nature portfolio

Peer Review File

Immobilization secondary to cell death of muscle precursors with a dual transcriptional signature contributes to the emu wing skeletal pattern

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors present a very interesting paper implicating mechanoregulation via muscle contraction (or rather lack of it) in the evolution of the vestigial emu wing. The importance of muscle propelled embryo movement on the emerging morphology of the skeletal system has been illuminated and emphasised in recent years by focussed work from a number of research groups. The implications of this relatively recently appreciated, fundamental aspect of musculoskeletal development for developmental plasticity and for the building of an integrated functional system are of utmost importance in conceptualising morphological evolution. The manuscript therefore presents an important example of how developmental plasticity could contribute to morphological change.

The paper makes two very interesting observations: 1) features of the emu vestigial wing align with the effects of decreased muscle movement following immobilisation of the chick model and 2) a population of muscle precursors in the proximal emu wing bud undergo cell death.

The authors build on a number of previously observed aspects of emu wing development and morphology to propose the hypothesis that changes in embryonic movement might contribute to evolutionary changes in the vestigial wing. They show that previously described variation of emu wing morphology extends to variation between left and right wings within individuals. They postulate that this may be due to lack of distal muscles in the embryonic wing. To reinforce this concept they carry out immobilisation studies in the chick to examine if intraindividual asymmetry is a feature of the phenotypic effects. In parallel they examine gene expression of marker genes in the developing emu wing and carry out single cell RNA sequencing identifying a population of cells with dual signatures typical of somite derived myogenic cells and LMP cells. These cells were localised to aggregates in the proximal forelimb bud where cell death detection is suggested, leading to the proposal that this is at least partially responsible for depleted muscle in the emu wing.

There are a number of points that need to be clarified. Specific aspects of the experimental procedures and results requiring attention are detailed below. In addition, and importantly, there are some statements through the manuscript that need to be qualified and tailored more appropriately to the results presented: there are incidences where statements overstep what is demonstrated by the study. All summary/concluding statements should be reviewed to ensure they are appropriate. For example it is stated in the abstract that the analyses has “revealed that immobilisation ….is responsible for the shortening and fusion of skeletal elements and their left-right intraindividual phenotypic variation”; at best the paper proposes a possible explanation for, rather then revealing…. Further down in the abstract it refers to “an atypical transcriptional signature of the emu wing”, but since the single cell RNA sequencing analysis was only carried out in the emu wing and not additionally either in the emu leg or the chick wing, a question can be raised as to the validity of stating categorically that this is an atypical transcriptional signature. Statements on lines 87, 178, 290 and 338 require adjustment and qualification.

Sentence starting on line 51 needs to be reworded.

Figure 1a: This appears to show the left and right limbs of two specimens. Clarify in the legend if this is correct. The legend states n=8, please clarify what is meant by this. The legend includes expansion of many more abbreviations and enumerations than are shown in the figure e.g. digit 2, dc, mc, p, x - remove

Figure 1e: state more clearly that this is chick.

Figure 1 legend: line 377 refers to "interspace" – is the term interzone intended? This is also used elsewhere in the text.

Figure 1: The diagram on the bottom right corner is not explained or used anywhere- remove if not explained/referenced

From line 119 the manuscript sets out the question of when distal muscle reduction occurs in the emu forelimb and describes reduced muscle at stages 35 and 37. Why are earlier stages (between 30 and 35) not examined to answer this question more definitively? It would be valuable to extend this study to stages between 30 and 35.

The methodology for assessing distal movements needs to be more clearly described in the supplementary material.

Line 132: the citations are incomplete. There is a large literature here so perhaps inclusion of a good review article would be valuable.

From line 137: chick embryo immobilisations are carried out from E9 and from E6 but the reasoning for this is not well explained and the text from lines 137 to 163 is very convoluted in places. The authors seem to suggest that while immobilisations from later stages have previously shown effects on skeletal rudiment length, earlier immobilisations have not. But this is not the case. Several studies have examined effects of earlier immobilisations and even a paper cited here as reporting changes to gene expression under earlier immobilisation also shows significant effect on skeletal length. Also similar studies in the mouse model from multiple groups show effects on skeletal length at equivalently early time points. The result presented here is a good and interesting finding- that immobilisation (from both E6 and E9) leads to asymmetric effects on left and right limbs- and this could be presented in a much more straightforward way. The separation into immobilisations at different time points, with references to pre and post cavitation that are not explained or easy to follow in the text, and the attempt to suggest incorrectly that effects on skeletal length have not been demonstrated previously from earlier immobilisation studies is not necessary. A much more straightforward presentation of the findings would be more correct, clearer and more valuable.

Line 157: reference to Figure 2c- this must be an error.

Sentence starting on line 167: has a significant reduction been shown? Rephrase or clarify what is meant by interspace.

Single cell RNA sequencing was carried out on emu forelimb field and later forelimb bud. However to reveal what may be specific to the derived morphology of the emu wing it would be valuable to

compare to emu hindlimb and ideally also chick forelimb but neither comparison is reported, limiting the value of the study and the extent of the conclusions that can be drawn. The analysis focussed in on a population of cells that are Pax3+ and Hand2+, localising aggregation of such cells in the proximal forelimb. One limb bud section shown in supplementary figure S12 suggests that there is no such population of cells in the chick but since this is an important point it would be valuable to support this conclusion more robustly, by a more extensive comparison to the emu hindlimb and/or chick wing.

The authors go on to show cell death markers in the emu forelimb, suggested to be in the region defined above. But are they specifically enriched in these cells? Can the authors elaborate on the quantification shown in Fig 5a and make a more explicit statement about the quantifications represented here? For the localisation data shown in Figs 5b, c and d, it is not evident to this reviewer how this localisation corresponds to the location of the cell population referred to earlier. Can the authors show a lower magnification image of the limb bud in Fig 5b so that the fuller context of the localisation can be seen? The image shown in Fig 5c does not appear to be a similar location to those shown in b and d. Can the authors explain and show a better reference frame for these localisations? It is important to show that these cells correspond to the Pax3+ Hand2+ cells, as represented in the graphical representation in Fig 5e.

The discussion misses an opportunity to deal with the wider importance of the contribution of such a mechanism to morphological evolution

Reviewer #2 (Remarks to the Author):

This manuscript introduces an important contribution to mechanisms that underlie emu wing reduction. The descriptive and manipulative data are well done and the manuscript is publishable in its current form without additional experimental work in my view. There are several interesting hypotheses that arise from the findings upon which the authors can consider expanding.

The authors' comparison of emu and chicken data is useful. An introductory word about the morphological and evolutionary similarity of the two species would also be useful.

In the manuscript, immobilisation is convincingly linked to skeletal variability and wing reduction with experimental support. At their discretion, the authors could further discuss the effect of deficient or insufficiently specified skeletal progenitors to wing reduction.

Is there any evidence in the scRNA-seq data or elsewhere that suggests whether the dual transcriptional signature cells:

arose from the somite or the lateral plate?

were specified to form any cartilage or bone?

were specified to distal muscle fates or to distal or posterior (consistent with Hand2) skeletal fates?

In other words, can the authors propose an explanation for the distally biased loss of muscle and bone?

The discussion linking the failure of myogenic precursors to clearly differentiate into muscle with the mitochondrial death pathway is interesting and raises additional hypotheses.

Another hypothesis the authors can consider raising is whether the disparate mechanisms that have been proposed for emu wing reduction that were listed by the authors such as alteration of patterning gene expression domains and proliferation could be downstream to insufficient specification and migration of precursors. Is there an experimental approach that can help to unify these mechanisms?

Do the authors presume the emu wing evolved due to a DNA variation that underlies dual specification of a subset of precursors?

Reviewer #3 (Remarks to the Author):

In this study, Tsuboi and colleagues delve into the mechanistic and developmental origins of reduced wings in Emu. Initially, utilizing the chicken model system and immobilizing muscles, the authors propose that the absence of distal muscle activity is likely responsible for the observed reduction in digit length. Subsequently, employing a combination of single-cell transcriptional approaches and in situ hybridization, the authors identify a unique LPM/myogenic cell population in developing limbs. The authors suggest that this particular cell population, eventually undergoing cell death, could be implicated in the lack of distal muscle.

This well-written manuscript convincingly demonstrates how the loss of muscle contraction during development can lead to a reduction in bone length and the observed asymmetry between the left and right limbs in Emu. However, the proposed developmental origin leading to the loss of distal muscle cells, that involve the existence of an LPM/muscle population undergoing apoptosis, appears intriguing but, in this reviewer's view, lacks support from the presented dataset.

The major concerns raised by the reviewer primarily revolve around the interpretation of single-cell analysis.

1. The authors base the LPM origin of some muscle cells on a small set of cells co-expressing Hand2 and Pax3. This hypothesis is questionable due to the low number of Hand2+ cells, suggesting that the identified cell population could originate form technical noise. Indeed, in both early and late stages when examining the UMAP, the muscle cluster is one of the lowest Hand2-expressing cluster.

2. Because of the above-mentioned limitation, performing a control experiment to prove the Emu-specific nature of the LPM/myogenic population would be essential. The authors could access publicly available single-cell data from other species like chicken or any other bird with a well-developed distal limb that could serve as a valuable control. A quantification of Hand2/Pax3 co-expressing cells in such a control model system should definitely show its Emu-specificity.

3. The presence of LPM/myogenic population at late stage, but not at early stage, could be explained by a late wave of Hand2 expression, that is unrelated to its activity as a LPM marker. Velocity analyses of single cell data could help identify the differentiation trajectory of these cells between early and late stage.

Minor Points:

- Figure 2 is unclear on several aspects. A- the transcription of Lbx1 at stage 18 in Emu (Fig2a) is not obvious to this reviewer. The authors could maybe pinpoint the region of interest in the figure. B- Figure 2b is challenging to interpret, a drawing of the limb and its axes could help the reader.

- Figure 3b: is Met the same gene the authors refer as C-met in the manuscript?

- The sentence on line 150 is unclear: in comparison to what are mechanosensitive genes altered?

Response to Reviewers:

We thank the reviewers for their valuable comments. Indeed, we agree that some points raised were unclear, and we have therefore used this feedback to make significant improvements to the manuscript, including new experimental results and re-writing of the text. All changes to the manuscript have been highlighted in yellow.

Response to Reviewer #1:

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The authors present a very interesting paper implicating mechanoregulation via muscle contraction (or rather lack of it) in the evolution of the vestigial emu wing. The importance of muscle propelled embryo movement on the emerging morphology of the skeletal system has been illuminated and emphasised in recent years by focussed work from a number of research groups. The implications of this relatively recently appreciated, fundamental aspect of musculoskeletal development for developmental plasticity and for the building of an integrated functional system are of utmost importance in conceptualising morphological evolution. The manuscript therefore presents an important example of how developmental plasticity could contribute to morphological change.

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procedures and results requiring attention are detailed below. In addition, and importantly, there are some statements through the manuscript that need to be qualified and tailored more appropriately to the results presented: there are incidences where statements overstep what is demonstrated by the study. All summary/concluding statements should be reviewed to ensure they are appropriate. For example it is stated in the abstract that the analyses has "revealed that immobilisation ….is responsible for the shortening and fusion of skeletal elements and their left-right intraindividual phenotypic variation"; at best the paper proposes a possible explanation for, rather then revealing…. Further down in the abstract it refers to "an atypical transcriptional signature of the emu wing", but since the single cell RNA sequencing analysis was only carried out in the emu wing and not additionally either in the emu leg or the chick wing, a question can be raised as to the validity of stating categorically that this is an atypical transcriptional signature.

-Thank you for your comments. We have carefully reviewed our concluding statements to ensure that they are appropriate. The suggested expressions in the abstract were also corrected (lines 33-35 and line 40). In addition, an analysis of the transcriptional profile of chicken wings in comparison to our emu dataset was added (Figure S12, S13).

Statements on lines 87, 178, 290 and 338 require adjustment and qualification.
Sentence starting on line 51 needs to be reworded.

-As suggested, we adjusted and qualified the suggested statements and sentences (lines 91-95, 191-194, 320-322, 368-373, 51-52).

Figure 1a: This appears to show the left and right limbs of two specimens. Clarify in the legend if this is correct. The legend states n=8, please clarify what is meant by this. The legend includes expansion of many more abbreviations and enumerations than are shown in the figure e.g. digit 2, dc, mc, p, x - remove

-We apologize for the inadequate explanation and errors. We examined the left and right limbs of eight specimens in total. Figure 1a presents two examples from these eight specimens. We now added this explanation to the text (lines 107-108) and figure legend (lines 393-394). We also corrected the errors in the legend (lines 394-395).

Figure 1e: state more clearly that this is chick.

-As suggested, we corrected the expression (line 409-410).

Figure 1 legend: line 377 refers to “interspace” – is the term interzone intended? This is also used elsewhere in the text.

-We apologize for the unclear explanation. We used the term “interspace” to describe the space between the ulna and the distal carpal/metacarpal of digit 3, not for the “interzone”. To avoid confusion, we removed this term from the text, and changed the color of the line segments from black to yellow in the figure. Additionally, the description of the y-axis in Figure 1f was updated from “Interspace distance” to “The distance between U and dc/d3” to more accurately convey the intended meaning (line 416).

Figure 1: The diagram on the bottom right corner is not explained or used anywhere- remove if not explained/referenced.

-As suggested, we removed the diagram on the bottom right corner.

From line 119 the manuscript sets out the question of when distal muscle reduction occurs in the emu forelimb and describes reduced muscle at stages 35 and 37. Why are earlier stages (between 30 and 35) not examined to answer this question more definitively? It would be valuable to extend this study to stages between 30 and 35.

-Thank you for your comments. We now examined the muscle pattern at stages 30 and 33 of emu and chicken forelimbs (lines 126-134; Figure S2a), as suggested.

The methodology for assessing distal movements needs to be more clearly described in the supplementary material.

-We apologize for our unclear explanation. We revised the methodology for assessing distal movements in “*Manipulation of embryo movement*” in the supplementary material, as suggested.

Line 132: the citations are incomplete. There is a large literature here so perhaps inclusion of a good review article would be valuable.

-As suggested, we included a review article (Pollard, McGonnell, Pitsillides, 2014 J Anat: Ref 27) in the citations (line 144).

From line 137: chick embryo immobilisations are carried out from E9 and from E6 but the reasoning for this is not well explained and the text from lines 137 to 163 is very convoluted in places. The authors seem to suggest that while immobilisations from later stages have previously shown effects on skeletal rudiment length, earlier immobilisations have not. But this is not the case. Several studies have examined effects of earlier immobilisations and even a paper cited here as reporting changes to gene expression under earlier immobilisation also shows significant effect on skeletal length. Also similar studies in the mouse model from multiple groups show effects on skeletal length at equivalently early time points. The result presented here is a good and interesting finding- that immobilisation (from both E6 and E9) leads to asymmetric effects on left and right limbs- and this could be presented in a much more straightforward way. The separation into immobilisations at different time points, with references to pre and post cavitation that are not explained or easy to follow in the text, and the attempt to suggest incorrectly that effects on skeletal length have not been demonstrated previously from earlier immobilisation studies is not necessary. A much more straightforward presentation of the findings would be more correct, clearer and more valuable.

-We apologize for the incorrect information. Now, we removed the introduction of the previous work and provided a more straightforward presentation, including separation according to pre and post cavitation stages, as suggested (line 148-180).

Line 157: reference to Figure 2c- this must be an error.

-As suggested, we corrected the error (line 163).

Sentence starting on line 167: has a significant reduction been shown? Rephrase or clarify what is meant by interspace.

-We apologize for our unclear explanation. A significant reduction of distance between the ulna and the distal carpal/metacarpal of digit 3 in immobilized forelimbs compared to control forelimbs was shown in the right graph of Figure 1f (line 175). As suggested, we clarified the explanation of y-axis to "Distance between U and dc/d3 (μm)".

Single cell RNA sequencing was carried out on emu forelimb field and later forelimb bud. However to reveal what may be specific to the derived morphology of the emu wing it would be valuable to compare to emu hindlimb and ideally also chick forelimb but neither comparison is reported, limiting the value of the study and the extent of the conclusions that can be drawn.

-We agree that comparing the sequencing data of the emu with those of the chicken would be valuable. We analyzed single-cell RNA sequencing data from stage 24 and 27 chicken forelimb buds (Feregrino, C. and Tshopp, P. Assessing evolutionary and developmental transcriptome dynamics in homologous cell types. Dev Dyn 251, 1472-1489 (2022)), and compared them with data from stage 25 emu forelimb buds. In the stage 25 emu forelimb buds, we identified clusters of muscle cells enriched with LPM cell markers (Figure S12). In contrast, in stage 24 and 27 chicken forelimb buds, *Hand2* expression was not observed, and the expression of other LPM markers were not enriched (Figure S13). This suggests that muscle progenitors with an LPM transcriptional signature are distinctive to emu forelimb buds (lines 263-271).

The analysis focused in on a population of cells that are Pax3+ and Hand2+, localising aggregation of such cells in the proximal forelimb. One limb bud section shown in supplementary figure S12 suggests that there is no such population of cells in the chick but since this is an important point it would be valuable to support this conclusion more robustly, by a more extensive comparison to the emu hindlimb and/or chick wing.

-Thank you for your comments. We agree that a more extensive comparison is valuable. We now examined additional chicken forelimb sections at stage 23 (Figure S16), alongside those previously assessed at stage 19 and 25. Our results confirm that no cell aggregation occurs at the proximal region in chicken forelimbs at stages 19, 23 and 25, and a subpopulation of cells exhibiting a dual transcriptional signature is a unique feature to emu forelimbs.

The authors go on to show cell death markers in the emu forelimb, suggested to be in the region defined above. But are they specifically enriched in these cells? Can the authors elaborate on the quantification shown in Fig 5a and make a more explicit statement about the quantifications represented here?

- We apologize for the inadequate description of the violin plots of the expression of *Bak1, Casp10* and *Apaf1*. Following your comments, we clarified that we cannot determine whether cell population expressing *Bak1, Casp10* and *Apaf1* shown in the violin plots are identical to the cell populations in which fragmentation of nuclei was observed (lines 305-307).

Regarding the quantification, the percentages of cells expressing *Bak1*, *Casp10* and *Apaf1* (Expression Level > 0.0) in Pax3+/Hand2- cells and Pax3+/Hand2+ cells are 23.5% and 44.3%, 15.9% and 27.9%, and 33.8% and 44.7%, respectively. Therefore, the percentages of cells expressing these cell death related genes are higher in Pax3+/Hand2+ cells than those in Pax3-/Hand2+ cells.

We acknowledge the complexities involved due to the nature of genes associated with cell death. The application of the Bonferroni correction often results in numerous cells exhibiting zero expression, primarily those that are viable, thereby complicating the accurate assessment of statistical significance. During our experimental procedures, most apoptotic cells were eliminated via density gradient centrifugation in the preparation stages of our single-cell expansions. As a result, a significant proportion of apoptotic cells were absent from the scRNA dataset, a common challenge when analyzing genes specific to cell death.

However, it has been demonstrated that the expression levels of certain cell death-associated genes, such as Bak1, Caspases, and Apaf1, increase in cells that about to die (Bowen et al., 2021 Cell Death Differ 28, 2083-2094; Balzer et al., 2022 Nat Commun 13, 4018). Therefore, we included violin plots comparing the expression levels of these genes, as we believed this information would be valuable to the readers. We conducted histological analyses to confirm that a population of muscle progenitors with dual LPM/myogenic identity is undergoing cell death (Fig. 5a, b, c). To avoid confusion among and to present the most convincing histological data, we have relocated the violin plots of Bak1, Casp10, and Apaf1 to the supplemental materials, thereby highlighting the histological data in the main figures.

For the localisation data shown in Figs 5b, c and d, it is not evident to this reviewer how this localisation corresponds to the location of the cell population referred to earlier. Can the authors show a lower magnification image of the limb bud in Fig 5b so that the fuller context of the localisation can be seen? The image shown in Fig 5c does not appear to be a similar location to those shown in b and d. Can the authors explain and show a better reference frame for these localisations? It is important to show that these cells correspond to the Pax3+ Hand2+ cells, as represented in the graphical representation in Fig 5e.

-As suggested, we now showed the lower magnification images of Figures 5a, b and c (Figure S17). We indicated that cells with pyknotic nuclei are found among Pax3+/Hand2+ co-expressing cells in Figure 4, S14 and S15.

The discussion misses an opportunity to deal with the wider importance of the contribution of such a mechanism to morphological evolution

-Thank you for your comments. We now included a discussion on the wider importance of contributions from a similar mechanism to morphological evolution (lines 382-389).

Reviewer #2 (Remarks to the Author):

This manuscript introduces an important contribution to mechanisms that underlie emu wing reduction. The descriptive and manipulative data are well done and the manuscript is publishable in its current form without additional experimental work in my view. There are several interesting hypotheses that arise from the findings upon which the authors can consider expanding.

The authors' comparison of emu and chicken data is useful. An introductory word about the morphological and evolutionary similarity of the two species would also be useful.

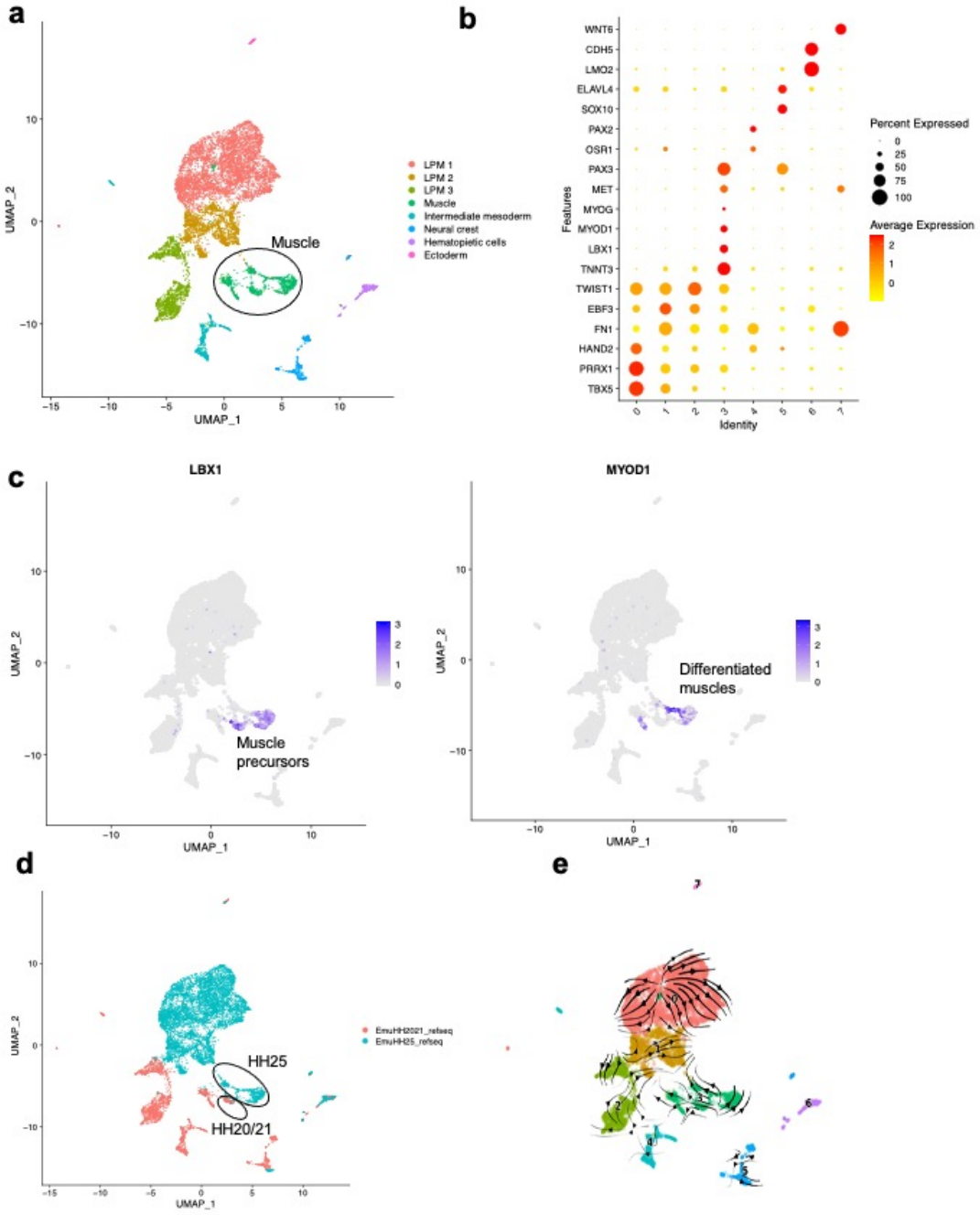
-As suggested, we provided the brief introduction about the morphology of these two species (lines 126-133).

In the manuscript, immobilisation is convincingly linked to skeletal variability and wing reduction with experimental support. At their discretion, the authors could further discuss the effect of deficient or insufficiently specified skeletal progenitors to wing reduction.

-Thank you for your suggestions. We now further discussed the effect of deficient or insufficiently specified skeletal progenitors to the reduction of wing skeletal elements (lines 368-373).

Is there any evidence in the scRNA-seq data or elsewhere that suggests whether the dual transcriptional signature cells:
arose from the somite or the lateral plate?

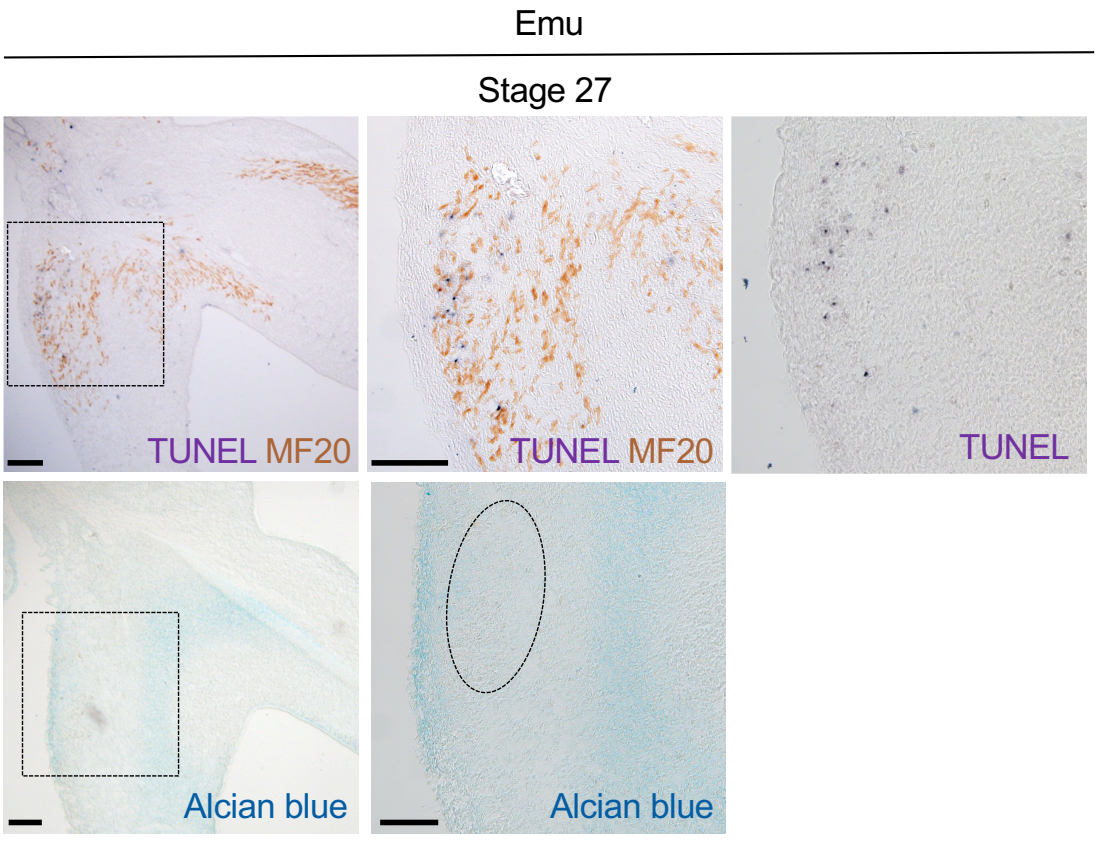
-Thank you for your comments. As you pointed out, it is important to accurately understand the differential trajectory of the Hand2+/Pax3+ cells. To this end, we merged the single-cell RNA sequencing data from emu forelimb buds at stages 20/21 and stage 25, as seen in the figure below, and conducted velocity analysis on the Hand2+/Pax3+ cells. Initially, we examined the velocity of muscle progenitors to determine if the differential trajectory could be accurately validated using this merged dataset. Our observations showed that muscle clusters composed of cells from both stages 20/21 and 25 (a-d). However, the muscle clusters were divided into clusters for each stage separately (d). Notably, the results of velocity analysis failed to capture the transition of the Lbx1-positive muscle progenitors at stage 20/21 into MyoD-positive differentiated muscle cells by stage 25 (e). This discrepancy likely arises from the considerable developmental defferences between stages of stages 20/21 and stage 25. Consequently, we concluded that it is difficult to identify the differentiation trajectory of Hand2+/Pax3+ cells using the velocity analysis with the current dataset.



a, A UMAP plot of stage 25 emu forelimb buds. b, Dot plots of subcluster marker gene expression of stage 25 data. c, The expression heatmap of marker genes is visualized on a UMAP plot. d, A UMAP plot colored by the origin of samples. e, Trajectory and velocity analyses of cell clusters. Note that the results of the Velocity analysis did not reflect the fact that Lbx1-positive muscle progenitors from stage 20/21 become MyoD-positive differentiated muscle cells by stage 25.

were specified to form any cartilage or bone?
were specified to distal muscle fates or to distal or posterior (consistent with Hand2) skeletal fates?
In other words, can the authors propose an explanation for the distally biased loss of muscle and bone?

-The reviewers raised important points. To examine these possibilities, we stained sections of forelimbs from later-stage embryos (serial sections of the forelimb buds used for Figure S15b) with Alcian blue (please see below), MF20 or TUNEL (Figure S18; same as the top panels in the figure below). Our results suggested that the cells with dual signatures died at proximal region before differentiating into muscles and were not specified to chondrocytes (please see below). We propose that a large population of muscle progenitors dies in the proximal region and fails to migrate toward the distal region (lines 311-315; Figure S18).



TUNEL and MF20 staining (top) and alcian blue staining (bottom) of stage 27 emu forelimb buds. Note that TUNEL-positive cells were surrounded by MF20-positive differentiated muscles but not by chondrocytes (indicated by a dotted circle). These

are serial sections of the forelimb buds used for Figure S15b; thus the TUNEL-positive proximal region is confirmed to be positive for Pax3 and Hand2. Scale bars, 100 μm.

The discussion linking the failure of myogenic precursors to clearly differentiate into muscle with the mitochondrial death pathway is interesting and raises additional hypotheses.

Another hypothesis the authors can consider raising is whether the disparate mechanisms that have been proposed for emu wing reduction that were listed by the authors such as alteration of patterning gene expression domains and proliferation could be downstream to insufficient specification and migration of precursors. Is there an experimental approach that can help to unify these mechanisms?

-We agree that understanding whether the mechanisms previously proposed can be linked to those we found is crucial. Young et al. (2019) observed distinct differences in *Fgf10* expression by stage 18. Conversely, in our study, we did not detect the cell population with dual transcriptional signatures (or their cell death) in stage 19 forelimb buds. Thus, it is likely that the reduction of *Fgf10* expression is not downstream of the emergence of the cell population with dual transcriptional signatures. While we cannot rule out the possibility that a deficiency in Fgf signaling is related to the emergence of this unique cell population, it is difficult to believe that a deficiency in Fgf signaling alone could cause a specific cell population to acquire a dual transcriptional signature. Thus, it is challenging to design experiments based solely on what is currently understood.

Do the authors presume the emu wing evolved due to a DNA variation that underlies dual specification of a subset of precursors?

-Thank you for your inquiry. We presume that the observed left-right intraindividual phenotypic variation in emu wings has arisen as a consequence of the reduction in mechanical stimuli subsequent to the cell death of a subset of muscle precursors. It is not clear what leads to this dual signature, nor what leads to the previously described Nkx2.5 expression in the emu myoblasts. It is possible that DNA variations in enhancer regions regulating one or more of the genes induced in emu are present; this is an exciting possibility for next studies.

Reviewer #3 (Remarks to the Author):

In this study, Tsuboi and colleagues delve into the mechanistic and developmental origins of reduced wings in Emu. Initially, utilizing the chicken model system and immobilizing muscles, the authors propose that the absence of distal muscle activity is likely responsible for the observed reduction in digit length. Subsequently, employing a combination of single-cell transcriptional approaches and in situ hybridization, the authors identify a unique LPM/myogenic cell population in developing limbs. The authors suggest that this particular cell population, eventually undergoing cell death, could be implicated in the lack of distal muscle.

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The major concerns raised by the reviewer primarily revolve around the interpretation of single-cell analysis.

1. The authors base the LPM origin of some muscle cells on a small set of cells co-expressing Hand2 and Pax3. This hypothesis is questionable due to the low number of Hand2+ cells, suggesting that the identified cell population could originate form technical noise. Indeed, in both early and late stages when examining the UMAP, the muscle cluster is one of the lowest Hand2-expressing cluster.

-We apologize for the inadequate explanation. It is not our intention to suggest that muscle cells co-expressing Hand2 and Pax3 originate from the LPM origin; rather, we believe that these cells exhibit dual transcriptional signatures characteristic of both LPM and somites. We agree with the reviewer that we cannot draw conclusions solely from the single-cell RNA seq data alone, given the limited number of Hand2+ cells. Consequently, we conducted HCR analyses. Our conclusions are drawn from observations of a subset of cells that exhibit a dual Pax3+/Hand2+ identity in emu forelimb buds (Figure 4c' and e). To avoid confusion, we removed violin plots showing

the expression levels of Pax3-/Hand2+ cells from Figure 3.

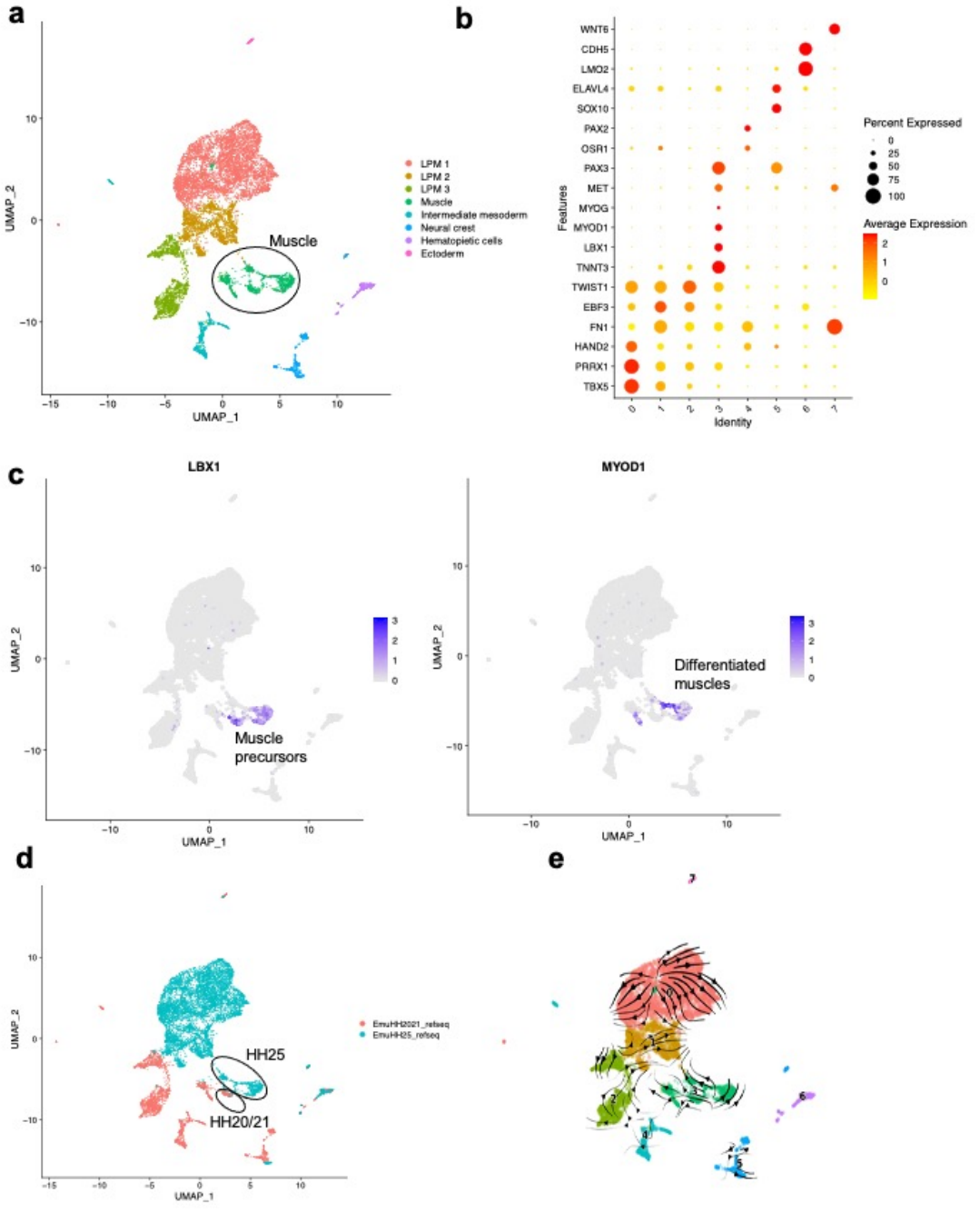
2. Because of the above-mentioned limitation, performing a control experiment to prove the Emu-specific nature of the LPM/myogenic population would be essential. The authors could access publicly available single-cell data from other species like chicken or any other bird with a well-developed distal limb that could serve as a valuable control. A quantification of Hand2/Pax3 co-expressing cells in such a control model system should definitely show its Emu-specificity.

-As suggested, we analyzed single-cell RNA-seq data from stage 24 and 27 chicken forelimb buds (Feregrino, C. and Tshopp, P. Assessing evolutionary and developmental transcriptome dynamics in homologous cell types. Dev Dyn 251, 1472-1489 (2022)) and compared them with data from stage 25 emu forelimb buds (Figures S12-13; lines 263-271). In the stage 25 emu forelimb buds, we found clusters of muscle cells enriched with LPM markers, such as Hand2, Prrx1, and Tbx5 (Figure S12). Conversely, in stage 24 and 27 chicken forelimb buds, Hand2 expression was not observed, and the expression of other LPM markers did not appear to be enriched (Figure S13). These results support our view that muscle progenitors exhibiting a transcriptional signature of the LPM are distinctive features of emu forelimb buds.

3. The presence of LPM/myogenic population at late stage, but not at early stage, could be explained by a late wave of Hand2 expression, that is unrelated to its activity as a LPM marker. Velocity analyses of single cell data could help identify the differentiation trajectory of these cells between early and late stage.

-Thank you for your comments. We merged single-cell RNA sequencing data from stages 20/21 and stage 25 emu forelimb buds to conduct further analysis. We observed muscle clusters composed of cells from both stages 20/21 and 25. However, the muscle clusters were divided into clusters for each stage separately (see below). As you can see, the results of the Velocity analysis did not reflect the fact that Lbx1-positive muscle progenitors from stage 20/21 become MyoD-positive differentiated muscle cells by stage 25 (see below). This discrepancy is likely because the developmental stages of stages 20/21 and stage 25 are too distant. Therefore, we concluded that it is difficult to identify the differentiation trajectory of Hand2+/Pax3+ cells using the velocity analysis with the current dataset. Consequently, we decided to histologically examine whether cells with a dual transcriptional signature are

present in the emu forelimb bud at earlier stages. We first conducted TUNEL analyses on the serial sections used for HCR analyses of stage 23 forelimb buds and found that the cell death in cells in the proximal region begins by stage 23 (Figure S17). Subsequently, we re-examined the HCR samples of stage 19 and 23, finding that a few cells in the proximal region were positive for Hand2 and Pax3 by stage 23, but not at stage 19 (Figure S14; lines 274-279).



a, A UMAP plot of stage 25 emu forelimb buds. b, Dot plots of subcluster marker gene expression of stage 25 data. c, The expression heatmap of marker genes is visualized on a UMAP plot. d, A UMAP plot colored by the origin of samples. e, Trajectory and velocity analyses of cell clusters. Note that the results of the Velocity analysis did not reflect the fact that Lbx1-positive muscle progenitors from stage 20/21 become MyoD-positive differentiated muscle cells by stage 25.

Minor Points:

- Figure 2 is unclear on several aspects. A- the transcription of Lbx1 at stage 18 in Emu (Fig2a) is not obvious to this reviewer. The authors could maybe pinpoint the region of interest in the figure.

-As suggested, we added the arrowheads in Figure 2a.

B- Figure 2b is challenging to interpret, a drawing of the limb and its axes could help the reader.

-As suggested, we added a drawing of the limb and added its axes (right panel in Figure 2b)

- Figure 3b: is Met the same gene the authors refer as C-met in the manuscript?

-We apologize for not using the same abbreviation for gene name . Yes, Met in Figure 3b is the same as cMet in the manuscript. To avoid confusion, we changed Met to cMet (Figure 3b).

- The sentence on line 150 is unclear: in comparison to what are mechanosensitive genes altered?

-We apologize for the poor explanation. This sentence was removed from the manuscript, as also suggested by the Reviewer #1.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

I have already commented positively on the valuable aspects of this paper so I will get to the point in reviewing the edits performed by the authors in response to earlier comments.

The authors have improved the manuscript in some respects but unfortunately they have engaged superficially in response to a number of the comments and therefore have not developed the content and presentation of the actual paper as they might have. Several responses are addressed by modifying supplementary data only which would leave readers of the paper with the same questions and doubts as this reviewer. The following highlights the original comments that have not be addressed sufficiently in my view. (note that the original comments reproduced here refer to line numbering in the original submission)

Line 132: the citations are incomplete. There is a large literature here so perhaps inclusion of a good review article would be valuable.

The authors have added one review article from 2014 (not recent) but have deleted a paper that they previously cited without explanation (see next comment). The papers cited are dated, with emphasis on the work from one research group among the more recent. The most recent is 2017 and most papers cited are pre 2010. Two very relevant, comprehensive reviews would be, for example, Felsenthal and Zelzer (2017) Development 144; 4271 and Murphy and Rolfe (2023) in Roles of Skeletal Muscle in Organ Development. The lack of comprehensive or careful treatment of this literature, which is central to the topic, even when highlighted as lacking through the review process, is very concerning.

The previous review, referring to the results section starting from original line 137, pointed out that 1) the presentation of the immobilisation data was made unnecessarily complicated and 2) that the presentation of early immobilisation affecting skeletal length as a novel finding was incorrect. The authors have simplified the presentation considerably which is an improvement but they continue to try to interpret early and late immobilisation treatments differently. It was pointed out previously that one of the papers cited by them actually contains data showing reduction in length following early immobilisation, however the authors response has been to remove the reference altogether. Although they no longer state/claim explicitly that this is a novel finding, it is presented as if it is by dividing the presentation into two parts - one supported by literature and the other not. The authors rebuttal states -"separation into pre and post cavitation stages as suggested"- but this was not suggested. I reiterate some key points from my previous critique and recommendation: "The authors seem to suggest that while immobilisations at later stages have previously shown effects

on skeletal rudiment length, earlier immobilisations have not. But this is not the case. Several studies have examined effects of earlier immobilisations and even a paper cited here as reporting changes to gene expression following earlier immobilisation also shows significant effect on skeletal length. Also similar studies in the mouse model from multiple groups show effects on skeletal length at equivalently early time points." The key and important result presented here is that immobilisation starting from both E6 and E9 timepoints leads to asymmetric effects on left and right limbs- and as stated before this could be presented in a much more straightforward way. The separation into immobilisations at different time points, with references to pre and post cavitation that are not explained or easy to follow in the text, and the attempt to suggest incorrectly that effects of early immobilisation on skeletal length have not been demonstrated previously, is not necessary. A much more straightforward presentation of the findings would be more correct, clearer and more valuable". This has not been achieved.

The following earlier comment has also not been addressed within the paper itself - the only concession made was to a supplementary figure which does not improve the apparent gap in the paper as presented

"The analysis focussed in on a population of cells that are Pax3+ and Hand2+, localising aggregation of such cells in the proximal forelimb. One limb bud section shown in supplementary figure S12 suggests that there is no such population of cells in the chick but since this is an important point it would be important to support this conclusion more robustly, by a more extensive comparison to the emu hindlimb and/or chick wing." In response the authors have added sections at an additional stage but within supplementary figure 16 only.

My previous critique raised a question about the quantifications shown in the previous Figure 5a and the response of the authors has been to remove the figure element.

The next point made in the previous review was that high magnification images shown in Figure 5 did not appear to be in a similar location (middle image looks different to those on left and right of it) and that it would be helpful to include a low power image for context. The response of the authors was to add more images in supplementary data and not to attempt to improve the clarity and presentation in the paper proper. This is not a fully engaged response to the suggestions made in the previous review and in this reviewer's opinion is not satisfactory.

In response to the very last point made, that the paper misses the opportunity to discuss the importance of the proposed mechanism for morphological evolution, the authors have added a couple of sentences. They refer appropriately to the one paper they have added to the reference list in the revised manuscript (ref 27). They also refer to reference 13 which is not appropriate here. I would expect reference to some seminal work on developmental plasticity such as that of Mary West-Eberhard, or interesting recent writings from authors such as Kim Cooper.

Reviewer #2 (Remarks to the Author):

The authors satisfactorily revised the manuscript and I have no further questions.

Reviewer #3 (Remarks to the Author):

The authors have satisfactorily answered my comments.

Response to Reviewer #1:

We appreciate the Reviewer #1's insightful comments. We apologize that some points raised were not explained well, and we have therefore used this feedback to make significant improvements to the manuscript, including new experimental results and re-writing of the text. All changes to the manuscript have been highlighted in yellow.

Reviewer #1 (Remarks to the Author):

I have already commented positively on the valuable aspects of this paper so I will get to the point in reviewing the edits performed by the authors in response to earlier comments.

The authors have improved the manuscript in some respects but unfortunately they have engaged superficially in response to a number of the comments and therefore have not developed the content and presentation of the actual paper as they might have. Several responses are addressed by modifying supplementary data only which would leave readers of the paper with the same questions and doubts as this reviewer. The following highlights the original comments that have not be addressed sufficiently in my view. (note that the original comments reproduced here refer to line numbering in the original submission)

Line 132: the citations are incomplete. There is a large literature here so perhaps inclusion of a good review article would be valuable.

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–**We deeply apologize for the oversight in deleting previously cited papers. This was an unintended error, and we have now re-included appropriate papers in our references. We also apologize for not including the more current review articles. We have now included the suggested reviews in introduction (lines 78, 81). Our initial**

emphasis on certain groups was primarily due to our focus on discussing works that concerned mostly avian and reptile forelimbs, especially regarding their effect on the shape of the skeleton. However, we acknowledge the importance of including a broader range of literature to provide a comprehensive overview of the topic. Thus, we have added more recent reviews as you suggested, ensuring our discussion now reflects the latest advances in the field. Thank you for highlighting this gap. We believe these updates offer a more balanced perspective.

The previous review, referring to the results section starting from original line 137, pointed out that 1) the presentation of the immobilisation data was made unnecessarily complicated and 2) that the presentation of early immobilisation affecting skeletal length as a novel finding was incorrect. The authors have simplified the presentation considerably which is an improvement but they continue to try to interpret early and late immobilisation treatments differently. It was pointed out previously that one of the papers cited by them actually contains data showing reduction in length following early immobilisation, however the authors response has been to remove the reference altogether. Although they no longer state/claim explicitly that this is a novel finding, it is presented as if it is by dividing the presentation into two parts - one supported by literature and the other not. The authors rebuttal states - "separation into pre and post cavitation stages as suggested"- but this was not suggested. I reiterate some key points from my previous critique and recommendation: "The authors seem to suggest that while immobilisations at later stages have previously shown effects on skeletal rudiment length, earlier immobilisations have not. But this is not the case. Several studies have examined effects of earlier immobilisations and even a paper cited here as reporting changes to gene expression following earlier immobilisation also shows significant effect on skeletal length. Also similar studies in the mouse model from multiple groups show effects on skeletal length at equivalently early time points." The key and important result presented here is that immobilisation starting from both E6 and E9 timepoints leads to asymmetric effects on left and right limbs- and as stated before this could be presented in a much more straightforward way. The separation into immobilisations at different time points, with references to pre and post cavitation that are not explained or easy to follow in the text, and the attempt to suggest incorrectly that effects of early immobilisation on skeletal length have not been demonstrated previously, is not necessary. A much more straightforward presentation of the findings would be more correct, clearer and more valuable". This has not been achieved.

–**We appreciate that the reviewer considers our text to have been improved. We have**

divided our immobilization experiments into pre and post cavitation stages to better discuss the effects on longitudinal growth (present after immobilization in both pre and post cavitation stages) and joint cavitation (present mostly in pre cavitation stages). This division was not meant to imply any claims of novelty; our novel data concerns the intraindividual variation after immobilization of chicken embryos, and how these phenotypes resemble the ones found in the vestigial emu wing. To clarify this point, we have added a new sentence meant to clarify this point: "Here, we will address these questions by first focusing on the effect of late stage (E10-18) immobilization on the longitudinal growth of individual skeletal elements, followed by examining how immobilization starting at pre-cavitation stages (E6-E10) could affect both growth and joint cavitation asymmetrically" (lines 148-151).

In addition, we apologize that, during rewriting, the reference "Pitsillides, A. A. Early effects of embryonic movement: 'a shot out of the dark'. J Anat 208, 417-431, (2006)." appeared to have been incorrectly removed. This reference had changed number from 24 to 13. Additional references have been added alongside the one mentioned by the reviewer (line 144).

The following earlier comment has also not been addressed within the paper itself - the only concession made was to a supplementary figure which does not improve the apparent gap in the paper as presented

"The analysis focussed in on a population of cells that are Pax3+ and Hand2+, localising aggregation of such cells in the proximal forelimb. One limb bud section shown in supplementary figure S12 suggests that there is no such population of cells in the chick but since this is an important point it would be important to support this conclusion more robustly, by a more extensive comparison to the emu hindlimb and/or chick wing." In response the authors have added sections at an additional stage but within supplementary figure 16 only.

–**We sincerely apologize for our incomplete explanation. Previously, we added HCR data of Pax3 and Hand2 in stage 23 chicken wings alone on Figure S16. This was because, in emu wings, we initially detected only a few Pax3 and Hand2 positive cells at stage 23. These signals became clearly recognizable only at stage 25, as Pax3 and Hand2 double-positive cells die soon after this period. By stages 26 and 27, only a small number of Pax3 and Hand2 double-positive cells were detected (Figure S15). To improve clarity for readers as suggested, we have now added merged images of HCR of Pax3 and Hand from stages 19, 23 and 25 chicken wings to Figure 4.**

Additionally, we performed HCR on stages 26 chicken wings and added these data in Figure S16.

We also apologize for not providing an explanation as to why we did not compare the emu wing data with the emu hindlimb data. The reason for this choice is twofold:

1. **The emu has evolved to specialize in running, resulting in significant adaptations in its leg muscles and bone structure. These specialized adaptations might introduce confounding variables, making the emu leg data an inappropriate control for our study on wing morphology.**

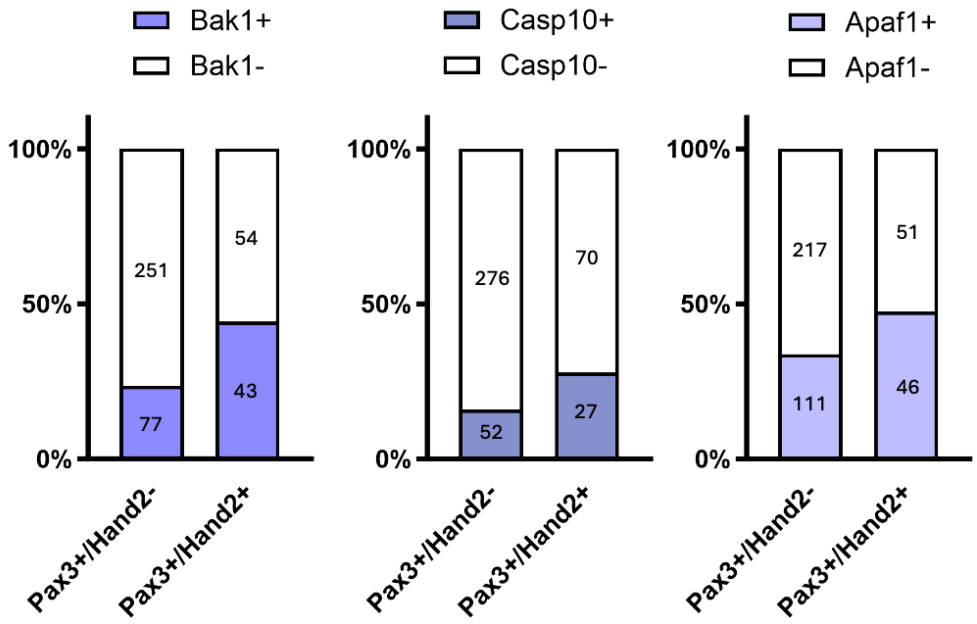
2. **We analyzed published single-cell RNA seq data on chicken wing buds at stages 24 and 27 (Figure S13). Therefore, using chicken wing data allows for a more accurate and meaningful comparison, leveraging these datasets to enhance the robustness of our findings. By comparing the emu wing data with chicken wing data, we ensure a more consistent and controlled analysis, thus providing clearer insights into the mechanisms underlying the characteristic features of emu wings.**

My previous critique raised a question about the quantifications shown in the previous Figure 5a and the response of the authors has been to remove the figure element.

–**We sincerely apologize that our response during the first round of review was unclear and did not meet your request. Initially, we did not include the quantifications of expression levels of apoptosis-related genes, because most cells actively undergoing cell death are typically eliminated during the cell preparation steps of scRNA-seq. The application of the Bonferroni correction in scRNA-seq often makes it difficult to accurate assessment of statistical significance, especially in case where there are zero-expressing cells. We intended to explain this in our response, but it seems we did not communicate this efficiently. We now provided this information in the text as follows: "…, albeit not statistically upregulated at expression level (Fig. 5a; Supplementary Table 3). Of note, most cells actively undergoing apoptosis have been eliminated from our scRNA-seq during cell preparation steps (density gradient centrifugation excludes apoptotic bodies) or bioinformatics quality control (exclusion of cells with mtDNA content > 15%)." (lines 309-313).**

While it is not standard practice to compare the number of cells in scRNA-seq studies,

we have quantified the numbers of cells expressing *Bak1*, *Casp10* and *Apaf1* (Expression Level > 0.0) in Pax3+/Hand2- cells and Pax3+/Hand2+ cells, for your reference. As shown in the attached graph, the percentages of cells expressing these cell death related genes are higher in Pax3+/Hand2+ cells than those in Pax3-/Hand2+ cells.



The numbers of cells expressing *Bak1*, *Casp10* and *Apaf1* in the muscle cluster of stage 25 emu forelimb scRNA-seq data. The numbers of cells expressing *Bak1, Casp10* and *Apaf1* (Expression Level > 0.0) in Pax3+/Hand2- cells and Pax3+/Hand2+ cells (23.5% and 44.3%, 15.9% and 27.9%, and 33.8% and 44.7%, respectively) in the muscle cluster of stage 25 emu forelimb scRNA-seq data. These data are summarized in the violin plots shown in Figure 5a.

Additionally, we have moved Figure 5a, which had been relocated to the supplementary data, back to the main figure as suggested.

The next point made in the previous review was that high magnification images shown in Figure 5 did not appear to be in a similar location (middle image looks different to those on left and right of it) and that it would be helpful to include a low power image for context. The response of the authors was to add more images in supplementary data and not to attempt to improve the clarity and presentation in the paper proper. This is not a fully engaged response to the suggestions made in the previous review and in this reviewer's opinion is not satisfactory.

–**We sincerely apologize for our misunderstanding of your suggestions. We initially believed that you were requesting the inclusion of low magnification images for Figure 5 b-d to better indicate the location of the enlarged areas, which led us to place these low magnification images in the supplementary data.**

In response to your feedback, and to enhance the clarity and presentation of our manuscript, we have now incorporated both the low magnification images and the corresponding enlarged images (Figure 5b-d and 5b'-d') directly into the main figure. Additionally, we have ensured that the magnifications and orientations of the images in Figure 5b-d are consistent.

In response to the very last point made, that the paper misses the opportunity to discuss the importance of the proposed mechanism for morphological evolution, the authors have added a couple of sentences. They refer appropriately to the one paper they have added to the reference list in the revised manuscript (ref 27). They also refer to reference 13 which is not appropriate here. I would expect reference to some seminal work on developmental plasticity such as that of Mary West-Eberhard, or interesting recent writings from authors such as Kim Cooper.

–**We are sorry that our discussion was still lacking. We have now considerably expanded our discussion about evolution, including by discussing the suggested writings (lines 392-404; 407-409).**

We hope this revision meets your expectations and provides a clearer context for our findings. Thank you for your patience and understanding.

Reviewer #2 (Remarks to the Author):

The authors satisfactorily revised the manuscript and I have no further questions.

Reviewer #3 (Remarks to the Author):

The authors have satisfactorily answered my comments.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have now addressed points raised and have adjusted the manuscript accordingly.

One detailed point arising in the passage added to the discussion: while reference 71 is appropriate where it is first used (sentence ending on line 400) but it is not appropriate as support for the following sentence (And finally, at organismal level, the “two-legged goat” example reveals how

401 a change in behavior led to morphological traits that phenocopied several evolutionary

402 novelties found in bipedal mammals, in a remarkable example of plasticity that is only

403 possible when the development of muscular and skeletal compartments is strongly

404 coordinated71). I presume this is an error that can be easily corrected.

Response to Reviewer #1:

We appreciate the Reviewer #1's thorough review and valuable comments.

Reviewer #1 (Remarks to the Author):

The authors have now addressed points raised and have adjusted the manuscript accordingly.
One detailed point arising in the passage added to the discussion: while reference 71 is appropriate where it is first used (sentence ending on line 400) but it is not appropriate as support for the following sentence (And finally, at organismal level, the "two-legged goat" example reveals how a change in behavior led to morphological traits that phenocopied several evolutionary novelties found in bipedal mammals, in a remarkable example of plasticity that is only possible when the development of muscular and skeletal compartments is strongly coordinated71). I presume this is an error that can be easily corrected.

－**We apologize for citing an inappropriate reference. We have now cited the correct reference (West-Eberhard M. J., 2005. J Exp Zool Pt B 304B, 610-618) as reference 72.**