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The livebearers platyfish and swordtails partially regenerate their hearts with persistent scarring

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eLife Assessment

This study presents **important** findings on how cardiac regenerative capacity diverges across species by examining heart repair in two species of livebearers, platyfish and swordtails. In contrast to zebrafish, the livebearer species show persistent scarring after cryo-injury, and the work highlights how lineage-specific anatomical and immunological traits may constrain regenerative competence. The study is **compelling**, the data are **convincing**, and the results contribute to our understanding of the mechanisms underlying heart regeneration across vertebrates.

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Abstract

Heart regeneration varies among vertebrates, with zebrafish serving as a reference species for efficient cardiac restoration. How this capacity diversified among teleosts is an emerging question, given the recent identification of non-regenerative cardiac repair in medaka and cavefish. Here, we investigate heart restorative capacity following cryoinjury in two livebearers, platyfish and swordtails (*Xiphophorus* species), belonging to the *Poeciliidae* family. We demonstrate that their hearts lack the vascularized compact myocardium, which is a ventricular layer implicated in the restorative response in zebrafish. After cryoinjury, both poeciliids failed to rapidly deposit fibrotic tissue that normally reinforces the damaged site. This deficiency led to striking wound protrusion reminiscent of pseudoaneurysm after myocardial infarction in humans. Although the remaining myocardium initially increased cell proliferation, subsequently deposited collagenous scar tissue permanently sealed the interrupted ventricle, preventing complete regeneration. Transcriptomic analysis revealed several divergently regulated pathways between cryoinjured hearts of zebrafish and platyfish, particularly in immune response regulation. This was validated by delayed leukocyte infiltration and prolonged inflammation in platyfish, compared to the rapid, resolved inflammatory response in zebrafish. Our findings demonstrate that *Xiphophorus* species have evolved hearts with compromised regenerative capacity, characterized by pseudoaneurysm-like protrusion and permanent scarring. These results reveal that evolutionary traits of phylogenetic lineages can fundamentally modulate regenerative competence among teleosts, with important implications for understanding the mechanistic basis of cardiac repair.

Highlights

- Viviparous poeciliids lack vascularized compact myocardium.
- Inflammation and fibrosis are delayed in the cryoinjured platyfish ventricle.
- Ventricular cryoinjury in *Xiphophorus* leads to pseudoaneurysm-like deformation.
- Failure to form a myocardial bridge results in permanent scarring.

Introduction

Efficient healing of heart injury is crucial for maintaining blood flow and ensuring survival. In most adult mammals, including humans, myocardial infarction damages ventricular muscle, which is subsequently replaced with a collagenous scar ^{1,2}. By contrast, some fish and amphibians, such as zebrafish and axolotls, can regrow a functional myocardium ³⁻⁶. There is therefore debate about why the regenerative capacity was lost during evolution of vertebrates ^{3,7-9}. Cautious interpretations are warranted because the zebrafish heart may not necessarily represent the exact ancestral prototype, given that teleosts and tetrapods are distinct evolutionary lineages ¹⁰.

Teleosts are a monophyletic taxon with a 300-million-year history, characterized by complex radiation resulting in over 30,000 extant species ¹¹⁻¹⁴. Interestingly, this extensive diversification led to the establishment of distinct types of hearts, as reported already 140 years ago by McWilliam (1885): “*The cardiac structure and blood-supply appear to present considerable diversity in different fishes. Thus, in the salmon, the dense outer part of the ventricular wall is of great thickness, comprising several longitudinal, circular and oblique layers...; in the cod’s heart there appears to be no distinct outer layer of dense muscular tissue ... And there are no coronary vessels to be discerned upon the surface as in the salmon, eel and others.*” ^{15,16}. Given that heart architecture and systemic factors are considered to impact restorative strategies ¹⁷⁻¹⁹, this quote suggests that various fish lineages should be included in studies prior to generalization. Indeed, the diversification of “the fish heart” could be linked to the diversification of regenerative responses. This phenomenon contrasts with “the mammalian heart”, which has the same anatomical architecture from mouse to whale ^{10,20}.

The zebrafish provides a leading animal model for efficient heart regeneration ^{7,19,21-23}. After cryoinjury, the damaged myocardium is significantly replaced within 30 days, although a full recovery may require additional months ²⁴⁻²⁷. The regenerative program can be restarted multiple times in the same individual, suggesting the robustness of the process ²⁸. Besides the myocardium, the endocardium, epicardium, vasculature, nerves and immune system are also actively involved in heart regeneration ²⁹⁻³⁴. Remarkably, recent studies have revealed that some teleost species from different phylogenetic orders, namely medaka and a cave-dwelling variant of the Mexican tetra, heal cardiac wounds through fibrosis ³⁵⁻³⁷. The correlation between heart type and heart regeneration remains poorly understood because only a handful of fishes have been analyzed after cardiac injury. A comparative approach might shed light on the evolution of this health-relevant trait within the monophyletic but diversified taxon of teleosts ³⁸⁻⁴⁰.

This study aims to assess cardiac regeneration in viviparous fishes from the family *Poeciliidae* of the order *Cyprinodontiformes*. We selected two members of the *Xiphophorus* genus, the platyfish (*X. maculatus*) and swordtail (*X. hellerii*), for several reasons. The order *Cypriniformes* (comprising zebrafish) and *Cyprinodontiformes* (comprising platyfish) occupy distant positions in the phylogenetic tree, given that their last common ancestor existed approximately 250 million years ago ^{11,12,41,42} (Figure 1A [↗](#)). Among poeciliids, *Xiphophorus* has attracted scientific interest for a century thanks to several peculiarities, including variation in pigmentation, susceptibility to melanomas, internal fertilization, sexual traits, geographical adaptability, evolutionary hybridization and radiation ⁴³⁻⁴⁸. The platyfish genome has been annotated, rendering it suitable for transcriptomic analysis ⁴⁹. Our laboratory has recently used this species to study fin regeneration, and we reported the presence of a few epichordally-derived principal rays in the caudal fin, which can be recognized as a rule-breaking trait among teleosts ^{50,51}. Despite this suite of interesting features, no *Cyprinodontiformes* or live-bearing fish have yet been examined for heart regeneration.

Here, we demonstrate the differences in the architecture and molecular components of the heart between zebrafish and *Xiphophorus* sp. and compare the restorative response after cryoinjury using immunofluorescence and transcriptomic analyses. Altogether, our study suggests that the livebearers can partially regenerate their heart, with persistent scarring. We propose that this

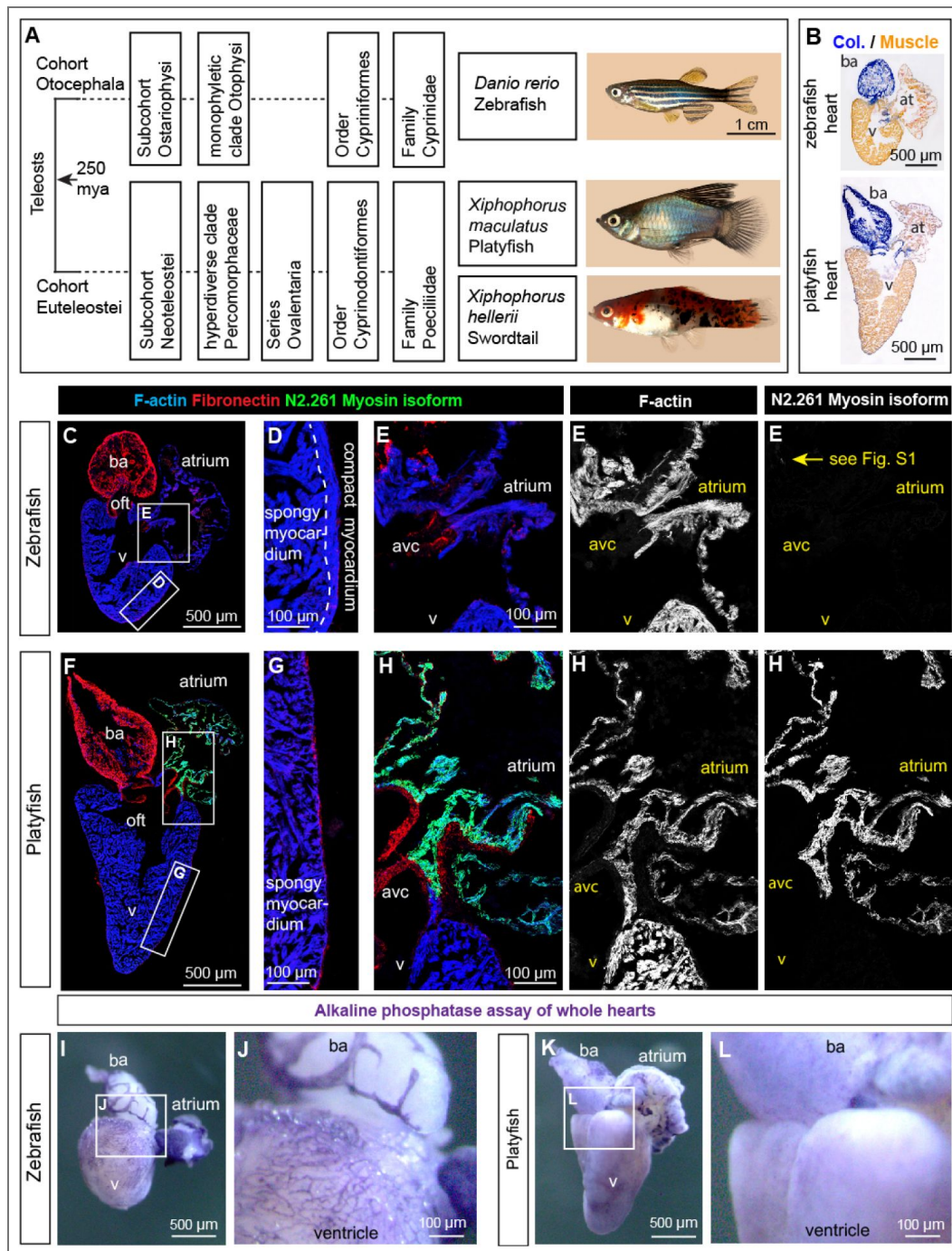


Figure 1. Platyfish possess a heart type lacking a compact vascularized myocardium.

(A) Simplified phylogenetic lineages of the relevant fish species, based on ¹¹. The clade of teleosts diversified into two main branches 250 million years ago (mya). The *Otophysi* clade contains more than one-third of fish species, including zebrafish and tetras ⁴¹. *Percomorphaceae* is a hyperdiverse clade described as “the bush at the top” of the fish phylogenetic tree ¹⁰⁰, which encompasses 55% of extant teleost diversity, including perciforms, cichlids and poeciliids ¹⁰¹. (B) AFOG staining of longitudinal heart sections of platyfish and zebrafish hearts. Col., collagen (blue). (C–H) Fluorescence staining of hearts shown in (B). The myocardium is F-actin-positive. Note the presence of the compact myocardium in zebrafish (D), and its absence in the platyfish (G). The N2.261 immunoreacts with a specific myosin isoform that is nearly absent in the uninjured zebrafish heart (C–E), except for a few fibers at the outflow tract (oft). In platyfish, this antibody immunolabels the atrium, suggesting evolutionary changes of myosins between the species. Fibronectin is an extracellular matrix protein present in the bulbus arteriosus. (I–L) The activity assay for alkaline phosphate reveals a dense network of blood vessels in zebrafish, but not in platyfish hearts. Frames depict the magnified areas shown on adjacent panels, labeled with the corresponding letter. avc, atrioventricular canal; at, atrium, ba, bulbus arteriosus; oft, outflow tract; v, ventricle. These conventions and abbreviations apply throughout all figures.

restorative mechanism most likely reflects the evolutionary innovations in ventricle type and the immune system. Including diverse species in regenerative biology has the potential to advance our knowledge of mechanisms driving the evolution of heart regeneration in vertebrates.

Results

Xiphophorus lacks vascularized compact myocardium in the ventricle

To establish baseline anatomical differences that might influence regenerative capacity, we compared heart morphology between adult zebrafish and platyfish. In teleosts, the heart consists of a single atrium and ventricle, which pumps blood to an elastic outflow chamber, the bulbus arteriosus. Longitudinal sections were stained with AFOG, labeling the bulbus arteriosus and valves with the collagen-binding blue dye, while the ventricle and atrium appear in beige (Figure 1B). The platyfish ventricle was longer than that in zebrafish, with a more pyramidal morphology and pointed apex. Swordtails displayed identical architecture (data not shown), indicating consistent ventricular differences between zebrafish and *Xiphophorus* species.

To assess molecular components, sections were labeled with three markers: phalloidin to visualize the F-actin-rich contractile tissue, anti-Fibronectin for connective tissue, and the N2.261 (embCMHC) antibody against a specific isoform of myosin (Figure 1C–H). In zebrafish, N2.261 has previously been identified as a marker of immature cardiomyocytes (CMs) in larvae and in regenerating myocardium^{52,53}. In the adult intact heart, only individual CMs were labeled near the outflow tract (Figure 1C, E; suppl. Figure S1A–C). In contrast, platyfish displayed N2.261 immunoreactivity throughout the entire atrium (Figure 1F, H; suppl. Figure S1D–F). Platyfish embryos reproduced this pattern, confirming its developmental origin (suppl. Figure S1H, I). Thus, the N2.261 recognizes different myosin types in zebrafish versus platyfish, revealing substantial evolutionary divergence in sarcomere proteins between these phylogenetic lineages.

Most critically, a fundamental structural difference was observed in the outer ventricular layer: zebrafish possessed vascularized compact myocardium, as previously shown⁵⁴, whereas platyfish completely lacked this regeneration-associated tissue layer (Figure 1D, G; suppl. Figure S1G). To examine vascularization, whole hearts were stained for alkaline phosphatase activity³⁷. The zebrafish ventricle was extensively covered with a vascular network, whereas platyfish lacked any comparable vascular pattern (Figure 1I–L). Podocalyxin-2 (Podxl2) immunostaining, which detects endothelial cell apical surfaces⁵⁵, confirmed the absence of ventricular vascularization in platyfish compared to zebrafish (suppl. Figure S1J–M). Together, these findings demonstrate that platyfish and zebrafish hearts differ fundamentally at both morphological and molecular levels, with *Xiphophorus* lacking key anatomical features associated with regenerative capacity.

Cryoinjured ventricles of *Xiphophorus* regenerate with deformation and partial scarring

Having established these fundamental anatomical differences, we next investigated how they influence cardiac repair responses. Cryoinjury creates reproducible damage through controlled freezing and thawing using a precooled probe⁵⁶ (Figure 2A). Examination of whole platyfish hearts at 7 days post-cryoinjury (7 dpci) revealed profound Phalloidin-negative tissue protruding from the ventricular wall, indicating extensive myocardial damage (Figure 2B). To evaluate regenerative dynamics, transversal sections of cryoinjured hearts were analyzed using AFOG staining across multiple time points. In zebrafish, transient collagenous tissue appeared between 7 and 14 dpci, detected by Aniline blue staining, as previously demonstrated^{25,26,57} (Figure 2C). In *Xiphophorus* species, however, the damaged area contained Fuchsin red-stained protein deposits at these time points, with minimal collagen present. This pattern suggests significantly delayed wound clearance in *Xiphophorus* compared to zebrafish.

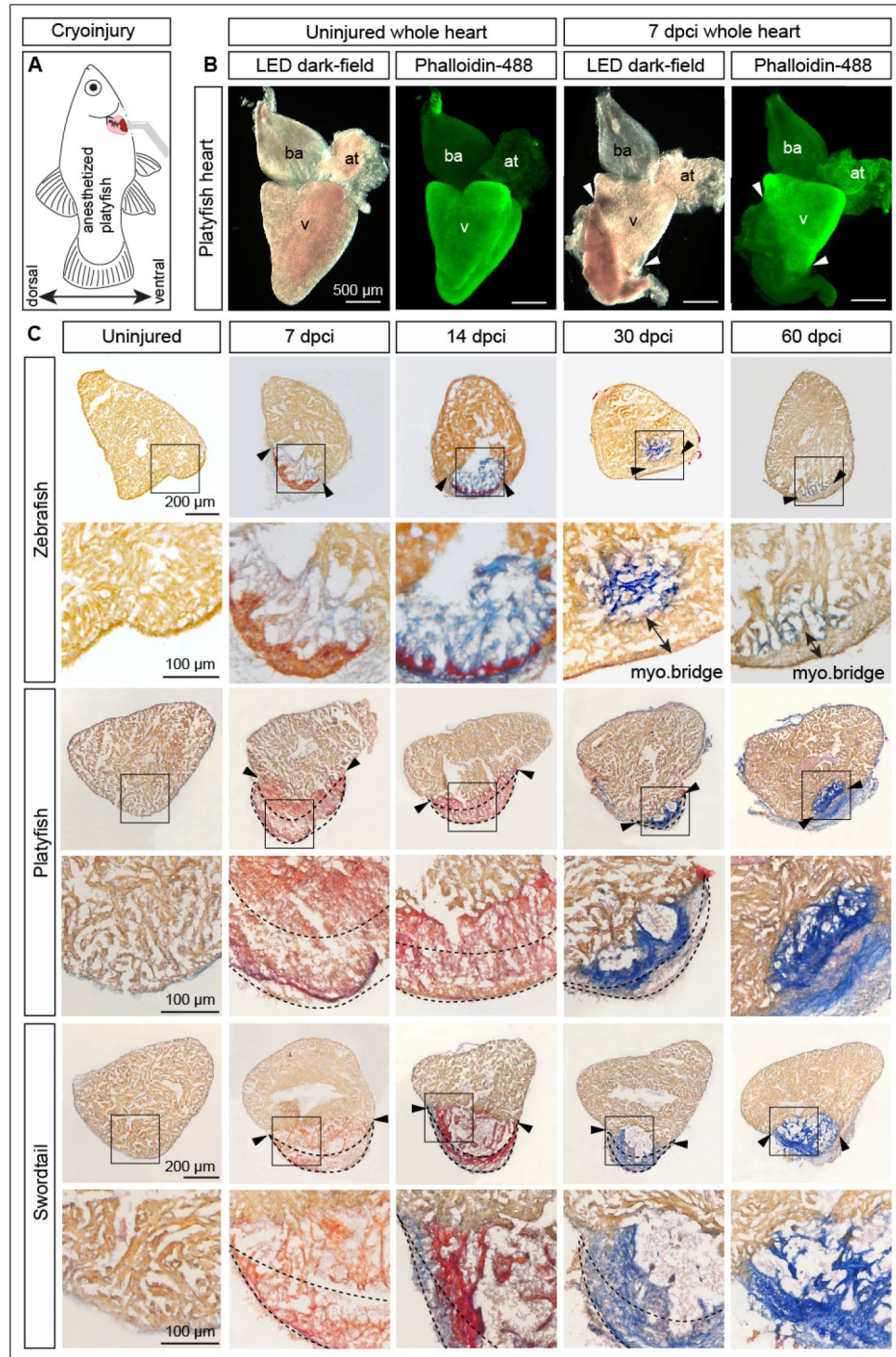


Figure 2. Cryoinjured ventricles in *Xiphophorus* fish display transient wound bulging and permanent scarring.

(A) Schematics of heart cryoinjury in platyfish. **(B)** Hearts from uninjured fish and at 7 days post-cryoinjury (dpi) stained with Phalloidin (green). The damaged area of the heart is revealed by a weak fluorescence signal in the absence of contractile cells. Arrowheads indicate the border between the intact myocardium and the wound. ba: bulbus arteriosus, at: atrium, v: ventricle. **(C)** AFOG staining of transversal ventricle sections of zebrafish, platyfish and swordtail, collected at different time points after cryoinjury. Intact myocardium (orange); fibrin and other protein deposits (red); collagen (blue). The arrowheads indicate the edge of the wound; double-ended arrows depict the myocardial (myo) bridge; the dashed line encircles the wound tissue that has expanded beyond the normal circumference of the heart (i.e. a pseudoaneurysm).

Strikingly, *Xiphophorus* wounds displayed marked bulging beyond the presumptive ventricular margin at 7 and 14 dpci (Figure 2B, C). This distinctive bulging phenotype closely resembled pseudoaneurysm formation, a pathological ventricular deformation that occurs after myocardial infarction in humans when the ventricular wall cannot maintain structural integrity^{58,59}. Given that this abnormality was absent in zebrafish and other non-regenerative species (medaka and cavefish)³⁵⁻³⁷, we concluded that collagen-deficient cryoinjured hearts are uniquely prone to deformation in *Xiphophorus*, revealing novel variations in teleost healing strategies.

In contrast to zebrafish, *Xiphophorus* species showed collagen deposition only beginning at 30 dpci, representing a significant delay in accessory fibrotic tissue formation. This collagenous pattern persisted at 60 and 90 dpci, demonstrating failure in matrix resorption (Figure 2C). The deposited collagen formed a distinctive belt-like structure, markedly different from the fine network observed in zebrafish hearts (Figure 2C; suppl. Figure S2B). Critically, the wound margin was sealed with a dense collagenous layer rather than the “myocardial bridge” structure that spans wounded myocardium in zebrafish^{60,61} (Figure 2C). We concluded that *Xiphophorus* species heal cardiac wounds through persistent scarring rather than regenerative repair.

To quantify deformation frequency, we categorized phenotypes into three groups: little to no wound, typical non-protruding wound, and protruding swollen wound (Figure 3A, see Methods). At 7 dpci, approximately 45% and 75% of platyfish and swordtail hearts, respectively, displayed pseudoaneurysm-like phenotypes. At 14 dpci, this proportion decreased by nearly half. At 30, 60, and 90 dpci, wound swelling was rarely observed; however, most hearts displayed persistent fibrosis, confirming impaired regeneration.

To quantify healing outcomes, platyfish hearts were stained with Phalloidin and anti-Fibronectin, and tissue volumes were calculated by measuring stained areas across all heart sections (Figure 3B). Total ventricle volume remained approximately 1 mm³ at all time points, indicating consistent heart sizes across experimental groups (Figure 3C). The slight increase at 7 and 14 dpci likely reflected injured area bulging. Wound volume, measured as the Phalloidin-negative ventricular portion, comprised 15% (SD ±10%) of total ventricle at 7 dpci, decreasing to 10% (±10%) at 14 dpci, 5% (±6%) at 30 dpci, and 2% (±2%) at 60-90 dpci (Figure 3D). Swordtails showed similar dynamics (suppl. Figure S3). Fibronectin quantification revealed progressive connective tissue decrease, paralleling wound volume reduction (Figure 3E). Importantly, Phalloidin staining confirmed absence of myocardial bridge formation, supporting AFOG findings. These quantitative data demonstrate that while *Xiphophorus* species achieve significant wound size reduction over time, this occurs through fibrotic sealing rather than true myocardial regeneration, as evidenced by persistent collagenous scarring and absence of regenerative myocardial bridge formation.

Cryoinjured ventricles show transcriptomic differences between zebrafish and platyfish

To identify molecular differences underlying distinct regenerative responses, we performed bulk transcriptomic analysis of ventricles at 7 dpci and uninjured controls. In each species, we identified genes with differential transcript abundance (Figure 4A, B; suppl. Table S1, S4). Interspecies comparison revealed that upon cryoinjury, 199 and 268 orthologous gene transcripts were more abundant in zebrafish than in platyfish, respectively (Figure 4C). Gene Set Enrichment Analysis (GSEA) revealed that tissue remodeling factors were enriched whereas metabolic regulators were reduced in both species (Figure 4D; suppl. Table 5). This suggests a similar response to disrupted homeostasis following heart injury^{23,62}. Interestingly, some differences between species were observed in the immune system, where signaling pathways of C-type lectin receptor, cytokine and Toll-like receptor were enriched only in zebrafish, but not in platyfish. Similarly, genes of GnRH, MAPK and TGFβ signaling pathways were increased only in zebrafish, whereas mTOR signaling and ErbB signaling factors were even decreased in platyfish.

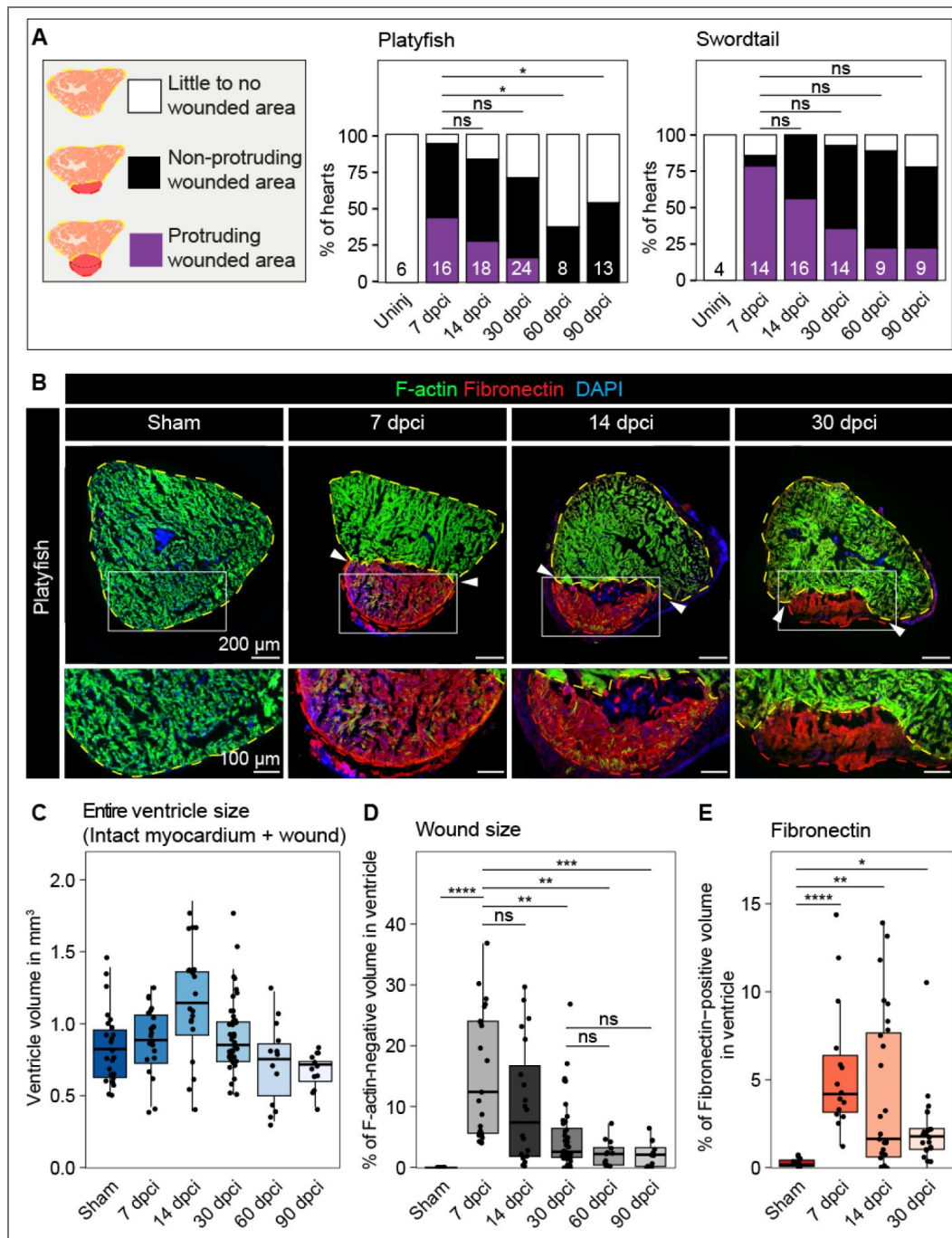


Figure 3. Partial restoration of the heart in platyfish and swordtail after cryoinjury.

(A) Classification of injury phenotypes based on AFOG staining, representatively shown in Figure 2C. The bar plots display the percentage of each category at different time points after injury in platyfish and swordtail. Numbers at the bottom of each bar correspond to biological replicates (fish). Pearson's chi-squared test with Holm's post hoc correction: ns, not significant; *, $p < 0.05$. (B) Fluorescence staining of transversal sections of platyfish hearts. Fibronectin-positive wound (red) contrasts with intact myocardium labeled by F-actin (green). Sham ventricles at 30 days post-thoracotomy serve as control. (C-D) Quantification of regeneration based on wound size and fibronectin deposition, as represented in (B). Kruskal-Wallis test followed by Dunn's test with Holm's post-hoc correction. Adjusted p-value: * < 0.05 , ** < 0.01 , *** < 0.001 , **** < 0.0001 . For C and D, n: Sham: 24; 7 dpci: 24; 14 dpci: 22; 30 dpci: 32; 60 dpci: 13; 90 dpci: 12. For E, n: Sham: 24; 7 dpci: 15; 14 dpci: 20; 30 dpci: 17.

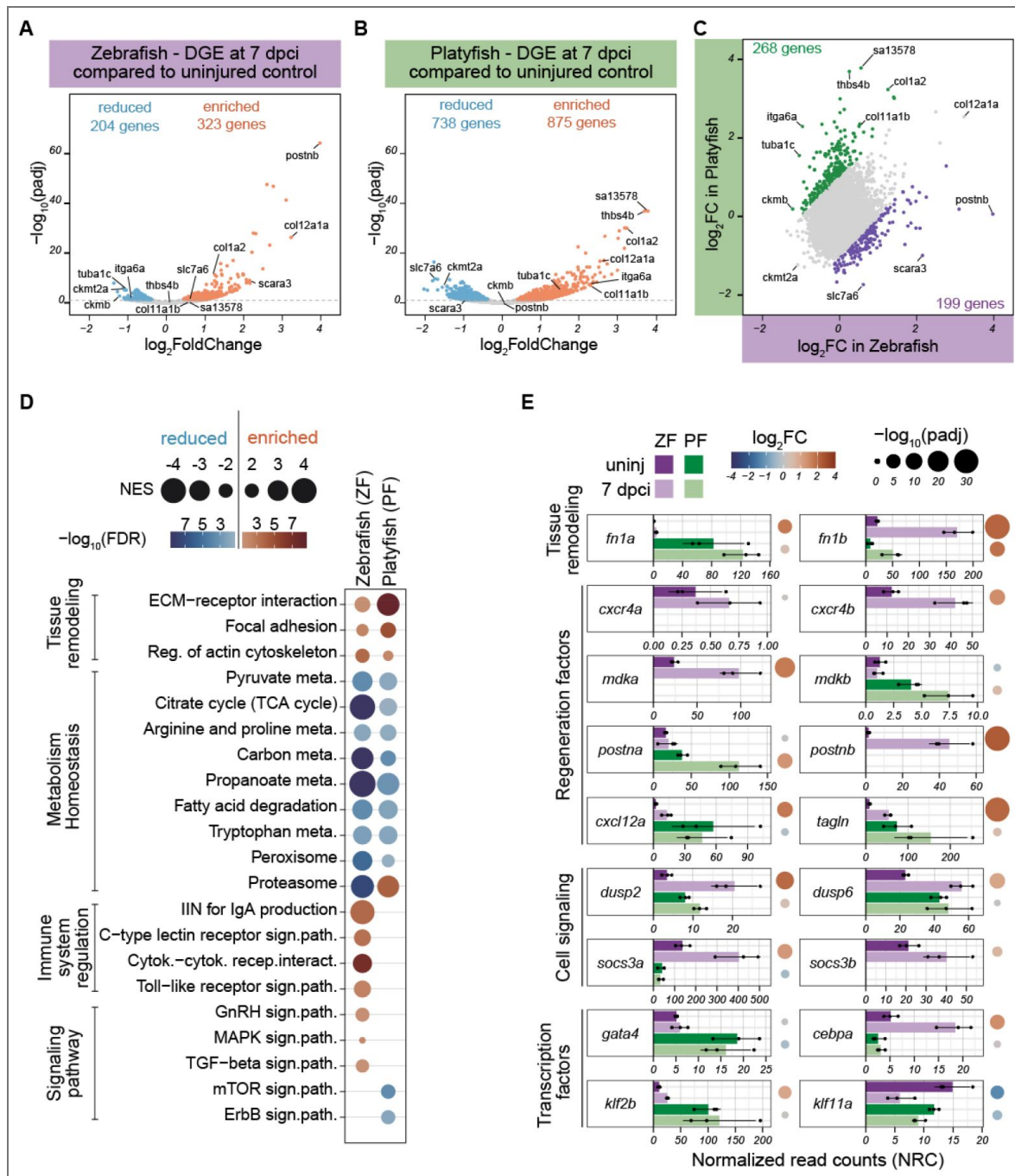


Figure 4. Comparison of bulk-RNA sequencing between zebrafish and platyfish reveals different tissue responses to cryoinjury.

(A-B) Volcano plots of transcriptomes in zebrafish (A) and platyfish (B) cryoinjured ventricles at 7 dpci, compared to uninjured control. The $\log_2$ fold change ($\log_2\text{FC}$) values show the ratio of gene transcript abundance in cryoinjured versus uninjured conditions. An adjusted p-value (padj) threshold of less than 0.05 was used to identify genes showing significant changes in transcript levels (decreased in blue and increased in orange). (C) Scatter plot comparing the $\log_2\text{FC}$ of the two RNA-seq analyses. Green represents gene transcripts that are more abundant in platyfish than in zebrafish upon cryoinjury, while purple represents the opposite situation. Highlighted genes are at least twice as abundant after cryoinjury in one species than the other. (D) A comparison of the Gene Set Enrichment Analysis obtained from the two species. Color intensity reflects the statistical significance of reduction (blue) or enrichment (orange) at 7 dpci, compared to uninjured control. Dot size corresponds to the magnitude of difference for each gene set. Selected gene sets have been organized into broader functional categories. (E) Transcript abundance comparison of orthologous genes between conditions (uninjured, dark color vs. cryoinjured, light color) and species (zebrafish, ZF, purple vs. platyfish, PF, green). Each point in the bar plot corresponds to the DESeq2-based TPM-like values (so-called Normalized read counts, see Methods) of the gene in one replicate. The $\log_2\text{FC}$ and $-\log_{10}(\text{padj})$ are those shown in (A) and (B). The orthologue genes are grouped into general categories.

To better understand these species-specific responses, we examined individual gene families in detail. The nomenclature of paralogous genes, which arose through the Teleost Genome Duplication, involves arbitrarily assigned “a” or “b” letters. In zebrafish and platyfish, we found numerous cases where different paralogous genes were predominantly expressed in the heart, as exemplified by *fibronectin* (*fn1a/b*), *periostin* (*postna/b*), or *midkine-a* (*mdka/b*) genes (Figure 4E). Here, the annotation of paralogs was considered when interpreting interspecies transcriptome comparisons. Analysis of specific signaling pathways revealed notable differences between species. In the chemokine cascade, transcripts of *cxcl12a* ligand were enriched in the zebrafish heart at 7 dpci, while their orthologues remained unchanged in platyfish (Figure 4E). Similarly, suppressors of cytokine signaling *socs3a/b*, and the dual specificity protein phosphatase family *dusp2* showed higher abundance in zebrafish cryoinjured hearts, compared to their platyfish counterparts. Among transcription factors involved in cell cycle regulation, *cebpa* was significantly and substantially enriched exclusively in zebrafish upon cryoinjury. These molecular factors may represent candidates associated with different regenerative responses in both species.

Based on our observation of delayed collagen deposition in the wounded heart at 7 dpci in platyfish (Figure 2C), we analyzed extracellular matrix-associated genes (suppl. Figure S4). No striking differences in *collagens* and *metalloproteinases* expression were detected between platyfish and zebrafish hearts. Thus, the delayed deposition of fibrotic tissue was not due to reduced *collagen* expression, but rather to post-transcriptional regulators. However, we found little expression of *tenascin C* (*tnc*) in platyfish, compared to zebrafish. The reduced *tenascin C* expression in platyfish hearts indicates fundamental species differences in extracellular matrix composition that may contribute to altered wound healing responses.

Delayed and persistent infiltration of leukocytes in the post-injured myocardium of platyfish

GSEA revealed distinct enrichment of immune-related gene sets, including Toll-like receptor, Neuregulin/ErbB, and cytokine signaling pathways, which were differentially expressed between zebrafish and platyfish (Figure 4D). As these pathways have been previously associated with cardiac regeneration⁶³⁻⁶⁶, we analyzed the abundance of gene transcripts specific to immune cell populations. Macrophage-related transcripts differed most prominently, with marked enrichment in zebrafish but not in platyfish (Figure 5A; suppl. Figure S5A-D).

To visualize immune cell infiltration patterns, we performed immunofluorescence analysis of platyfish sections using antibodies against Myeloperoxidase (Mpx or Mpo) for neutrophils and L-plastin for phagocytes/macrophages, respectively⁶⁷⁻⁶⁹ (suppl. Figure S5E-G). In sham controls, both markers remained consistently low at all time points (Figure 5B-E). At 7 dpci, the number of Mpx-positive neutrophils was significantly higher in cryoinjured ventricles compared to sham-operated controls (Figure 5D). In the intact myocardium, their numbers increased slightly between 7 and 14 dpci, then returned to near sham-operated levels. In contrast, within the wound, neutrophil numbers doubled between 7 and 14 dpci and remained elevated at 30 dpci. A gradual increase throughout the entire ventricle was also observed for L-plastin-positive phagocytes (Figure 5E). Between 7 and 30 dpci, their numbers doubled in the intact myocardium and tripled within the wound.

Comparison of the kinetics of these two cell populations revealed distinct temporal patterns. In the intact myocardium, neutrophil numbers rose rapidly but transiently, whereas phagocytes increased more slowly and continuously, remaining elevated even at 30 dpci (Figure 5F). Within the wound, neutrophil infiltration occurred rapidly following cryoinjury, reaching a plateau at 14 dpci, while L-plastin-positive cells increased markedly only at later stages, at 14 and 30 dpci (Figure 5G). Critically, these leukocytes persisted in the injured tissue at 30 days after cryoinjury. This prolonged inflammatory response differs dramatically from zebrafish, where leukocytes are nearly absent after 14 dpci^{62,70-72}, indicating that platyfish exhibit delayed resolution of the inflammatory response.

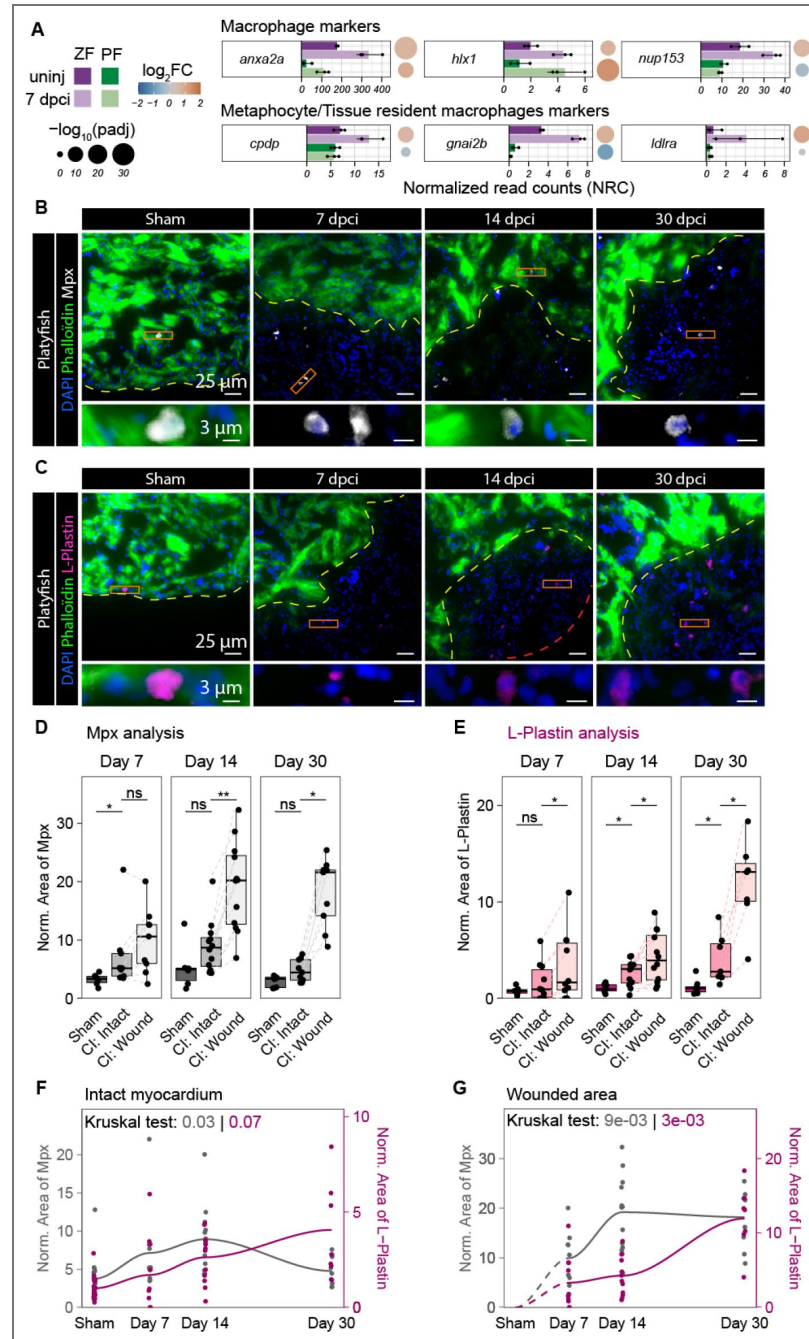


Figure 5. Delayed recruitment of immune cells during platyfish heart repair.

(A) A comparison of the abundance of macrophage-specific transcripts. For details, see legend to Figure 4 and Methods. (B-C) Immunofluorescence analysis of neutrophils (Mpx-positive cells, B) and phagocytes (L-plastin-positive cells, C) following cryoinjury at 7, 14, and 30 dpci. Control sections are from uninjured ventricles of sham-operated fish at each time point. The sham image corresponds to 30 dpt. (D-E) Quantification of Mpx-positive area (D) and L-plastin-positive area (E), corresponding to the images representatively shown in B and C. In the cryoinjured ventricles (CI), quantifications were assessed separately in the intact myocardium (CI: Intact) and the wounded area (CI: Wound). Statistical comparisons of Sham vs. CI: Intact were done with unpaired Wilcoxon tests, as different animals were in each group, whereas comparisons between the intact myocardium and the wound of the same cryoinjured hearts were based on a paired Wilcoxon test. Holm's post-hoc correction was applied for multiple comparisons. Adjusted p-value: * $P < 0.05$, ** $P < 0.01$. (F-G) Recruitment kinetics of neutrophils (grey, left Y-axis) and phagocytes (purple, right Y-axis) in the intact myocardium (F) and wounds (G) of ventricles at 7, 14, and 30 dpci. 'Sham' corresponds to all data from the sham-operated ventricle at different time points and represents the baseline. Kruskal-Wallis statistical tests were used to compare the different time points for each marker.

Cardiomyocyte proliferation increases mainly in the first phase of regeneration

Zebrafish cardiac regeneration relies on the dedifferentiation and proliferation of CMs within the border zone myocardium (also called peri-injury zone) at a distance of 100 μm from the injury margin (Figure 6A [52,53](#)). To examine CM-specific responses, we filtered our bulk transcriptomic analysis for genes linked to cardiac cell proliferation and dedifferentiation, based on scRNA-seq analysis data from three different studies [73-75](#). Proliferation markers such as *chaf1a*, *rrm2*, and *stmn1a* were enriched in both species at 7 days post-cryoinjury (dpci) (Figure 6B [53](#)). However, analysis of CM dedifferentiation-related transcripts, including *kcnh6a*, *idb3a*, and *nppb*, provided no evidence for the ability of platyfish CMs to dedifferentiate (Figure 6B [53](#)), suggesting fundamental differences in the cellular plasticity.

To assess CM proliferation dynamics in platyfish, we performed PCNA immunofluorescence analysis combined with Tropomyosin (TPM) and DAPI staining, followed by quantification within the remote myocardium, border zone and wounded area (Figure 6A [53](#)). Sham control with thoracotomy did not significantly increase PCNA staining, compared to the uninjured condition (Figure 6C, D [53](#); suppl. Figure S6A, B [53](#)). At 7 dpci, PCNA-positive nuclei were significantly enriched in both the remote myocardium and the wounded area (suppl. Figure S6B [53](#)). At 14 dpci, the number of proliferating cells declined, reaching baseline levels at 30 dpci. Analysis of PCNA/TPM-double positive nuclei showed the same temporal trend, with high enrichment at 7 dpci followed by a gradual decrease (Figure 6C, D [53](#)). Notably, PCNA/TPM-positive nuclei in the border zone myocardium reached levels comparable to those in the remote myocardium. Thus, cryoinjury induced transient CM cell cycle re-entry throughout the entire ventricle, not restricted to the border zone, as observed in zebrafish [53,76](#).

To track DNA-replicating over extended periods, we adapted the BrdU approach previously used in zebrafish, where treatment from day 7 to day 30 after cryoinjury resulted in approximately 60% of labeled CMs in the regenerated tissue [52](#) (Figure 6E [53](#)). We applied this approach to platyfish that were subjected to BrdU treatment starting at day 3, followed by heart collection at 7, 14 and 30 days after surgery (suppl. Figure S6C [53](#)). A peak of DNA synthesis in CMs was detected on day 7 (suppl. Figure S6E [53](#)). This increase was equally pronounced in the remote myocardium and border zone, confirming that the CM cell cycle re-entry was not spatially restricted to the border zone, unlike in zebrafish. At 14 and 30 dpci, the number of BrdU-positive CMs dropped dramatically in both regions (Figure 6F [53](#), suppl. Figure S6D, E [53](#)), indicating that the CM proliferative response occurred exclusively during the initial phase, consistent with PCNA analysis.

Importantly, unlike zebrafish, neither the wounded area nor the border zone of cryoinjured platyfish heart showed evidence of substantial BrdU labeling at later time points (Figure 6F [53](#)). Together, BrdU assay and PCNA expression analyses demonstrate that while CMs do re-enter the cell cycle in platyfish, this process is transient and not sustained by continuous cell proliferation during advanced regeneration phases. This pattern contrasts sharply with the prolonged proliferative response observed in successfully regenerating zebrafish hearts, suggesting that insufficient CM proliferation contributes to the incomplete regenerative response in platyfish.

Discussion

Sampling diverse phylogenetic lineages can provide new insights into the distribution of cardiac regeneration among teleosts [77,78](#). This study revealed that platyfish and swordtails, which represent live-bearing poeciliids, evolved a ventricle lacking vascularized compact myocardium, which is present in the zebrafish [54](#). Thus, zebrafish and *Xiphophorus* possess fundamentally distinct heart types.

In platyfish and swordtail hearts, the reparative phase within the first 2 weeks after cryoinjury occurred without collagen deposition and was associated with protrusion beyond the chamber circumference. This indicates that the fibrotic matrix provides essential mechanical support for

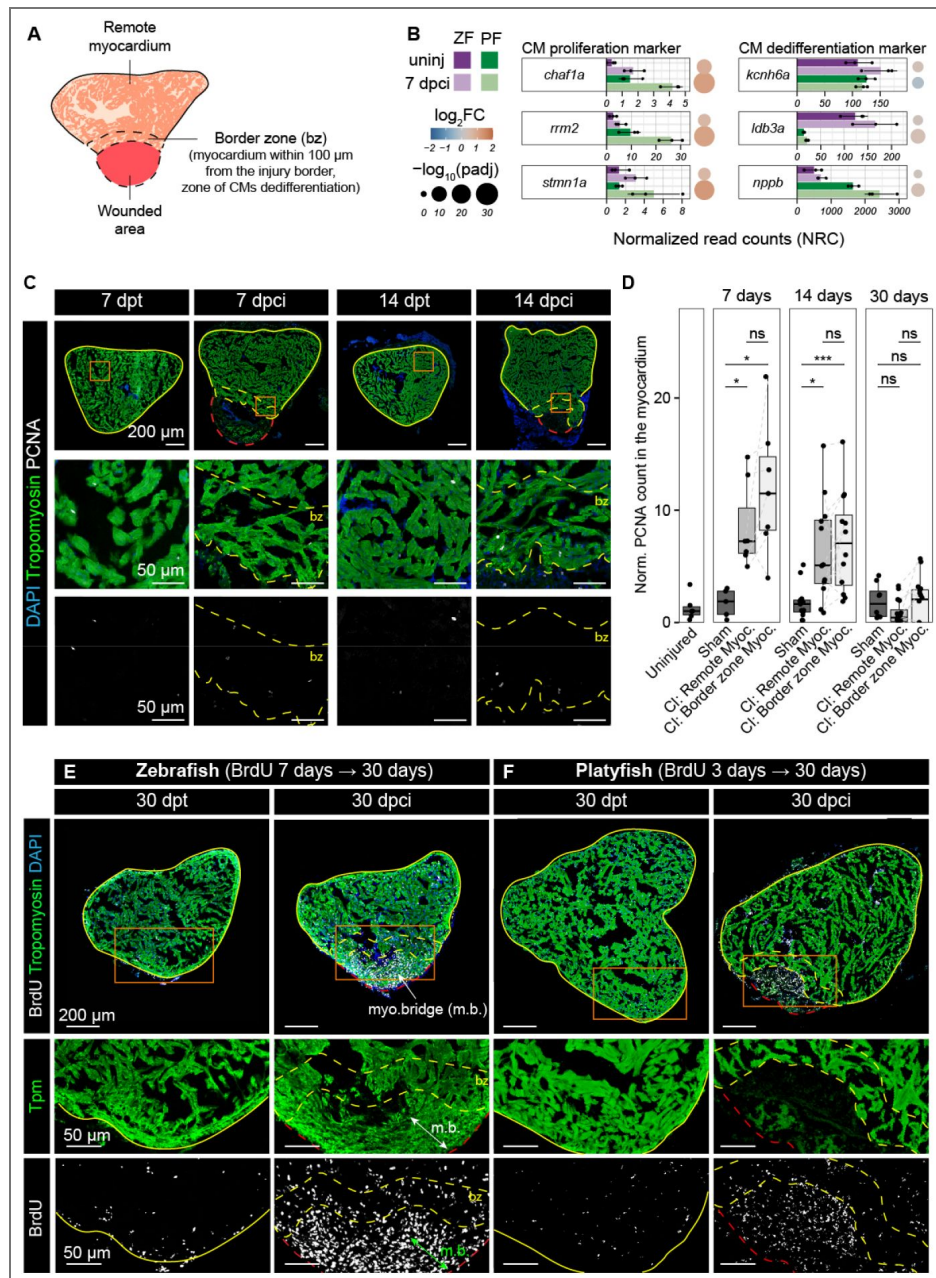


Figure 6. Ventricular cryoinjuries trigger transient activation of cardiomyocytes.

(A) Schematic illustration of the three analyzed areas: tropomyosin-negative wounded area, a border zone myocardium within 100 μ m from the injury border, and a remote myocardium distant from the border zone. (B) Comparison of the abundance of orthologous transcripts defined as markers of CM proliferation and dedifferentiation. For further details, see legend to Figure 4 and Methods. (C) PCNA localization analysis of sham-operated platyfish (7 dpt and 14 dpt) and cryoinjured platyfish (7 dpci and 14 dpci). The orange frame of the top images depicts the area magnified in the middle images. The bottom images show the same magnification, displaying only PCNA. bz: border zone. (D) Quantification of proliferating cells in the Tropomyosin-positive myocardium at different time points and different regions of the heart. Sham vs. CI: Remote Myoc. and CI: Remote Myoc. vs. CI: Border zone Myoc. were compared using unpaired and paired Wilcoxon tests, respectively, followed by Holm's post-hoc correction for multiple comparisons. Adjusted p-value: * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001. (E-F) BrdU labeling of zebrafish (E) and platyfish (F) for 23 days (from day 7 to 30 days post-surgery) and 27 days (from day 3 to 30 post-surgery), respectively. Ventricular sections were counterstained with DAPI and Tropomyosin. In zebrafish, the initial wounded area is defined based on myocardium appearance and the high concentration of BrdU-positive nuclei. The myocardium bridge (m.b.) is shown with the double-headed arrow. A myocardial bridge is not observed in platyfish. For C, E, and F, dashed red lines encircle the outer border wound, whereas dashed yellow lines surround the border zone (bz).

wounds within the blood-pumping ventricle, consistent with studies in zebrafish ^{27,79,80}. A similar bulging phenomenon has been reported in zebrafish treated with an inhibitor of TGF β signaling ⁷⁹. Blocking this pathway prevented collagen and tenascin C deposition, which are typically abundant in the wounds of normally regenerating hearts. Interestingly, in platyfish, TGF β signaling was not activated after injury, and tenascin C was not enriched, which may explain the molecular basis of the pseudoaneurysm phenotype. Further investigation is needed to understand how extracellular matrix proteins contribute to the healing of the damaged ventricle.

In the advanced phase, between 2 and 4 weeks after cryoinjury, fibrosis filled the damaged area more densely than in zebrafish. These dynamics correlated with enhanced CM proliferation mainly in the initial phase before scar deposition. Although wound size decreased after 30 days, platyfish failed to form the myocardial bridge that interconnects the interrupted ventricle. Instead, a fibrotic scar sealed the wound margin. The failure to form the myocardial bridge could be a direct consequence of the missing compact myocardium and its underlying intramyocardial connective tissue ⁸¹. Thus, *Xiphophorus* species display compromised myocardial regeneration combined with partial scarring.

Transcriptomics and immunofluorescence analyses of platyfish ventricles revealed delayed and persistent inflammation, characterized by the prolonged retention of macrophages and neutrophils in injured tissue. In zebrafish, macrophages can contribute directly to scar formation via cell-autonomous collagen deposition ⁷⁰. Thus, poor initial immune cell infiltration might explain the delayed fibrosis observed in platyfish. This finding correlates with the absence of coronary vasculature that facilitates cell circulation. In zebrafish, blood and lymphatic vessels promote wound clearance and provide inductive factors for heart regeneration ^{29,31,82-85}. Thus, the avascular heart of platyfish appears less compatible with regeneration, although tissue remodeling may lead to decreased wound size. The importance of immune responses in reactivating and promoting CM proliferation is widely recognized for cardiac regeneration. Whether the suboptimal immune response in platyfish is solely responsible for the lack of sustained CM proliferation, or whether other factors are also involved, requires further investigation.

Studies of other fish species suggest that inflammation and organismal physiology significantly impact cardiac regeneration ^{35,36}. An inappropriate immune response prevents heart regeneration in medaka, and this effect can be rescued by stimulating Toll-like receptor signaling ³⁷. In platyfish, pathway enrichment analysis showed that this signaling was not induced, similar to medaka. Thus, certain mechanisms might be common between medaka and platyfish hearts, which both lack vascularized compact myocardium. However, some fish possess a vascularized compact layer; nevertheless, they fail to regenerate their heart. This is the case for a cave-dwelling population of the Mexican tetra, which likely lost regenerative competence through metabolic adaptations to life in a dark and ecologically limited habitat ⁸⁶. Thus, systemic and environmental factors may create variations in cardiac regeneration. This situation contrasts with caudal fin regeneration, which appears less sensitive to such modulations, and is broadly conserved across diverse teleosts, including medaka, cavefish and *Xiphophorus* species ^{51,78,87-90}. One difference might be that in teleosts, the caudal fin bauplan is more conserved than ventricular architecture.

What might be the advantage of having a ventricle without coronary vasculature and compact myocardium, given that the vascularized heart is considered ancestral in fish evolution, and the compact layer is prominent in athletic fish such as tuna ^{91,92}? One aspect could be linked to health, because dependence on blood vessels might pose risks to the organ. For example, migratory salmonids exhibit coronary lesions, as demonstrated in several studies ⁹³. The consequences of such lesions on ischemic states may correlate with premature aging. The risk of coronary pathology is obviously non-existent in the avascular heart type of *Xiphophorus*, which might have a reduced predisposition to cardiovascular diseases.

In humans, myocardial infarction can lead to complications characterized by changes in ventricular geometry, such as pseudoaneurysm formation ^{58,59}. Cardiac deformations are life-threatening, and the underlying mechanisms are not yet fully understood. It is tempting to

speculate that the cryoinjured heart of *Xiphophorus*, which is also prone to deformations, could serve as a model with biomedical significance for understanding pseudoaneurysm pathophysiology.

Materials and methods

Resources

Phylogenetic information was based on the updated classification of bony fishes, inferred using molecular and genomic data ¹¹. The number of species in taxa was taken from FishBase (<https://www.catalogueoflife.org> ¹²). The references for chemical reagents, antibodies, and computer software are listed in the [Supplementary Materials](#) ¹³.

Animal strains

Xiphophorus hellerii (swordtail) and *Xiphophorus maculatus* (platyfish) at approximately 3.5 cm standard length were purchased from a commercial aquarium fish vendor (Aqualand, Renens/Lausanne, Switzerland). Wildtype zebrafish were from the AB strain, bred in our fish facility. Fish were of random sex and aged between 4 months and 1 year. Fish housing animal procedures were approved by the cantonal veterinary office of Fribourg. All assays were performed using different animals randomly assigned to experimental groups. The exact sample size (n) is described for each experiment on the graphs or in the figure legends.

Ventricular cryoinjury procedure

Fish were first immersed in an analgesic solution of 5 mg/L lidocaine for 45 min, then in an anesthetic solution of 0.6 mM tricaine for a few minutes. The anesthetic state was verified before each procedure. Ventricular cryo-injuries were performed according to our established video protocol ⁵⁶. Briefly, anesthetized fish were placed dorsal side down on a damp sponge under a stereomicroscope, with the ventral side exposed. Following incision of the thoracic skin, a stainless steel cryoprobe pre-cooled in liquid nitrogen was applied to the ventricle for 20-23 s. To terminate the freezing process, water was poured over the ventricle. The probe was then removed, and the fish was immediately returned to water. Sham operations consisted of thoracotomy alone, i.e., incision of the thoracic skin without cryoprobe application. Fish were monitored until spontaneous movement resumed and sub-sequently observed for several hours.

Heart collection and fixation

Fish were euthanized in 300 mg/L buffered tricaine for a few minutes. The euthanized state was verified before each procedure. As described in our protocol ⁹⁴, the ventral side was reopened, and the heart was extracted. Hearts were rinsed with PBS, fixed in 2% formalin overnight at 4°C, rewashed in PBS, and equilibrated in 30% sucrose at 4°C for at least 24 hours. Hearts were then embedded in tissue freezing medium and cryosectioned at 12 µm. Sections were collected on Superfrost Plus slides, dried for ~1 h at room temperature, and stored at -20°C in tight boxes.

BrdU treatment

For the indicated time periods, 4 to 5 fish in 1 L of system water were treated with 50 mg/L of BrdU. The treatment was changed every 2 days with fresh stock solution. On the day of water change, 5 mg/mL of BrdU was prepared by dissolving the appropriate weight of BrdU in demineralized water under gentle agitation at 37°C. The solution was then diluted 100-fold in system water and well mixed before introducing the fish.

Immunofluorescence analysis

Sections were first permeabilized with 0.3% Triton X-100/PBS for 10 min, then blocked with blocking buffer (5% goat serum/0.3% Triton X-100/PBS) for 1-2 hours at room temperature. Sections were then incubated overnight at 4 °C with primary antibodies diluted in blocking buffer. After washing with 0.3% Triton X-100/PBS, sections were incubated with fluorophore-conjugated

secondary, diluted in blocking buffer for 1-2 hours at room temperature, followed by several wash steps with 0.3% Triton X-100/PBS. Nuclei were counterstained with DAPI, and in some experiments, muscle was also counterstained with Phalloidin-CruzFluor-488. Primary and secondary antibodies are listed in (Supplementary Materials [S1](#)). Finally, the slides were mounted using a custom glycerol-based mounting medium. Depending on the experiment, sections were counterstained with Phalloidin-CruzFluor-488 during a third incubation step, which lasted 1 hour at room temperature.

For BrdU and PCNA immunofluorescence analyses, additional antigen retrieval steps were performed between the permeabilization and blocking steps. To reveal BrdU labeling, slides were treated with 2 N HCl/0.3% Triton X-100/PBS for 45 min at room temperature. For PCNA immunofluorescence analysis, slides were treated with pre-warmed sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) in a pressure cooker at maximum pressure for 3 min.

AFOG staining

Aniline blue, acid fuchsin, and orange-G (AFOG) staining was performed as described ^{79,95}. Briefly, sections were fixed with 10% formalin for 15 minutes at room temperature, washed in 0.3% Triton X-100/PBS for 10 minutes, and incubated in pre-warmed Bouin's fixative for 2.5 hours at 56°C, followed by one hour at room temperature. After washing with tap water, sections were stained with AFOG solution (3 g acid fuchsin, 2 g Orange G, 1 g aniline blue, 200 mL acidified distilled water, pH 1.1) and rewashed with distilled water. Finally, the sections were dehydrated through several baths of increasing ethanol concentrations, treated with xylene, and mounted with Entellan. The chemical dyes label fibrin/protein deposits in red, collagen in blue, and muscle in orange. Sections were imaged using the DM6B Leica microscope.

Alkaline phosphatase staining

For whole mount alkaline phosphatase staining, hearts were fixed for 1h at room temperature under gentle agitation with 2% formalin/PBS, washed three times 10 min with PBS, equilibrated in 2 mL alkaline buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 0.1% Tween20) for 45 min at room temperature. The alkaline phosphatase reaction is triggered by adding 1.7 µL NBT and 1.75 µL BCIP per milliliter of alkaline buffer. After 7 minutes, the staining reaction was stopped by three PBS washes for 10 minutes each, and the samples were imaged as soon as proper vessel staining was revealed. Hearts were photographed with a Leica M205 FA stereo microscope.

Image acquisition with a confocal microscope

Confocal images were acquired using a Leica SP5 microscope equipped with diode, argon, diode-pumped solid-state, and helium-neon lasers. Different objectives were used: Plan Apo 20×/0.75 multi-immersion, Plan Apo 40×/1.3 oil, and Plan Apo 63×/1.3 glycerol immersion. An electronic zoom factor of 1.5 was typically applied. Excitation was performed with 405, 488, 561, and 633 nm lasers. Fluorescence detection was achieved using hybrid detectors in photon counting mode or PMT detectors, with the following detection ranges: 415–480 nm, 498–550 nm, 571–630 nm, and 643–780 nm. Laser intensity, gain, offset, and detection ranges were adjusted to avoid bleed-through between channels, which was verified for each acquisition. Complete images were assembled by stitching together multiple adjacent fields to create composite panoramic views. Note that BrdU and PCNA colocalization analysis were achieved from confocal images.

Image acquisition with a widefield microscope

Histological and fluorescent staining were imaged using a fully automated upright Leica DM6B widefield microscope equipped with a Lumencore SOLA Light Engine as the fluorescence illumination source and a Leica CTR6 LED for transmitted light illumination. The following objectives were used: Plan Fluotar 10×/0.32 dry, Plan Apochromat 20×/0.8 dry, and Plan Apochromat 40×/0.95 dry. For fluorescence, excitation and emission were achieved using CFT filter cubes, allowing for the detection of DAPI, FITC, Cy3, and Cy5 fluorophores. Image acquisition was

performed using three different cameras. A Hamamatsu Orca Fusion C14440-20UP sCMOS and a Leica DFC9000GT cCMOS for fluorescence imaging, and a Leica DMC5400 color CMOS camera for brightfield images. The system was controlled using LAS X Navigator software.

Stereomicroscope imaging of whole hearts

Whole hearts were photographed using a Leica M205 FA stereomicroscope equipped with a K3 camera. Samples were imaged at 16× magnification in a Petri dish containing 1% solidified agarose, either after fixation in PBS or after dehydration in 30% sucrose/PBS solution. Illumination was provided from below using transmitted light, and the incidence of the light was manually adjusted to achieve high-contrast images.

Image Analysis

To obtain representative data, multiple sections from each heart at each time point were imaged and analyzed. Quantification was performed using Adobe Photoshop CS6, Fiji/ImageJ, and R software for image processing, data analysis, and graph generation. Custom ImageJ macros were developed for colocalization and area analysis across multiple regions of interest (ROIs). The main steps of the macro are outlined below; the full script is available upon request.

Definition of Regions of Interest and ROI area/volume calculation

For each channel, background subtraction was performed using the rolling ball algorithm with an appropriate radius, followed by brightness adjustment. To facilitate the specific selection of stained areas with the “Analyze Particles” function (see below), images were slightly blurred, and the watershed algorithm was applied to separate individual nuclear signals and to segment the ventricular myocardium, thereby excluding internal lumens or holes from area measurements.

Using DAPI and muscle staining, both the entire heart section and the wound area were manually delineated. The macro then automatically defined the border zone as a 100 µm-wide band extending from the wound edge into the intact remote myocardium, provided that the wound area had been previously outlined. The areas of the different ROIs were saved for downstream analysis.

For ventricle and wound volume calculation, all sections from a single heart were analyzed as described above to obtain the area of the remote myocardium and the wound in each section. These areas were then summed and multiplied by the section thickness (12 µm) and the number of slides prepared from a single heart (8 slides) to estimate the total volume. To calculate the wound volume as a percentage of the whole ventricle (Figure 3 [↗](#)), the calculated wound volume was divided by the total ventricular volume.

Segmentation and Mask Extraction

A manual thresholding strategy was applied to each channel to generate binary masks representing the signal of interest. The different ROIs were then extracted from these masks. Using the separated mask images, particle analysis was performed on the channels of interest using appropriate size and circularity parameters.

Area Quantification – L-Plastin, Mpx, Fibronectin

The total area of all selected particles in each channel of interest was measured. For L-Plastin and Mpx analysis, the total particle area was normalized by dividing by the area of the corresponding ROI. For fibronectin, all sections from a single heart were analyzed, and the approximate volume of fi-bronectin was calculated (see previous section). This value was then divided by the calculated ventricular volume to obtain the approximate fibronectin volume as a percentage of the whole ventricle. Each data point corresponds to the average value from several non-consecutive sections of the same heart, multiplied by a scaling factor of 1000 to obtain values greater than 1.

Colocalization Analysis – BrdU and PCNA

Particles defined in the DAPI channel that overlapped by at least 50% of their area with the mask of the channel of interest were considered colocalized. If a DAPI-defined particle met this criterion for two channels, it was counted as positive for both markers. “Normalized count in CMs” corresponds to the total number of nuclei positive for all three channels (BrdU+, TPM+, DAPI+ or PCNA+, TPM+, DAPI+) divided by the total number of nuclei positive for DAPI and TPM. “Normalized count” corresponds to the total number of nuclei positive for two channels (BrdU+, DAPI+ or PCNA+, DAPI+) divided by the total number of DAPI-positive nuclei. Each data point represents the average value from several non-consecutive sections of the same heart, multiplied by a scaling factor of 1000.

Quantification of Pseudoaneurysm

Based on AFOG and Phalloidin/DAPI staining of heart sections, each ventricle was classified into three categories: (1) no or minimal injury, (2) clearly visible injury, and (3) injury bulging beyond the normal circumference of the heart. Two independent analyses were performed by two different investigators, from cryoinjury to image analysis. For one of them, image classification was performed blindly by another lab member. The data presented in this paper combine the results from both analyses.

RNA extraction, library preparation and sequencing

For each zebrafish and platyfish species, at 7 days post-cryoinjury, 24 hearts from uninjured fish and 24 from cryoinjured fish were collected. Ventricles were dissected from the atrium and bulbus arteriosus, following a brief treatment with 5mg/mL heparin to minimize blood cell contamination. Eight ventricles were pooled per sample, resulting in three biological replicates per condition. Samples were rapidly frozen on dry ice in Eppendorf tubes containing a single steel bead, then stored at -80 °C until further processing.

Tissues were lysed using a TissueLyser LT in 75% TRIzol/RNase-free water, until homogenization was complete. RNA was isolated and purified using the Qiagen RNeasy Plus Micro Kit. An on-column DNase digestion (Qiagen, RNase-free DNase set) was performed to eliminate genomic DNA contamination. RNA quantity was assessed with a NanoDrop spectrophotometer, and quality was evaluated using an Agilent TapeStation. cDNA was synthesized and amplified using the SMART-Seq Low Input RNA Kit for Sequencing. RNA-seq libraries were prepared from total RNA using the TruSeq Stranded mRNA kit (Illumina) and sequenced on an Illumina HiSeq3000 system. RNA sequencing data have been deposited at GEO: GSE305467.

Raw data processing and differential gene expression analysis

The first analyses were run in R. The quality of the RNA-seq data was assessed using fastqc and RSeQC. The reads were mapped to the reference genome (Danio_rerio.GRCz11.94 for zebrafish and *Xiphophorus maculatus*.X_maculatus-5.0-male.95 for platyfish) using HiSat2. FeatureCounts was used to count the number of reads overlapping with each gene as specified in the genome annotation. The Bioconductor package DESeq2 was used to test for differential gene expression between the experimental groups. DESeq2-normalized gene expression was visualized on a volcano plot and a scatter plot. ClusterProfiler was used to identify gene ontology terms containing an unusually high number of differentially expressed genes. Gene set enrichment analysis (GSEA) was run in ClusterProfiler using genesets from KEGG ⁹⁶ and MSigDb ⁹⁷. An interactive Shiny application was set up to facilitate the exploration and visualization of the RNA-seq results and is available on demand. References and versions of the packages used are available in the Supplementary Data.

Cross-species RNA-seq analysis

Orthologous gene correspondence between platyfish and zebrafish was established by merging data from the Ensembl and Orthogene databases, resulting in a dataset containing 15,609 orthologs ([supplementary Table S1](#)).

To identify transcripts specific to cell types or cellular behaviors, three single-cell RNA-seq datasets were utilized:

1. scRNA-seq data from 1- and 3-days post-fertilization (dpf) zebrafish, and from various adult tissues (blood, brain, caudal fin, eye, gill, heart, intestine, kidney, liver, muscle, ovary, pancreas, skin, spleen, swim bladder, and testis). Only genes from the following clusters were considered: embryonic macrophage, T cell, embryonic muscle cell, cardiomyocyte (CM), immune cell, muscle cell, granulocyte, neuron, and macrophage [98](#).
2. scRNA-seq data from hearts of adult zebrafish, including both control (uninjured, no sham) and cryoinjured (3-, 7-, and 30-days post-cryoinjury) samples. In this analysis, all genes from atrial and ventricular CMs were merged into a single CM cluster [74](#).
3. scRNA-seq data from MPEG1.1+ cells isolated from the epidermis, gill, intestine, liver, heart, and brain of 6-month-old Tg(*mpeg1.1:DsRedx*) zebrafish [75](#).

Genes present in more than one cluster across these datasets were filtered out, resulting in a new dataset containing 2,104 genes distributed across 34 cell clusters ([supplementary table S2](#)). This dataset was then merged with the orthologous gene dataset, yielding a final set of 1,294 genes ([supplementary Table S3](#)).

Comparison of Transcriptome Upon Cryoinjury Between Species

For scatter plot analysis, log₂ fold change values calculated by DESeq2 were compared between one-to-one orthologous genes. A difference of 1 in the log₂ fold change was arbitrarily defined as a threshold to highlight genes differentially enriched upon cryoinjury.

To compare transcriptomes between conditions and species, DESeq2-normalized values for each gene were divided by the sum of all DESeq2-normalized values from a single replicate and multiplied by 10⁶ as a scaling factor. This normalization, similar to TPM (Transcripts Per Million), ensures that all sequenced libraries are scaled to the same size, allowing for direct comparison of transcript abundance levels of orthologous genes between conditions and species. This dataset was merged with the previous dataset containing clustered orthologous genes ([supplementary Table S4](#)). Genes shown in the figures were selected based on their normalized expression levels, variability between replicates, differences between species and conditions, and existing knowledge about zebrafish ventricular regeneration.

Plot and statistical analysis

Analyses were performed using custom scripts for R and ImageJ available on request. Versions of the different software and packages to make the figure are available below.

Data availability

RNA sequencing data have been deposited at GEO: GSE305467. All data generated or analyzed during this study are included in the manuscript and supporting files; source data files have been provided for all figures.

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Additional information

Ethics approval

This study complied with all relevant ethical regulations. Zebrafish were bred, raised, and maintained in accordance with the FELASA guidelines ⁹⁹. The animal housing and all experimental procedures were approved by the cantonal veterinary office of Fribourg, Switzerland.

Author contributions

Investigations, all authors
 Data curation, VH, LR, SB, HL, RB
 Formal analysis, VH, LR, HL, RB
 Conceptualization, AJ, SB
 Visualization, VH, LR, SB
 Supervision, AJ, SB, RB
 Writing - original draft, VH, AJ;
 Writing - review & editing, all authors

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Additional files

Supplemental figures and material [↗](#)

Sup. Table S1. [↗](#)

Sup. Table S2. [↗](#)

Sup. Table S3. [↗](#)

Sup. Table S4. [↗](#)

Sup. Table S5.1. [↗](#)

Sup. Table S5.2. [↗](#)

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Peer reviews

Reviewer #1 (Public review):

Summary:

How the regenerative capacity of the heart varies among different species has been a long-standing question. Within teleosts, zebrafish can regenerate their hearts, while medaka and cavefish cannot. The authors examined heart regeneration in two livebearers, platyfish and swordtails. Interestingly, they found that these two fish species lack the compact myocardium layer that contains coronary vessels. Furthermore, these fish form a "pseudoaneurysm" after cryoinjury without initial deposition of fibrotic tissues. However, delayed leukocyte infiltration and prolonged inflammation lead to permanent scar tissue in the injured heart. Although their cardiomyocytes can also proliferate, platyfish and swordtails can only regenerate partially. The authors argue that the restorative mechanism of platyfish and swordtails likely reflects "evolutionary innovations in the ventricle type and the immune system".

Strengths:

The authors took advantage of the annotated genome of platyfish to perform transcriptomic analyses. The histological analyses and immunostaining are beautifully done.

Minor Weaknesses:

Transcriptomic analysis was only done for one time point. Different time points could be included to validate whether some processes occur at different time points. But this can be done in the future for more detailed studies."

<https://doi.org/10.7554/eLife.109441.1.sa2>

Reviewer #2 (Public review):

This manuscript by Hisler, Rees, and colleagues examines the cardiac regenerative ability of two livebearer species, the platyfish and swordtail. Unlike zebrafish, these species lack cortical myocardium and coronary vasculature. Cryoinjury to their hearts caused persistent scarring at 60 and 90 days post-injury and prevented most of the myocardium from regenerating. Although the wound size progressively shrinks and fibronectin content decreases, the myocardial wall does not recover. Transcriptomic profiling at 7 dpi revealed significant differences between zebrafish and platyfish, including alterations in ECM deposition, immune regulation, and signaling pathways involved in regeneration, such as TGF β , mTOR, and Erbb2. Platyfish exhibit a delayed but chronic immune response, and although some cardiomyocyte proliferation is observed, it does not appear to contribute to myocardial recovery significantly.

Overall, this is an excellent manuscript that tackles a crucial question: do different fish lineages have the ability to regenerate hearts, or is this capability limited to a few groups? Therefore, this work is relevant to the fields of cardiac regeneration and comparative regenerative biology for a broad audience. I am very enthusiastic about expanding the list of species tested for their heart regeneration abilities, and this study is detailed and rigorous, providing a solid foundation for future comparative research. However, there are several aspects where additional work could significantly strengthen the manuscript.

Major comments

(1) Title selection

The title the authors chose suggests that platyfish and swordtails "partially regenerate," but I do wonder how much these animals truly regenerate. This may be a semantic discussion and a matter of personal preference. Still, based on other significant work on regenerative capacity (see, for example, the landmark cavefish regeneration paper PMID: 30462998 or work on medaka PMID: 24947076), the persistence of such a prominent fibrotic scar would be considered a minimal regenerative capacity. Measuring this "partial regeneration" more precisely by comparing zebrafish with platyfish and swordtails would also greatly strengthen the comparisons made here - see below.

The same can be said about line 152-153 - do these hearts "regenerate" with deformation and partial scarring, or would it be more fair to say that they are "healed" or "repaired" with a process that involves fibrosis?

(2) Cross-species comparisons

Having two species of livebearers strengthens the findings of this paper, but the presentation of results from both species is inconsistent. For example, the reader should not be asked to assume that the architecture of the swordtail ventricle is similar to that of the platyfish (line 125). The same applies to the presence or absence of coronary vessels (Figure 1), the reduction in wound area over time (Figure 3), and the immune system's response (Figure 5). Most importantly, the authors miss an opportunity to move from qualitative observations to quantifying the "partial regeneration" phenotype they observe. Specifically, providing a side-

by-side comparison between these new species and zebrafish would help define the extent of differences in regeneration potential. For instance, in Figure 6, while the authors provide excellent quantification of PCNA staining in platyfish, these data are less meaningful without a direct comparison with zebrafish results. The same applies to Figures 6E and 6F - although differences are noted, quantifying these results would enable a more rigorous assessment of the process.

(3) Lack of coronary vasculature

There is a growing body of evidence highlighting the importance of the coronary vessels during zebrafish heart regeneration (PMIDs: 27647901, 31743664). Surprisingly, this finding has not been integrated or discussed in the context of this literature.

The results of the alkaline phosphatase assay and anti-podocalyxin-2 staining appear inconsistent. Specifically, in Supplementary Figure 1L-M, we can see some vessels covering the bulbus arteriosus and also what appears to be a signal in the ventricle. However, in Figures 1 K and 1L, we cannot see any vessels, even in the bulbus. The authors should also be more rigorous and add a description of how many animals were analyzed, their ages, and sizes. In zebrafish, the formation of the coronary arteries appears to depend on animal size and age. With the data provided, we cannot say whether this is a one-time observation or a consistent finding across many animals at different ages and across both species.

The link between livebearers' responses and pseudoaneurysms is overstated. This work is already extremely relevant without trying to make it medically oriented.

<https://doi.org/10.7554/eLife.109441.1.sa1>

Author response:

Reviewer #1:

Minor Weaknesses:

"Transcriptomic analysis was only done for one time point. Different time points could be included to validate whether some processes occur at different time points. But this can be done in the future for more detailed studies."

Our response regarding time points of transcriptomic analysis:

We appreciate this constructive suggestion. We fully agree that performing RNA-seq at multiple time points would provide valuable insights into the temporal dynamics of molecular pathways during cardiac regeneration. However, given that our study represents the first comprehensive characterization of cardiac regeneration in poeciliids, we deliberately focused our resources on establishing the foundational framework, including morphological, cellular, and initial transcriptomic analyses between zebrafish and platyfish. Expanding to multiple time points would constitute a substantial additional study that, while scientifically valuable, would extend beyond the scope of this initial characterization.

We will acknowledge this limitation in the Discussion and indicate that temporal transcriptomic profiling is an important direction for future investigation.

Reviewer #2:

(1) Title selection

Our response regarding the use of the term “partially regenerate” in the title and results:

We thank Reviewer 2 for this important point regarding the terminology used to describe the cardiac response in platyfish and swordtails. We agree that the term "partially regenerate" may overstate the regenerative capacity of these species, particularly given the persistence of a substantial collagenous scar at the injury site. The reviewer is correct that, based on established criteria in the field, including the landmark studies on cavefish (PMID: 30462998) and medaka (PMID: 24947076), the presence of such prominent fibrotic scarring would be more appropriately characterized as limited or minimal regenerative capacity rather than partial regeneration.

While we observe a significant reduction in wound volume at 30 dpci and some degree of tissue remodeling, we acknowledge that the persistent scarring and incomplete myocardial recovery more accurately reflect a healing or repair process rather than true regeneration. We therefore agree with the reviewer's suggestion to revise our terminology throughout the manuscript.

We will revise the title to: "The livebearers platyfish and swordtails heal their hearts with persistent scarring." We will also modify other relevant sections of the Results and Discussion to consistently describe these processes as "healing" or "repair" rather than "regeneration", while still acknowledging the biological changes that do occur (wound contraction, remodeling, limited cardiomyocyte proliferation). This revised framing better aligns our work with the established terminology in the comparative cardiac regeneration literature and more accurately represents the phenotype we observe.

We believe this change will strengthen the manuscript by providing a more precise characterization of the cardiac response in these species and facilitating clearer comparisons with other model systems.

(2) Cross-species comparisons

Our response regarding the inconsistent presentation of results for different species:

We thank the reviewer for recognizing that our conclusions regarding the regenerative capacity of livebearers are strengthened by including two poeciliid species, platyfish and swordtails. We agree that presenting results more consistently across both species will significantly improve the manuscript. We acknowledge that our current presentation creates a burden on the reader by asking them to assume similarities between species without providing supporting data. While we initially focused primarily on platyfish due to its superior genome annotation (critical for our transcriptomic analyses), we recognize that this approach left important gaps in the manuscript.

We will address this by generating comprehensive supplementary figures that present swordtail data alongside platyfish for key findings. Specifically, we will add a complete anatomical characterization of swordtail ventricle architecture, demonstrating the structural similarities to platyfish that underpin our comparative conclusions. We will also perform quantification of wound area reduction and immune response dynamics over time in swordtails, allowing direct comparison between species.

We clarify that we did perform detailed analyses of swordtail heart anatomy during our initial studies, which revealed remarkable similarity to platyfish. However, space constraints in Figures 1 and S1 (which already span full pages with zebrafish-platyfish comparisons) prevented us from including these data in the original submission. We now recognize that explicitly presenting these data is essential for the reader to evaluate our conclusions.

Our response regarding quantification and comparison with zebrafish:

We appreciate the reviewer's suggestion to move beyond qualitative observations toward rigorous quantification of the "partial regeneration" phenotype. As suggested by the reviewer for the PCNA analysis, we will provide direct quantitative comparisons with published zebrafish regeneration studies, including data from several relevant studies and our own lab's work. This comparison will delineate the extent of differences in proliferative response between complete regenerators (zebrafish) and limited regenerators (poeciliids).

These additions will transform our descriptive observations into quantitative assessments that rigorously define the incomplete healing phenotype in poeciliids relative to complete regeneration in zebrafish. We believe these changes will substantially strengthen the manuscript and address the reviewer's concerns about comparative rigor.

| (3) *Lack of coronary vasculature*

Our response regarding inconsistencies in vascularization data:

We thank the reviewer for his/her comment regarding our data on the absence of coronary vasculature in the platyfish heart. The reviewer noted differences between alkaline phosphatase (AP) enzymatic staining and anti-Podocalyxin-2 immunofluorescence staining. We would like to clarify that these observed differences are not inconsistencies but rather reflect the distinct specificities of these two complementary approaches.

Alkaline phosphatase staining is selective for arterial branches and capillaries in the heart (PMID: 13982613; PMID: 9477306; PMID: 8245430; PMID: 3562789; PMID: 29023576; PMID: 28632131) and revealed a typical vascular pattern in the bulbus arteriosus and ventricle in zebrafish but not in platyfish. Anti-Podocalyxin-2 staining displayed a vessel-like pattern in zebrafish but not in platyfish. However, in both species Podocalyxin staining also labeled other types of non-vascular structures. This is expected given that Podocalyxin is a cell surface sialomucin with broader expression beyond blood vessels, including the endocardium (PMID: 19142011) and certain neuronal populations, in addition to other non-cardiac tissue types (PMID: 19578008; PMID: 3511072; PMID: 34201212).

We will revise the manuscript to emphasize this distinction and clarify our rationale: we deliberately employed Podocalyxin-2 staining as a complementary, less selective approach to corroborate our alkaline phosphatase findings. In platyfish, the convergent evidence from both methods (the absence of typical vascular structures with a selective AP staining and the detection of only non-vascular patterns with the broader marker Podocalyxin-2) strengthens our conclusion that platyfish hearts lack a conventional coronary vascular network.

Our response regarding reproducibility:

The assays were performed independently by two researchers at different stages of the study using two different batches of adult platyfish. The results were consistent in both assays, and we are therefore confident in the reproducibility of our findings.

Our response regarding citations of references on revascularization:

We thank the reviewer for recommending the studies PMID: 27647901 and PMID: 31743664 that revealed the importance of rapid revascularization during heart regeneration in zebrafish. We will be pleased to integrate these works to present our data in the appropriate context of current knowledge.

Our response regarding a link to pseudoaneurysms:

We appreciate the reviewer's feedback regarding the link to pseudoaneurysm. We agree that the primary contributions of our work stand on their own merit, and we will revise the text

to present the livebearer findings more cautiously without overstating their potential medical relevance. We will focus on the intrinsic biological significance of our findings.

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