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Letter to the editor Open Access

Cavefish mount a rapid and sustained regenerative response following skeletal muscle injury

Dear Editor,

Physical injury and tissue damage are prevalent throughout the animal kingdom, with the ability to regenerate quickly and efficiently providing a selective advantage. In most vertebrates, skeletal muscle possesses a remarkable ability to regenerate, making it a valuable model for investigating the cellular processes underpinning tissue regeneration. Following damage, skeletal muscle mounts a complex regenerative cascade centered around dedicated muscle stem cells, i.e., satellite cells. These satellite cells are, in turn, regulated by both resident and infiltrating innate immune cells of myeloid lineage, which play a crucial role in activating and guiding the transition of satellite cells towards maturation (Chen et al., 2020; Tidball, 2017; Tidball & Villalta, 2010). While critical for muscle regeneration, the innate immune system is energetically costly, resulting in many species decreasing immune investment under nutrient-limited environments. Whether this reduced investment results in a decreased capacity to mount a regenerative response following tissue damage remains unclear. Here, we utilized an emerging evolutionary model, the Mexican tetra (*Astyanax mexicanus*), to investigate the consequences of shifts in immune system investment on skeletal muscle regeneration.

The Mexican tetra (*A. mexicanus*) is a single species comprised of both river-dwelling surface fish and cave-dwelling cavefish. Approximately 160 000 years ago, ancestral surface fish colonized the surrounding caves, resulting in the establishment of multiple cave populations. These caves are completely devoid of light, with limited primary producers and poor biodiversity. These different selective pressures have contributed to the evolutionary emergence of many cave-specific morphological and physiological adaptations (Olsen et al., 2023). For example, we previously found that the diminished diversity of macroparasites in certain caves affected the evolutionary development of immune investment strategies and sensitivity of the immune system to immunological stimuli (e.g., lipopolysaccharides; LPS) in cavefish (Peuß et al., 2020). The hematopoietic niche of cavefish contains fewer innate immune cells, such as neutrophils and monocytes, compared to surface fish, and this reduction in proinflammatory cells is compensated by a prolonged proinflammatory response through stronger sustained expression of proinflammatory cytokine genes, such as *il6*, *tnfα*, *il1β*, and *g-csf*, upon exposure to LPS (Peuß et al., 2020). Similarly, heart injury in *A. mexicanus* can result in an elevated immune response in cavefish relative to surface fish — a phenomenon thought to underlie their inability to fully regenerate cardiac tissue (Stockdale et al., 2018). Based upon this observation, we explored whether a similar decrease in regenerative potential exists in cavefish skeletal muscle following injury by analyzing transcriptional responses after cardiotoxin injection in both surface fish and cavefish.

To characterize the regenerative response in *A. mexicanus*, we injected the skeletal muscle of fish with the necrotic agent cardiotoxin, which is known to induce rapid and dramatic muscle necrosis (Seger et al., 2011; Supplementary Figure S1). Subsequently, we collected skeletal muscle samples at 1, 7, and 14 days post injury (dpi) and performed bulk RNA-sequencing (Figure 1A). Our findings revealed a more robust gene expression response in cavefish compared to surface fish at the 1 dpi, 7 dpi, and 14 dpi timepoints following cardiotoxin injection. Specifically, we identified 2 936 differentially expressed genes (DEGs) at 1 dpi in cavefish, with a marked decrease at 7 dpi (944 DEGs) and 14 dpi (1 656 DEGs). Surface fish exhibited a comparable pattern, albeit with a reduced number of DEGs at 1 dpi (1 851 DEGs), followed by a substantial decrease at 7 dpi (673 DEGs) and 14 dpi (no DEGs). Hierarchical clustering analysis further supported these findings, with 1 dpi samples exhibiting a distinct clustering pattern compared to the other timepoints (Figure 1B). Collectively, these results demonstrate that cardiotoxin injection elicits a robust change in gene expression in the skeletal muscles of both cavefish and surface fish, with cavefish displaying a more extreme and sustained response.

Given our recent findings that cavefish exhibit a reduced investment in the innate immune system (Peuß et al., 2020) — an essential component of muscle regeneration — we next explored gene expression changes underlying pro- and anti-inflammatory signaling. We first performed Gene Ontology enrichment analysis of all DEGs at each timepoint and, as expected, identified multiple enriched pathways related to the immune system. Specifically, at 7 dpi, surface fish exhibited increased enrichment in several immune-related pathways, including “immune response” (P=0.0014), “adaptive immune response” (P=0.0016), and “positive regulation of immune response” (P=0.006) (Supplementary Figure S2A). Cavefish showed similar increases in immune-related pathways, including “immune response” (P=0.0116) and “defense response” (P=0.0116), albeit at 1 dpi (Supplementary Figure S2B). As shown in Figure 1C, most genes within these pathways exhibited an increase in expression following

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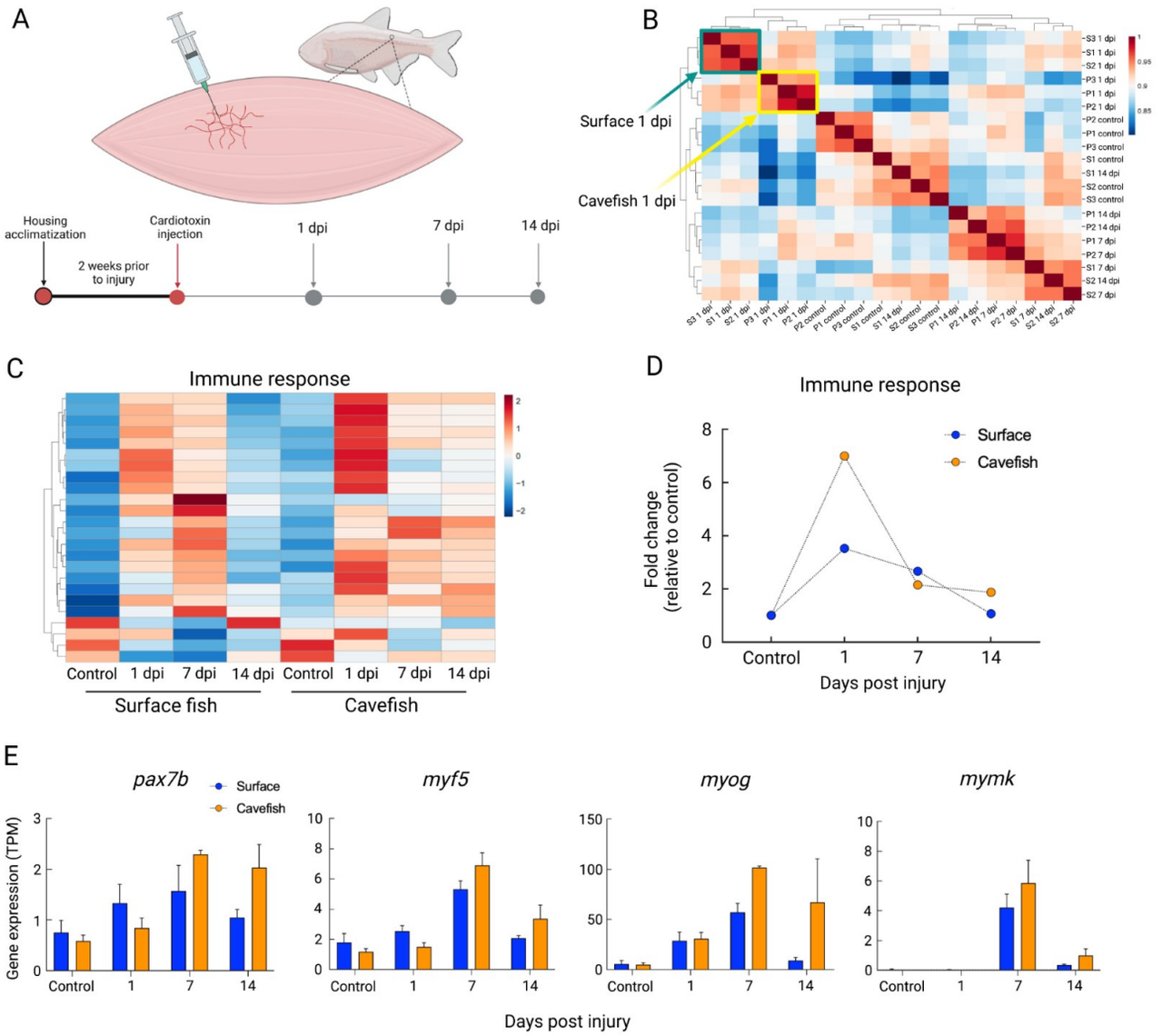

Figure 1 Muscle regeneration in *A. mexicanus* cavefish and surface fish

A: Schematic of cardiotoxin injection and tissue collection at 1 dpi, 7 dpi, and 14 dpi. B: Hierarchical clustering heatmap of each sample, with specific emphasis on 1 dpi in both surface fish (green) and cavefish (yellow). Individual sample identifiers are labeled on the x- and y-axes. C: Heatmap of genes associated with immune response pathway in surface fish at 7 dpi and corresponding genes in cavefish. D: Average fold-change in genes associated with immune response pathway (from Figure 1C). E: Relative gene expression of canonical satellite cell markers in transcripts per million (TPM).

injection, with the most significant changes observed at 1 dpi and 7 dpi, before decreasing to baseline at 14 dpi, albeit to a lesser degree in cavefish. This suggests the possibility of prolonged immune signaling following muscle damage. Notably, when considering all genes associated with the "immune response" pathway, cavefish displayed a greater increase in expression compared to surface fish (Figure 1D).

In addition to genes associated with the "immune response" pathway, we also characterized classical markers involved in inflammation, including the cytokines *il6*, *tnfα*, *il1β*, and *g-csf* (*gcsfa*), which have previously been shown to be differentially regulated between cavefish and surface fish (Peuß et al., 2020). Consistent with previous findings, the cavefish exhibited lower basal expression compared to surface fish (Supplementary Figure S2C). Interestingly, at 1 dpi, cavefish showed a robust 19-fold, 37-fold, 9-fold, and 6-fold increase in the expression levels of *il6*, *tnfα*, *il1β*, and *g-csf*, respectively, surpassing the 4-fold, 5-fold, 2-fold, and 1.1-fold increases observed in surface fish (Supplementary Figure S2D). These findings support our previous research showing that cavefish possess fewer innate immune cells (as indicated by lower baseline expression) but exhibit heightened sensitivity following inflammatory stimulus compared to surface fish, at least within the local site of injection (Peuß et al., 2020).

Efficient coordination of the inflammatory response following muscle injury is critical for proper functional satellite cell dynamics and regeneration resolution. Given that cavefish showed a more robust inflammatory response relative to surface fish following cardiotoxin injection, we next determined how this influences satellite cell dynamics, specifically central genes involved in satellite cell quiescence (*pax7a/b*), activation/proliferation (*myf5/myod*), differentiation (*myod/myog*), and ultimate myoblast fusion (*mymk*) (Chen et al., 2020; Millay et al., 2013). As expected, the expression

of many of these genes increased in both surface fish and cavefish following cardiotoxin injection. Surprisingly, cavefish showed a more robust and sustained increase in expression compared to surface fish. For example, cavefish exhibited a significant increase in *pax7b* at 7 dpi and 14 dpi, whereas the increase in surface fish did not reach statistical significance (Figure 1E). Additionally, cavefish exhibited an increase in the expression levels of *myf5* (7 and 14 dpi) and *myog* (1, 7, and 14 dpi) following injury, whereas surface fish only showed an increase in *myog* at 7 dpi (Figure 1E). Moreover, *mymk* showed a robust increase at 7 dpi and 14 dpi in cavefish, but only increased at 7 dpi in surface fish. Consistent with the immune system findings, cavefish demonstrated a more robust and sustained increase in genes orchestrating satellite cell dynamics following injury, suggesting heightened sensitivity to external stimuli. Histological analyses further supported these findings, with cavefish showing similar, if not more rapid, muscle regeneration than surface fish. Indeed, when extending muscle collection to 16 dpi, we observed a minimal portion of cavefish skeletal muscle undergoing regeneration relative to surface fish (Supplementary Figure S1B). However, caution is warranted when interpreting these findings due to the small sample size and challenges associated with assessing regeneration in relatively small tissue sections (10 μm) compared to bulk RNA-sequencing of larger muscle tissue samples (100 mg).

Overall, our study provides evidence suggesting that cavefish skeletal muscle initiates a "hyper-sensitive" inflammatory response following local muscle injury. We reasoned this heightened response could result in satellite cell dysregulation, as observed in cavefish cardiac injury models. However, in contrast to our expectations, cavefish demonstrated a more robust increase in markers associated with satellite cell proliferation, differentiation, and fusion compared to surface fish. This suggests that cavefish may exhibit heightened sensitivity to injury stimuli, resulting in a greater reliance on their satellite cell pool compared to surface fish. Although these results serve as an initial step in understanding cavefish skeletal muscle regeneration, further investigations are required to validate the significance of our gene expression-level observations in relation to muscle regeneration. While histological characterization of muscle regeneration supported similar, if not greater, regeneration in cavefish, larger sample sizes and additional timepoints are necessary to establish the robustness of these findings.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

L.O. and N.R. designed the research. L.O., H.H., and F.X. performed the research. L.O. and H.H. analyzed the data. L.O., S.K., and N.R. wrote the paper, with input from all other authors. All authors read and approved the final version of the manuscript.

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