



Limb regeneration exhibit both deterministic and stochastic patterns

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Dear Editor,

The salamander is renowned for its ability to regenerate the limbs perfectly, yet it is often overlooked that it can also experience abnormal limb regeneration issues. Unravelling the factors contribute to imperfect regeneration would enable us to design strategies circumventing them to improve the regeneration outcome. In this study, we confirmed that aging is a predictable factor that leads to abnormal limb regeneration. However, repeated amputations in young animals resulted in random phenotypes in each limb regeneration naturally, indicating the involvement of stochastic factors. Global gene expression analysis revealed greater variations among the abnormal regenerations compare to the normal ones, indicating certain unpredictable inherent mechanisms could cause deviations and abnormalities during limb regeneration. The salamander limb regeneration exhibit both deterministic and stochastic patterns that may provide adaptive advantages in nature.

Limb regeneration has been studied in salamanders for many years and the underlying mechanisms regulating this unique process are still being discovered. All along, researchers have focused on the salamander's ability to regenerate limbs perfectly, while often overlooking the fact that abnormal regenerations can also occur naturally. Previous studies identified many genetic, biochemical, physical, and environmental factors that are necessary for a successful limb regeneration because perturbation any of them would lead to abnormal or failed regeneration.¹ On the other hand, the salamanders would also exhibit abnormal limb regenerations with reduced fidelity and mild defects even in undisturbed conditions. For instance, the patterning defects have been documented in the skeletal muscle of the seemingly successfully regenerated limb.² Furthermore, detailed limb histological analysis suggested that abnormalities occur even in larval salamanders at the first regeneration.³ Additionally, paleontological evidence from the fossil record suggests that both normal and abnormal limb regeneration may have been ancient features of these animals dating back at least 300 million years.⁴ It indicates that certain degrees of deviations from the perfect limb regeneration process may have conferred some adaptive advantage in response to environmental pressures. Understanding the mechanisms underlying both normal and abnormal limb regeneration can instruct the design of new therapies for human regenerative medicine.

Conducting repeated treatments in the same individual under identical settings could provide a paradigm to identify the intrinsic conservations and deviations underlying the biological process of interest. In the case of salamander limb regeneration, studying the variability in phenotype and gene expression among repeated limb amputations could provide insights into the deterministic or stochastic factors that might contribute to both normal and abnormal limb regeneration outcomes. The repeated amputations in newt (*Notophthalmus viridescens*)⁵ and axolotl (*Ambystoma mexicanum*)⁶ showed that the fidelity of regeneration deteriorates following many successive amputations. These studies indicate that the regeneration is not always predictable, but nonetheless does not address the concept of whether certain intrinsic fixed or random factor(s) caused the abnormalities following repeated insults.

In this study, comparative analysis of repeated amputations in juvenile and adult animals confirmed that aging is a deterministic factor causes abnormal regeneration. However, stochastic regeneration patterns were also observed in young animals when aging factor was excluded. Instead of exactly replicate the pre-amputation limb, each regeneration seems to try to copy a fixed template but with random variations. This stochasticity is also reflected in the high variability of limb morphology and gene expression observed in abnormal regenerating limbs compared to normal ones.

We first performed amputations at the forelimbs of juvenile (≈5month) and

adult (>3years) salamanders (*Pleurodeles waltl*). The regeneration process was recorded and the regenerative outcome were evaluated as normal (four fingers clearly separated, normal length) and abnormal (oligodactyly or polydactyly, fingers not clearly separated, fingers too long or too short, etc.). Regenerative decline with aging has been evidenced in a variety of organs and species,⁷ and we also observed abnormalities during limb regeneration in older salamanders (Figure 1A), confirmed that aging is a definitive factor causes abnormal limb regeneration.

To avoid the aging effect and evaluate the existence of stochastic factors underlying limb regeneration, we performed repeated amputations in the juvenile animals under identical conditions. We found that overall abnormal regenerations account for about half of the cases irrespective of rounds of regeneration (Figure 1B). However, the majority of animals show random and varied patterns during consecutive regenerations, with no consistent pattern or trend detected in the same individual (Figure 1C). For example, when a previous limb regenerates normally, the next limb may appear to be abnormal. In contrast, when a previous limb regenerates abnormally, the next one may revert to a normal phenotype. The transitions between normal and abnormal appears to be totally random. There is no definitive factor identified as responsible for these unpredictable regeneration phenotypes, since all experimental conditions were kept constant. It is likely that there could be unknown stochastic internal factors that contribute to the variability observed in limb regeneration phenotypes.

Patterning plays a vital role in the development and regeneration of limbs. We utilized the image analysis techniques to compare the morphology and structure of limbs from development and regeneration to see if there are consistent patterns exist. We first combined multiple limb images to obtain a "development model" and a "regeneration model" separately, and observed clear differences in the overall morphology between the two models. Specifically, the development model showed less variation and more distinct fingertips compared to the regeneration model, which exhibited a more heterogeneous and less distinguishable fingertip distribution (Figure 1D). These findings suggest that while the regeneration process can produce functional limbs, it is not able to faithfully replicate the identical morphologies of the original limbs. Both the standard deviation analysis and Violin plots on the development and regeneration datasets indicate the variations of regeneration limbs was significantly greater than that of development (Figure 1E). We could attribute these increased variations to the underlying stochastic factors associated with regeneration. Furthermore, we performed RNA-seq on selected normal and abnormal regenerated limbs and the distance matrix analysis of the transcriptomes revealed much greater variations and fluctuations among the abnormal limbs than the normal ones (Figure 1F). Thus, the stochastic factors may drive the regeneration program to deviate away from normal patterns.

Finally, we compared the transcriptome datasets of development and regeneration samples and found that the difference between regeneration and development is significantly bigger than between normal and abnormal regenerations (Figure 1F). It indicates that the process of regeneration, regardless of stochastic factors, is distinct from limb development. These findings align with previous studies that have identified altered cellular programs during limb regeneration in comparison to development, particularly in the bone.⁸ Overall, the results indicate that the limb regeneration may follow a unique template that is not established during development, although there are random variations within the regeneration process.

Our study revealed both deterministic and stochastic factors underlying the limb regeneration in salamanders. Aging is one of the most decisive factors that cause dysfunction in adult stem cells and deterioration in tissue repair in

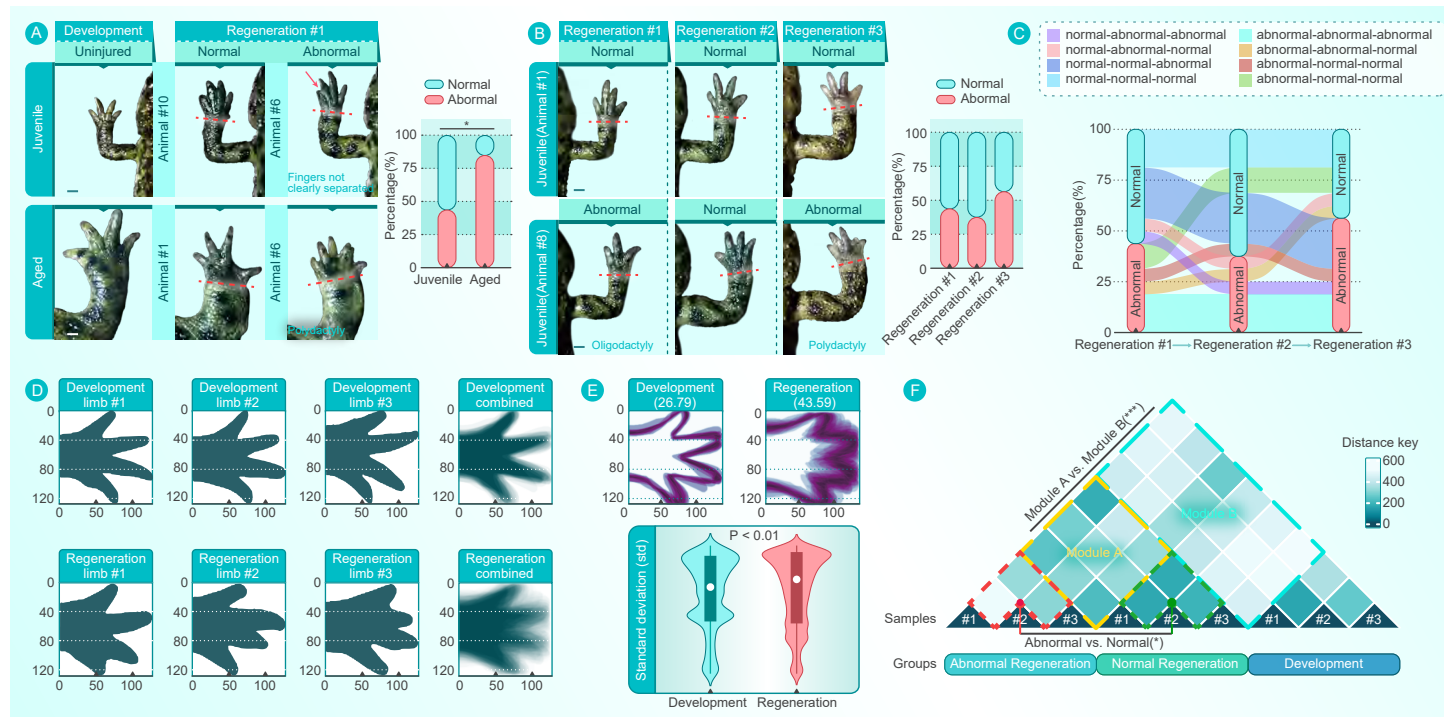


Figure 1. Both deterministic and stochastic patterns were found during limb regeneration (A) The representative pictures showing the limb development, normal and abnormal regenerations in juvenile and aged animals. The dotted lines indicate the amputation plane. The right panel shows the proportion of normal and abnormal regenerations in young and old animals. The red arrow marks the areas where the fingertip distinction is not obvious. $n=20$ limbs for aged group, $n=16$ limbs for juvenile group. Scale bar: 1 mm. Fisher's Exact Test; $*p < 0.05$. (B) Representative pictures showing consecutive limb regenerations in the same limb from the same animal. Please note that both normal and abnormal regenerations can occur in the same limb. The right panel shows the proportion of normal and abnormal limb regenerations in young animals upon repeated amputations. $n=16$ limbs for juvenile group. Scale bar: 1 mm. (C) The alluvial diagram showing the phenotype transitions of the same limb throughout three consecutive amputations. The proportion of phenotype transitions are indicated on the left axis. (D) Representative images showing the limb development and regeneration patterns. The overlapped (combined) images of limb development and regeneration were shown, respectively. Please note that the limbs from development exhibit more consistent and distinguishable patterns, whereas the limbs from regeneration show obscure and varied patterns. (E) The standard deviation analysis (top) of the development and regeneration datasets. The average pixel standard deviation in the developmental images (26.79) is significantly smaller than that in the regeneration images (43.59). Violin plot (bottom) of the pixel differences in standard deviation (std) images between development and regeneration images shows that the pixel std in regeneration images is significantly higher than the development. Mann-Whitney U test; $p < 0.01$. (F) The distance matrix analysis of the transcriptomes from development, normal regeneration and abnormal regeneration samples. The color depth denotes the Euclidean distance between samples in the triangular matrix heatmap. The dark means more similar and white means more different. The red dotted area denotes comparisons among abnormal regenerations and the green dotted area denotes comparisons among normal regenerations. The yellow dotted area (Module A) denotes comparisons among all regenerations including normal and abnormal. The blue dotted area (Module B) denotes comparisons between development and regeneration. Please note that the variations were significantly larger in abnormal regenerations than in normal regenerations. The variations between development and regeneration (Module B) were significantly bigger than that within regenerations (Module A). Two-sample t-test; $*p < 0.05$; $***p < 0.001$.

many species and this is also the case in salamander limb regeneration. On the other hand, stochastic factors could also affect the quality of limb regeneration since the same animal exhibited random patterns in repeated regenerations under identical conditions. However, in *Notophthalmus viridescens*⁵ and *Ambystoma mexicanum*⁶, prolonged and repeated limb amputations resulted in the increased abnormal regenerations. We reason that the extend of injury is less severe in this study (amputation at the more distal zeugopod instead of the proximal stylopod), would enable the animal to recover more efficiently and thus easily exhibit the intrinsic variations among the repetitions. Furthermore, we performed fewer rounds of amputations than the previous studies and it might not have reached the turning point that deterministic factors (e.g., aging) override the stochastic factors.

The random switches between normal and abnormal phenotypes during repeated regenerations suggest that each regeneration was attempting to copy a fixed template with variations, rather than rebuilding the previous structure that was just lost. This is best exemplified by the corrective role of regeneration: previous limb defects were often rectified in the next regeneration. The final regeneration quality seems to be determined by the accuracy of gene expression in resemblance to the normal or perfect regenerations. The exact intrinsic causes of these gene expression deviations that contribute to the stochastic patterns of limb regeneration remained to be identified. Nevertheless, such deviations may provide animals with an advantage for adapting to different and harsh environments.

For animal experiments, Iberian ribbed newts *Pleurodeles waltl* were maintained in filtered tap water at 22°C under 12h/12h light cycles. We randomly selected full-sibling juvenile (approximately 5 months) and adult newts (3

years), respectively. The animals were housed individually and maintained in strictly identical conditions throughout the experiments. The animals were amputated at zeugopod of either left or right forelimbs with a surgical scalpel. The amputation plane was right in the middle of the zeugopod and the amputation angle were aligned to be precisely vertical to the proximal-distal direction. The animals were housed in individual boxes with excessive food and handled with extreme care to do not disturb regenerating limbs when feeding and taking pictures. The polydactyly, oligodactyly, unseparated fingers, web-fingered were all classified as abnormal. The animal experiments were not randomized. All images were derived from more than 10 different individuals.

Image datasets were collected from developing and regenerating limbs. The scikit-image Python package was used to adjust the size of images to a uniform size of (128, 128). To compare the differences between the limb development and regeneration image datasets, the mean pixel values of the two datasets were calculated, and the different areas between the two average images were visualized by subtracting one from the other. In addition, to quantitatively compare the variation in pixel intensity between the two datasets, per-pixel standard deviation was calculated, and the Mann-Whitney U test was performed to test for significant differences. All the codes involved in the above analysis can be found here: <https://github.com/Nanguage/Regeneration>.

For RNA-seq, samples were selected from third regeneration (normal and abnormal) and developmental individuals. There are three individuals for each condition. After RNA extraction and library construction, the samples were sequenced using the Illumina NovaSeq 6000 platform. After removal of low

quality reads, the trinity software suite was used for further analysis.⁹ The transcriptome integrating various tissues of the newt *Pleurodeles waltl* was used as reference transcriptome for calculation of expression values.¹⁰ The R package 'sva' was used to remove batch effects on isoform expression values. The expression matrix after removal of batch effects was performed using the `dist()` function in R to calculate the Euclidean distance between samples.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

ETHICAL STATEMENT AND PATIENT CONSENT

All procedures were carried out in accordance with the Institutional Animal Care and Use Committee of Huazhong Agricultural University (ethics approval No.: 2018-0125).

DATA AND CODE AVAILABILITY

All the codes involved in the above analysis can be found here: <https://github.com/Nanguage/Regeneration>. The RNA-seq data were deposited in PRJNA987856 at NCBI.