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Altered metabolic state impedes limb regeneration in salamanders

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ABSTRACT

Salamanders are unique among tetrapods in their ability to regenerate the limbs throughout life. Like other poikilothermic amphibians, salamanders also show a remarkable capacity to survive long periods of starvation. Whether the physiological reserves necessary for tissue regeneration are preserved or sacrificed in starved salamanders is unknown. In the current study, we maintained Iberian ribbed newts (*Pleurodeles waltl*) under extreme physiological stress to assess the extent of regeneration and identify the molecular and cellular changes that may occur under such conditions. After 19 months of complete food deprivation, the animals exhibited extensive morphological and physiological adaptations but remained behaviorally active and vigilant. Autophagy was elevated in different tissues and the transformed gut microbiota indicated remodeling of the intestinal tract related to autophagy. Upon limb amputation in animals starved for 21 months, regeneration proceeded with progenitor cell proliferation and migration, leading to limb blastema formation. However, limb outgrowth and patterning were substantially attenuated. Blockage of autophagy inhibited cell proliferation and blastema formation in starved animals, but not in fed animals. Hence, tissue autophagy and the regenerative response were tightly coupled only when animals were under stress. Our results demonstrate that under adverse conditions, salamanders can exploit alternative strategies to secure blastema formation for limb regeneration.

Keywords: Starvation; Stress; Autophagy; Regeneration; Salamander

INTRODUCTION

Salamanders exhibit a remarkable ability to repeatedly regrow various tissues and organs throughout their life cycles. Rebuilding a lost body appendage, such as limb or tail, is mainly achieved by epimorphic regeneration, including the formation of a transient regenerative blastema and subsequent outgrowth of complex structures (Joven et al., 2019; Yun, 2015). Therefore, extensive cell activation and proliferation are required for rebuilding missing tissue during epimorphic regeneration (Liu et al., 2021). Although we lack direct measurements of the energetic costs of appendage regeneration in salamanders, adequate nutrient supply and energy flow are prerequisites for new cell synthesis during

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blastema formation and tissue regeneration (Eming et al., 2017). Indeed, injured animals adapted to hypermetabolic and catabolic states favor body protein catabolism for new protein and cell synthesis (Love et al., 2014). Thus, elevated nutritional needs and food intake are frequently observed during tissue regeneration (Cheng & Yilmaz, 2021).

Relative to birds and mammals, however, ectothermic amphibians and reptiles have low-energy systems characterized by low energy flow and highly efficient biomass conversion (Kristín & Gvoždík, 2012; Pough, 1983). In the wild, salamanders are often subjected to intermittent food supplies over long periods due to their harsh habitat environments (Carvalho et al., 2010; Hervant et al., 2001). As such, many species have developed a remarkable tolerance to prolonged periods of complete food deprivation and can survive long bouts of starvation without hibernation (Hervant et al., 1999; Mali et al., 2013). For instance, the cave-dwelling olm (*Proteus anguinus*) can survive food deprivation for exceptionally long periods (up to 96 months) with no signs of illness or hibernation (Hervant et al., 2001). It is likely that these animals can adjust their metabolism and utilization of physiological reserves when they encounter long-term starvation.

However, several questions arise regarding whether starved animals can still effectively regenerate their limbs after amputation and whether the energy reserves necessary for tissue regeneration are preserved or sacrificed. Tail regeneration has been tested in starved larval-stage salamanders, but under a relatively short duration of starvation (22 days), with catch-up growth known to contribute substantially to regeneration in fast-growing animals (Twitty & Delanney, 1939). In the current study, we investigated whether starved adult salamanders can still regenerate their limbs, and if so, identify the mechanisms that may contribute to tissue regeneration under extreme environments. Moreover, it remains unclear how energy and resources are made available to dividing cells during regeneration in starving animals. Thus, we examined Iberian ribbed newts (*Pleurodeles waltl*), which exhibit the widest known spectrum of regeneration abilities among adult vertebrates (Elewa et al., 2017; Yun et al., 2021). These salamanders are easily bred in the laboratory, with well-characterized transcriptome and genome assemblies (Joven et al., 2015, 2019) and genetically modified strains (Cai et al., 2019; Hayashi & Takeuchi, 2016). Here, the animals were maintained under food deprivation for 21 months and limb regeneration ability was examined. We found that the starved animals initiated limb regeneration by efficiently forming a blastema but failed to regrow a patterned limb. Hence, starvation does not hinder blastema formation but does inhibit limb outgrowth and patterning. In addition, cellular autophagy was elevated in the starved animals, which may provide energy and resources to sustain the massive cell proliferation required to construct a blastema. Autophagy inhibition experiments showed that autophagy was necessary for blastema formation in starved animals, but not in fed animals. Therefore, complementary strategies exist for inducing regeneration in salamanders under adverse conditions.

MATERIALS AND METHODS

Animal husbandry, limb amputation, and chemical administration

Iberian ribbed newts were maintained in tap water at 22–24 °C under natural light cycles. All animals used in this study were females and more than one year old. Newts in the normal feeding group were fed with frozen blood worms and pellets every three days. The starved animals were kept in separate boxes individually and water was changed once a week. Limb amputations were performed on anesthetized animals with a clean sterile scalpel. The animals in the autophagy inhibition treatment group were intraperitoneally injected with chloroquine at a concentration of 100 mg/kg. For EdU-labelling, animals were injected intraperitoneally with 50 mg/kg. All procedures were carried out in accordance with the Institutional Animal Care and Use Committee of Huazhong Agricultural University (ethics approval No.: 2018-0125).

Biochemical and physiological index measurements

Oxygen consumption was measured at 22 °C in ultrapure water inside a sealed box. Oxygen depletion in the air was monitored with an oxygen detector (AC8100, SMART SENSOR, China) and oxygen in the water was measured with a dissolved oxygen analyzer (DO850, APERA, China). Blood glucose was tested using a blood glucose level kit (BC2490, Solarbio, China) based on glucose oxidase levels. Liver and muscle glycogen levels were tested using a glycogen assay kit (BC2490, Solarbio, China) based on the anthrone method according to the manufacturer's instructions. In short, glycogen in tissue was extracted with a strong alkaline and reacted with anthracene ketone and then measured with a spectrophotometer. Liver and muscle triglycerides were tested using a triglyceride content detection kit (BC0620, Solarbio, China). Briefly, triglycerides were extracted with isopropyl alcohol and saponified with potassium hydroxide to produce glycerol. The glycerol was oxidized by periodate to generate formaldehyde and then condensed with acetyl acetone to emit light at 420 nm wavelength. EnSpire multifunctional spectrophotometer (Perkin Elmer, USA) was used to measure the intensity of specific light.

16S rRNA sequencing (rRNA-seq) and data analysis

The intestines of starved animals were completely empty. To avoid sampling bacteria introduced from food in the control animals, we gently removed intestinal content and collected only the intestine for processing. The intestines were cut into small pieces and homogenized in cold sterile 0.1×phosphate-buffered saline (PBS) using an autoclaved mortar and pestle. The mixtures were centrifuged (4 °C, 200 *g*, 5 min) and the suspensions were filtered with a 100 μm nylon filter. The filtrate was centrifuged (4 °C, 21 500 *g*, 20 min) to collect the bacteria. The cells were collected to isolate metagenomic DNA using a TIANamp Stool DNA Kit (DP180622, Tiangen, China) according to the manufacturer's instructions. The obtained DNA was submitted to Majorbio (China) for 16S rRNA-seq using the Illumina PE300 sequencing system (Illumina, USA). Two samples from the starved group and three samples from the control group were sequenced.

Sequencing data are listed in Supplementary Tables S1, S2. All reads were mapped and clustered into operational taxonomic units (OTUs) at a 97% similarity threshold in Qiime v1.9.1 (Bolyen et al., 2019). Taxonomies were assigned to classify each OTU at the phylum to species level using the SILVA 138 reference database (Quast et al., 2013). Alpha and beta diversity parameters were calculated from qualified OTU data in Qiime. Principal coordinate analysis (PCoA) was performed with the Bray-Curtis distance matrix. Based on Greengene ID corresponding to each OTU, family information from the Clusters of Orthologous Genes (COG) corresponding to each OTU was obtained and the functional abundance spectrum of each COG was calculated in PICRUSt2 v2.2.0 (Douglas et al., 2020). The 16S rRNA-seq data were submitted to the NCBI database under BioProjectID PRJNA749907 and the Genome Sequence Archive (GSA) database under accession No. PRJCA006821.

Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

The brain, heart, liver, intestine, and limb muscles were collected and snap-frozen in liquid nitrogen. Trizol reagent was used for tissue RNA extraction. The cDNA synthesis was performed with a PrimeScript^{TM} RT reagent kit (Takara, Japan), and qPCR analysis was performed with qPCR reagent (ABclonal, USA) and CFX96/384 fluorescent quantitative PCR instrument (BioRad, USA). The PCR primer sequences are listed in Supplementary Table S3. Relative qPCR was employed to examine autophagy marker gene expression in different tissues.

Immunofluorescence and transmission electron microscopy

Tissue samples were fixed with 4% formaldehyde, dehydrated with 30% sucrose, and then frozen with liquid nitrogen after optimal cutting temperature compound (OCT, CellPath Ltd., UK) embedment and sectioned at −30 °C. Sections (6–8 μm) were thawed at room temperature and fixed in 4% formaldehyde for 5 min. The sections were then blocked with 10% goat serum in 0.1% Triton-X for 30 min at room temperature. For LC3B staining, slides were incubated with rabbit anti-LC3B primary antibody (NB100-2220, Novus Biologicals, USA) overnight at 4 °C, followed by secondary antibodies for 1 h at room temperature. Antibodies were diluted in blocking buffer at 1:500. EdU staining was performed by incubating the sections with 100 mmol/L Tris, 1 mmol/L $CuSO_4$, 10 mmol/L fluorescent azide, and 100 mmol/L ascorbic acid for 30 min. Once all washing steps were completed, the coverslips were counterstained with 1–10 μg/mL 4',6-diamidino-2-phenylindole (DAPI). A confocal fluorescence microscope was used to examine and image the sections. For electron microscopy, the forelimbs (2 mm×2 mm size) were collected and immediately placed into the electron microscope fixator (Servicebio, China) at room temperature for 2 h, then stored at 4 °C for preservation. Leica EM UC7 ultramicrotome (Leica, Germany) was used for the sections and HITACHI HT7800 120 kV transmission electron microscope (HITACHI, Japan) was used to take photos. For EdU^+ nuclei and LC3B quantification, 1 in every 20 serial sections was selected and three images from each section were taken, with counting normalized to $DAPI^+$ cells. The number of autophagosomes in the electron microscope was normalized to the number of myofibers. Animal numbers are provided in each figure legend.

Statistical analysis

The animal experiments were not randomized. No samples were excluded from statistical analysis. Data are shown as mean±standard error of the mean (*SEM*). Statistical analyses were performed using GraphPad Prism v8.0, and the unpaired two-tailed *t*-test was used. Differences were considered statistically significant at $P<0.05$, $P<0.01$, and $P<0.001$.

RESULTS

Physiological adaptations after long-term starvation

Two-year-old animals were divided into two groups, i.e., starvation group (deprived of food for 19 months) and control group (fed normally). Starvation caused a steady reduction in body weight from the start of food deprivation. At the end of 19 months, the starved animals weighed only 40% of the control animals with normal feeding (Figure 1A). Body length (snout to vent length) in the control animals remained constant during treatment, but decreased by 11% in the starved animals (Figure 1B). The starved animals remained active during the entire experimental period, with no hibernation or illness observed. Although there was a striking difference in body morphology, the starved animals were still highly vigilant and moved actively in water (Supplementary Video 1), indicating that they maintained maneuverability and healthy status. Nevertheless, the starved animals showed significantly decreased oxygen consumption compared to the fed animals, suggesting their metabolic rate was reduced (Figure 1C).

Next, we measured and compared several physiological and energy indices in the starved and fed groups. Blood glucose levels were significantly reduced in the starved animals and were only 37% of normal levels after 19 months of food deprivation (Figure 1D). However, glycogen storage in the liver and muscle was comparable to that in the control animals (Figure 1E). This is in striking contrast to fasting in mammals, where blood glucose is maintained at a certain level at the expense of glycogen stores in the liver and skeletal muscle (McCue, 2010). Triglycerides are another major form of energy storage. We found notable fat deposition in various locations of the abdominal cavity in the normally fed controls, but no fat deposition in any part of the body in starved animals. In addition, the content of triglycerides in the liver and muscle was reduced to negligible levels in animals after 19 months of starvation (Figure 1F).

Effects of starvation on microbial communities

Diets play a dominant role in shaping gut microbiota and altering host metabolism (Kolodziejczyk et al., 2019). Calorie restriction or fasting can remodel the host microbiota considerably and may benefit host health and longevity (Catterson et al., 2018; Cignarella et al., 2018). However, food deprivation for a long period may impose profound challenges to microbial communities and gastrointestinal health

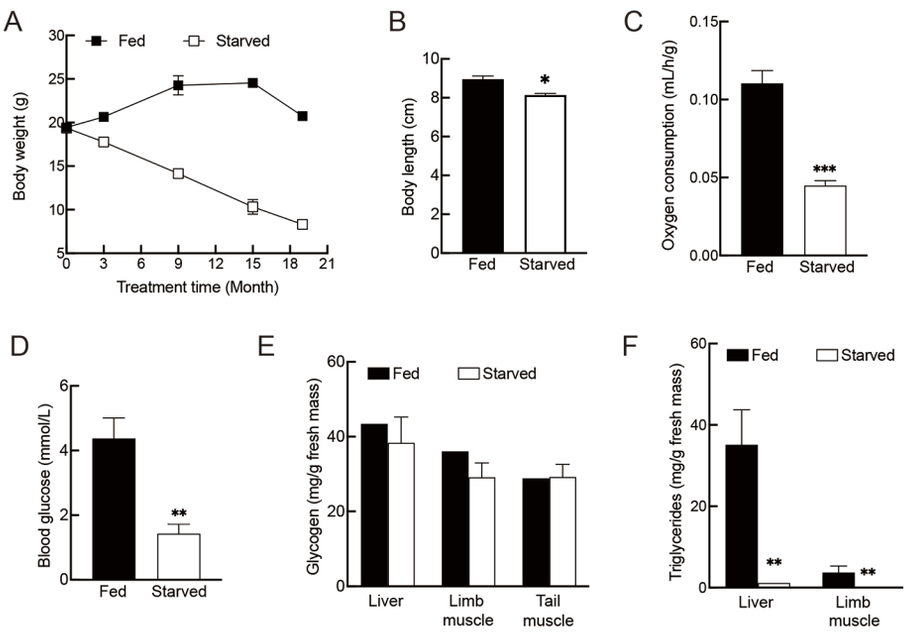

Figure 1 Physiological alterations after long-term starvation

Two-year-old female newts were divided into starvation (deprived of food for 19 months) and control groups (fed normally). A: Body weights of fed and starved newts. B: Body lengths (mouth to vent) of fed and starved newts. C: Oxygen consumption of fed and starved newts. D: Blood glucose levels of fed and starved newts. E: Glycogen content in liver, limb muscle, and tail muscle of fed and starved newts. F: Triglycerides in liver and limb muscle of fed and starved newts. Results are mean±*SEM*, *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$, after *t*-test, $n=5$ in starvation group, $n=6$ in control group.

(Sonnenburg & Sonnenburg, 2014; Xia et al., 2014). We assessed whether the microbial community was maintained or diminished in animals in response to deprivation of exogenous substrates for an extended period. We sampled the intestines of the starved and normally fed newts and performed 16S rRNA-seq to analyze microbiota composition.

The 16S rRNA-seq generated 236 101 sequences from a total of two starved and three fed samples (Supplementary Tables S1, S2). All clean data were mapped into OTUs. All 354 OTUs from the two groups were subsequently classified into 15 phyla, 26 classes, 61 orders, 95 families, 157 genera, and 202 species. The microbiome taxonomic comparisons between the starvated and fed groups at the phylum and class levels are shown in Figures 2A and 2B. Proteobacteria, Bacteroidota, and Firmicutes were the three most abundant bacterial phyla in both groups, consistent with previous microbiota studies in other salamanders (Demircan et al., 2018; Walker et al., 2020). At the class level, the starved animals had a higher proportion of Gammaproteobacteria but a lower proportion of Alphaproteobacteria compared to the fed animals, whereas the other major classes, such as Bacteroidia and Clostridia, were similar. Alpha diversity analysis also indicated that microbial community richness was similar between the starved and fed groups (Supplementary Figure S1A). Hence, the long-term starved animals managed to preserve their general gut microbiota without supplementary substrates.

However, Bray-Curtis-based beta diversity analysis showed that the bacterial community composition diverged considerably between the starved and fed animals (Supplementary Figure S1B). Furthermore, the analysis of similarities (ANOSIM) showed that the difference between the starved and fed groups was greater than the difference within each group (Supplementary Figure S1C). The control group had 326 OTUs but the starved group had only 227 OTUs, with an overlap of 199 OTUs between them (Figure 2C). Thus, during starvation, the animals appeared to lose one-third of their gut microbial species. Next, COG information corresponding to each OTU was obtained and the abundance of each COG in each group was calculated. We listed the top 10 COG categories that showed the largest difference in enrichment between the two groups and inferred the functional relevance for the host animal based on the eggNOG database (Tatusov et al., 2001) (Figure 2D). As expected, COG categories related to energy metabolism, such as “carbohydrate transport and metabolism”, “energy production and conversion”, and “translation, ribosomal structure, and biogenesis” were reduced in starved animals. In contrast, several COG categories related to autophagy were increased in the starved animals, including “post-translational modification, protein turnover, chaperones” and “intracellular trafficking, secretion, and vascular transport”. We hypothesized that these enriched bacterial species generated during starvation may be associated with autophagy in the host animal.

Elevation of autophagy in different tissues of starved animals

To examine autophagy levels in starved animals, we sampled different tissues and evaluated the expression of autophagy markers by RT-qPCR, immunohistochemistry, and electron

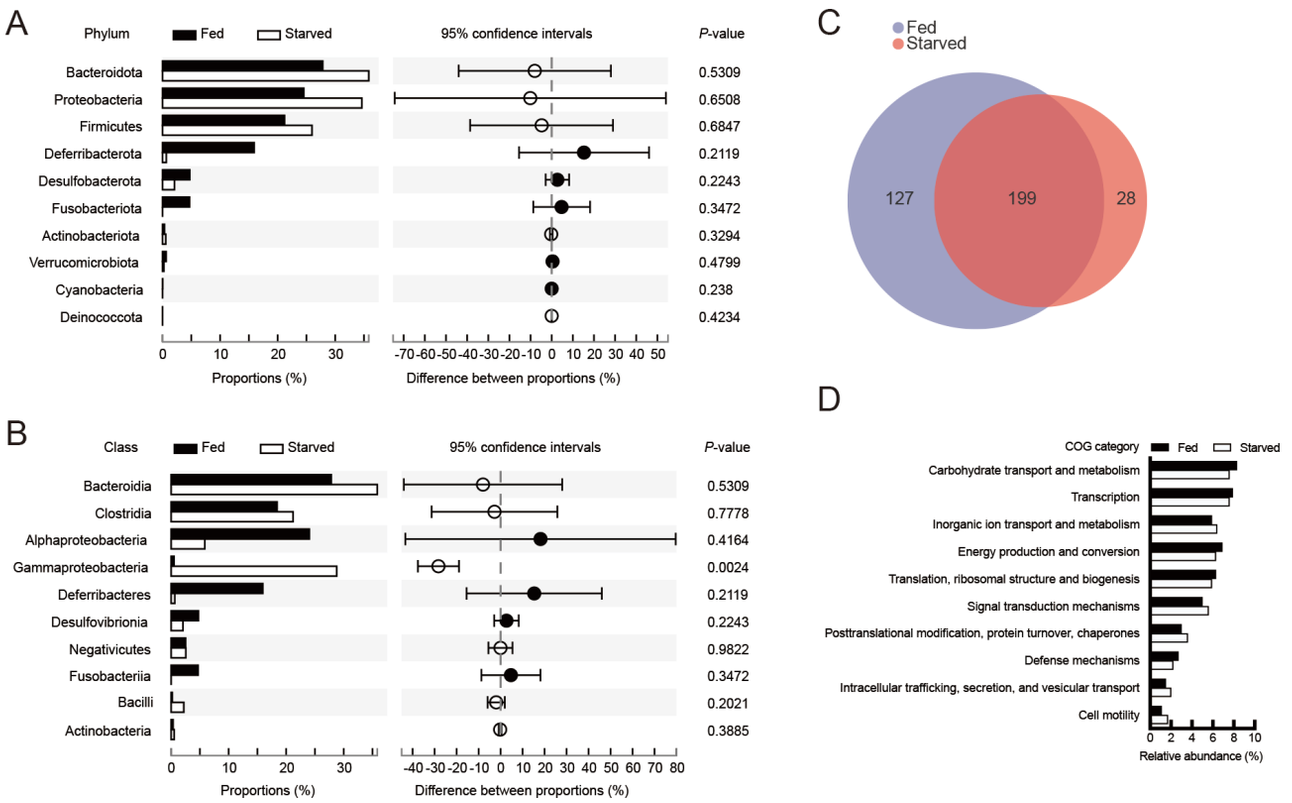

Figure 2 Taxonomic and functional transcriptomic changes in gut microbiota of newts during long-term starvation

A: Two-year-old female newts were starved for 19 months, after which gut microbiota (16S rRNA-seq) were analyzed. Relative abundance (percentage) of top 10 microbiome taxa at phylum level between fed and starved newts. B: Relative abundance of top 10 microbiome taxa at class level between fed and starved newts. C: Operational taxonomic unit (OTU) overlap between fed and starved newts. All OTUs from different repeats of each group were combined. Note: starvation group lost many OTUs compared to control group. D: Top 10 COG categories showing largest difference in enrichment between groups, with inferred functional relevance shown.

microscopy. Three classical autophagy-related genes, i.e., *Ulk1* (Unc-51-like autophagy activating kinase), *Atg12* (autophagy-related 12), and *Atg3* (autophagy-related 3) (Mizushima et al., 2010), were detected by relative RT-qPCR in different tissues, including the brain, heart, liver, intestinal tract, and forelimb. In general, all three autophagy marker genes showed higher expression in the different tissues of the starved animals. In particular, all three genes were significantly up-regulated in the starved livers (Figure 3A). We also focused on the autophagy levels in the limb tissue due to the unique limb regeneration abilities in salamanders. Microtubule-associated protein-1 light chain 3 (LC3/LC3B) is a ubiquitin-like protein involved in the formation of autophagosomes and is a commonly used marker for autophagosome visualization (Barth et al., 2010). Frozen forelimb sections were prepared for LC3B immunofluorescence staining. Results showed that autophagy in the forelimb tissues increased during starvation (Figure 3B), in agreement with the qPCR results. We verified the forelimb autophagy levels by analyzing the distribution of autophagosomes via transmission electron microscopy. We found significantly more autophagosome bodies in the limb muscle samples of starved animals (Figure 3C). Taken together, our results indicate that autophagic activity is elevated in the limbs and other tissues of starved animals.

Blastema formation and morphogenesis in starved animals upon limb amputation

Due to their unique limb regenerative ability, we investigated whether starved salamanders can still regenerate limbs under such stressful conditions. The animals were starved for 21 months and subjected to limb amputation. Wound healing, blastema formation, and limb outgrowth and patterning were recorded and compared to the fed controls at the same time intervals (Figure 4A). No food was provided over the course of limb regeneration to the starved group. As shown in Figure 4B, the starved animals were able to regenerate the limb, but regrowth was much slower and outgrowth length was much shorter compared to the fed animals. We carefully examined the regenerating limbs at different time points after amputation, and while blastema formation appeared normal, limb outgrowth and patterning were delayed in the starved animals. At the end of the regeneration experiment at 226 days after amputation (dpa), distinct digits were visible in the fed animals, but there was no sign of digit demarcation or individualization in the starved animals (Figure 4B). Thus, it appears that starved animals could adequately initiate blastema formation during the early phase of limb regeneration but could not efficiently sustain limb outgrowth due to the constraint of depleted resources during starvation.

Blastema formation requires massive proliferation and

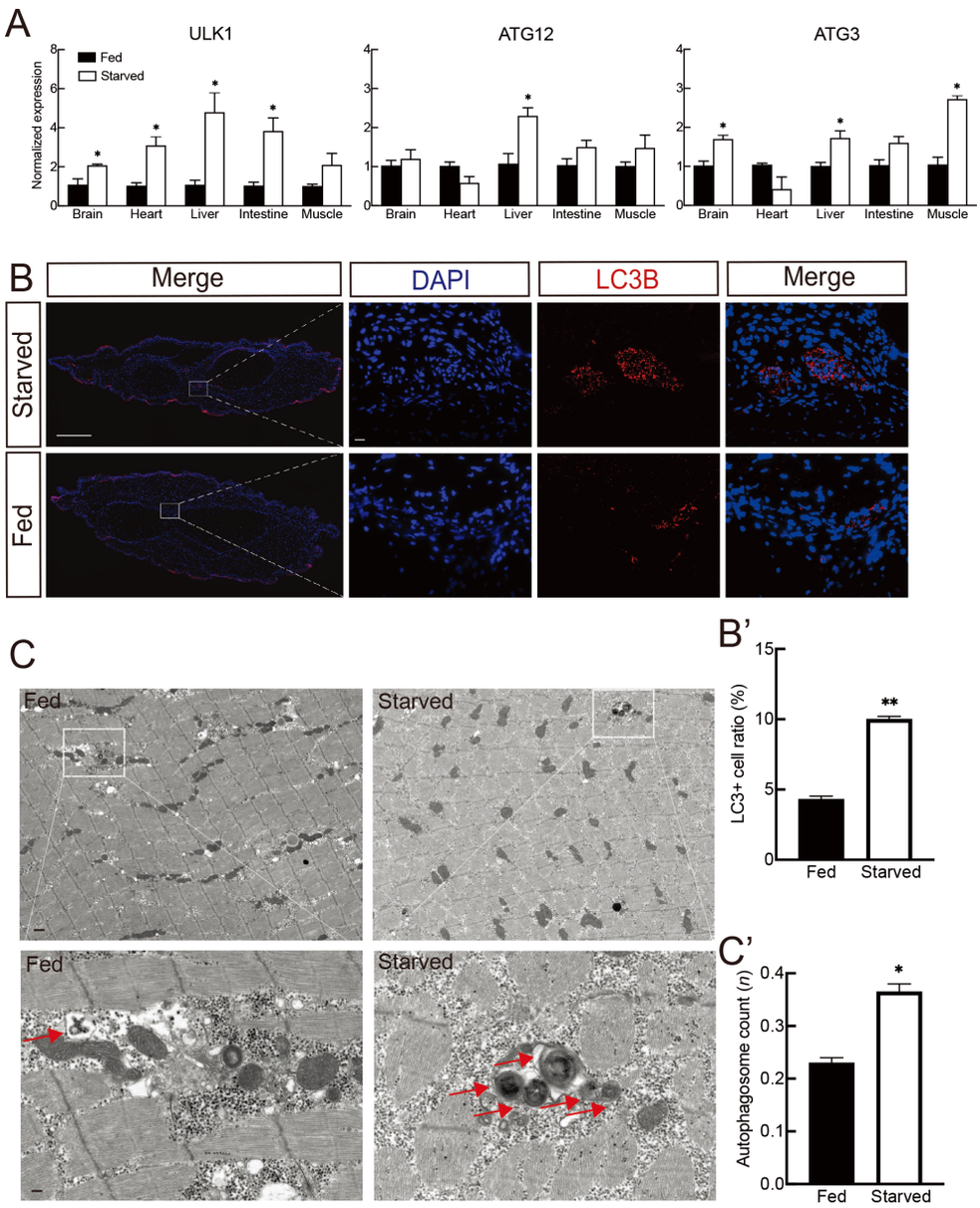

Figure 3 Autophagy is elevated in different tissues of starved newts

A: Two-year-old female newts were starved for 19 months, after which autophagy levels were analyzed. qPCR results of expression of three autophagy-related genes (*ULK1*, *ATG12* and *ATG3*) in five tissues, including brain, heart, liver, intestine, and limb muscle. B, B': Immunofluorescence staining and quantification showed that LC3B protein expression was elevated in limbs of starved newts compared to fed newts. Scale bar: left: 500 μm; middle and right: 20 μm. C: Representative transmission electron microscopy images showing distribution of autophagosomes (red arrows) in limbs of fed and starved newts. Scale bar: top: 5μm; bottom: 1 μm. C': Three limbs from three individual newts from each group were selected and average number of autophagosomes per myofiber was calculated. Data are mean±*SEM*, *: $P<0.05$; **: $P<0.01$, after *t*-test, $n=3$ in each group.

migration of progenitors to the amputation plane. Therefore, we evaluated cell proliferation dynamics during blastema formation by EdU labeling (Figure 4C). Results showed robust cell proliferation in both starved and fed animals during the early stages of limb regeneration (Figure 4D). Hence, injury-induced cell activation and proliferation were not compromised by the absence of nutrients in the long-term starved animals. Thus, other compensatory systems may contribute to maintaining normal cell proliferation upon injury.

Blockage of autophagy inhibits cell proliferation and blastema formation

The exceptional potency of cell proliferation and blastema formation in salamanders during extreme starvation was intriguing. One obvious possibility is that autophagy may provide energy and raw materials to support progenitor cell proliferation, leading to blastema formation (Rabinowitz & White, 2010). To test this, we blocked autophagy before and during limb regeneration in both starved and fed animals using

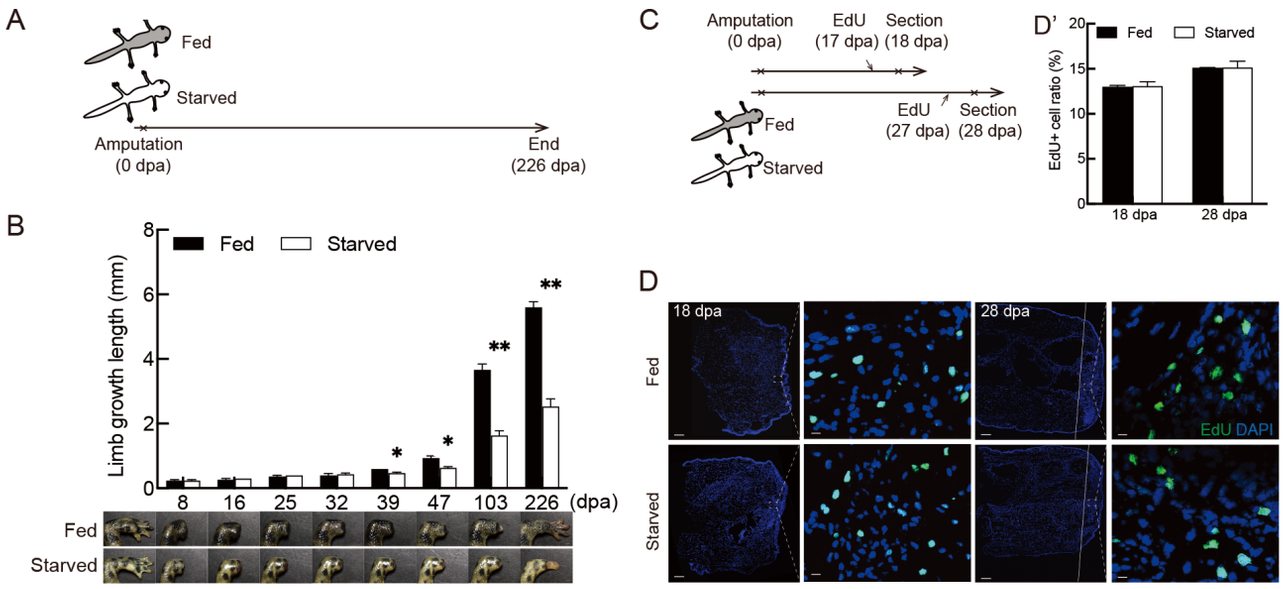

Figure 4 Normal blastema formation but retarded limb outgrowth and patterning in starved newts

A: Schematic of forelimb regeneration experiments. Two-year-old female newts were starved for 21 months and then subjected to limb amputation. B: Blastema and limb outgrowth length were measured at multiple time points over entire regeneration period in fed and starved animals (data are mean±*SEM*, *n*=5 in each group). Representative pictures of limbs are shown. Scale bar: 1 000 μm. C: Schematic of EdU injection and forelimb regeneration experiments. D: Representative images show EdU^+ in stump region at 18 days post amputation (dpa) and blastema region at 28 dpa. Solid lines indicate amputation plane. D': Cell proliferation index was counted (data are mean±*SEM*, *n*=5 in each group). Scale bar: overview: 500 μm; magnification: 20 μm.

chloroquine (Figure 5A), a classical inhibitor of autophagy that blocks the binding of autophagosomes to lysosomes (Mauthe et al., 2018). We first confirmed that autophagy can be effectively inhibited by chloroquine treatment in salamanders (Supplementary Figure S2). In the starved animals, autophagy inhibition significantly reduced cell proliferation (Figure 5B) and regenerative blastema length (Figure 5C). Therefore, during the early phase of limb regeneration in the starved animals, cell proliferation was dependent on tissue autophagy to provide energy and material. As autophagy is also implicated in tail regeneration in zebrafish and geckos under normal homeostasis (Varga et al., 2014; Zhang et al., 2016), we further explored the role of autophagy during limb regeneration in fed animals under the same chloroquine treatment regime (Figure 5D). Surprisingly, there were no differences in cell proliferation or blastema length between the chloroquine-treated and control groups (Figure 5E, F). Thus, our findings indicate that inhibition of autophagy does not have an impact on cell proliferation or limb regeneration in normal fed animals. Therefore, autophagy is an adaptive response to starvation that ensures effective blastema formation during limb regeneration.

DISCUSSION

To the best of our knowledge, the current study presents the longest trial of complete food deprivation ever recorded among tetrapods under well-controlled experimental conditions. The animals were active, and no hibernation or illness was observed throughout the entire starvation period. Available data indicate that intermittent starvation, such as fasting, can extend lifespan in many organisms and protect against age-related diseases, including in mammals (Honjoh et al., 2009; Mitchell et al., 2019). One prevailing theory is that short-term starvation or fasting promotes the rejuvenation and replenishment of adult stem cells (McLeod et al., 2010), such as intestinal (Mihaylova et al., 2018) and hematopoietic stem cells (Cheng et al., 2014). Nevertheless, homeothermic animals such as birds and mammals cannot withstand prolonged starvation lasting several days or weeks because of the high energy demands for maintaining body temperature and metabolism (Hohtola, 2012; Yoda et al., 2000). Therefore, it is unclear to what extent fasting should proceed to rejuvenate adult stem cells before body health is compromised by the detrimental effects of malnutrition (Buono & Longo, 2018). In contrast, poikilothermic animals can tolerate prolonged starvation by gradually and effectively adjusting energy demands and metabolic adaptation in the body (Marsden, 1973). For instance, planarians can handle prolonged starvation by shrinking their body size and then growing immediately when food becomes available (Felix et al., 2019). Interestingly, planarian starvation leads to an enrichment of stem cells with longer telomeres, which can benefit future regrowth and regeneration (Iglesias et al., 2019). However, tissue regeneration in starved or starved-refed animals has not been carefully examined (Brøndsted, 1953). Whether stem cells can cope with the acute demand of massive cell proliferation required for tissue regeneration in starved animals is unknown. Therefore, we performed a long-term food deprivation study in salamanders and examined their regenerative response, given their high regeneration capacity among vertebrates.

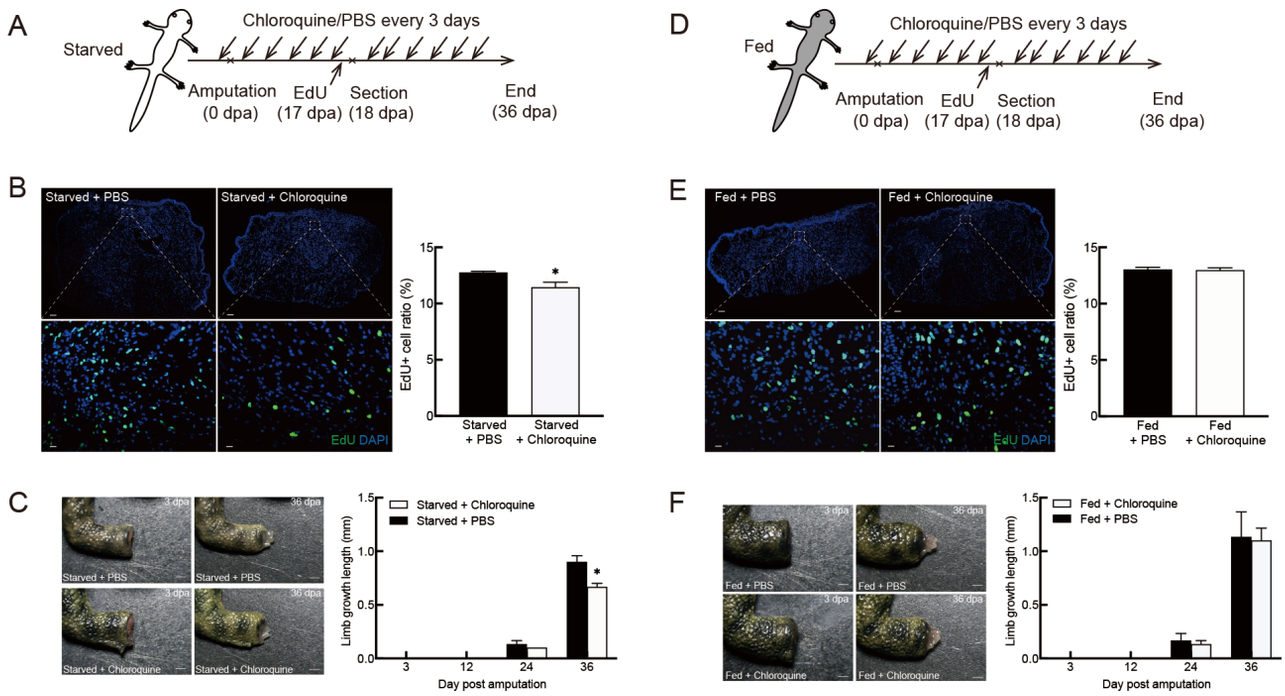

Figure 5 Blockage of autophagy inhibits cell proliferation and blastema formation in starved but not in fed animals

A: Schematic of autophagy inhibition experiments in starved animals. One-year-old female newts were starved for 12 months and then subjected to limb amputation. B, C: Representative images of EdU staining (B) and blastema (C) are shown. B: EdU staining and cell proliferation calculations between chloroquine (CHQ)-treated and control (PBS) newts under starvation. Scale bar: top: 500 μm; bottom: 20 μm. C: Forelimb blastema length was measured at multiple time points between chloroquine-treated and control newts under starvation. Scale bar: 1 000 μm. D: Schematic of autophagy inhibition experiments in normal fed animals. E: EdU staining and cell proliferation calculations between chloroquine-treated and control newts under normal feeding. Scale bar: top: 500 μm; bottom: 20 μm. F: Forelimb blastema length was measured at multiple time points between chloroquine-treated and control newts under normal feeding. Scale bar: 1 000 μm. Data are mean±*SEM*, *: P<0.05; **: P<0.01, after t-test, n=4 in starvation group, n=5 in control group.

Salamanders are powerful models for understanding the regenerative mechanisms of complex body parts (Brockes & Kumar, 2008). Whether long-term starved salamanders can still regenerate limbs is unknown. Here, after 19 months of complete starvation, the animals showed profound loss of body mass and depletion of fat storage, which are typical animal responses to food deprivation (McCue, 2010). We measured two major forms of biological energy reserves in the body, i.e., glycogen and lipids, to explore the strategies used by the newts to endure extreme starvation. As expected, the blood glucose levels were markedly reduced (37% of normal levels), suggesting that the animals may lower overall metabolism to cope with hypoglycemia. Lower blood glucose levels during starvation have also been observed in several fish (Furné et al., 2012; Pérez-Jiménez et al., 2012) and amphibian species (Hervant et al., 2001; Merkle & Hanke, 1988) but not in birds or mammals (Aronoff et al., 2004; Hohtola, 2012), indicating that reducing blood glucose may be a common strategy for ectothermic vertebrates to survive extreme starvation. One intriguing observation of the starved newts in this study was their normal liver glycogen content, consistent with previous study on frogs (*Rana esculenta*) deprived of food for 18 months (Grably & Piery, 1981). In contrast, a substantial decline in liver glycogen content has been reported in several species subjected to relatively short-term starvation, such as African lungfish (6 months) (Frick et al., 2008), *Xenopus laevis* (3 months) (Merkle & Hanke, 1988), and mice (36 hours) (Jensen et al., 2013). Hence, we measured glycogen and lipid content in tissues from newts starved for only 3 months and found that liver glycogen was indeed reduced at the beginning of starvation but later recovered (Supplementary Figure S3). Lipid content in the liver and muscle declined continuously, supporting the assumption that animals may restore tissue glycogen by increasing lipid consumption, as reported in the Pyrenean brook salamander (*Euproctus asper*) (Hervant et al., 2001) and African lungfish (*Protopterus annectens*) (Frick et al., 2008). Whether this gradual shift from carbohydrate- to lipid-dominant metabolism is an adaptation to extreme starvation and prolonged nutritional stress needs further study.

How gut microbiota change in response to extreme starvation is another interesting question, as gut bacteria need nutritional substrates that are missing over the extended period of starvation. Several studies have demonstrated dynamic alterations in microorganisms in the intestines of fish (Tran et al., 2018; Xia et al., 2014), frogs (Tong et al., 2021) and mice under starvation (Kohl et al., 2014). Through microbial function inference analysis, we identified several pathways closely related to autophagy processes in the host that were enriched in starved animals. Autophagy is up-

regulated in animals when they need to generate nutrient substrates and energy during starvation (Chera et al., 2009; Kang et al., 2007; Mizushima et al., 2004). We evaluated a panel of autophagy markers and found that autophagy is indeed elevated in a variety of tissues in starved salamanders. The physiological adaptations, gut microbiota remodeling, and tissue autophagy features of the starved salamanders may have important implications for human health during fasting.

Tissue regrowth during limb regeneration is an inherently anabolic process that requires an adequate supply of energy, amino acids, and nucleic acids to construct new cells for new tissue (Love et al., 2014). Under normal circumstances, animals cope with the increased energy demand by ample nutrition storage and intake. However, limb regeneration may be challenging for animals enduring prolonged starvation as their metabolism and energy storage are extremely low. Unexpectedly, we found that the starved animals could still mount a massive cell proliferation response to support blastema formation upon limb amputation. Given that tissue regeneration and cell proliferation are biosynthetic in nature, evoking autophagy to yield energy to support anabolic pathways is an advantageous strategy for starved animals with poor energy reserves (Chera et al., 2009; González-Estévez et al., 2007; Lampert & Gustafsson, 2020). To explore the role of cellular autophagy during limb regeneration in starved animals, we manipulated autophagy levels by pharmacological means and assessed its impact during regeneration. As expected, cell proliferation and blastema formation were severely disrupted when autophagy was blocked in starved animals. Thus, autophagy appears to be strictly required for blastema formation when animals are under adverse conditions. Surprisingly, when autophagy was blocked using the same pharmacological tools, normal fed animals regenerated limbs efficiently. These results disagree with previous reports that suggest autophagy is beneficial for appendage regeneration in zebrafish (Varga et al., 2014) and geckos under normal conditions (Zhang et al., 2016). We speculate that under normal circumstances, salamanders have sufficient energy and resources for limb regeneration, but during periods of extreme starvation, their energy reserves are reduced, thereby lowering the threshold for autophagy mobilization. In other words, starvation-induced stress increases animal dependency on autophagy to sustain cell proliferation and regeneration. Therefore, salamanders possess a latent autophagic capacity that can be triggered during tissue regeneration under adverse circumstances.

Our results demonstrated the existence of a variety of strategies for blastema formation during limb regeneration in salamanders. Nutritional status and physiological condition of the animal can influence the plasticity of the mechanism for initiating limb regeneration. This study also draws attention to the possibility of different regenerative strategies, which can be harnessed for tissue repair to survive harsh environments.

DATA AVAILABILITY

16S rRNA-seq data were deposited in the NCBI under BioProject ID PRJNA749907 and the Genome Sequence Archive (GSA) database under accession No. PRJCA006821.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Z.L.P. conceived and designed the study. Z.L.P. prepared the data. B.X.Y., R.M.R., Y.L.L., and H.C. analyzed the data. Z.L.P. and H.W. wrote the paper. All authors read and approved the final version of the manuscript.

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