 and year. To avoid misleading data (frost periods in winter without functional effect on seed regeneration) we only use the water potential data during the growing season (from April to September, avoiding below 0ºC) in 2022 and 2023. Clarification has been made in the L 149 - 157 and in the figure 3 caption. Furthermore, we have included more data points, collected after the initial submission of the manuscript, now data from all four summits is included and shown in figure 3B”.

Table 1 is somewhat confusing because it makes it seem like there were two different experiments with their own levels of replication and unique data -- one focusing on storage treatment, and a separate focusing on water potential. In reality, it seems like all water potentials were applied to all subpopulation x storage combinations to result in a single dataset… and these replicates contributed to the analysis of storage treatment, water potential, or both assessments (i.e. you did not have totally unique sets of replicates for different analyses).  I am wondering if you even need Table 1, or if you could just drop it – I think I had a clearer understanding of the study design and analysis from the manuscript text and Table 2 (which clearly shows how the treatments were applied across different subpopulations. You could also clarify in the data analysis section by simply stating the number of subpopulations/replicates that contributed to each analysis you describe.

We agree that table 1 might not be necessary, nevertheless it was a Journal requirement specified in the submission guidelines. Therefore, we would be happy to remove Table 1 from the manuscript, if the editor agrees. In any case, we have added the clarification of the number of subpopulations that contributed to each analysis in L 236-239 and 280-281.

L297 -298 I assume you included all possible subpopulations and replicates for this analysis, whether data was available for only one or both storage conditions (fresh or AR)? Maybe you could clarify that. I’m also curious whether patterns hold if you reduce this analysis to only the six subpopulations for which fully-crossed data are available (i.e. both storage conditions and all water potentials)?  Have you distributed the storage treatments (fresh, AR or both) across subpopulations evenly such that there is unlikely to be bias based on which subpopulations received only a ‘fresh’ or ‘AR’ assignment (for the 12 receiving only one storage treatment)?

Yes, the reviewer is right in his/her assumption, and to make this clear we have included a clarification in L 280-289.

As suggested by the reviewer we have repeated the analysis with the reduced dataset (fully-crossed subpopulations, N = 6), and the model showed concordant results with the ones previously reported for the full dataset. Seeds in the after-ripening storage treatment showed significantly higher germination than fresh seeds, confirming a certain degree of dormancy in fresh seeds. Details of these results have been added in Table S3 in Supporting Information.

We carefully distributed the storage treatments across subpopulations to include representation from all summits in both storage treatments. We clarified this distribution in L 282-285.

L299 I had to do a little bit of digging to understand that you are estimating ‘median’ water potential as described by Bradford et al. (1990) rather than base water potential of any particular germination subpopulation (which some studies report using). Bradford’s approach is referenced in other parts of the paper, but it would be helpful to give details on the specific metric you are estimating here (i.e. median base water potential), where you are describing the details of the hydrotime analysis. Also, does the median base water potential represent an equivalent metric to the mean base water potential described by Bradford et al. (1990) as an estimate of base water potential for the 50th germination percentile? Why estimate the median instead of the mean?

We appreciate the detailed comment. There was a slight term confusion in the manuscript. We specifically estimated the base water potential using the estimate for the 50th germination percentile following Bradford et al. (1990), but we referred to this metric as the “median” instead of the “mean”. We have clarified the methods and the terminology in L 293 - 296, adding the details of the metric used in our research.

Table 2. The conceptual significance of sigma is never described in the paper, and the other parameters are not so easy to interpret (theta, R2) unless one has a background in hydrotime modeling or read Bradford very carefully. I think a bit more description of these parameters, even in the figure caption would be very helpful to readers (e.g., sigma represents the standard deviation of the base water potential estimate, no?). If it is possible to show the figures underlying the estimated parameters from Table 2, I think that would be even more helpful to interpret these metrics and how they are derived (particularly as I expect future studies use this paper and R package to estimate base water potential). I would suggest adding these to the supplement along with an explanatory caption that describes the estimated metrics.

We agree with the Reviewer's comment. We have added a description of the different parameters used in the caption of Table 2 L 767-773. We also have included the figures underlying the estimated parameters in Figure S2 in Supporting Information.

Table 2 Several estimates of base water potential are positive – why is that, and is that physically/ ecologically feasible? Bradford et al. 1990 describe 0MPa as the upper limit for base water potential, and as far as I can tell  from the supporting figures, all of the seedlots did reach at least ~50% germination at 0MPa. I expect that positive estimates could result from attempts to model median base water potential from observed data with relatively little germination (and few germination percentages near or above 50%)... But would it be more appropriate to assume a maximum base water potential of 0MPa, or to integrate a maximum base water potential of 0MPa into the modeling process in order to avoid positive estimates?

We agree with the reviewer’s point, the positive base water potential values are an artefact created when the hydro-time models are applied to seed populations with final germination below or close to 50% (see updated figure 1 of supporting information). We have repeated the models, but fixing the maximum base water potential to 0 MPa (clarification added in methods L 315-318) and the results did not change.

L301-303  In the text, can you briefly describe the process by which seedr models and returns base water potential? I have not used this package and am unsure whether there are options to choose different model types or specifications when estimating base water potential, or how it combines germination data from multiple replicates. Including brief details in the text would be helpful.

Thank you for your suggestion, we have included step by step explanations in L 297-312. There are no options to choose different model specifications to estimate base water potentials in the hydrotime model (although there are different options when considering thermal time), a more detailed explanation of the options is provided in the online repository where vignettes are also available, (<https://github.com/efernandezpascual/seedr>) as well as in the package documentation (https://CRAN.R-project.org/package=seedr).

L308 I would spell this out – ‘We found a significant interaction between storage and GDD’

Done as requested.

L308-301 Is it necessary to repeat separate, new models for each treatment? Consider using post-hoc comparisons for your full model that offer insight into significantly different treatment combinations (e.g., via the emmeans package in R). These can also be specified to adjust for the multiple comparisons you are making, which may be more appropriate for statistical power than simply rerunning additional, separate models with the same dataset.

Thank you for the suggestion. As we have only 2 levels of storage treatment, we don’t think it is necessary to apply a post hoc test for this factor. The significant interaction already allows us to interpret that the relationship between ψb and GDD was significantly different in fresh seeds vs. after-ripened seeds, and also explicitly tested this difference between storage treatments details in L 352 Then, doing separate models for each separate dataset (the fresh and the after ripened seeds) allows us to focus on that relationship in each storage treatment, without the confounding effect of storage treatment. We clarified the justification in lines L 324-329.

L311 Dickman et al. 2019 (which is cited in the references) included seed mass as a covariate in all models to account for maternal effects. I wonder whether it is worth looking at a similar approach here, to understand not only whether base water potential varies by seed mass, but if seed mass alters or explains the relationship between base water potential and GDD?

Thank you for the comment. In an initial version of the manuscript, we did check the relationship between seed mass and base water potential, but, as the results showed not significant effect of seed mass, we decided to do not include it in the final version. After careful consideration, we agree that seed mass might be an important covariate, which can help to understand wider implications of our results. Thus, now we report the results (L 360-368).

L340-342 Although it does appear that base water potential tends to be lower for after-ripened seeds, don’t the results of your model suggest that this is not a significant difference? (Table S4) If you formally tested this, the analysis results should be clearly stated here. However, I realize this text refers to patterns for the six subpopulations tested in both storage conditions. If you suspect that these results are different (something I was also wondering above, L297), then perhaps consider formalizing and reporting results from a reduced  analysis of storage effects for those six subpopulations.

We appreciate the detailed comment of the reviewer we indeed formally tested this differences in base water potential between storage treatments (first without considering GDD) and results indicate significant differences in base water potential between storage treatments, considering both the full dataset and the subset of six subpopulations (p-value <0.001), details of these analysis have been added in Results L 352 . Nevertheless, when GDD is included in the model, results remain the same (with the full dataset or only the 6 fully crossed subpopulations) with a significant interaction between GDD and storage treatment and when analysed separately to test the strength of the relationship only in after ripened seed, higher GDD correlates with lower germination base water potential. Model results have been added in Table S4 in Supporting Information and clarification has been made in methods L 324 - 329.   
  
L401-405 Can you include some suggestions for how we could test alternative hypotheses, or refine mechanistic understanding, moving forward?

We have added some alternatives we considered to improve our knowledge, for example: to collect seeds from extreme base water potential plots, then do reciprocal sows and control germination in rainfall episodes of diverse intensity while measuring soil base water potential. This idea has been added in discussion L 479-483.   
  
L406-424 Maternal effects are not explicitly mentioned here, but I might discuss their potential importance too, and again, suggest reconsidering an analysis of whether and how they may play a role (by integrating seed mass a covariate in models).  
We checked seed mass covariant and added it in the models, we found no direct significant maternal effects in germination base water potential even though seed mass might be influencing base water potential through its significant relationship with GDD. See methods L 330 reported results in L 360-368 and discussion L 396-402.

L444-446 True, but an alternative approach is to sow seeds in the field and simulate/predict germination timing based on your estimated hydrotime parameters and measured soil water potential to understand how well your lab-estimated parameters and hydrotime models do at predicting field germination outcomes. I think that hydrothermal parameters are potentially very useful and important as species or population-level traits, yet a major limitation continues to be a lack of evidence that they contribute to meaningful differences in germination time in the field.  I think this represents a significant point for discussion (see also next comment).

We agree it would be very interesting to test this hypothesis in the field. We added this to the discussion (L 467-469).

Discussion point: Although there is a significant, negative relationship between GDD and base water potential for after-ripened seeds, the range of base water potentials range from -0.35 to -0.55 MPa, with 5% of values falling into the narrow range of -0.40 to -0.48Mpa. Is this range of variation likely to translate into meaningful differences in germination time in the field? I think the authors should clearly mention the observed range of variation in the discussion, at minimum, and if possible, try to make some interpretations about the likely impacts to germination dynamics at their site based on the observed microclimatic data that they have (Figure 3).

Thank you for the suggestion. We think this is interesting and we have developed this idea. We have calculated, from our data in the field, how much time it takes to go from -0.55 to -0.35 in two different time periods; from 1st of July and from 1st of August (the two months with more drought). Results showed very little (few hours) or non-existent time differences, meaning that once a rain episode happens, the soil water potential spikes very rapidly, surpassing the germination base water potential range limits. These results suggest a limited ecological significance of base water potential in the field. We added this discussion in L 419 - 426.

L447 to 451 expand on points mentioned above, but L451-453 has less context. Can you offer more about whether and how base water potential for germination should be related to other aspects of seed regeneration? Was it related to dormancy in the current study?

We have expanded the discussion in L 484 - 493. The differences to reach germination base water potential, thus the time when germination can start, might be influencing seedling development times, considered highly vulnerable, as well as rehydration cycles in the soil seed bank, which can modify seed soil persistence and viability.   
  
L454-461 I echo my comments from above with a reminder here that this study shows an important trend, but cannot directly demonstrate that base water potential influences fitness, success, or performance in the field, or that this level of observed variation translates to meaningful differences in germination timing that will influence outcomes in different climatic contexts. It is an important first step, and these are all future directions of study. So I might pare these statements back, or build clear support for them throughout the discussion with more direct evidence from other studies, if they exist.

We agree and have toned down the statements.

Reviewer: 2

Comments to the Corresponding author  
This manuscript details a highly important and understudied aspect of seed ecology research – the water thresholds for required for germination – linking field conditions with a laboratory study and using PEG to regulate the amount of water available for germinating seeds.

We appreciate the Reviewer 2 positive opinion about our research. We appreciate all the comments and suggestions, which have been incorporated in the manuscript and discussed in the comments down below. We think the new manuscript has improved through the reviewing process.

The Introduction is well structured and written and out the study into context, including couching the topic in terms of its functional importance and referring to the theory behind hydro-time models. The hypothesis and rationale for the study is well developed, however I would have preferred some more specifically articulated aims for the study, to indicate the details of the various components of the study.

We agreed and have explicitly added the aim of the study in L 117 - 118.   
  
The Methods are clear and suitable to test the hypotheses. Did you also consider whether cold-stratification would be useful to alleviate dormancy in this species – given that some seeds in the wild may persist under snow through winter and then germinate the following growing season?

Yes, we did consider it, nevertheless, we had previous knowledge available from a germination phenology experiment mimicking field temperatures for the same species, now under review in another peer-reviewed journal. In that experiment, the focal species germinates to 100% in autumn conditions between September and November, most of the seeds within the first two weeks without the need for cold stratification. Considering these results, we decided that cold stratification might not be a relevant factor regulating dormancy phenology in the field. More clarification has been added in methods L 228 - 232.   
  
In the germination experiment at 20C with the PEG solutions, did condensation (water droplets) appear inside the petri dishes, and if so, how did you deal with this issue given that the you’re trying to control the amount of water available to the seeds? Also, please explain how you tested that the water potentials in the petri-dishes in this experiment to ensure they were accurate.

We appreciate the comment and have added more details in methods L 256 - 257. Condensation did not happen due to the daily checks being done, nevertheless each petri dish was not open for more than a few seconds a day, so we assume low effects of evaporation because the germination experiment only lasted 28 days. Even if some evaporation happened, we assume that the effect will be similar in all petri dishes, and our manuscript focuses on the relative differences between treatments and seed lots.

Is there a way to visually represent the Bradford hydro-time model results? The patterns across the field collections (sub-populations) aren’t very clear in a table.

We have added the hydro-time model figures for each subpopulations in Figure S2 in supporting information.

The Discussion section is insightful and again highlights the importance of the work in the context with other studies of the abiotic factors restricting alpine seed germination under natural conditions. The caveats of the study are also not unexpected and do not detract from the overall results of the study. Reminding readers to carefully translate laboratory results to field conditions is wise, given the natural variation that occurs in the field.

We appreciate the positive comment.

On line 450, change ‘sows’ to ‘sowing’

Done as requested.  
  
A really nice study. Well done

Thank you for all your positive comments.