nature portfolio

Peer Review File

An archetype and scaling of developmental tissue dynamics across species

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This is an interesting study attempting to find a common morphometric plan underlying the development of hindlimbs in two vertebrate model organisms which differ in size, shape and developmental timing. It combines mathematical modelling with cell tracking in vivo, and builds on a previous study of the chicken hind limb bud. The study uses a very clever method of labelling many. small groups of cells in the mesenchyme along with mathematical modelling which supports an "archetype" for stage 52 to 54. This stage interval is about 5 days of Xenopus tadpole development (starting 9 days after the buds appear, and finishing as digits begin to condense). At these stages the mesenchyme anterior-posterior axis is well established, and cells fated to form the stylopod, zeugopod and autopod can be identified (Tschumi 1957). Effectively, the study and model covers the period when zeugopodal cartilage elements condense and when digits and interdigits are being defined in the autopod. The latter has been recently shown to be recapitulated in in vitro culture in mice, indicating self-organising ability of the limb bud mesenchyme (Fuiten et el PMID: 36994104)The model is backed up with some nice RNAscope data of anterior posterior marker hox genes. There are also of course more interdigital regions in frog hindlimb (5 digits) vs. chick hindlimb (4 digits) and I can't see this reflected in the model or discussed. Major: The paper would benefit from some more background into the model to be more accessible to biologist readers. The discussion is brief and includes no citations, so is more a conclusion. I think at the least it would be good to address the limitations of this model in terms of the stages it covers, and perhaps to expand the discussion to include reference to the work of others – e.g. What is known about cell division rate and orientation in these models, or in other vertebrates, and how would this direct morphogenetic growth in a comparable way? Additionally I suggest changes that I think would assist with broad readership appeal as "minor" below.
Minor:
Throughout: species names in italics
Abstract: include full species names for both chicken and Xenopus
line 71 I am not familiar with the exact meaning of "tissue deformation" in this context, and think it would be more accessible to wider readership if this was briefly explained.
Line 84: similarly, what is a tissue deformation map?
Line 114: can you explain why "on the dorsal ventral boundary plane" and what this means? Is the grid focussed somehow so that epithelial cells are not triggered to express GFP? The image in figure 2A (and S1) is really nice but I cannot tell if it is mesenchymal only – were two lasers used to focus on a single spot, and only where they meet the heat shock promoter activates?
Line 120/121 state the Nieuwkoop and Faber stages used?
Line 135: Xenopus limb bud stages are points on a continuum, but how did you accurately subdivide each stage in to 10th stages?
Line 136 state HH stages
Line 139 citations are needed to support this "Xenopus limb buds include the prospective autopod (toe-to-ankle), zeugopod (lower leg), and stylopod (upper leg) regions. In contrast, chick limb buds contain mainly the former two, while the stylopod is embedded in the trunk.
Line 173 gene names in italics
Line 229 "we can intuitively see…" please explain what we are supposed to be seeing because I cannot make this conclusion from the coloured vectors in figure 3C and D. Are there even interdigits present at stage 52? Maybe this refers to another figure?
Paragraph starting line 236: what criteria did you use/match to set the clock for each

organism (e.g cartilage condensation, gene expression?)

Line 487 H2O2
Line 499 I'm still not understanding what you mean by dorsal ventral boundary: the mesenchyme does not really have such a boundary (unlike the epithelial AER?) – does this mean cells were labelled in the centre relative to the DV axis, where lmx1b positive and negative cells meet? Could you perhaps illustrate this better with a figure?
Line 669 Gene names in italics

Figure 1: I find this legend to be a bit overlong, it should really be re-written to just describe what is shown and the rest can be incorporated into the text? In particular, having a study aim in the legend seems out of place.

Figure 2 The Xenopus A-P axis is posturally inverted: shh marking the ZPA clearly shows this for the stages of this study: Endo et al 1997 PMID: 9186057, Keenan and Beck 2016 PMID: 26404044, Wang et al 2015 PMID: 26527308. I can see you've inverted the limbs in this figure to account for this but it should be stated.
I'm not clear on how the maps are made- 1D or is information captured from multiple layers and stacked? If the former then how do you account for cells that move or divide dorsoventrally in the mesenchyme?
Figure 3 rather than using left and right, fro C and D maybe label all the panels ? In the legend, the final statement "Note that the dynamics for the prospective autopod and zeugopod are compared" at the end does not seem to obviously relate to anything shown in the figure?

Reviewer #2 (Remarks to the Author):

ms review
NCOMMS-23-19472-T

The goal of the paper is to explore whether there are conserved 'archetypal' growth dynamics in organs detectable among species. The authors produce tissue deformation maps through developmental stages of chick and Xenopus for comparison. For comparisons of tissue dynamics among species, they propose rescaling tissue deformation and synchronizing developmental clocks. They conclude that chicks and xenopus share A-P axis asymmetry, as well as the pattern of cell division contributing to elongation of the limb bud.

The study is a valuable contribution to the limb development in describing details of shape change and dynamics over ontogeny and attempting to devise a method for comparisons that helps to understand underlying principles.

My main comments here are about this study's comparison of species. The study lacks a necessary discussion of anuran limb development as qualitatively different than amniotes (chick), first because anuran limb development derives from different tissues and by a different initiating process. The elongation of the frog limb may indeed independently recapitulate the amniote limb developmental characteristics, but the fundamental developmental difference of having the thyroid-dependent metamorphosis in frogs drive limb

initiation, rather than limb buds derived from the early axis/embryonic lateral mesoderm, is not mentioned.

Secondly, the authors conclude - line 403 - "the spatiotemporal expression patterns of hox genes essential for limb
development were also found to be conserved"

I wonder about this, as the anuran hind limb zeugopod to autopod boundary is complicated by the proximal tarsal modification into an additional hindlimb segment; along with this morphology, the HoxA11- HoxA13 boundary distinguishing the zeugopod is modified. See: Blanco, M. J., Misof, B. Y., Wagner, G. P., Blanco, M. J., Misof, B. Y., & Wagner, G. P. (1998). Heterochronic differences of Hoxa-11 expression in Xenopus fore-and hind limb development: evidence for lower limb identity of the anuran ankle bones. Development genes and evolution, 208(4), 175.

Thirdly, the 'paddle' formation in the autopod of anurans at digit formation is also qualitatively different from the amniotes.

from: Fabrezi, M, Goldberg, J, Pereyra, MC. 2017. Morphological variation in anuran limbs: Constraints and novelties. J. Exp. Zool. (Mol. Dev. Evol.) 328B: 546– 574.

"Cameron and Fallon (1977) noted how the patterns of digit formation of amphibians differed from that of amniotes. In amphibians, there are no interdigital zones of massive cell death during digit formation. Rather the digits appear to arise by differential proliferation of interdigital and digital cells. Each digital primordium first grows and enlarges, and then it segments to form the interphalangeal joints and the precise number of phalanges (Sanz-Ezquerro and Tickle, 2003). In amphibians and lizards, this process seems to be different from mammals and birds because, even when phalanges differentiate by segmentation, there is no continuous primordium dividing up into as many phalanges. Instead, phalanges appear as cartilaginous condensations that grow in size and then segment to form the next phalange in a proximo-distal direction (Fig. 6)."

All these features would affect the shape dynamics described.
Given that the purpose of the paper is to provide a method for comparing species differences, the acknowledgement of the fundamental differences of highly derived forms requires a review of evidence for homology as a starting point. The differences in gross developmental contexts shouldn't be ignored.

That said, I think this study could potentially provide evidence for similar tissue dynamics *in spite of* the big differences between anurans and chicks, and this discussion is completely missing. For example, if a mouse and chick were compared, there is a strongly supported developmental homology that is 'tinkered' with to create the differences among species, so that the tissue dynamics synchronization is more easily interpretable for similarity and difference from an evolutionary perspective. However, if they find similarities between amniote and anuran limb development, it possibly says more about a deeper question of how evolution is constrained by tissue dynamics even in the case where the 'limb program' is redeployed in a different context (i.e., the post-larval metamorphosis). I would think this is especially important for the stated goal of finding "archetype" forms for organs. (I think they are really assessing is an archetypal ontogeny, not just the resulting form..?)

What an archetype is begs some other questions, which would be worthwhile to present

here. Why are they similar? Any tissue dynamics-based archetype description would need additional discussion of biophysics as a mechanistic contribution adding to this primarily morphological comparison. It could strengthen the discussion.

The conclusion that the pattern of cell division ("cell flow") contributing to elongation of the limb bud is an important point overall as it contradicts the paradigm that the distal cells are where elongation primarily arises.
Also see
Young, J. J., & Tabin, C. J. (2017). Saunders's framework for understanding limb development as a platform for investigating limb evolution. Developmental biology, 429(2), 401-408.

Both D'arcy Thompson's and this study are missing the third dimension of shape, which is critical in cartilage condensation initiation and digit development.

Reviewer #3 (Remarks to the Author):

This manuscript presents a timely exploration of a significant research question, namely, how to establish a standard that facilitates quantitative comparisons of tissue dynamics in homologous organs across different species. The strength of this study lies in its proposal of an approach to map morphogenetic dynamics from one organ to another using a space-time coordinate system. These achievements are made possible through the integration of cutting-edge measurement technologies, advanced data analysis techniques, and a solid foundation in mathematical knowledge. The discovery of the conservation of rescaled tissue dynamics in developing limb buds between tadpoles and chicks may not be considered ground-breaking in itself. However, it is crucial to acknowledge the significance of this finding in light of the absence of rigorous scientific evidence until now, primarily due to the lack of the proposed approach utilized in this study.

The presented results are compelling and well-aligned with the scope of this study. The limitations are adequately described, addressing the essential aspects. While I believe the manuscript is generally of high quality, I have a few suggestions for minor revisions that will further improve the clarity and impact of their work. These suggestions should not require additional experiments or major modifications. While additional experiments to test the robustness and extensibility of this approach under perturbations such as small molecule inhibitors or low temperatures could be envisioned, it is understandable that including them may delay the timely publication, which should be avoided. Expanding the Discussion section, as suggested below, will enhance the overall significance of the results. I believe that incorporating these revisions will significantly enhance the manuscript and make it even more impactful.

1) While the discussion addresses the interpretation of the results, it is recommended to expand on the potential implications of the methods and findings for future research or practical applications in the field. For instance, the authors briefly mention organoid maturation as a target topic for applicability on line 416, but a more detailed explanation would provide greater clarity to the readers. Suggesting potential applications in more depth

would be advantageous for a wide range of readers. Additionally, including a paragraph that highlights the limitations of the study would further enhance its practical implications.

2) There are several suggestions to enhance the clarity and structure of the manuscript. Some statements should be revised to ensure they are within the proper scope. For example, it is important to state in the Abstract that their approach is applicable only a phase when the tissue morphogenesis exhibits simple elongation in the limb bud, as described in the Results section. This will provide readers with a clear understanding of the limitations of the study, as mentioned in my comment #1. Another suggestion is to consider shifting the last section in the Results to the Discussion. This section appears to be more of the authors' views and hypotheses, and may be better suited for the Discussion section rather than being presented as a part of rigid results.

3) Figure 4B: The legend states that the white points indicate the closest chick stage to each Xenopus stage, and the black points indicate the Xenopus to chick stage. However, in Figure 4B, the representation of white and black points seems to be opposite to the description in the legend. Please verify the correctness.

4) Figure 4E: The authors mentioned that the choice of initial times for each target affects the level of similarity, but they only show the case when the Xenopus stage is fixed. It would be beneficial to present the results in a round-robin manner, considering different initial times for each target. Also, it would be helpful to provide a brief explanation or interpretation as to why the difference level is smaller at chick stage 21 compared to earlier or later stages. The authors should describe what the error bars represent and provide the number of samples used in the analysis in the legend.

5) Figure 5: The authors claim a high degree of similarity in ξ-space (line 353), which is an important result. It would be beneficial to quantify the level of similarity to provide a more quantitative assessment of the observed similarity.

6) Materials and Methods, line 441: "All animal handling was performed under appropriate anesthesia ..". It is recommended to provide a detailed description of the anesthesia protocol used in the study for the purpose of reproducibility.

Replies to reviewers' comments

First, we would like to thank all the reviewers for their very valuable comments and questions. We have listed our responses to the individual comments below.

Replies to Reviewer #1's Comments

Reviewer #1 (Remarks to the Author):

This is an interesting study attempting to find a common morphometric plan underlying the development of hindlimbs in two vertebrate model organisms which differ in size, shape and developmental timing. It combines mathematical modelling with cell tracking in vivo, and builds on a previous study of the chicken hind limb bud. The study uses a very clever method of labelling many. small groups of cells in the mesenchyme along with mathematical modelling which supports an "archetype" for stage 52 to 54. This stage interval is about 5 days of Xenopus tadpole development (starting 9 days after the buds appear, and finishing as digits begin to condense). At these stages the mesenchyme anterior-posterior axis is well established, and cells fated to form the stylopod, zeugopod and autopod can be identified (Tschumi 1957). Effectively, the study and model covers the period when zeugopodal cartilage elements condense and when digits and interdigits are being defined in the autopod. The latter has been recently shown to be recapitulated in in vitro culture in mice, indicating self-organising ability of the limb bud mesenchyme (Fuiten et el PMID: 36994104)The model is backed up with some nice RNAscope data of anterior posterior marker hox genes. There are also of course more interdigital regions in frog hindlimb (5 digits) vs. chick hindlimb (4 digits) and I can't see this reflected in the model or discussed.

Major: The paper would benefit from some more background into the model to be more accessible to biologist readers. The discussion is brief and includes no citations, so is more a conclusion. I think at the least it would be good to address the limitations of this model in terms of the stages it covers, and perhaps to expand the discussion to include reference to the work of others – e.g. What is known about cell division rate and orientation in these models, or in other vertebrates, and how would this direct morphogenetic growth in a comparable way? Additionally I suggest changes that I think would assist with broad readership appeal as "minor" below.

<u>Reply to Reviewer #1's major comments:</u>

Thank you very much for your valuable comments. First, please let us clarify the usage of the term "model". In this study, we used a Bayesian statistical model to reconstruct smooth tissue deformation maps from noisy cell trajectory data, where the term "deformation map" means the positional correspondence of each point within a tissue at different time points or the trajectory of each point by

1

37 which deformation characteristics can be calculated. Thus, the spatiotemporal patterns of quantified

38 local tissue deformation characteristics (heatmaps in Fig. 2) and the cell flows under the ξ coordinate

39 system (Fig. 3) are real values, not virtual (model) values. In this sense, the difference in the number

40 of digits between species does not need to be accounted for *a priori* to calculate these quantities. In

41 addition, as stated in the original manuscript,

42 "Anatomically, the interspecies differences in the relative lengths of the tarsals and zeugopods and the

43 number of formed digits begin to appear around $t_{Xenopus}=53.5$ (*xenopus*) and $t_{Chick}=27$ (chick). Thus,

44 these highly consistent cell trajectories in the ξ-space suggest that temporal changes in the relative

45 positions of cells within the developing tissues are well conserved regardless of the state of cartilage

46 differentiation."

47 After the basic skeletal patterns are formed, species-specific tissue deformation begins to appear.

48 Regarding this point, we have added the following sentence in the Results section of the revised

49 manuscript:

50 "For example, regarding cell/tissue behavior during digit formation within the paddle-like autopod

51 region, amphibians and amniotes have been reported to have qualitative differences in the rate of cell

52 death in the interdigital zone and in the segmentation processes of digits (31)."

53

54 Furthermore, the following text regarding the quantified spatiotemporal patterns of tissue deformation

55 characteristics has been added to the Results section:

56 Regarding A-P asymmetric growth, "Further, in our previous study on chick limb development, we

57 showed that this A-P asymmetric area growth rate at the tissue level is quantitatively consistent with

58 the positional dependence of the cell cycle time (14)." Regarding the almost homogeneous elongation

59 rate along the P-D axis, "This fact indicates that, similar to the chick case (14), the P-D elongation of

60 a *Xenopus* limb bud cannot be explained by the classical model that limb bud elongation is caused

61 primarily by proliferation of distal cells (25-27, and see also (24) that nicely reviews the history of

62 "proliferation gradient" model); it should be noted that the factors that drive this anisotropic local

63 tissue deformation remain unknown."

64

65 We have substantially revised the Discussion section in accordance with the three reviewers'

66 comments. The major modifications are as follows:

67 (i) We have added the following sentences describing the limitations of our methodology (please see

68 the subsection "Representation of space-time information and limitations"):

69 "With respect to time (τ), we propose a method for achieving synchronization of developmental time

70 between species based on geometrical information, i.e., cell flow under the ξ-coordinate system. The

71 method has a limitation that it functions effectively only when the transformation of time coordinates

72 between species adheres to the conditions of being both bijective and continuous. These conditions

73　can be compromised when significant interspecies differences in the flow arise; in the case of chick

74　and *Xenopus* limb development, (geometrical) synchronization was not possible after the phase when

75　basic digit patterning was established. We should note that this does not necessarily imply only a

76　negative aspect, because the method conversely could also serve to detect the emergence of

77　interspecies differences in tissue dynamics, as shown above."

78

79　(ii) We have also modified the text regarding future challenges:

80　"In this study, we decomposed a tissue deformation map as the product of the average growth $\mathbf{L}(t)$

81　(i.e., species-specific tissue size and aspect ratio at time *t*) and the rescaled tissue dynamics $\xi(\xi_0, \tau)$

82　(Eq. 1) and focused on similarities in the latter. However, how the former, species-specific tissue size,

83　is determined remains a critical issue because it not only satisfies pure scientific interest but also is

84　closely linked to applied research. For example, current organoids can reproduce the differentiated

85　states of individual organs, but remain considerably distant from being fully functional, mature organs

86　of adequate size (37). Identifying the factors that determine interspecies differences in the size of

87　homologous organs will introduce the possibility of regulating the size of immature organoids. A group

88　of genes associated with species differences whose expression patterns are not conserved in the τ-ξ

89　coordinate system will provide clues to elucidate the molecular mechanisms responsible for organ size

90　determination."

91

92　(iii) The following three items have also been added to the Discussion section in response to

93　suggestions from other reviewers.

94　"Spatially, positions in the rescaled space (ξ), rather than those on an absolute scale, would be encoded

95　as gene expression vectors in a manner common to different species. Changes in tissue size (or more

96　precisely, average deformation $\mathbf{L}(\tau)$) are cancelled out in ξ-space, which allows direct comparison of

97　spatial representations within a tissue at different time points. This perspective will be crucial for

98　advancing the previous theoretical frameworks on positional-information coding in static fields (33,

99　34) to encompass dynamic scenarios."

100

101　"As described in the Introduction, the trigger/timing of limb development are very different between

102　chick and *Xenopus* (Keenan and Beck, Dev. Dyn., 2016). Despite such qualitative differences, it is

103　surprising that tissue dynamics are well conserved, which suggests that the evolution of limb

104　morphogenesis is constrained by common physical processes as well as conserved signaling pathways

105　and gene expression patterns across species. Clearly, such an interdisciplinary understanding is

106　necessary to determine what an archetype of tissue dynamics is (including knowing if an archetype

107　itself really exists). In the context of limb development, clarifying the physicochemical factors

108　responsible for the nearly uniform elongation rate along the P-D axis and asymmetric

109 growth/deformation along the A-P axis, as revealed in our analysis (Figs. 3**C-H**), will be an important

110 clue to understanding the constraints."

111

112 "Especially, since three-dimensional (3D) features of organ morphology become more pronounced in

113 the later stages of development, 3D map reconstruction would be critical. In the context of limb

114 development, at stages following the establishment of the skeletal patterning (e.g., after St. 30 in the

115 chick case), the shapes of all digits become more distinct, while simultaneously assuming a more 3D

116 arrangement relative to each other. Thus, extending the analysis to a 3D analysis will be essential for

117 a deeper understanding of species differences in tissue dynamics."

118

119

120 Minor:

121 *Throughout: species names in italics*

122 **Reply**: We have corrected all mentions of species names so that they are in italics.

123

124 *Abstract: include full species names for both chicken and Xenopus*

125 **Reply**: We have added this information in the revised manuscript.

126

127 *line 71 I am not familiar with the exact meaning of "tissue deformation" in this context, and think it*

128 *would be more accessible to wider readership if this was briefly explained.*

129 **Reply**: Thank you for your comment. We have added a brief explanation of tissue deformation as

130 follows:

131 (original)

132 ... the tissue deformation dynamics during animal development (6).

133 (revised)

134 ... the tissue deformation dynamics during animal development that include spatio-temporal patterns

135 of area/volume change of each local tissue piece and the extent/direction of its stretching or shrinking

136 (6).

137

138 *Line 84: similarly, what is a tissue deformation map?*

139 **Reply**: We have added a brief explanation of a tissue deformation map as follows:

140 (original)

141 … to obtain quantitative tissue deformation maps for the developmental processes of organs…

142 (revised)

143 … to obtain quantitative tissue deformation maps (i.e., the positional correspondence of each point

144 within a tissue at different time points or the trajectory of each point by which deformation

4

145   characteristics can be calculated) for the developmental processes of organs…

146

147   *Line 114: can you explain why "on the dorsal ventral boundary plane" and what this means? Is the*
148   *grid focused somehow so that epithelial cells are not triggered to express GFP? The image in figure*
149   *2A (and S1) is really nice but I cannot tell if it is mesenchymal only – were two lasers used to focus on*
150   *a single spot, and only where they meet the heat shock promoter activates?*

151

152   **Reply**: Thank you for this comment. For convenience, here we were referring to the frontal section at
153   the mid dorsoventral (D-V) level where the cross-sectional area of a limb bud becomes maximal as
154   the "D-V boundary plane". To avoid any confusion, we have decided not to use the term "dorsal-
155   ventral boundary" in the revised manuscript. The focus on that plane is because the distinctive event
156   of cartilage formation during limb development occurs around the D-V boundary, not at the
157   dorsal/ventral end. Regarding GFP induction, laser irradiation can be focused to a certain D-V level to
158   some extent, but each spot can become elongated in the D-V direction. However, we found that the
159   shape of each spot was well maintained during our measurement time interval (around 24 hours), and
160   that even if we changed the focus of the microscope in the D-V direction, the spot position hardly
161   changed in the plane spanned by the A-P and P-D axes. This means that the D-V dependence of the
162   in-plane deformation is sufficiently small, at least in the measurement interval. As for this point, we
163   have added the explanation in the Materials and Methods as follows:
164   "Since our aim was to compare the 2D deformation dynamics on the frontal plane at the mid D-V level
165   with the maximal area of a limb bud in *Xenopus* with that previously reported for chick (14), we
166   focused the irradiation within this plane (Fig. 2**A**). The heat-shock treatment was performed as
167   described in our previous work (20). The diameter of a single labeled spot on the plane was typically
168   20-30 μm, a size equivalent to a few cells. Although laser irradiation can be focused to a certain D-V
169   level to some extent, each spot could become elongated in the D-V direction. However, we found that
170   the in-plane shape of each spot was well maintained during our measurement time interval (around 24
171   hours), and that even if we changed the focus of the microscope in the D-V direction, the spot position
172   hardly changed in the plane. This means that the D-V dependence of the in-plane deformation is
173   sufficiently small, at least in the measurement interval."

174

175   *Line 120/121 state the Nieuwkoop and Faber stages used?*

176

177   **Reply**: The staging method we adopted is described in the statement immediately following. In a
178   previous study, we proposed an objective staging method based on the contour shape of a limb bud,
179   and we adopted that method here. This was because the developmental rate of *Xenopus*, a cold-blooded
180   animal, varies significantly, and there is not always a precise alignment between stage values and

181   actual time. This point was stated in the Materials and Methods:

182   "Staging of each individual was based on a previously proposed morphometric staging method (21);

183   briefly, digitized outlines of the limb buds were approximated using elliptic Fourier descriptors, and a

184   continuous stage value, not discrete values as in traditional staging (e.g., 51 and 52), was assigned to

185   each individual based on its coefficients. In this study, this morphometric stage was denoted by

186   t_{Xenopus} ."

187

188   *Line 135: Xenopus limb bud stages are points on a continuum, but how did you accurately subdivide*

189   *each stage in to 10th stages?*

190

191   **Reply**: As stated above, *Xenopus* staging was based on a previously proposed morphometric method.

192   Briefly, digitized outlines of the limb buds were approximated using elliptic Fourier descriptors, and

193   a continuous stage value, not discrete values as in traditional staging (e.g., 51 and 52), was assigned

194   to each sample based on its coefficients. For each individual, the positional coordinates of the

195   fluorescently labeled spots were measured at 2 to 4 time points approximately every 24 hours, and the

196   coordinates at timepoints between the measurements were obtained by linear interpolation. That is, as

197   shown in Fig. S1C, for each sample, we obtained cell trajectory data for a time window represented

198   as a line segment on the number line. Samples containing cell trajectory data within each subdivided

199   interval were used to reconstruct the deformation dynamics during that interval. This point is explained

200   in the Materials and Methods as follows:

201   "Staging of each individual was based on a previously proposed morphometric staging method (21);

202   briefly, digitized outlines of the limb buds were approximated using elliptic Fourier descriptors, and a

203   continuous stage value, not discrete values as in traditional staging (e.g., 51 and 52), was assigned to

204   each individual based on its coefficients. In this study, this morphometric stage was denoted by t_{Xenopus} .

205   Each limb bud was resized to a previously determined typical value corresponding to its morphometric

206   stage. The spatial coordinates of the fluorescently labeled spots were measured at 2 to 4 time points

207   approximately every 24 hours for each individual, and the coordinates at the timepoints between the

208   measurements were obtained by linear interpolation. The period from t_{Xenopus} =50.6 to t_{Xenopus} =54.4

209   was divided into nine roughly equally spaced time intervals (each interval corresponded to a 0.4-0.5

210   stage increment), and data from the individuals included in each interval were integrated to reconstruct

211   the tissue deformation map for that interval."

212

213   *Line 136 state HH stages*

214

215   **Reply**: Yes, we based our chick staging on the Hamburger-Hamilton table. A stage value was assigned

216   to each sample using increments of 0.5 (not necessarily integers) based on incubation time. In our

217 previous study, we quantified the deformation map for every 12-hour interval. Thus, each heatmap

218 shown in Fig. 2E is the result of each 12-hour interval. Since the developmental rate of chickens,

219 which are warm-blooded animals, closely matched real time, we staged them based on real time rather

220 than morphology. This point is stated in the Materials and Methods as follows:

221 "For chick hindlimb development, we used a previously reported tissue deformation map

222 $x = \phi_{Chick}(X,t)$ (14). The only minor modification from the previous study was that we changed the

223 staging method. In the previous study, a tissue deformation map was quantified for every 12-hour

224 interval, and for each time point, we assigned the integer value of the Hamburger-Hamilton stage with

225 a shape closest to that observed at the time point. In the present study, staging was performed using

226 increments of 0.5 (not necessarily integers) based on incubation time."

227

228 *Line 139 citations are needed to support this "Xenopus limb buds include the prospective autopod*

229 *(toe-to-ankle), zeugopod (lower leg), and stylopod (upper leg) regions. In contrast, chick limb buds*

230 *contain mainly the former two, while the stylopod is embedded in the trunk.*

231

232 **Reply**: We modified the corresponding sentences as follows: "*Xenopus* limb buds include the

233 prospective autopod (toe-to-ankle), zeugopod (lower leg), and stylopod (upper leg) regions (22). In

234 contrast, as shown later, based on the inverse mapping of cartilage patterns, chick limb buds contain

235 mainly the former two, while the stylopod is embedded in the trunk."

236

237 *Line 173 gene names in italics*

238

239 **Reply**: We have made this correction.

240

241 *Line 229 "we can intuitively see…" please explain what we are supposed to be seeing because I cannot*

242 *make this conclusion from the coloured vectors in figure 3C and D. Are there even interdigits present*

243 *at stage 52? Maybe this refers to another figure?*

244

245 **Reply**: Thank you for this comment. The colored vectors represent the movement of the limb bud

246 mesenchymal cells when observed in the ξ coordinate system. What we wanted to state was that, in

247 the internal tissues of a limb bud, both species share a similar flow oriented from the posterior to the

248 anterior side, while the direction of the arrow around the limb bud boundaries (e.g., anterior boundary)

249 is not necessarily similar. We have modified the text as follows:

250 (original)

251 "we can intuitively see that the cell trajectories of the internal tissues that will form future skeletal

252 structures are more consistent between species than those near the tissue boundaries (Fig. 3**C**, **D**)"

253 (revised)

254 "we can intuitively see that the cell trajectories (the flow patterns in the ξ coordinate system) of the

255 internal tissues that will form future skeletal structures are more consistent between species (i.e.,

256 basically oriented from posterior to anterior) than those near the tissue boundaries (Fig. 3**C**, **D**)"

257

258 *Paragraph starting line 236: what criteria did you use/match to set the clock for each organism (e.g*

259 *cartilage condensation, gene expression?)*

260

261 **Reply**: Thank you for this comment. We introduced the concept of a common clock, which is

262 represented as an abstract one-dimensional curve, and we regarded the developmental stages of each

263 species as time coordinates of the common clock. Then, determining the correspondence of the

264 developmental stages between species (i.e., synchronizing the developmental times) means giving the

265 coordinate transformations between those time coordinates. Here, we devised a method of

266 synchronization (or coordinate transformation) based solely on the geometric information, i.e., tissue

267 deformation dynamics, not on changes in cellular states such as gene expression or cartilage

268 condensation. As shown in Fig. 3, we defined a spatial coordinate system, ξ, in which tissue

269 deformation is represented as a cell flow. We synchronized the developmental time by adjusting the

270 scale interval of the time axis of both species so that the difference between cell trajectories starting

271 from the same initial position was as small as possible. The introduction of mathematical concepts is

272 inevitable for a more precise definition of synchronization. The main text is limited to an intuitive

273 explanation, while the Materials and Methods text provides a more rigorous explanation (please see

274 the subsection "Common clock T and synchronization between species"). Regarding this point, the

275 main text states the following:

276 "As a way to find the composite map that relates stages between chick and *Xenopus* (denoted by

277 $\psi_C \circ \psi_X^{-1}$ and $\psi_X \circ \psi_C^{-1}$), we aligned the scales of their temporal axes such that the mean difference

278 in cell trajectories from the same initial position in the ξ-space is minimized (see Materials and

279 Methods for details)."

280

281 *Line 487 H2O2*

282 **Reply**: We have rewritten this correctly as H_2O_2.

283

284 *Line 499 I'm still not understanding what you mean by dorsal ventral boundary: the mesenchyme does*

285 *not really have such a boundary (unlike the epithelial AER?) – does this mean cells were labelled in*

286 *the centre relative to the DV axis, where lmx1b positive and negative cells meet? Could you perhaps*

287 *illustrate this better with a figure?*

8

288   **Reply**: As explained above, here we were referring to the frontal section at the mid dorsoventral (D-

289   V) level where the cross-sectional area of a limb bud becomes maximal as the "D-V boundary plane".

290   To avoid any confusion, we have decided not to use the term "dorsal- ventral boundary" in the revised

291   manuscript. We have modified Fig. S1 to illustrate this.

292

293   *Line 669 Gene names in italics*

294   **Reply**: We have made this correction.

295

296   *Figure 1: I find this legend to be a bit overlong, it should really be re-written to just describe what is*

297   *shown and the rest can be incorporated into the text? In particular, having a study aim in the legend*

298   *seems out of place.*

299

300   **Reply**: Thank you very much for this comment. We have revised the legend of Figure 1 accordingly.

301   As you pointed out, we agree that including the study aim is out of place; thus, we deleted it. We then

302   confirmed that the rest of the text corresponds to the figure.

303

304   *Figure 2 The Xenopus A-P axis is posturally inverted: shh marking the ZPA clearly shows this for the*

305   *stages of this study: Endo et al 1997 PMID: 9186057, Keenan and Beck 2016 PMID: 26404044, Wang*

306   *et al 2015 PMID: 26527308. I can see you've inverted the limbs in this figure to account for this but*

307   *it should be stated.*

308

309   **Reply**: Thank you for this comment. At your suggestion, we have stated the following in the legend

310   of Fig. 2: "Note that the images of the *Xenopus* limb bud, except for the top-left photo in panel (**A**),

311   are inverted in the A-P direction to have the posterior side facing downward."

312

313   *I'm not clear on how the maps are made- 1D or is information captured from multiple layers and*

314   *stacked? If the former then how do you account for cells that move or divide dorsoventrally in the*

315   *mesenchyme?*

316

317   **Reply**: As stated above, we are focusing on the 2D deformation dynamics of mesenchymal tissue on

318   the frontal plane at around the mid D-V level where the cross-sectional area of a limb bud becomes

319   maximal. In addition, even when each spot became elongated in the D-V direction, we found that the

320   shape of each spot was well maintained during our measurement time interval (around 24 hours), and

321   that when we changed the focus of the microscope in the D-V direction, the spot position hardly

322   changed in the plane. This means that the D-V dependence of the in-plane deformation is sufficiently

323   small, at least in the measurement interval. Another important piece of information is that tissue-level

324   deformation is not calculated from the movement of cells within each spot, but from the change in the

325   relative positions among the labeled spots. Therefore, if the relative positional changes between spots

326   over the entire limb bud are reproducible, the deformation dynamics can be correctly reconstructed.

327   In that sense, our data are highly reproducible among samples, and the resultant reconstructed tissue

328   deformation dynamics are reliable. Regarding the latter point, we have added the following sentences

329   in the Materials and Methods:

330   "It should be noted that tissue-level deformation is not calculated from the movement of cells within

331   each spot, but from the change in the relative positions among the labeled spots. Therefore, if the

332   relative positional changes between spots over the entire limb bud are reproducible, the deformation

333   dynamics can be correctly reconstructed. In that sense, our data are highly reproducible among

334   samples, and the resultant reconstructed tissue deformation dynamics are reliable."

335

336   *Figure 3 rather than using left and right, fro C and D maybe label all the panels ? In the legend, the*

337   *final statement "Note that the dynamics for the prospective autopod and zeugopod are compared" at*

338   *the end does not seem to obviously relate to anything shown in the figure?*

339

340   **Reply**: According to this comment, we relabeled Fig. 3 (please see Fig. 3 and its legend in the revised

341   manuscript). The final statement, "Note that the dynamics for the prospective autopod and zeugopod

342   are compared" is related to the figure because the flow pattern in the ξ coordinate system changes

343   depending on the region to be analyzed within the limb bud (as analyzed in Fig. 4H). We have slightly

344   modified that sentence as follows:

345   (original)

346   "Note that the dynamics for the prospective autopod and zeugopod are compared."

347   (revised)

348   "The cell flows in the ξ coordinate system shown in panels (**C**) and (**D**) correspond to the

349   morphogenesis of the region consisting of prospective autopods and zeugopods."

350

351

352

Replies to Reviewer #2's Comments

Reviewer #2 (Remarks to the Author):

ms review

NCOMMS-23-19472-T

The goal of the paper is to explore whether there are conserved 'archetypal' growth dynamics in organs detectable among species. The authors produce tissue deformation maps through developmental stages of chick and Xenopus for comparison. For comparisons of tissue dynamics among species, they propose rescaling tissue deformation and synchronizing developmental clocks. They conclude that chicks and xenopus share A-P axis asymmetry, as well as the pattern of cell division contributing to elongation of the limb bud. The study is a valuable contribution to the limb development in describing details of shape change and dynamics over ontogeny and attempting to devise a method for comparisons that helps to understand underlying principles.

My main comments here are about this study's comparison of species. The study lacks a necessary discussion of anuran limb development as qualitatively different than amniotes (chick), first because anuran limb development derives from different tissues and by a different initiating process. The elongation of the frog limb may indeed independently recapitulate the amniote limb developmental characteristics, but the fundamental developmental difference of having the thyroid-dependent metamorphosis in frogs drive limb initiation, rather than limb buds derived from the early axis/embryonic lateral mesoderm, is not mentioned.

Reply: First, we would like to sincerely thank you for your very valuable comments. We completely agree that stating the differences in gross developmental contexts is biologically very important in comparing chickens and frogs. We have modified the manuscript to reflect these comments as much as possible.

According to this comment, we have added the following sentences in the Introduction and Discussion sections:

(Introduction)

"Both species share many developmental characteristics, including major signaling and gene expression patterns, whereas the trigger and timing of development are clearly different. Limb buds of amniotes including chick develop concurrently with the main body axis formation of an embryo and arise from the lateral plate mesoderm. In contrast, limb development in *Xenopus* proceeds as one of the thyroxine (thyroid hormone)-dependent events in metamorphosis after embryonic stage (17), and the precise origin of a limb bud is difficult to determine (18). Through the comparison of

11

389 homologous organs with such qualitative differences, we inquired into the existence of archetypal

390 tissue dynamics."

391 (Discussion)

392 "As described in the Introduction, the trigger/timing of limb development are different between chick

393 and *Xenopus*. Despite such qualitative differences, it is surprising that tissue dynamics are well

394 conserved, which suggests that the evolution of limb morphogenesis is constrained by common

395 physical processes, as well as conserved signaling pathways and gene expression patterns across

396 species. Clearly, such an interdisciplinary understanding is necessary to determine what an archetype

397 of tissue dynamics is (including knowing if an archetype itself really exists). In the context of limb

398 development, clarifying the physicochemical factors responsible for the nearly uniform elongation rate

399 along the P-D axis and asymmetric growth/deformation along the A-P axis, as revealed in our analysis

400 (Figs. 3C-H), will be an important clue to understanding the constraints."

401

402 *Secondly, the authors conclude - line 403 - "the spatiotemporal expression patterns of hox genes*

403 *essential for limb development were also found to be conserved"*

404 *I wonder about this, as the anuran hind limb zeugopod to autopod boundary is complicated by the*

405 *proximal tarsal modification into an additional hindlimb segment; along with this morphology, the*

406 *HoxA11- HoxA13 boundary distinguishing the zeugopod is modified. See:*

407 *Blanco, M. J., Misof, B. Y., Wagner, G. P., Blanco, M. J., Misof, B. Y., & Wagner, G. P. (1998).*

408 *Heterochronic differences of Hoxa-11 expression in Xenopus fore-and hind limb development:*

409 *evidence for lower limb identity of the anuran ankle bones. Development genes and evolution, 208(4),*

410 *175.*

411

412 **Reply**: Thank you for this comment. We read the above paper, and we now have a better understanding

413 of the differences between forelimbs and hindlimbs. In this paper, the expression of *HoxA11* in the

414 hindlimb was examined by RNAscope, and we found that the obtained pattern was somewhat different

415 from that examined by ordinary *in situ* hybridization in the Blanco 1998 paper. For example, in the

416 Blanco 1998 paper, the expression in later stages was very weak, whereas our results show a more

417 regional pattern with a broad, strong signal in the zeugopod. We think that this difference is due to

418 detection sensitivity. Regarding the comparison of expression patterns in chick and *Xenopus* hindlimbs,

419 as shown in the right panel of Fig. 5B, the range of *Hoxa11* expression in ξ-space was very similar in

420 both species, at least when basic limb skeletal patterning was done. In contrast, the somewhat

421 ambiguous overlapping of the Hoxa11 expression ranges between species in the early stages is

422 probably because those stages correspond to the transient period in which the distal end of the *Hoxa11*

423 expression region shifts from the tip of limb bud to the more proximal side.

424 We have added the following sentences in the Results.

425 "It should be noted that the overlap of *Hoxa11* expression ranges of both species was somewhat
426 ambiguous in the early stages, which is probably because the stages correspond to the transient period
427 in which the distal end of the *Hoxa11* expression region shifts from the tip of the limb bud to the more
428 proximal side."

429

430 Lastly, the differences between the forelimb and the hindlimb described in the Blanco 1998 paper are
431 so important that we will take them into consideration when studying the similarities/differences in
432 those tissue dynamics in future.

433

434 *Thirdly, the 'paddle' formation in the autopod of anurans at digit formation is also qualitatively*
435 *different from the amniotes.*
436 *from: Fabrezi, M, Goldberg, J, Pereyra, MC. 2017. Morphological variation in anuran limbs:*
437 *Constraints and novelties. J. Exp. Zool. (Mol. Dev. Evol.) 328B: 546– 574.*
438 *"Cameron and Fallon (1977) noted how the patterns of digit formation of amphibians differed from*
439 *that of amniotes. In amphibians, there are no interdigital zones of massive cell death during digit*
440 *formation. Rather the digits appear to arise by differential proliferation of interdigital and digital cells.*
441 *Each digital primordium first grows and enlarges, and then it segments to form the interphalangeal*
442 *joints and the precise number of phalanges (Sanz-Ezquerro and Tickle, 2003). In amphibians and*
443 *lizards, this process seems to be different from mammals and birds because, even when phalanges*
444 *differentiate by segmentation, there is no continuous primordium dividing up into as many phalanges.*
445 *Instead, phalanges appear as cartilaginous condensations that grow in size and then segment to form*
446 *the next phalange in a proximo-distal direction (Fig. 6)."*
447 *All these features would affect the shape dynamics described. Given that the purpose of the paper is*
448 *to provide a method for comparing species differences, the acknowledgement of the fundamental*
449 *differences of highly derived forms requires a review of evidence for homology as a starting point. The*
450 *differences in gross developmental contexts shouldn't be ignored.*

451

452 **Reply**: We have added the following sentence in the Results section:
453 "For example, regarding cell/tissue behavior during digit formation within the paddle-like autopod
454 region, amphibians and amniotes have been reported to have qualitative differences in the rate of cell
455 death in the interdigital zone and in the segmentation processes of digits(31)."

456

457 *That said, I think this study could potentially provide evidence for similar tissue dynamics *in spite*
458 *of* the big differences between anurans and chicks, and this discussion is completely missing. For*
459 *example, if a mouse and chick were compared, there is a strongly supported developmental homology*
460 *that is 'tinkered' with to create the differences among species, so that the tissue dynamics*

461 *synchronization is more easily interpretable for similarity and difference from an evolutionary*
462 *perspective. However, if they find similarities between amniote and anuran limb development, it*
463 *possibly says more about a deeper question of how evolution is constrained by tissue dynamics even*
464 *in the case where the 'limb program' is redeployed in a different context (i.e., the post-larval*
465 *metamorphosis). I would think this is especially important for the stated goal of finding "archetype"*
466 *forms for organs. (I think they are really assessing is an archetypal ontogeny, not just the resulting*
467 *form..?)*
468 *What an archetype is begs some other questions, which would be worthwhile to present here. Why are*
469 *they similar? Any tissue dynamics-based archetype description would need additional discussion of*
470 *biophysics as a mechanistic contribution adding to this primarily morphological comparison. It could*
471 *strengthen the discussion.*
472
473 **Reply**: We appreciate this valuable comment. As described above, according to the comments received,
474 we have added the following sentences in the Discussion section:
475 "As described in the Introduction, the trigger/timing of limb development are very different between
476 chick and *Xenopus*. Despite such qualitative differences, it is surprising that tissue dynamics are well
477 conserved, which suggests that the evolution of limb morphogenesis is constrained by common
478 physical processes, as well as conserved signaling pathways and gene expression patterns across
479 species. Clearly, such an interdisciplinary understanding is necessary to determine what an archetype
480 of tissue dynamics is (including knowing if an archetype itself really exists). In the context of limb
481 development, clarifying the physicochemical factors responsible for the nearly uniform elongation rate
482 along the P-D axis and asymmetric growth/deformation along the A-P axis, as revealed in our analysis
483 (Figs. 3C-H), will be an important clue to understanding the constraints."
484
485 *The conclusion that the pattern of cell division ("cell flow") contributing to elongation of the limb*
486 *bud is an important point overall as it contradicts the paradigm that the distal cells are where*
487 *elongation primarily arises.*
488 *Also see*
489 *Young, J. J., & Tabin, C. J. (2017). Saunders's framework for understanding limb development as a*
490 *platform for investigating limb evolution. Developmental biology, 429(2), 401-408.*
491
492 **Reply**: We have added the following sentence:
493 "This fact indicates that, similar to the chick case (14), the P-D elongation of a *Xenopus* limb bud
494 cannot be explained by the classical model that limb bud elongation is caused primarily by
495 proliferation of distal cells (25-27, and see also (24) that nicely reviews the history of "proliferation
496 gradient" model)"

14

497

Both D'arcy Thompson's and this study are missing the third dimension of shape, which is critical in cartilage condensation initiation and digit development.

500

Reply: We agree with this comment. In this study, we analyzed the two-dimensional tissue deformation dynamics of mesenchyme in the frontal plane at around the middle of the D-V axis, where basic skeletal patterning occurs. At stages following the establishment of the skeletal patterning (e.g., after St. 30 in the chick case), the shapes of all the digits become more distinct, while simultaneously assuming a more three-dimensional arrangement relative to each other. Species differences in the morphology and the proportion of anatomical structures will be stronger in later stages, and thus extending the analysis to a 3D analysis will be essential for a deeper understanding of species differences in tissue dynamics. We have added the following text in the Discussion section:

509

"Especially, since three-dimensional (3D) features of organ morphology become more pronounced in the later stages of development, 3D map reconstruction would be critical. In the context of limb development, at stages following the establishment of the skeletal patterning (e.g., after St. 30 in the chick case), the shapes of all digits become more distinct, while simultaneously assuming a more 3D arrangement relative to each other. Thus, extending the analysis to a 3D analysis will be essential for a deeper understanding of species differences in tissue dynamics."

516

517

518

519

Reply to reviewer #3's comments:

Reviewer #3 (Remarks to the Author):

This manuscript presents a timely exploration of a significant research question, namely, how to establish a standard that facilitates quantitative comparisons of tissue dynamics in homologous organs across different species. The strength of this study lies in its proposal of an approach to map morphogenetic dynamics from one organ to another using a space-time coordinate system. These achievements are made possible through the integration of cutting-edge measurement technologies, advanced data analysis techniques, and a solid foundation in mathematical knowledge. The discovery of the conservation of rescaled tissue dynamics in developing limb buds between tadpoles and chicks may not be considered ground-breaking in itself. However, it is crucial to acknowledge the significance of this finding in light of the absence of rigorous scientific evidence until now, primarily due to the lack of the proposed approach utilized in this study.

The presented results are compelling and well-aligned with the scope of this study. The limitations are adequately described, addressing the essential aspects. While I believe the manuscript is generally of high quality, I have a few suggestions for minor revisions that will further improve the clarity and impact of their work. These suggestions should not require additional experiments or major modifications. While additional experiments to test the robustness and extensibility of this approach under perturbations such as small molecule inhibitors or low temperatures could be envisioned, it is understandable that including them may delay the timely publication, which should be avoided. Expanding the Discussion section, as suggested below, will enhance the overall significance of the results. I believe that incorporating these revisions will significantly enhance the manuscript and make it even more impactful.

Reply: Thank you very much for your positive comments. Below is a list of responses to your suggestions.

1) While the discussion addresses the interpretation of the results, it is recommended to expand on the potential implications of the methods and findings for future research or practical applications in the field. For instance, the authors briefly mention organoid maturation as a target topic for applicability on line 416, but a more detailed explanation would provide greater clarity to the readers. Suggesting potential applications in more depth would be advantageous for a wide range of readers. Additionally, including a paragraph that highlights the limitations of the study would further enhance its practical implications.

Reply: The Discussion section has been substantially revised in accordance with the comments

16

556   received.

557   (i) We have added the following sentences in "Future challenges" subsection of the Discussion section:

558   "In this study, we decomposed a tissue deformation map as the product of the average growth $\mathbf{L}(t)$,

559   that determines species-specific tissue size and aspect ratio at time t, and the rescaled tissue dynamics

560    $\xi(\xi_0, \tau)$ (Eq. 1) and focused on similarities in the latter. However, how the former, species-specific

561   tissue size, is determined remains a critical issue because it not only satisfies pure scientific interest

562   but also is closely linked to applied research. For example, current organoids can reproduce the

563   differentiated states of individual organs, but remain considerably distant from being fully functional,

564   mature organs of adequate sizes (37). Identifying the factors that determine interspecies differences in

565   the size of homologous organs will introduce the possibility of regulating the size of immature

566   organoids."

567

568   (ii) We have also added the following sentences describing the limitations of our methodology to the

569   Discussion section (please see the subsection "Representation of space-time information and

570   limitations"):

571   "With respect to time (τ), we propose a method for achieving synchronization of developmental time

572   between species based on geometrical information, i.e., cell flow under the ξ-coordinate system. The

573   method has a limitation that it functions effectively only when the transformation of time coordinates

574   between species adheres to the conditions of being both bijective and continuous. These conditions

575   can be compromised when significant interspecies differences in the flow arise; in the case of chick

576   and *Xenopus* limb development, (geometrical) synchronization was not possible after the phase when

577   basic digit patterning was established. We should note that this does not necessarily imply only a

578   negative aspect, because the method conversely could also serve to detect the emergence of

579   interspecies differences in tissue dynamics, as shown above."

580

581   (iii) In addition, the following three items have also been added to the Discussion section in response

582   to suggestions from other reviewers.

583   "Spatially, positions in the rescaled space (ξ), rather than those on an absolute scale, would be encoded

584   as gene expression vectors in a manner common to different species. Changes in tissue size (or more

585   precisely, average deformation $\mathbf{L}(\tau)$) are cancelled out in ξ-space, which allows direct comparisons of

586   spatial representations within a tissue at different time points. This perspective will be crucial for

587   advancing the previous theoretical frameworks on positional-information coding in static fields (33,

588   34) to encompass dynamic scenarios."

589

590   "As described in the Introduction, the trigger/timing of limb development are very different between

591   chick and *Xenopus*. Despite such qualitative differences, it is surprising that tissue dynamics are well

17

conserved, which suggests that the evolution of limb morphogenesis is constrained by common physical processes, as well as conserved signaling pathways and gene expression patterns across species. Clearly, such an interdisciplinary understanding is necessary to determine what an archetype of tissue dynamics is (including knowing if an archetype itself really exists). In the context of limb development, clarifying the physicochemical factors responsible for the nearly uniform elongation rate along the P-D axis and asymmetric growth/deformation along the A-P axis, as revealed in our analysis (Figs. 3C-H), will be an important clue to understanding the constraints."

"Especially, since three-dimensional (3D) features of organ morphology become more pronounced in the later stages of development, 3D map reconstruction would be critical. In the context of limb development, at stages following the establishment of the skeletal patterning (e.g., after St. 30 in the chick case), the shapes of all the digits become more distinct, while simultaneously assuming a more 3D arrangement relative to each other. Thus, extending the analysis to a 3D analysis will be essential for a deeper understanding of species differences in tissue dynamics."

2) There are several suggestions to enhance the clarity and structure of the manuscript. Some statements should be revised to ensure they are within the proper scope. For example, it is important to state in the Abstract that their approach is applicable only a phase when the tissue morphogenesis exhibits simple elongation in the limb bud, as described in the Results section. This will provide readers with a clear understanding of the limitations of the study, as mentioned in my comment #1. Another suggestion is to consider shifting the last section in the Results to the Discussion. This section appears to be more of the authors' views and hypotheses, and may be better suited for the Discussion section rather than being presented as a part of rigid results.

Reply: According to this comment, we have modified the manuscript as follows:

(i) Regarding the limitation of the proposed method, we have added an explanation in the Discussion section as stated above. We have also modified the Abstract as follows:

(original)

"... We found that tissue dynamics are well conserved across species under this space-time coordinate system, and that the tissue dynamics of both species are mapped with each other through a time-variant linear transformation in real physical space,..."

(revised)

"... We found that tissue dynamics are well conserved across species under this space-time coordinate system, at least from the early stages of limb development through the phase when basic digit patterning was established. For this developmental period, we also revealed that the tissue dynamics of both species are mapped with each other through a time-variant linear transformation in real

18

628   physical space,..."

629

630   (ii) Secondly, according to the reviewer's suggestion, we have shifted the last section in the Results to

631   the Discussion.

632

633   *3) Figure 4B: The legend states that the white points indicate the closest chick stage to each Xenopus*

634   *stage, and the black points indicate the Xenopus to chick stage. However, in Figure 4B, the*

635   *representation of white and black points seems to be opposite to the description in the legend. Please*

636   *verify the correctness.*

637

638   **Reply**: Thank you for this comment. We revised the legend as follows:

639   (Original)

640   "The white and gray points/curve indicate the closest chick stage to each *Xenopus* stage, which defines

641   the map $\psi_C \circ \psi_X^{-1}$. The black and gray points/curve show the closest *Xenopus* stage to each chick

642   stage, defining the map $\psi_X \circ \psi_C^{-1}$."

643   (Revised)

644   "The white and gray points/curve indicate the correspondence of each *Xenopus* stage to the closest

645   chick stage, which defines the map $\psi_C \circ \psi_X^{-1}$. The black and gray points/curve show the

646   correspondence of each chick stage to the closest *Xenopus* stage, defining the map $\psi_X \circ \psi_C^{-1}$."

647

648   *4) Figure 4E: The authors mentioned that the choice of initial times for each target affects the level of*

649   *similarity, but they only show the case when the Xenopus stage is fixed. It would be beneficial to*

650   *present the results in a round-robin manner, considering different initial times for each target. Also, it*

651   *would be helpful to provide a brief explanation or interpretation as to why the difference level is*

652   *smaller at chick stage 21 compared to earlier or later stages. The authors should describe what the*

653   *error bars represent and provide the number of samples used in the analysis in the legend.*

654

655   **Reply**: In this revision, we have analyzed the case when the chick stage is fixed. As shown in the

656   original manuscript, we found that chick stage 21 was the best when the *Xenopus* stage was fixed at

657   50.6, so in an additional analysis, we fixed the chick stage at 21 and changed the *Xenopus* stage. As a

658   result, the best agreement of trajectories between species was found at 50.6 (51.1 was equally good).

659   Therefore, in this study, we chose 50.6 (*Xenopus*) and 21 (chick) as the initial time combination. Note

660   that as shown in the heatmap in Fig. 4B, the closest chick stage to *Xenopus* stages 50.6 and 51.1 is 21.

661   This is thought to be because the relative positions of cells within the tissue do not change very much

19

662   between 50.6 and 51.1.

663

664   The blue area of the heatmap narrows (like an hourglass) at around chick stage 23-24 and *Xenopus*

665   stage 52-52.5. In those stages, AP-asymmetric tissue growth clearly appears, and it greatly affects the

666   change in the rescaled cell position in the ξ space (i.e., cell flow), so those time windows should

667   correspond between the two species. Therefore, it is necessary to choose an initial time combination

668   from the stages before those windows. Also, St21 and St50.6 are considered to be a good combination

669   for the initial time because the PD-AP aspect ratios of the limb bud are similar.

670

671   In response to the reviewers' comments, we have revised the statistics somewhat in this revision. The

672   error bar shows the variation in the distance between species for 24 combinations of α and β values

673   when the region to be analyzed within the *Xenopus* limb bud is parameterized by α, β, and θ, as shown

674   in Figure 4H. For α, values corresponding to the position around the boundary between the prospective

675   zeugopod and stylopod were selected, and for β, values corresponding to the orientation around the A-

676   P axis were selected. The sizes of the error bars indicate that the interspecific distance is robust for

677   changes in the values of α and β.

678

679   Based on the above, the following sentences have been added to the revised manuscript.

680   (i) In the Results subsection "Cross-species synchronization of developmental clocks":

681   "Further, the blue area of the heatmap narrows (like an hourglass) at around chick stage 23-24 and

682   *Xenopus* stage 52-52.5. In those stages, A-P asymmetric tissue growth clearly appears, and it greatly

683   affects the change in the rescaled cell position in the ξ space (i.e., cell flow), meaning that those time

684   windows should correspond between the two species."

685

686   (ii) In the Materials and Methods section:

687   "Here, we examined the following two cases: First, the initial time for *Xenopus* was fixed at t_{Xenopus}

688   =50.6, and four initial times, t_{Chick} =20, 21, 23, 24, were tested in the calculations for the chick cell

689   trajectories in the ξ-space. For each initial value for chick, $\min_{\theta}[\eta(\alpha,\beta,\theta)]$ was calculated for 24

690   different parameter sets (α,β), where values corresponding to the position around the boundary

691   between the prospective zeugopod and stylopod were chosen for α, and values corresponding to the

692   orientation around the A-P axis were chosen for β. As a result, t_{Chick} =21 was the best fit for the

693   initial time corresponding to t_{Xenopus} =50.6 (Fig. 4**E**). In Fig. 4**E**, the medians and standard deviations

694   are shown for the 24 sets of (α,β). The standard deviations are not large, showing the robustness

695   of the values of $\min_{\theta}[\eta(\alpha,\beta,\theta)]$. Second, we performed a similar analysis for the case in which

696   the initial time for chick was fixed at t_{Chick} =21. The five initial times, t_{Xenopus} =50.6, 51.1, 51.6, 52.0,

697   52.4 were tested in the calculations for the *Xenopus* cell trajectories in the ξ-space. For each initial

698   value for *Xenopus*, $\min_\theta[\tilde{\eta}(\alpha,\beta,\theta)]$ was calculated for the same 24 parameter sets for (α,β)

699   as above, where $\tilde{\eta}$ is defined as follows:

700    $$\tilde{\eta}(\alpha,\beta,\theta)=\left\langle\delta(t^k_{\text{Chick}},t^{k,\text{CtoX}}_{\text{Xenopus}}(\alpha,\beta,\theta))\right\rangle_k,$$

701    $$t^k_{\text{Chick}}\in[21,30.5].$$

702   Consequently, the best agreement of trajectories between the two species was found at $t_{\text{Xenopus}}=50.6$

703   (51.1 was equally good). Based on these results, we chose $t_{\text{Xenopus}}=50.6$ and $t_{\text{Chick}}=21$ as the initial

704   time combination. Note that the aspect ratios (P-D vs A-P) of the limb buds (for the prospective

705   autopod and zeugopod) of both species are similar at $t_{\text{Xenopus}}=50.6$ and $t_{\text{Chick}}=21$."

706

707   *5) Figure 5: The authors claim a high degree of similarity in ξ-space (line 353), which is an important*
708   *result. It would be beneficial to quantify the level of similarity to provide a more quantitative*
709   *assessment of the observed similarity.*

710

711   **Reply**: After careful consideration, we have decided to describe only qualitative similarities here. As
712   mentioned in the Discussion section, recent advances in spatial transcriptome techniques are
713   remarkable. In fact, we have recently started a spatial transcriptome analysis of chick and *Xenopus*
714   limb development for the purpose of evaluating the similarity of expression patterns in the τ-ξ
715   coordinate system between the two species in a quantitative and genome-wide manner. Clear data on
716   this point will be obtained in the future.

717

718   *6) Materials and Methods, line 441: "All animal handling was performed under appropriate*
719   *anesthesia ..". It is recommended to provide a detailed description of the anesthesia protocol used in*
720   *the study for the purpose of reproducibility.*

721

722   **Reply**: Thank you for your comment. We have added accurate chemical information about anesthesia
723   as follows:
724   "0.05% tricaine methanesulfonate dissolved in Holtfreter's solution (60 mM NaCl , 0.6 mM KCl, 0.9
725   mM CaCl$_2$, 2.4 mM NaHCO$_3$)".

726

727

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have provided a helpful response to both the major and minor points raised in the first round of review, and amended the manuscript accordingly. I am happy to recommend publication of this revised version.

Reviewer #2 (Remarks to the Author):

Reviewing the comments from the revision of this manuscript, the authors have attended to all of them fairly carefully so I would support publication. The only thing I would strongly suggest is recognizing that the word 'conservation' as they use it implies that the trait of interest is not changed from the shared ancestor (i.e. they say the limb development process is conserved between frog and chick), while in this case, I believe evolutionary convergence is at least equally likely. It doesn't lessen the results that similar processes are involved and that those processes are present because of deeply conserved genetic, cellular and tissue properties, but the way the frog limb evolved coincident with a novel life history with metamorphosis, is unknown, therefore convergence should be mentioned in the discussion as an alternative explanation in addition to simply perpetuated conservation of limb developmental processes. I think the frog development literature supports this (the refs I suggested earlier). This perspective might also slightly change the discussion in other places, something for the authors to consider for consistency through the manuscript.

Second - a comment on line 503-504. I suggest adding '"species-specific" features of organ morphology' to clarify.

Reviewer #3 (Remarks to the Author):

The authors clarified all of my questions and concerns. I believe that the manuscript is now much improved and ready for publication. The revisions made have significantly enhanced the clarity and overall quality of the work.

Reply to the reviewer 2's comments

Reviewing the comments from the revision of this manuscript, the authors have attended to all of them fairly carefully so I would support publication. The only thing I would strongly suggest is recognizing that the word 'conservation' as they use it implies that the trait of interest is not changed from the shared ancestor (i.e. they say the limb development process is conserved between frog and chick), while in this case, I believe evolutionary convergence is at least equally likely. It doesn't lessen the results that similar processes are involved and that those processes are present because of deeply conserved genetic, cellular and tissue properties, but the way the frog limb evolved coincident with a novel life history with metamorphosis, is unknown, therefore convergence should be mentioned in the discussion as an alternative explanation in addition to simply perpetuated conservation of limb developmental processes. I think the frog development literature supports this (the refs I suggested earlier). This perspective might also slightly change the discussion in other places, something for the authors to consider for consistency through the manuscript.

<u>Reply to the comment (1)</u>
Thank you very much for this advice. We have added the following sentences into the Discussion section of the second revised manuscript:
"It should be noted that, in the context of evolution, it is important to exercise caution when using the term 'conservation.' Generally, the high similarities in tissue dynamics and associated genes make it plausible that these processes have been conserved without change from a common ancestor. However, it is essential to remain open to the possibility that they may have independently evolved through convergence."

Second - a comment on line 503-504. I suggest adding '"species-specific" features of organ morphology' to clarify.

<u>Reply to the comment (2)</u>
We have added the word in the Discussion section.