THE UNIVERSITY OF WESTERN AUSTRALIA

Centre for Evolutionary Biology

School of Biological Sciences (M092)

Dear Professor Phillips,

We thank you for the opportunity to submit a revised version of our manuscript entitled “Geographic variation in adult and embryonic desiccation tolerance in a terrestrial-breeding frog (version BIORXIV/2018/314351). We appreciate the interest that you and the two reviewers have taken in our manuscript and the valuable and constructive criticism you have all provided. Please find our responses to your comments and those of both reviewers, in detail, below.

Recommender (Prof Phillips)

This is a solid piece of work on an important and fascinating topic. It is a large dataset, is well reported, and the methods and analysis are appropriate. All reviewers saw the value of the work and none found any serious flaws. Consequently, all reviewers' comments are in the nature of suggestions for improvement. I would encourage the authors to consider the reviewer's comments and, where improvements can be made, revise or clarify.

In particular, I thought Lohr's suggestion for the discussion to focus less on mechanism and more on broader implications (targeted gene flow, local adaptation, conservation) was good advice. Some meditation is warranted on how the plasticity you uncovered is relevant to these broader themes.

Gaitan-Espitia's review was particularly thorough, and he raises some interesting thoughts. Being a little more precise with your use of "selection" and being clear that you have evidence for local adaptation, rather than having measured selection per se will address several of his concerns. It would also be useful to report the random effects and residual variance for the fitted models. Doing so gives us more information about the model fit, but also provides information pertaining to maternal/paternal effects, and provides hints about how heritable the traits are. I also wondered about a formal heritability analysis, but felt that the manuscript is already substantial, and there might not be sufficient family groups in many of the populations for robust estimation of heritability. Some information on timing of oviposition would be useful in the methods (how synchronised was their breeding?). Finally, it is worth discussing briefly the role of behaviour (and sexual dimorphism in behaviour and traits) and how these might affect your conclusions.

Response: We fully agree with the recommendations of Prof Phillips and the two reviewers and have addressed them in the manuscript. Please see our responses to the individual points in the sections below. Specifically, we have expanded the final paragraph of the discussion (reviewer 2, comment 4), we are more precise with the term selection (e.g. reviewer 1, comment 7), we provide additional information regarding the timing of oviposition (reviewer 1, comment 5) and we discuss the role of behaviour in buffering selection (reviewer 1, comment 1).

Reviewer #1: Juan Diego Gaitan-Espitia

General comments

Overall, this work of Rudin-Bitterli and colleagues is well written and the experiments and analyses seem to be well executed and replicable. Methods were carefully described, particularly for the experimental approach and the in-vitro fertilisation. The results are short and clear, and the discussion is, in general, easy to follow. Before recommending the MS, however, I invite the authors to address the following comments. I hope these comments and suggestions will serve to improve the quality and impact of this investigation:

1) During the introduction and the discussion, the authors made a strong emphasis on the potential role of directional selection shaping the phenotypic differences on desiccation tolerance in natural populations. This is a valid argument. However, there is almost no information or discussion about geographic clines in selection and/or the role of behaviour buffering selection. These two factors are quite important for the interpretation of the results as: 1) selection can change across spatial and temporal scales shaping different fitness landscapes across the species distribution; and 2) behaviour plays a fundamental role buffering the strength and direction of selection on natural populations, particularly for traits related to desiccation tolerance and evaporative water loss in amphibians (behavioural selection of microhabitats; see for instance Mitchell & Bergmann (2016) (or in lizards Li et al., 2018). In fact, the importance of behaviour and selection of microhabitats for the burrows, are factors that require some attention in the discussion.

Response: This is an excellent point. Indeed, females of other terrestrial-breeding frog species can assess the quality of oviposition sites, and we have now added a new paragraph to the discussion to discuss this topic (L591-607).

2) If I understood well the experimental design used for the within-population crosses, then the authors have the possibility of performing some additional quantitative genetic analyses using a North Carolina II design to disentangle the genetic basis of the traits analysed (heritabilities and perhaps genetic correlations), and the parental/maternal contribution to these traits.

Response: We agree it would be interesting to incorporate quantitative genetic analyses. However, the experimental design of this study does not allow the disentanglement of further sources of variation, as the within-population crosses were set-up according to a maternal half-sib design (not a factorial NCII or indeed paternal half-sibling). Specifically, this design precludes estimates of sire variance, and thus the ability to calculate additive genetic variance, required for estimates of heritability.

3) I am curious about the maternal effects on desiccation tolerance of embryos and hatchlings. There were some effects of ovum size on some of the traits. How was ovum size distributed among populations?

Response: This information is now provided in Table 1 (L933).

4) Desiccation tolerance was focused on adult males and based on this the authors made an extrapolation to the population level. It would be interesting to include some discussion about the potential responses of this trait on females (What have been found in other species?). Water availability may represent a more relevant/important *physiological constraint for females than males, due to the allocation of energy and fluids into eggs.*

Response: We agree it would be interesting to discuss potential differences in dehydration and rehydration rates between the sexes. Unfortunately, water balance has almost exclusively been studied in male anurans. This may be due to the relative ease of collecting males, but we are aware of authors actively excluding females from their studies due to ovarian eggs adding disproportionally to body weight (e.g. see Krakauer 1970). We agree with the reviewer, water availability may represent a more relevant physiological constraint for females due to their allocation of fluids into eggs. Alternatively, however, females may dehydrate more slowly due to their larger size (an allometric effect), particularly when gravid. In the absence of available data, we would prefer to not discuss potential differences between the sexes in the manuscript.

5) It is not clear if the breeding season is synchronised across the species range or if there are some mismatches among populations. This may induce differences in the maturity of the eggs during the experiments.

Response: Breeding is highly influenced by local rainfall patterns and there may be differences of 2-6 weeks in the timing of peak breeding activity between populations. In the current study, all fertilisations were initiated between the 12th and 26th of May 2016, and we have now added this information into the methods section (L221-L222). It is unlikely that temporal variation in the breeding peak would have affected the maturity of the eggs used in our experiments, as females of this species enter the breeding chorus once they are ready to mate (often appearing *en masse* at the breeding site when breeding conditions are ideal), and this is when we collected them. Further, all females responded to the hormone treatment well, ovulation was readily induced and fertilisation success was very high (above 90%) in all crosses, indicating that the eggs obtained were mature.

6) In the discussion (line 405) the authors stated that their findings (intra-specific variation on desiccation tolerance consistent with the environmental/climatic cline) may reflect (in part) cryptic speciation. I am not convinced about this idea based on the experimental design used. Moreover, I did not find arguments in the text supporting this statement… populations genetically structured are not equivalent to cryptic species, and there is not phenotypic divergence (sensu stricto) among populations. There is variability and differences on plastic responses, but not in opposite directions. Moreover, it is hard to tell if this argument is correct, as the authors developed a common garden experiment with 1 generation in which maternal and environmental effects are still present on phenotypic responses.

Response: We agree and have removed this argument from the discussion (L415-L421), as the unpublished work we had described has now been published (Cummins et al. 2019, as cited in the manuscript).

7) In the discussion (lines 436-437), the authors stated that "Together these results are consistent with patterns of directional selection for lower dehydration rates in areas where water is frequently scarce". Although I understand the reasoning behind that, I am not confident with this argument because: 1) The experiment was not designed to measure selection; 2) responses in the lab can be dramatically different to responses on the field; 3) selection varies across temporal and spatial scales; 4) *behaviour in some cases buffers selection. Perhaps a reciprocal transplant experiment may reveal some clues about this…*

Response: We agree and have now removed this sentence from the discussion. Please also see our response to this reviewer's first comment.

Minor comments

How did the authors know that animals were acclimated after 2-5 days?

Response: Due to the second part of the experiment requiring males to be euthanized to harvest their sperm, we were limited in the time we were able to allow males to adjust to laboratory conditions. The time frame of 2-5 days is in line with other studies that have investigated water balance in frogs. However, we have now reframed the wording within the methods section and replaced the word "acclimate" with "maintained at", as we cannot confirm that frogs were acclimated after 2-5 days (L143-144).

Line 186 – Similarly to similar

Response: This change has been implemented (L195).

Reviewer #2: Jennifer N. Lohr

This is a review of the paper "Geographic variation in adult and embryonic desiccation tolerance in a terrestrial-breeding frog" from authors Rudin-Bitterli, Evans and Mitchell at the University of Western Australia.

Summary:

In this study Rudin-Bitterli et al. investigate the intra-specific variation present regarding desiccation tolerance in one species of terrestrial-breeding frog in Australia. They use six populations of frogs originating from locations with different amounts of annual rainfall and also perform crosses between the populations. I agree with the authors that intra-specific variation in an important factor for conservation efforts and in need of more extensive study.

I found it a well-written manuscript with appropriate methods and statistics. The authors measure many traits in detail, which adds a lot of power to the study. Below are a few points, which I think might help to improve the quality and clarity of the manuscript.

Major Points:

1. Purpose of crosses not stated in abstract – also in the introduction/discussion it might be good to address the value of your crosses. There was a lot of effort that went into this part of the study and so it should be stated more directly what knowledge we gain from this instead of just measuring the tolerance of adults.

Response: We have now added this information both to the abstract (L11-12) and the introduction (L97-102).

2. Statistics: authors seem to make use of the proper tests, R-packages and normalization methods beforehand. Maybe state at the beginning of the methods how normality was check for all data and not just in the second section – I assume the authors also did Q-Q plots for the dehydration/rehydration data, etc.

Response: Yes, all data were checked for normality prior to analysis but we failed to mention this for the dehydration/rehydration data. This information has now been added (L302-304).

Maybe one question about the statistics for rehydration/dehydration rates. Here the authors present ANOVA results for the 6 populations and lin reg results by rainfall measure. Could the authors maybe comment if it is justified to do both test separately with independent P-values? As far as I understand the data set each population is also one rainfall measurement, so in fact these two tests are actually the same and this is a bit of double reporting (i.e. not independent hypotheses)? In this case it might be best to report your ANOVA and then give an R2 value using the same model as your ANOVA. Then it is only one test and you are just reporting an effect size.

Response: We agree and have now removed the regression analysis and Figures 3B and 3C.

3. Tests were performed on males only. It is stated in the methods that females are harder to obtain. Could the authors add a note about the expected effects in females and what is known from other studies?

Response: This comment has been addressed in an earlier section (pages 4 & 5). As water balance has almost exclusively been studied in males, it is difficult to predict the effects in females. Females may dehydrate more slowly due to their larger size (an allometric effect), particularly when gravid. Alternatively, however, water availability may represent a more relevant physiological constraint for females due to their allocation of fluids into eggs. In the absence of available data, we would prefer to not discuss potential differences between the sexes in the manuscript.

4. Discussion: there is a rather large amount of text on mechanisms in the discussion. While this is very interesting, it seems not to be either the focus of this paper nor the points to which your results can be best applied. The final summary paragraph of the discussion is actually the interesting part. Here you compare your results to other similar ones and discuss phenotypic plasticity and local adaptation – in your frogs.

I think it might improve the interest of the manuscript to expand on this last paragraph a bit and to place your evidence for local adaptation within the general framework of conservation biology as a whole, not just your species of frogs. Specifically, the last sentence of your abstract states "We emphasise the importance of considering geographic variation in phenotypic plasticity when predicting how species will respond to climate change." And also in the introduction you talk about how "In particular, information on the environmental sensitivity of range-edge populations will be vital for understanding future changes in species distributions, since it is at range edges where colonisations and extinctions primarily occur as the climate changes". Both interesting points. I think it would be nice to come back to this in the discussion and relate your results to those from other species to try to speak to these challenges.

Response: This is an excellent point. The last paragraph has now been expanded (L629-L634).

Minor Points:

1. line 175-176: "The eggs of each female were divided equally into five groups and fertilised separately with sperm from one of five males." Does this mean each combination of 1-5 males, or randomly 1-5 males – clarify.

Response:

The eggs of each female were always fertilised with sperm from five males (never <5 males) which were chosen at random. To achieve this, the eggs of each female were divided equally into five groups, and then each group was fertilised separately with the sperm from one of the five males. We have now clarified this within the methods section (L185).

2. line 186 – change similarly to similar

Response: This change has been implemented (L195)

3. line 204 – change de-ionised to deionised to match with other usage in text

Response: This change has been implemented (L213)

4. line 221-222: homogenised soil, previously collected from a P. guentheri breeding site – was different soil collected from the various sample sites or all from one site? Maybe state that in the methods since you are looking for between site differences the origin of the soil may also have an effect.

Response: All soil originated from a single *P. guentheri* site (Pinjar), as a water-retention curve had been developed for this specific soil type (Eads et al. 2012). We have now clarified this in the methods section (L233-234).

5. line 249: "Swimming performance was recorded 6 - 12 hours after hatching on a subset of hatchlings" - was recorded ***for*** *a subset?*

Response: This change has been implemented (L266).

6. line 407: ***A*** *recent genetic analysis*

Response: This section has been removed in response to an earlier comment (cryptic speciation argument, page 4).

The University of Western Australia
M092 Perth WA 6009 Australia

T +61 8 6488 2010

F +61 8 6488 1029

E tabitha.rudin-bitterli@research.uwa.edu.au