02-Dec-2022   
  
Dear Dr. Mueller,   
  
First...thanks for the manuscript!   
  
Manuscript ID IOB-2022-043 entitled "Metamorphosis imposes variable constraints on genome expansion through effects on development" which you submitted to the Integrative Organismal Biology: A Journal of the Society for Integrative and Comparative Biology, has been reviewed.  The comments of the reviewers are included at the bottom of this letter.   
  
The editors are really excited about his work and we can see a few places where the work can be clarified and improved. I look forward to seeing your revision, which we will send out to at least one of the reviewers.    
  
To revise your manuscript, log into https://mc.manuscriptcentral.com/iob and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions."  Under "Actions," click on "Create a Revision."  Your manuscript number has been appended to denote a revision.   
  
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IMPORTANT:  Your original files are available to you when you upload your revised manuscript.  Please delete any redundant files before completing the submission.   
  
Because we are trying to facilitate timely publication of manuscripts submitted to the Integrative Organismal Biology: A Journal of the Society for Integrative and Comparative Biology, your revised manuscript should be uploaded along with your response to the reviewers (in a separate file) by such time as RSV, pandemics, vaccines, and the stressors of the holiday season allow you the mental energy to fully appreciate the reviewers' comments.   
  
If it is not possible for you to submit your revision in this time frame please contact our office (icbjournal@sicb.org).   
If after a possible extension, you're unable to upload the revision in a reasonable date, we may have to consider your paper as a new submission.   
  
Once again, thank you for submitting your manuscript to the Integrative Organismal Biology: A Journal of the Society for Integrative and Comparative Biology and I look forward to receiving your revision.   
  
Sincerely,   
Dr. Adam Summers   
Editor in Chief, Integrative Organismal Biology: A Journal of the Society for Integrative and Comparative Biology   
IOB\_editor@sicb.org   
  
Associate Editor   
Comments to the Author:   
This is an interesting manuscript, and I especially appreciate the care you have taken in explaining your hypotheses and grounding them in biological observation, as well as the extensive supplementary methods explaining your code.  The reviewers have made constructive comments about the manuscript's structure, and reviewer 2 in particular commented on potential issues with the statistical analysis that need to be addressed. Being a little more careful in interpretation, and explaining why, will strengthen the manuscript's potential to serve as a model for future phylogenetic comparative analyses.

**We appreciate these comments and those of both reviewers, and we have incorporated them in revising our manuscript. We agree that they strengthen the work substantially overall.**  
  
Reviewer: 1   
  
Comments to the Author   
This paper provides a great study on genome size evolution in salamanders while also making clear, broader points about the utility of the featured comparative methods. This combination of conceptual points (especially around language) + case study, make this paper a really useful addition to the field. The introduction was incredibly clear and most of my minor comments are simply suggestions for clarifying language and methods.

**Thank you for this positive assessment of our work and for the comments overall.**  
  
In the methods, some of the information in the “Stochastic Genome Expansion” section seems highly relevant to the overarching hypotheses of the paper and could be moved into the introduction to provide key context.   
I don’t think this is something the authors “need” to move, just a suggestion.

**Thank you for this suggestion, which was also made by Reviewer 2. We have moved this section into the Introduction.**  
  
215: How many miniaturized taxa were excluded? And how is miniaturized defined here?

**This is a good question, and it was also raised by Reviewer 2. Miniaturization is defined as mean SVL < 35 mm, per Decena-Segarra et al. 2020. Miniaturization, genome size, and biological size in a diverse clade of salamanders. The American Naturalist 196(5):634-48. We excluded 20 species based on this criterion. We added this to the manuscript as well as suggesting that the interaction between miniaturization and life history in shaping genome size is an important target for future research.**   
  
Line 278: A suggestion that “we do not” should be “we did not”

**We have followed this suggestion.**  
  
540: you mention that “ direct developers do not have smaller genome size equilibria than gradual metamorphosers, suggesting that any constraint imposed by energetic limitation in direct developers is weak.”   
This is one potential interpretation but another is that this finding suggests energetic limitation is not constraining (or at all affecting) genome size, regardless of being a direct developer or not.

**Great point. We have revised the manuscript to indicate that our results are consistent with energetic limitation being weak or absent.**  
  
The discussion has a lot of great information but is a bit harder to follow than the introduction. My suggestion (which is a “suggestion” not a “requirement for publication”) is to:   
More explicitly explain what is known about the connection/associations between TE accumulation and the three vulnerabilities here. This is a critical assumption to how hypotheses were devised and interpreted for the study.   
Use the really clear and helpful relationships between the development strategies and the vulnerabilities in Table 4 to make clear predictions of how your results for both “pull” and “stochastic” measures in Table 2 support (or not) the likelihood that each variability affects genome size evolution.   
Most of this information is definitely in the text but there are so many different strategies, taxonomic examples, and results, that the clear connections among these points aren’t easy for a reader to digest.   
One related thought is to bring up the justification for these hypotheses earlier in the paper, so that the discussion can simply focus on how the results support (or not) the distinctions among these development modes in relation to the three vulnerabilities. But I think there are a few ways to effectively deliver this information and I defer to the authors on their preferred writing outline.

**We really appreciate this thoughtful set of comments. In truth, we experimented with many ways of structuring the discussion (as well as the introduction) while drafting this manuscript, and we believe we have converged on the best way to explain what is admittedly a complex body of work.** **We appreciate the reviewer deferring to our preference here.**  
  
627:  “our results support the idea that loss of metamorphosis has released constraints against genome expansion in paedomorphs.”   
I think a really interesting and useful feature of this paper is the thoughtful use of language in relation to the OU parameters. But at times the discussion mentions “release from constraints” without addressing the added assumption that without constraints to keep genomes small, TEs will inevitably accumulate. I think keeping these points hand-in-hand when discussing paedomorphs is important because if it was ONLY a release from constraints, we might assume we would see increased stochasticity in addition to genome expansion. But instead you just see the deterministic pull, which is really cool! But should be clearly distinguished from other interpretations folks may have with the phrase “released from constraint”.   
As a whole, the paper is very clear in this distinction. But specific points in the discussion could be misconstrued and a few extra clarifying words could prevent that and make the points clearer for readers.

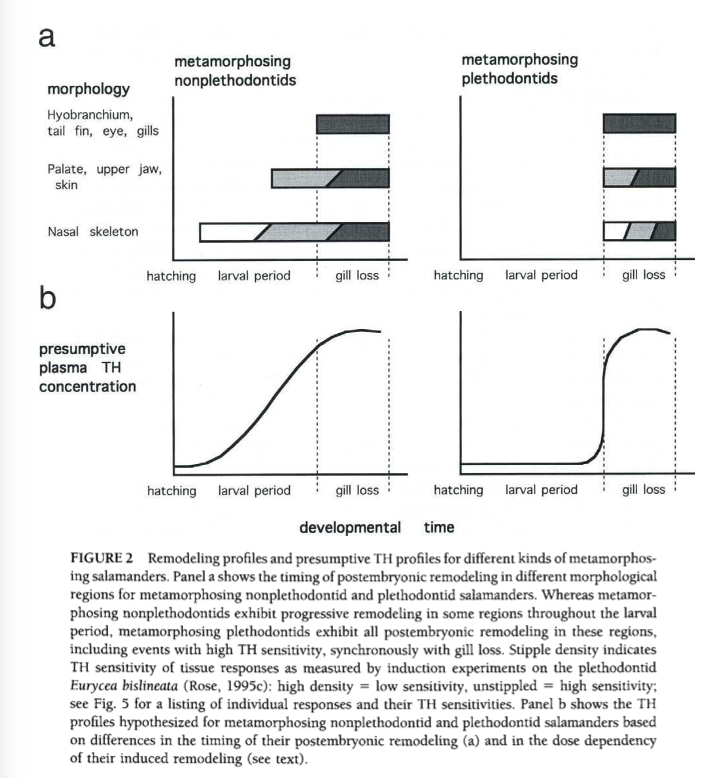
**Thank you for this excellent point – we have clarified in the Discussion that we are talking specifically releasing constraint against biased stochastic genome expansion.**   
  
  
Reviewer: 2   
  
Comments to the Author   
Overview   
Overall, I found this manuscript to be interesting, clear, well-written and easy to follow.  The methodological point about interpreting theta as something other than a selective optimum is one with very general applications.  The supplemental files are excellent.  Thus, overall, I believe that this manuscript will make a valuable contribution to the literature.     
  
However, I also have several questions and comments about data coding, models, model interpretation and use of the term ‘constraint’.   I believe that addressing these will lead to significant improvement in an already good manuscript, and will also reduce the chances that results will be misinterpreted by others.     
  
Questions about data analysis and interpretation   
Significant statistical issue   
The most serious problem with this manuscript is a statistical issue that pervades most uses of phylogenetic comparative analyses.   See Case Study II of Uyeda et al. (2018), https://doi.org/10.1093/sysbio/syy031, for a more detailed explanation of the statistical issue as well as possible ways to address it.  Even though this article focuses on examples where a trait originates just once, the statistical problem is not limited to these cases.  At a minimum, the authors should also explain the limitations of the models they used—in particular the fact that a clade-specific effect could easily be interpreted as a life-cycle specific effect, because the only possible explanations are based on life cycle.  Better   
  
l. 372-375 provides an example of the kind of overinterpretation of the results that results from ignoring this statistical issue.  In this case, life history was the only biological trait that was allowed to explain variation in the deterministic equilibrium for genome size.  In instances like this, if there is substantial variation across the phylogeny for other reasons, they are prone to be misinterpreted as reflecting life history variation.  The parametric bootstrapping approach is not sufficient to control for this.  (Example: suppose a new deterministic equilibrium evolved in the ancestor of a large clade, which predominantly had a different life history than other taxa.  In many cases, this would favor the more complex model, and would thus be interpreted as evidence that the life history feature explained variation.)

**Thank you for raising this important point, and we agree that it is critical not to overstate the strength of conclusions that can be drawn from our analyses. We have added text to explain these limitations of our models, as suggested by the reviewer. We also added a citation to the Uyeda et al. 2018 paper discussed by the reviewer.**

Data questions   
E. quadridigitata: could not figure out why its metamorphosis is coded differently from that of other plethodontids with larval forms (e.g., Semlitsch 1980—occurs at a fixed point)

**This coding was a mistake on our part and we are very glad to have had it spotted. We have fixed this error. Fortunately, it had no effect on the results, only slightly shifting quantitative values (e.g., of parameter estimates).**  
  
l. 192-193: More discussion of this category is needed, as well as better description of the evidence used in deciding how to classify different species.  For example, Rhyacotriton has a life history/ecology that seem very plethodontid-like, yet the genus is classified as gradual metamorphosers, whereas almost all biphasic plethodontids are classified as abrupt.  What justifies this difference?  What about Dicamptodon, where decreased feeding led to increased probability of metamorphosis (opposite the pattern expected for gradual metamorphosis)?  (See Coriell, S. 2003. Environmental factors affecting paedomorphosis in the Pacific giant salamander, Dicamptodon tenebrosus. Unpubl. Master's thesis,. Humboldt State University. Arcata, California) 

**This is a really important point, and we are grateful to the reviewer for raising the need to clarify this. The distinction between gradual and abrupt metamorphosers follows the work of Chris Rose (e.g. Rose CS. 1999. Hormonal control in larval development and evolution—amphibians. In. The Origin And Evolution Of Larval Forms: Elsevier. p. 167-VI. as well as the others we cite). We have included a figure from the 1999 paper below. We revised the manuscript to make it clear that “gradual, synchronous” and “abrupt” are words taken from Rose et al. to make it more clear that these categories are established in the literature, and that they reflect differences in the rapidity of thyroid hormone level increase. We also fixed the miscoding of *E. quadridigitata*, which would understandably have led to confusion about these regimes. Finally, we also added that abrupt, synchronous metamorphosis has evolved only within the plethodontidae.**



l. 196-197: check reference (as statement is about salamanders as a whole whereas cited reference is about plethodontids specifically) 

**We have clarified that this form of metamorphosis only evolved within the plethodontids, which should clarify why this is the correct citation. Please also see our response to the comment above.**

Analysis questions/suggestions   
l. 214-216: This seems a bit like picking and choosing the data one wants to include.  To what extent is it clear which taxa are miniaturized (e.g. physical size and biological size are different because of differences in genome size)?  What happens if miniaturized taxa are included?  If all miniaturized taxa are direct developing, then this could be tested as an additional category in the model. As an alternative, biological size or a bivariate miniaturized/not miniaturized character could be included in the analysis.

**This is a good question, and it was also raised by Reviewer 1. Miniaturization is defined as mean SVL < 35 mm, per Decena-Segarra et al. 2020. Miniaturization, genome size, and biological size in a diverse clade of salamanders. The American Naturalist 196(5):634-48. We excluded 20 species based on this criterion. We added this to the manuscript as well as suggesting that the interaction between miniaturization and life history in shaping genome size is an important target for future research.**   
    
  
l. 253-255: This seems wrong.  A single-optimum OU model (a simpler model than a multiple-optimum model) is possible.

**Thank you for pointing this out. We have corrected the manuscript to clarify that we meant the simplest multi-regime OU model.**

l. 258-271: Given the expectation of mutational bias, an additional non-selective model should also be tested, a biased random walk (e.g., Gill et al.  https://doi.org/10.1093/sysbio/syw093).

**Thank you -- this is a really interesting point, and we realized in response to this comment that we needed to be clearer about our model choices. Our null model (random evolution, BM) is expected to capture neither of the two directional forces we hypothesize are acting on genome size (i.e. biased stochastic genome expansion and constraint against large genome sizes). Our remaining models are meant to capture the hypothesized balance between these two forces. We can definitely see the logic of adding a biased random walk model and appreciate the value of the Gill et al. approach; in our case, it would capture one of the two hypothesized forces, but not the other. However, in reality, it would require a substantial effort to recode all of our models such that they could incorporate this additional comparison, and we do not think that this effort is warranted by the additional information it would yield. We did make several edits to the manuscript to clarify this, including 1) the addition of the Gill et al. citation and mention of biased BM as an interesting target for future research, and 2) the deletion of a somewhat tangential mention of the relative values of BM and biased BM models in modeling unconstrained genome expansion. We also clarified in the methods that BM models neither of the deterministic forces we hypothesize are acting on genome size, and made other small clarifications throughout. Again, we truly appreciate this suggestion and hope that these edits will clarify our model choices and what their comparison reveals.**

Other data interpretation questions   
Overall, I found some of the argumentation around direct development unpersuasive.  Specific examples are below:   
l. 95-96: metamorphic remodeling within the egg: what is the evidence that this involves greater change than normal embryonic development?  (This would need to be true to support the reasoning used here.)  The extent to which developmental trajectories recapitulate larval development followed by metamorphosis varies greatly across direct-developing salamanders.  See, for example, Wake and Hanken Int. J. D BioI. 40:859-869 (1996).  Additionally, time spent in eggs is highly variable across salamanders.  Some direct developers spend longer in eggs than is spent in embryonic development + metamorphosis  (excluding larval growth phase).

**Thank you for this comment. Here, the argument we are making is based on a comparison between direct developing lineages and paedomorphic lineages. The key point here is that, overall, paedomorphic lineages undergo fewer events of metamorphic remodeling than do direct developers. We revised the manuscript to make this clearer. We completely agree that there is variation in ontogeny across direct-developing lineages, and this comment helped us see that we should cite this work here as well as in the Discussion (where we had it cited previously). We have made that improvement.**

l. 173-176: On what basis are direct developers assumed to pay higher fitness costs for developmental errors than biphasic taxa are?  (Presumably the same arguments would lead to the conclusion that developmental errors would be common in larvae as well.)

**Good question. We are talking here specifically about the developmental errors associated with decreased cell numbers in direct developers, where metamorphic structures form in a much smaller developing organism. We expand this more in the discussion. More generally, we want to reiterate that at this stage in the manuscript, we are making predictions; we modified the language a bit to make this more evident.**

l. 533-540: On what basis is direct development of a juvenile within the egg assumed to require greater energetic resources than development of a larva (which, in many species, has fully formed all of its major organs, etc.)?

**Good question. We are not making any claims about overall energetic requirements, and we agree with the reviewer that the one raised here would be difficult to justify. We are talking specifically about vulnerability to energetic limitations (which we detail earlier in the manuscript) – the organism running a risk of being unable to meet its energetic needs.**

l. 569-574: similarity to insects.  An alternative interpretation of this similarity to insects strikes me as more plausible (and in line with a main point of this manuscript), namely that holometabolous species are vulnerable during metamorphosis in a way that hemimetabolous species are not (because the former are non-feeding and non-motile), and thus there may be selection to reduce time spent in metamorphosis.

**Great point. We have added this alternative interpretation to the manuscript.**

l. 575-576: It’s unclear to me why having fewer cells/less tissue means that less coordination is required.  Here’s a simple example: suppose that some transformation requires contact between an epithelium and a mesenchyme; if both of these are small, it would seem to require greater precision for them to come into contact than if both were already large tissues.

**We are considering cell-cell coordination across the entire developing tissue, which we posit is a simpler problem of spatiotemporal coordination in a small versus large tissue. We can see the logic of the reviewer’s comment, but we think it applies in the case of two small groups of cells “finding” each other within a larger total tissue field.**

l. 600: from context here, one would expect that Desmognathus are (all) direct developers. Also, the restriction to eastern Plethodon seems artificial and designed to add unjustified weight to the cited evidence, as there’s no reason to expect that the ontogenetic repatterning in western Plethodon will be any different than in eastern Plethodon, despite their much larger genome sizes. 

**This is a good point. We have revised the manuscription to avoid adding unjustified weight to this limited empirical data, and we included discussion of western Plethodon.**

l. 602-604: Why does the difference have to be due to differences in developmental error rather than other factors?

**We toned down the language in the manuscript and now state that the differences *are consistent with* differences in developmental error.**

l. 640-641: similarity in genome size of gradual metamorphosers and direct developers: of course, many of these are closely related (and indeed, the paedomorphs that are closely related also have similar genome sizes)—these thoughts should be reevaluated in light of the main statistical concern.

**We see the reviewer’s concern, but we would counter with the fact that it is the abrupt metamorphosers that are the closer relatives of the direct developers, not the gradual metamorphosers.** **It is true that the paedomorphs that are more closely related to metamorphosers have smaller genome sizes, and this is to be expected because of the shorter branch lengths associated with these instances of paedomorphosis (and thus less time spent evolving under the paedomorphic regime).**  
------------------------------------------------------------------------------------   
l. 138-152 (with implications elsewhere in the ms.) The reasoning about metamorphic vulnerabilities appears to also predict that developmental speed may be under much stronger selection in some species/clades/environments than others.  For example, warm temperatures in tropics may weaken selection against genome size expansion, as the extra time spent in a vulnerable life stage will be lower, thus decreasing the strength of selection.

**This is an interesting point that is well worth considering. We offer that temperature differences are also likely to affect other variables beyond just developmental speed that may well be relevant here (i.e. performance, energetics, and developmental error). We do not view predictions here as straightforward, and to give them the depth of research and discussion that they deserve is beyond the scope of the current manuscript. We thank the reviewer for this thought-provoking idea.**    
  
l. 604-607: Is there really sufficient statistical power to detect differences in variance of the stationary distribution?  It appears that variance is correlated with sample size in the data.

**This is a good point, and we have added a caveat to reflect this in the manuscript.**

l. 668-676: variation in variance/noise intensity: I recognize that a model with multiple noise intensity parameters provided a better fit, but to what extent is there evidence for significant differences between specific life history types?  Many of the 95% CIs in table 3 overlap.

**This result is supported by the parametric bootstrap model comparison assessing the power to discriminate among models presented in Figure 2f: p = 0.002, power = 0.958.**   
  
l. 673-675: the claim here, about ‘variable constraints’, makes it sound like the authors are suggesting that there are multiple different deterministic equilibria or optima, but that does not seem like an appropriate interpretation of the models.

**This is another good point. We have clarified the manuscript to raise the possibility that the large variance reflects true differences in constraint among direct-developers that were not captured by our models and are a potential target of future research.**

Fig. 3: Please be specific about the stationary distribution, in particular that it’s the stationary distribution expected if the ancestral value is at the optimum (=deterministic equilibrium).

**We have made this addition to the Figure Legend.**    
  
Table 3: There must be a better way to show this information—either bar graphs or some other table format

**We imagine that when the table is formatted for publication, it will be easier to read.**   
  
***Please note that we respond to all of the individual comments about constraint together****,* ***below:***  
Use of the term ‘constraint’   
I appreciate it that the authors are clear about what they mean by ‘constraint’ (l. 76-77).  Nonetheless, they appear to always (or almost always) be equating it to selection (although often selection on a character that is connected to genome size, rather than directly on genome size) rather than to non-adaptive causes.  Thus, using the term selection throughout instead would help prevent misinterpretations resulting from the many different meanings constraint has—both across fields, and to individual researchers.  At a minimum, constraint and selection shouldn’t be use together implying that they are two separate phenomena, when the only form of constraint that fits is selection.  When both are used, examples of non-adaptive sources should be given, or alternatively, reformulating in terms of selection would lead to greater clarity.     
  
l. 75: here, ‘constraint’ appears synonymous with selection (i.e., time-limited metamorphosis selects against genome size increase).     
l. 116: “constraint set by other aspects of organismal biology”—here, the only meaning of constraint I see is selection.     
l. 243-4, l. 248, l. 263, l. 265, 1. 269, l. 270: Other cases where it’s unclear what would count as constraint that isn’t selection (even though both are mentioned, suggesting they are distinct).   
l. 304: ‘metamorphosis selects against genome expansion’ seems accurate     
l. 610: another place where it’s unclear what non-selective constraints might apply   
l. 627: ‘released constraint’ = relaxed selection   
l. 646, l. 687: other places where ‘selection’ would seem to be clearer.   
  
Other comments:   
l. 77: “indirect selection on a correlated trait”: rephrase, since the scenario is one in which there is direct selection on the correlated trait (leading to changes in the focal trait).

**We really appreciate the reviewer pointing out that our use of “constraint” was not as clear as it should be. We agree this is critically important. The reviewer is correct that we are referring to direct selection on a correlated trait (e.g. duration of metamorphosis) indirectly shaping the focal trait (i.e. genome size). We clarified this in the manuscript** **where we first define constraint in the Introduction and subsequently throughout the manuscript as needed.**

l. 124-136: This reads like background; it does not describe methods that were used.  Thus, I recommend moving the relevant information to the introduction (or potentially discussion).

**Thank you for this suggestion, which was also made by Reviewer 1. We have moved this section into the Introduction.** 

l. 218: change ‘need’ to ‘ability’

**We have followed this suggestion.**  
  
l. 615: is it ability or rate that decreases?

**Good question. We have clarified in the manuscript that it is both rate and, in some cases, the ability to do so at all that decrease.**   
  
l. 683; change ‘variation’ to ‘values’

**We have followed this good suggestion**.  
  
Table 1: note that bar graphs of genome size are slightly misaligned (too high) relative to tree & species names   
  
Table 2: Add LnL and number of parameters for each model