Cavefish Increase Red Blood Cell Development and Reprogram Metabolism as Adaptations to Environmental Hypoxia

Running Title: Cavefish erythrocytes and hypoxia

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Summary

Astyanax mexicanus cavefish adapt to life in hypoxic cave environments by evolving the capacity to increase red blood cell numbers during early development and reprogramming metabolism to favor anaerobic processes.

Abstract

The teleost *Astyanax mexicanus* is a single species with surface dwelling (surface fish) and cave dwelling (cavefish) morphs. Constructive and regressive traits have evolved in cavefish as adaptations for survival in perpetual darkness. In addition to darkness, cavefish must cope with de-oxygenated aquatic environments. Blood cell quantification and expression of hematopoietic marker genes indicated that cavefish have more erythrocytes than surface fish, and that this increase has a developmental basis. In contrast to zebrafish and other teleost embryos, in which erythrocyte formation is restricted to the posterior lateral mesoderm, the anterior as well as the posterior lateral mesoderm is involved in red blood cell formation in *Astyanax* embryos, and both of these hematopoietic domains are expanded in cavefish embryos. Erythroid development in the anterior mesoderm may be a pre-adaptation for surface fish to successfully colonize hypoxic cave environments. We also show that cavefish are less sensitive to phenylhydrazine-induced erythrocyte ablation than surface fish, suggesting a functional advantage of increased red blood cells. By mimicking a hypoxic cave environment in the laboratory, we further demonstrate that cavefish respond to hypoxia differently than surface fish. Surface fish with fewer red blood cells use overall metabolic depression to counteract hypoxia, whereas cavefish with larger numbers of erythrocytes respond to hypoxia by switching to anaerobic metabolism. These results suggest that cavefish may have adapted to hypoxic environments by enhancing the capacity to form erythrocytes and reprogramming metabolism.

Introduction

It is critical to know how organisms adapt to novel environments in order to understand evolution. We study evolution in the Mexican tetra *Astyanax mexicanus*, a single species consisting of surface-dwelling (surface fish) and cave-dwelling (cavefish) morphs. Surface fish colonized subterranean waters in the Sierra de El Abra region of Mexico (Mitchell et al., 1977; Gross, 2012) during the late Pleistocene (Fumey et al., 2018; Herman et al., 2018), and their cavefish descendants evolved numerous traits adapting them for life in perpetual darkness. Cavefish adaptations include constructive traits, such as more taste buds and cranial neuromasts, increased olfactory capacity, and fat deposits, which facilitate feeding and survival during times of low food input, and regressive traits, such as decreased metabolic rates and loss of eyes, which may be important in energy conservation (Yamamoto et al., 2004; Moran et al., 2014; 2015, Varatharasan et al., 2009; Espinasa et al., 2014; Yoshizawa et al., 2014; Hüppop, 1986; Xiong et al., 2018). Although the unifying theme of cave habitats is complete darkness and the absence of primary productivity (Culver and Pipan, 2009), measurements of abiotic factors indicate that there are additional environmental constraints: caves often contain groundwater severely depleted in oxygen (Malard and Hervant, 1999). For example, oxygen reductions of 50% or more have been measured in Tinaja and Pachón Caves in the Mexican Sierra de El Abra (Rohner et al., 2013, Ornelas-Garcia et al., 2018), suggesting that hypoxia could be an important driver of evolutionary changes in *Astyanax* cavefish. However, little is known about how cavefish have adapted to hypo-oxygenated waters.

In vertebrates, oxygen is bound to hemoglobin and transported through the body by red blood cells. Increasing erythrocyte production is a well-documented strategy for coping with hypoxia at high altitude (Haas, 2013), but the role of red blood cells in adaptation to hypoxic caves is unknown. Hematopoiesis, the process in which erythrocytes and other blood cells are formed during embryogenesis, occurs in two waves in vertebrates: the primitive and definitive waves (Davidson and Zon, 2004; Paik and Zon, 2010). In zebrafish and other teleost embryos, the

first or primitive wave occurs in anterior and posterior lateral mesoderm beginning during the tailbud stage. However, different hematopoietic cell types are produced anteriorly and posteriorly. The anterior lateral mesoderm (ALM) undergoes myelopoiesis to form macrophages, and some of these later differentiate into neutrophils and microglia (Herbomel et al., 1999; Herbomel et al., 2001; Le Guyader et al., 2008), whereas the posterior lateral mesoderm (PLM) undergoes erythropoiesis to form primitive erythrocytes. Subsequently, the PLM converges into the intermediate cell mass, where the precursors of endothelial cells lining the first blood vessels, primitive erythrocytes, and myeloid cells will ultimately differentiate. The second or definitive wave of hematopoiesis begins later in development when hematopoietic stem and progenitor cells are formed in the aorta-gonad-mesonephros (AGM), and these cells seed the caudal hematopoietic tissue and form the posterior blood islands, where definitive erythrocytes begin to differentiate (Paik and Zon 2010; Gore et al., 2018). Eventually, definitive hematopoiesis moves to the thymus and head kidneys in adults. The primitive and definitive waves of hematopoiesis are potential targets for adaptive changes induced by hypoxia.

Hematopoiesis is directed by a series of developmental-stage specific transcription factors producing different types of blood cells (Davidson and Zon, 2004; Carroll and North, 2014). For example, the *growth factor independence 1* (*gfi1*) gene, which encodes a transcriptional repressor, is important for primitive hematopoiesis at several developmental stages (Wei et al., 2008; Cooney et al., 2013; Moore et al., 2018). In mice *gfi1* has been shown to regulate many different genes through chromatin modification (Möröy et al., 2015). Expression of the zebrafish homolog *gfi1aa* occurs throughout the period of embryonic segmentation, first in the PLM, then in the intermediate cell mass, and finally in the AGM (Wei et al., 2008, Cooney et al., 2013), where it directs the development of hemogenic endothelia into hematopoietic stem and progenitor cells, which ultimately differentiate into red blood cells (Moore et al., 2018). *Gfi1aa* is also expressed in the ALM, albeit weakly and only for a short time (Wei et al., 2008). The expression of *gfi1aa* and other

hematopoietic genes during both waves of hematopoiesis provide excellent markers for studying early red blood cell development.

Oxygen is required in aerobic organisms to produce energy, and insufficient oxygen leads to the activation of an evolutionarily conserved transcriptional response (Majmundar et al., 2010). The responses to oxygen deprivation are coordinated by activation of the hypoxia inducible factors (HIFs), a family of transcription factors controlling a large number of downstream target genes involved in promoting many different responses to hypoxia (Haase, 2013). HIFs function in metabolic tissue homeostasis, balancing oxygen consumption, Reactive Oxygen Species (ROS) production, and generation of NAD^+ by activating transcription of target genes central to reprogramming a metabolic shift (Wheaton and Chandel, 2011). *Astyanax* cavefish (Hüppop, 1986; Moran et al., 2014, Aspiras et al., 2015) and other cave-adapted animals (Bishop et al., 2004) have evolved reduced metabolic rates to conserve energy in the cave environment. However, it is unknown whether metabolic changes in cavefish include a switch from aerobic to anerobic metabolism as a consequence of the hypoxic environment.

In the present investigation, comparison of *Astyanax* morphs revealed that cavefish embryos develop significantly more erythrocytes than surface fish, and that this increase is mediated by the expansion of primitive hematopoietic domains into the ALM of cavefish embryos. Although minor compared to cavefish, red blood cell formation was also noted in the surface fish ALM, suggesting the potential importance of erythrocyte enhancement for colonization of hypoxic cave environments. Our results also revealed a stronger response to laboratory-induced hypoxia in cavefish than surface fish, which involves upregulation of HIF dependent genes and metabolic genes, signaling a switch from oxygen dependent processes to glycolysis and fermentation. These studies suggest that hypoxia may be one of the driving forces of adaptive evolution in cavefish.

Materials and methods

Biological materials

Astyanax mexicanus surface fish and cavefish were obtained from laboratory stocks descended from collections in Balmorhea Springs State Park, Texas and Cueva de El Pachón, Tamaulipas, Mexico respectively. Fish were raised in a constant flow culture system as described previously (Jeffery et. al. 2000). Embryos were obtained by natural spawning and reared at 23°C. Fish handling and husbandry protocols were approved by the University of Maryland Animal Care and Use Committee (IACUC #R-NOV-18-59) (Project 1241065-1), and conformed to National Institutes of Health guidelines.

Quantification of circulating blood cells

Developing larvae were immobilized at 36-40 hours post-fertilization (hpf) with 2 μg ml^{-1} tricaine (Western Chemical, Inc, Ferndale, WA, USA) and placed in water on a concave microscope slide. Blood flow was imaged for 10 seconds using a stereoscope (Olympus SZX12) with 50x magnification and a 5 MP, Color, AmScope Microscope Eyepiece Camera (MD500). Recordings were captured with Photobooth on a Macbook computer via USB (OS 10.13.6). Videos were rendered into separate frames in Adobe Photoshop (CC2017, Adobe Inc., San Jose, CA, USA) and processed and analyzed in Fiji (Schindelin et al., 2012). After conversion to 32 bit, a data image showing the movement of particles was created by subtracting two frames 0.15 seconds apart. This data image was used in the plugin TrackMate (Tinevez et al., 2017) to analyze the number of moving blood cells in a region of interest (ROI) covering 50% or more of the blood stream over the yolk (150x150 pixels). Using the LoG detector, moving blood cells were automatically detected by setting the "blob diameter" to 5 and the threshold to 2. An automatic quality threshold was used to obtain the number of circulating blood cells and checked against the recording for accuracy.

Red blood cell staining

Red blood cells were stained with 0.6 mg ml^{-1} o-dianisidine (Sigma-Aldrich, St. Louis, MO, USA), 0.01 M sodium acetate pH 4.5, 0.65 % H_2O_2 for 15 min in the dark (Iuchi and Yamamoto, 1983). The stained embryos were rinsed in phosphate buffered saline (PBS), fixed in 4% paraformaldehyde (PFA), and imaged as described below for *in situ* hybridization.

In situ hybridization

Genes were cloned from a 24 hpf surface fish cDNA library and 10 hpf, 24 hpf and 30 hpf cavefish cDNA libraries using the pCRII TOPO dual promoter vector (ThermoFisher Scientific, Waltham, MA, USA) and the following primers: *hbb2* (24 hpf SF; ENSAMXG00000031275: 5'-gcaggacaagtagaaacctcaaagtc-3' and 5'-tttcgtaagggcagagcctaca-3'), *gfi1aa* (24 hpf CF; ENSAMXG00000006669: 5'-gaaggtctgcgctcgtgatatt-3' and 5'-agttatccgcggtgtgaacag-3'), *lcp1* (36 hpf CF; ENSAMXG00000012855: 5'-aggccttcagcaaagttgatgtg-3' and 5'-ttcaggtcctctgcaccgatatt-3'), *lmo2* (10 hpf CF; ENSAMXG00000032986: 5'-ggcctctacaatcgagaggaaa-3' and 5' taccaagttgccgtttagtttgg-3'). DIG labeled probes were made using SP6 or T7 transcription kits (Roche, Mannheim, Germany) from linearized plasmid or, in the case of *gfi1aa,* from a PCR product made with the cloned cDNA as template and the above mentioned primers, after a RNA-polymerase promoter site was added to the 5' end.

In situ hybridizations were performed as previously described (Ma et al., 2014). Briefly, stored (-20°C) 4% PFA fixed and methanol dehydrated embryos were rehydrated into PBS stepwise, fixed with 4% PFA, digested with proteinase K, fixed with 4% PFA, and hybridized with probes at 60°C for 16 hours. Un-hybridized probe was removed by SSCT stringency washes followed by incubation in MABT blocking solution (Roche) and subsequently with anti-DIG-AP Fab fragments (Roche). Embryos were thoroughly rinsed in MABT buffer and PBS, equilibrated in AP buffer, and stained with BM-Purple (Roche). The stained embryos were imaged and photographed on a Zeiss Discovery V20 stereoscope with a Zeiss AxioCam HRc camera.

Quantitative real time polymerase chain reaction

RNA was extracted from 30 embryos with Trizol (ThermoFisher) and treated with RNase-free DNase I (ThermoFisher). The DNase I reaction was stopped, and the RNA was cleaned and concentrated by phenol/chloroform extraction and precipitated by ammonium acetate and ethanol. Poly(A)-primed cDNA was made with SuperScript III First Strand Synthesis SuperMix (ThermoFisher) and used in quantitative real time polymerase chain reaction (qPCR) with Takara SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara Bio USA Inc., Mountain View, CA, USA) and LC480 (Roche). The *rpl13a* gene was used as a reference gene. The primers used in qPCR analysis are shown in (Table 1).

For surface and cavefish comparisons, ΔCt for each gene was calculated by subtracting the average Ct value of the reference gene *rpl13a*. For comparison of gene expression in cavefish to surface fish at different time points, ΔΔCt was calculated by subtracting the average ΔCt of surface fish from each ΔCt of cavefish. ΔΔCt values for cavefish compared to surface fish for different time points were then used for comparison between time points. Statistical analysis was done for each gene using a one-way ANOVA followed by pairwise comparison with Tukey HSD (N=3)($p<0.05$).

For comparison of gene expression between hypoxia to normoxia, ΔΔCt values were calculated by subtracting the average ΔCt at normoxia from each ΔCt at hypoxia for cavefish and surface fish. These ΔΔCt values were then compared between cavefish and surface fish with a Student's t-test for each gene, and significance was determined for an overall value of $p<0.05$ using the Bonferroni correction (8 genes; $p_{adjusted}<0.0063$) (N=3) (Rosenthal and Rosnow, 1991). For graphical representation, the fold change was calculated as $2^{-(\Delta\Delta Ct)}$, where values >1 show an increase and values <1 a decrease. Variation was expressed as the range of fold change $2^{-(\Delta\Delta Ct+stdev\Delta\Delta Ct)}$ for the upper value or $2^{-(\Delta\Delta Ct-stdev\Delta\Delta Ct)}$ for the lowest value.

Red blood cell ablation with phenylhydrazine

Phenylhydrazine (PHZ; Sigma-Aldrich, St. Louis, MO, USA) was used to ablate red blood cells (Houston et al., 1988). At 12 hpf, 20 embryos were placed in clean fish system-water containing a particular concentration of PHZ or clean fish system water as a control. Embryos were incubated with PHZ for 28 hours when blood circulation became visible over the yolk. Embryos were fixed and used for *in situ* hybridization with a *hbb2* probe or live video imaging. Measurements of tail length were made in Fiji and differences tested with a two-way ANOVA ($p<0.05$), ($N=10$) and Tukey-Kramer for individual comparisons ($p<0.05$). Quantification of red blood cells was done as described above, and the reductions expressed as percent of the average of the untreated specimens. Differences were tested with a two-way ANOVA, ($p<0.05$), ($N=10$) and Tukey HSD.

Laboratory emulation of a low oxygen cave environment

Oxygen was reduced by heating clean fish system water and pouring it into a 60cm long, 0.9 cm diameter plastic tube (Tygon® S3™ B-44-4X Food, Milk and Dairy Tubing, United States Plastic Corp., Lima, OH, USA). The water was 50°C at time of capping, which equates to about a 50% reduction in oxygen (Benson et al., 1979). The tubes were cooled to room temperature, 40 embryos in 0.5 ml of normoxic water were placed inside each tube within 30 seconds after removing the caps, and the tubes were re-capped airtight with silicone stoppers (Versilic® Silicone Stoppers, United States Plastic Corp.). Because of the low diameter to length ratio of the tube, oxygen diffusion back into the water was minimal.

Embryos were exposed to low oxygen by incubating the plastic tubes at 23°C for 22 hours beginning at 16 hpf. After removal from the tubes, embryos were assayed for morphological changes, and the number of blood cells was quantified as described ($N = 8$ for each morph). For extraction of RNA, embryos were exposed to hypoxia and transferred into 1 ml of Trizol within 3 min after being removed from the tubes. As controls, normoxic conditions were created by filling tubes with water at room temperature, placing embryos in the tubes, and capping the tubes.

Results

Cavefish have more erythrocytes than surface fish

To test the possibility that cavefish cope with hypoxic environments by producing more red blood cells, we compared erythrocytes between surface fish and cavefish larvae in several different ways. First, we conducted direct observations of blood cell numbers. The circulatory system is still partially open when blood begins to circulate at about 34 hpf, permitting blood cells to be imaged as they flow from the posterior cardinal vein over the surface of the yolk mass toward the heart (Fig. 1A-D). The number of circulating blood cells was quantified in this region in sequential video frames using Fiji image analysis software. This quantification revealed about twice as many circulating blood cells in cavefish as in surface fish (Fig. 1B-E). Second, we compared erythrocytes in surface fish and cavefish by determining the expression of *hemoglobin subunit beta-2-like* (*hbb2*) using *in situ* hybridization, and *hbbe2*, an *Astyanax* embryonic β-hemoglobin most similar to zebrafish *hbbe,* for qPCR (Ganis et al., 2012) (Fig. 1F-H). *In situ* hybridization showed more *hbb2* staining in cavefish compared to surface fish, which was especially evident in the anterior yolk mass at 36 hpf (Fig. 1F), and *hbb2* staining was also more extensive in the posterior region of cavefish larvae at 84 hpf (Fig. 1G). The qPCR results showed increased *hbbe2* mRNA levels in cavefish compared to surface fish at 10 and 24 hpf, and particularly high differences were detected at 10 hpf, near the beginning of primitive hematopoiesis (see also below) (Fig. 1H). Third, we evaluated expression of the *gfi1aa* gene, which encodes a transcription factor essential for hematopoiesis (Cooney et al., 2013), by qPCR (and *in situ* hybridization; see below) during surface fish and cavefish development. The results showed increased *gfi1aa* mRNA levels in cavefish compared to surface fish at 24 hpf (Fig. 1H), near the beginning of definitive hematopoiesis. These results provide three complementary lines of evidence supporting the enhancement of red blood cells in cavefish.

Expansion of erythropoietic domains in cavefish embryos

To understand the developmental basis of erythrocyte increase in cavefish, we compared the timing and spatial expression of *hbb2*, *gif1aa,* and other

hematopoiesis marker genes by *in situ* hybridization during surface fish and cavefish embryogenesis.

As in zebrafish (Brownlie et al., 2003; Wei et al., 2008), *hbb2* and *gif1aa* were initially expressed in the intermediate cell mass of the posterior lateral mesoderm (PLM) in *Astyanax* surface fish and cavefish embryos during primitive hematopoiesis, and expression subsequently progressed to the posterior blood islands (Fig 2A, C). We also detected *gfi1aa* expression in the anterior lateral mesoderm (ALM) (Wei et al., 2008), but in striking contrast to zebrafish, in which ß-hemoglobin expression and erythropoiesis are restricted to the PLM, *gif1aa* and *hbb2* staining were also detected in the ALM in both surface fish and cavefish embryos (Fig 2A). Furthermore, *gfi1aa* and *hbb2* staining in the ALM and PLM at 14 hpf and in the aorta-gonad-mesonephros (AGM) at 26 hpf were stronger in cavefish than in surface fish (Fig. 2A, C). These results, which are supported by qPCR quantifications (Fig 1H), confirm enhanced development of erythrocytes in cavefish and show that primitive erythropoiesis occurs in both the ALM and PLM in *Astyanax* embryos.

In zebrafish, the ALM undergoes myelopoiesis, rather than erythropoiesis, to form macrophages, which disperse throughout the embryo (Herbomel et al., 1999). To determine if the *Astyanax* ALM also contains macrophage progenitors during the period of *hbb2* and *gif1aa* expression, *in situ* hybridizations were performed at 15 hpf using the *l-plastin1* (*lcp1*) gene, a marker for differentiated macrophages (Herbomel et al., 2001). This stage is equivalent to the 13-17 somite stage in zebrafish, when macrophage migration is seen over the yolk mass (Herbomel et al., 1999). The results showed that *lcp1* expressing cells were distributed throughout the ventral body and yolk masses of surface fish and cavefish embryos, implying that macrophages had already differentiated and migrated away from the ALM (Fig 2B). Therefore, it is likely that the erythrocyte progenitors in the *Astyanax* ALM represent an independent hematopoietic lineage.

To further explore erythropoiesis in surface fish and cavefish embryos, we performed *in situ* hybridizations with the *lim domain only 2* (*lmo2*) gene, a marker of developing blood cells (Patterson et al., 2007). The results confirmed that both

anterior and posterior regions are responsible for blood cell development in surface fish and cavefish embryos, and also showed that *lmo2* expression and erythrocyte production is expanded in the ALM and PLM during primitive erythropoiesis in cavefish.

In summary, the results indicate that the increase in cavefish red blood cells is due to the enhancement and expansion of erythropoiesis in the ALM and PLM during embryonic development.

Cavefish are more resistant to red blood cell ablation than surface fish

To understand the role of erythrocytes in early development, we compared the responses of surface fish and cavefish embryos to red blood cell ablation induced by phenylhydrazine (PHZ) (Houston et al., 1988). In these experiments, embryos were incubated with 1, 2, or 4 mg L^{-1} PHZ from 12 to 40 hpf, the effects on erythrocyte number were determined by *in situ* hybridization with *hbb2*, red blood cells were quantified by video counting (Fig. 1), and morphological changes were assayed by microscopy (Fig. 3A-D). In both surface fish and cavefish embryos, *hbb2* staining decreased as a function of increased PHZ concentration (Fig. 3A). Erythrocyte staining was minimal in surface fish at 2 mg L^{-1} PHZ, whereas considerable *hbb2* staining remained in cavefish at this concentration. Quantification reinforced this result, and showed that cavefish have more circulating blood cells remaining after PHZ ablation than surface fish (Fig. 3B). Observation of living embryos showed surface fish treated with 1 or 2 mg L^{-1} PHZ developed edemas, which were visible as effusions displacing a portion of the yolk near the ventrally located heart (Fig. 3C). In contrast, cavefish did not develop edemas at these concentrations (Fig. 3C), although edemas were eventually observed at 4 mg L^{-1} PHZ. Furthermore, surface and cavefish with ablated red cells showed axial defects, most notably swollen notochords and shortened and ventrally bent tails (Fig 3C). Tail length was reduced and the proportion of surface fish larvae with shorter tails was increased as a function of PHZ concentration, and the extent of tail reduction was higher in surface fish than in cavefish exposed to the same PHZ concentration (Fig. 3D). These results show that cavefish are less sensitive than

surface fish to red blood cell ablation, possibly because of their increased erythrocyte numbers. The results also suggest that erythrocytes are required for normal *Astyanax* development.

Cavefish fare better than surface fish in hypoxic environments

Cave waters are commonly low in oxygen, therefore we hypothesized that an increase in red blood cells may be advantageous for combating hypoxia. To test this hypothesis, a cave-like hypoxic environment was emulated in the laboratory by comparing the development of surface fish and cavefish embryos raised in inert plastic tubes filled with hypoxic water (Fig. 4A). Embryos were placed in tubes at 16 hpf, incubation was continued for 22 hours, and larvae were assayed for survival, red blood cell numbers, and morphological differences. Video analysis of blood flow over the yolk (Fig. 1) showed a mean of 125 +/- 38 erythrocytes in cavefish compared to 45 +/- 33 erythrocytes in surface fish, suggesting that the differences between cavefish and surface fish erythrocytes were not changed by hypoxia, although the total numbers of blood cells may have been somewhat reduced. Most surface fish and cavefish (38 of 40 for each morph) survived in hypoxic environments, however, all surface fish exposed to hypoxic tubes developed edemas, although no axial problems were evident, whereas edemas were absent in all cavefish larvae raised in hypoxic tubes. These results suggest that cavefish, which have more blood cells than surface fish, are better able to cope with hypoxic conditions than surface fish.

Cavefish upregulate hypoxia related genes and reprogram metabolism in a hypoxic environment

To compare the molecular responses of surface fish and cavefish to the emulated hypoxic environment described above, qPCR analyses were performed using marker genes for hypoxia and hypoxia-related metabolic changes. We examined *hif1al*, a homologue of zebrafish *hif3a* that is transcriptionally regulated by the HIF1 hypoxia master regulator (Pasanen et al., 2010), and other genes controlled by HIF1 (Iyer et al., 1998, Kim et al., 2006, Cui et al., 2017): *hexokinase 1*

(*hk1*), which is involved in glycolysis, *lactate dehydrogenase a* (*ldha*), which is involved in fermentation, and *pyruvate dehydrogenase kinase1* (*pdk1*), which is involved in inhibiting the TCA cycle in mitochondria. In addition, genes were assayed that are functionally associated with hypoxia or hypoxia induced processes, but not known to be controlled by HIF1: *ATP-dependent 6-phosphofructokinase* (*pfkma*), which is involved in glycolysis (Ptashne et al., 1983), *lactate dehydrogenase b* (*ldhb*) and *monocarboxylate transporter1* (*mct1*), which are involved in fermentation (Ždralević et al., 2018; Miranda-Gonçalves, 2016), and *pyruvate dehydrogenase kinase 2* (*pdk2*), which functions similarly to *pdk1* but is regulated by hormones and insulin signaling pathways (Jeong et al., 2012).

In these experiments, we compared expression of the genes listed above in surface fish and cavefish raised in tubes containing hypoxic water or normoxic water. Figure 4B shows expression of these genes in surface fish and cavefish as fold change of hypoxic over normoxic conditions. Expression of *hif1al* was up-regulated more than 2-fold in cavefish under hypoxic conditions, indicating a strong response to hypoxia, whereas under the same conditions surface fish showed a change of *hif1al* of less than one fold, indicating a decrease in expression (Fig. 4B a). Cavefish appear to downregulate mitochondrial function as a response to hypoxia, as shown by about 2.5 and 5.5 fold increases in *pdk1* and *pdk2* expression respectively, while in surface fish this inhibition of mitochondrial function is reduced, as shown by 0.5 or less fold change in expression (Fig. 4B b). The approximate 19 fold increase in *hk1* and 5.5 fold increase in *pfkma* expression in cavefish under hypoxic relative to normoxic conditions suggested that cavefish increase glycolysis during hypoxia, probably to compensate for the reduction of ATP synthesis by mitochondria, compared to about a 0.5 fold change, indicating suppression of glycolysis, in surface fish (Fig. 4B c). Additionally, *ldha* and *ldhb* were up-regulated about 2 and 3 fold and *mct1* by about 3 fold in cavefish raised in hypoxic compared to normoxic waters, suggesting fermentation of pyruvate and continuation of glycolysis by generating NAD^+, while in surface fish a change of less than 1 fold of these genes suggested a reduction in fermentation capacity (Fig. 4B d).

In summary, the results show that cavefish respond to hypoxia by increasing expression of hypoxia and hypoxia-related metabolic genes while surface fish react to hypoxia by suppressing these genes (Fig. 4B), suggesting that cavefish have a more robust response to hypoxia than surface fish. Furthermore, the results provide evidence that surface fish and cavefish respond differently to hypoxia. Surface fish appear to counteract hypoxia by continuing to undergo oxidative phosphorylation, whereas cavefish reprogram metabolism to increase glycolysis and anaerobic metabolism as responses to hypoxia.

Discussion

Proliferation of red blood cells is one of the classic responses to reduced oxygen levels in vertebrates. For example, humans show a temporary increase in the production of erythrocytes during acclimation to high altitudes (Haase, 2013). In *Astyanax*, we found that cavefish embryos develop more red blood cells than surface fish embryos when raised under normoxic laboratory conditions. This conclusion is based on three lines of evidence: (1) direct counts of red blood cells, (2) increased expression of the *hbb2* and *hbbe2* globin genes, and (3) hyper-expression of the *gfi1aa* gene, a crucial regulator of hematopoiesis. Hematopoiesis occurs in two waves in teleosts and other vertebrates: the primitive and definitive waves (Davidson and Zon, 2004; Paik and Zon, 2010). Increased *hbb2, hbbe2,* and *gfi1aa* expression was observed during embryogenesis and after larval hatching. Transfusion experiments indicate that blood cells circulating during the first four days of zebrafish development are primitive erythrocytes (Weinstein et al., 1996). If this is also the case in *Astyanax*, then our findings suggest that surface fish and cavefish larval blood cells are primitive erythrocytes derived from the first wave of hematopoiesis. Nevertheless, we also found stronger expression of the marker genes in the AGM and caudal hematopoietic tissue, which are involved in definitive hematopoiesis, implying that the second wave is also expanded in cavefish. We conclude that cavefish have evolved a permanent enhancement in red blood cells as an adaptation for survival in hypoxic cave waters.

Using *hbb2*, *gfi1aa*, and *lmo2* genes as markers of hematopoiesis and erythropoiesis, red blood cell development could be traced back to the early stages of surface fish and cavefish embryogenesis. The *lmo2* gene was a particularly useful indicator for hematopoietic origins because it encodes a transcription factor critical for early hematopoietic development and is essential for erythropoiesis (Yamada et al, 1998; Warren et al., 1994). The expression patterns of *Imo2* and other marker genes indicate that the primitive erythrocytes of cavefish and surface fish are derived from two different hematopoietic domains, one located anteriorly in the ALM and the other posteriorly in the PLM. Importantly, the anterior and posterior embryonic domains show expanded marker gene expression in cavefish compared to surface fish. It is noteworthy that expansion of the posterior hematopoietic

492 domain can be induced in zebrafish by overexpressing LMO2 together with its
493 partner transcription factor SCL/TAL1 (Gering et al., 2003), suggesting that teleosts
494 have the innate ability to modulate blood cell numbers during development.
495 Expansion of erythropoiesis into the ALM during *Astyanax* primitive
496 hematopoiesis contrasts strikingly to zebrafish, medaka, and other teleosts, where
497 embryonic erythropoiesis is confined to the PLM, and the ALM is devoted to
498 myelopoiesis (Detrich et al., 1995; Govoni et al., 2005, Moriyama et al., 2010).
499 Evidence from the macrophage marker *lcp1* (Herbomel et al., 2001) indicated that
500 myelopoietic precursors had already differentiated into macrophages as erythrocyte
501 progenitors were being formed in the ALM of early *Astyanax* embryos, and thus are
502 distinct from anteriorly derived erythrocytes. Cell tracing studies in zebrafish have
503 shown that blood precursor cells produce either erythrocytes or macrophages, but
504 not both cell types (Warga et al., 2009), indicating an opposing relationship between
505 the erythroid and myeloid cell populations. However, it has been reported that this
506 relationship can be reversed by knockdown of the myeloid-controlling transcription
507 factor PU.1, which induces blood precursors of the ALM to form erythrocytes
508 instead of macrophages (Rhodes et al., 2005). Apparently, teleosts are potentially
509 able to undergo erythropoiesis in both the ALM and PLM, but in most species this
510 capacity is normally confined to the PLM. In *Astyanax*, the balanced relationship
511 between myeloid and erythroid cell specification appears to have tilted toward the
512 production of erythrocytes. However, the development of erythrocytes in the ALM is
513 not necessarily at the expense of fewer myeloid cells because there is also an overall
514 expansion of the ALM (and PLM) in *Astyanax* embryos, which presumably includes
515 the specification of more blood precursor cells. The existence of erythrocyte
516 forming capacity in the ALM of surface fish, albeit smaller than in cavefish, suggests
517 that *Astyanax* may be pre-adapted for colonizing hypoxic cave environments.
518 Specification of cells in the primitive blood lineage can be traced back to their
519 positions during gastrulation in zebrafish (Kimmel et al., 1990): ALM macrophages
520 are originally derived from progenitors on the dorsal side of the embryo, and PLM
521 erythrocytes originate from the ventral side of the embryo (Davidson and Zon,
522 2004; Warga et al., 2009). Erythrocyte progenitors corresponding to the ALM and

PLM are also likely to be positioned on the dorsal or ventral sides respectively in gastrulating *Astyanax* embryos. Therefore, increased erythropoiesis in the ALM suggests that a relative ventralization of hematopoietic development has evolved in cavefish compared to surface fish. This possibility is supported by the expansion of erythroid development in zebrafish by overexpression of the ventralizing factors *bmp2b* and *bmp7* during the gastrula period (Lengerke et al., 2008) and is in line with evidence implicating evolutionary changes in dorsal-ventral axis determination based on modified Spemann organizer activity in cavefish embryos (Ren et al., 2018; Ma et al., 2018).

Teleosts show considerable diversity in utilizing hemoglobin and red blood cells for oxygen transport during development: some species begin hemoglobin synthesis during embryogenesis, whereas others postpone hemoglobin and erythrocyte production until late in larval development (Wells, 2009). In the present investigation, the ability to ablate red blood cells in a concentration dependent manner with PHZ allowed determination of the functional significance of embryonic red blood cells during early *Astyanax* development and their enhancement in cavefish embryos. Surface fish embryos with most red blood cells ablated by PHZ develop abnormally, exhibiting edemas and axial (notochord, tail formation) defects, but cavefish exposed to the same PHZ concentrations and exhibiting residual red blood cells do not show any edemas and axial defects are less severe. These results suggest that red blood cells are necessary for oxygen transport at early stages of *Astyanax* development and that larger numbers of erythrocytes are advantageous in cavefish. This is contrary to the situation in zebrafish, where PHZ ablation shows that red blood cells are not required during the first few weeks of development under normoxic conditions (Pelster and Burggren, 1996), although under severe hypoxic conditions zebrafish embryos use red blood cells to increase the supply of oxygen (Rombough and Drader, 2009). Furthermore, pelagic teleosts, such as the halibut (Pittman et al, 1990) and spot (Govoni et al., 2005), do not produce hemoglobin until just before metamorphosis, and icefish do not produce any red blood cells throughout life (Ruud, 1954). These differences may be explained by size, *Astyanax* larvae are larger than zebrafish larvae, and thus may be more

554 resistant to simple oxygen diffusion, the probability of exposure to intermittent
555 hypoxia, which is rare in pelagic teleosts, or high oxygen solubility at very low water
556 temperatures in icefish habitats. The requirement of red blood cells for normal
557 development in *Astyanax* provides additional support for the possibility that early
558 formation of erythrocytes could be a pre-adaptation for the successful colonization
559 of hypoxic cave waters.

560 Teleosts have evolved different strategies to cope with environments
561 depleted in oxygen, including changes in gill morphology (i.e. crucian carp, Sollid et
562 al., 2003), behavioral avoidance of hypoxic waters (i. e. woolly sculpins, Congleton,
563 1980), and altered metabolism (i. e. mesopelagic fishes, Torres et al., 2012). It is
564 well known that *Astyanax* cavefish exhibit reduced metabolic rates compared to
565 surface fish (Hüppop, 1986; Moran et al., 2014; Aspiras et al., 2015), but the effects
566 of hypoxia on metabolic programs have not been previously determined. The
567 approach used to determine the effects of hypoxia on metabolism was to assay
568 expression changes in key genes underlying the different metabolic states and
569 outcomes in surface fish and cavefish embryos confined to hypoxic tubes in the
570 laboratory. The hypoxic tubes closely emulated hypoxic conditions in Sierra de El
571 Abra cave waters by permanent exposure to about 50% oxygen levels (Rohner et al.,
572 2013, Ornelas-Garcia et al., 2018). Surface fish, which do not have as many red
573 blood cells as cavefish, suppress metabolism in response to low oxygen, as indicated
574 by decreased expression of glycolytic and anaerobic genes. Reduced metabolism is
575 an evolutionary strategy for limited survival in hypoxic environments (Regan et al.,
576 2017), a strategy that could suffice for surface fish because hypoxia exposure may
577 be intermittent and/or they may be able to counteract low oxygen by moving to
578 normoxic waters. In contrast, upregulated expression of key glycolytic,
579 fermentation, and anaerobic genes suggested that cavefish react to hypoxia by
580 shifting to anaerobic metabolism, which may be enabled by more red blood cells. To
581 counteract the accumulation of lactate they also increase the efflux of lactate out of
582 cells as suggested by increased expression of a lactate transporter. This strategy
583 may be adaptive for life in hypoxic cave waters because metabolic depression is
584 likely to be detrimental over time and cavefish are unable to escape by moving to

more highly oxygenated waters. Our studies reveal a key difference between surface fish and cavefish in metabolic reprograming as a response to low oxygen.

In conclusion, the present investigation has revealed two cavefish adaptations that may have evolved to better sustain life in hypoxic cave waters. First, cavefish have evolved enhanced development of red blood cells and thus may have increased their capacity to carry and distribute essential oxygen to tissues and organs. Second, cavefish have coped with more permanent hypoxic conditions by evolving the potential to reprogram metabolism toward anaerobic processes. Together, these changes could be key contributors for successful adaptation of cavefish to hypoxic environments.

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Competing interests

The authors declare no competing or financial interests

Author contributions

CMW and WRJ conceived the project. CMW performed the experiments. CMW and WRJ wrote the manuscript.

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953 **Figure Legends**
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955 **Figure 1. Cavefish have more erythrocytes than surface fish**. (A). Red
956 blood cells flowing out of the cardinal vein over the yolk to the heart in surface (SF)
957 and cavefish (CF) embryos visualized by o-dianisidine staining. (B-E). The number
958 of circulating blood cells (arrowheads) in a ROI (B, yellow box) visualized by
959 subtraction of two video frames 0.15 seconds apart (C and D) and quantified using
960 the plugin TrackMate (Fiji) (D, purple dots). N = 8. Error bars: SD. Asterisk: $p < 0.05$.
961 Statistical analysis using Two-tailed Student's t test. Scale bar in A is 100μm, A-D are
962 the same magnification. (F, G). *In situ* hybridizations showing more extensive *hbb2*
963 staining in cavefish (CF) than surface fish (SF) at 36 hpf and 84 hpf. Scale bars in F
964 and G are 200 μm. (H). qPCR quantification of *gfi1aa* and *hbbe2* expression in
965 developing cavefish (CF) and surface fish (SF). N=3. Error bars: range of fold
966 change. Asterisks: $p<0.05$. Statistical analysis by one-way ANOVA followed by
967 Tukey HSD.
968
969 **Figure 2. *In situ* hybridizations showing expression of hematopoietic**
970 **marker genes in the posterior and anterior lateral mesoderm of surface fish**
971 **and cavefish embryos.** (A). At 14 hpf *gfi1aa* and *hbb2* are expressed in the anterior
972 lateral mesoderm (arrowheads) in cavefish (CF) and surface fish (SF) embryos, and
973 staining is expanded in CF. (B). At 14 hpf *lcp1* is expressed in macrophages
974 dispersed in SF and CF embryos. (C). At 26 hpf *gfi1aa* and *hbb2* expression are
975 increased in the posterior blood islands (PBI) in CF compared to SF. (D). At 14 hpf
976 *lmo2* expression is increased in both the anterior lateral mesoderm and posterior
977 lateral mesoderm in CF and SF embryos, and staining is expanded in CF. Scale bar in
978 A and C is 200μm; magnification is the same in A, B and D.
979
980 **Figure 3. Red blood cells are required for normal development**. (A). *In*
981 *situ* hybridization with the *hbb2* gene marker in embryos treated with
982 phenylhydrazine (PHZ) shows that only a few circulating erythrocytes are left at 1
983 mg L^{-1} PHZ (black arrowhead) and absent at 2 mg L^{-1} PHZ in surface fish (SF),
984 whereas erythrocytes are still present in cavefish (CF) treated with the same PHZ
985 concentrations. Most erythrocytes disappeared from CF embryos treated with 4 mg

L^{-1} PHZ. Scale bar is 200 µm. (B). The number of remaining circulating red blood cells (RBC) was determined as in Fig. 1. Quantification of blood cells conducted as in Fig. 1A-E showed that CF were less sensitive to PHZ treatment than SF. Error bars: SD. Asterisks: $p<0.05$. N=10 Statistical analysis by two-way ANOVA. (C). PHZ ablation of red blood cells has more severe effects on SF than CF development, inducing edemas (white arrowhead), swollen notochords and bent tails. Scale bar is 200 µm.; magnification is the same in the top four frames. Scale bar is 200 µm in bottom frame. (D). PHZ has larger effects on SF than CF tail development. N = 10. Asterisks: $p<0.05$. Statistical analysis by two-way ANOVA and Tukey HSD pairwise comparisons, whiskers are 5%, line is mean, dot is outlier.

Figure 4. Expression changes in hypoxia related and metabolic genes in surface fish and cavefish embryos exposed to emulated cave-like hypoxic environments. (A). Hypoxic cave environments were emulated by placing embryos in 60 cm long inert plastic tubes filled with water of approximately 50% oxygen content. Left: Tube emulating hypoxic cave-like environment. Scale bar: 5 cm. Right: Magnified region of tube showing embryos (arrow heads). (B). Comparison of hypoxia and metabolic gene expression by qPCR in cavefish (CF) and surface fish (SF) embryos exposed to emulated hypoxic versus normoxic conditions. Upregulation of (a) *hif1al* hypoxia related gene, (b) *pdk1* and *pdk2* gene markers of the TCA cycle, (c) *hk1* and *pfkma* gene markers of glycolysis, and (d) *ldha*, *ldhb*, and *mct1* marker genes of fermentation by hypoxia in CF compared to downregulation of all these genes in SF embryos by hypoxia. N = 3. Statistical analysis by two tailed Student's t-tests with Bonferroni adjustment, Asterisks: $p_{adjusted} < 0.0063$. Error bars: range of fold changes.

Table 1: Primer sequences used for gene expression analysis with qPCR

1017

Rpl13a	GeneID: 103025160	ENSAMXG 00000033532	caagtactgctgggcccaaag	aggaaagccaggtacttcaatttgtt
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Hbbe2*		ENSAMXG 00005017210	taaatccctctgcagggctctgat	cctgatcacctccggattagccataata
Hif1al	GeneID: 103027586	ENSAMXG 00000008564	tgcctcacctgcttctaactct	agctgtattctcctctggcttga
Hk1	GeneID: 103028521	ENSAMXG 00000012670	ctcaatcggctgaaggacaacaa	agccgtcgagaatactgtggat
Pfkma	GeneID: 103038352	ENSAMXG 00000012908	aggaggtgatgctcaaggaatga	agccctcgtgaacgaagaaga
Ldha	GeneID: 103047177	ENSAMXG 00000032467	tgtggtgtccaacccagttgata	agccctcgtgaacgaagaaga
Ldhb	GeneID: 103033414	ENSAMXG 00000007636	atcgtggctgacaaggactact	tcctctgcacaaggttgagtct
Mct1	GeneID: 103033445	ENSAMXG 00000000023	ttctcttcgagacgctgatgga	gtacctatcgtttaatgcaccagtag
Pdk1	GeneID: 103033744	ENSAMXG 00000039808	tcctcaaccagcacactcttct	agtgacacgacagtgaggatcaa
Pdk2	GeneID: 103046317	ENSAMXG 00000010438	agatggtgcagagctggtat	aacaacatcgttgtgtcggttct

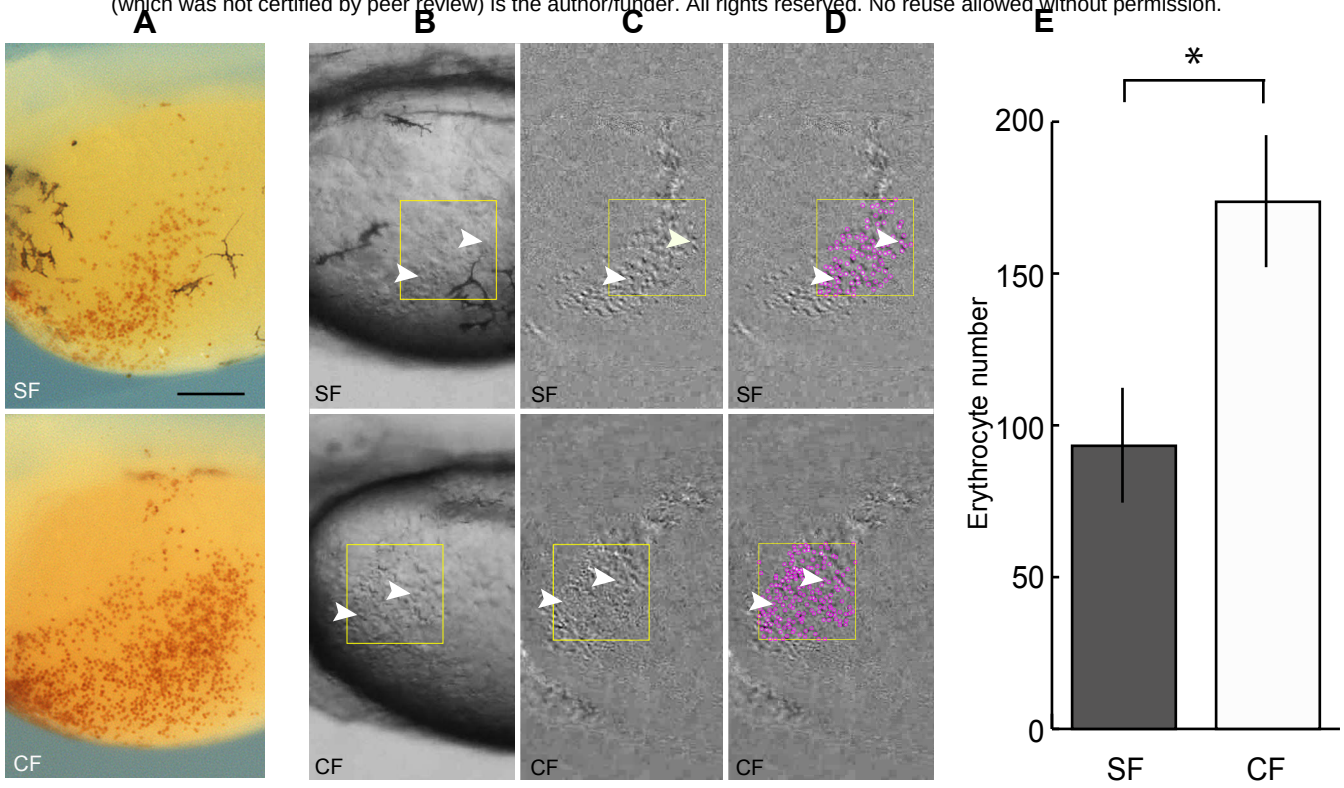
1018

1019 *blasts to Pachon cavefish genome but also cloned from surface fish in this study

1020

1021

1022



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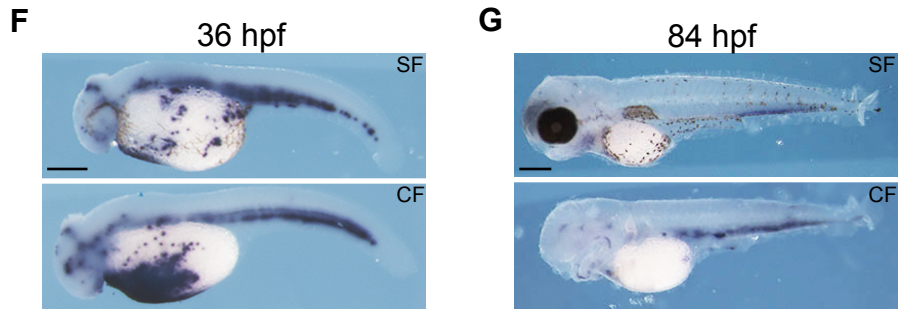

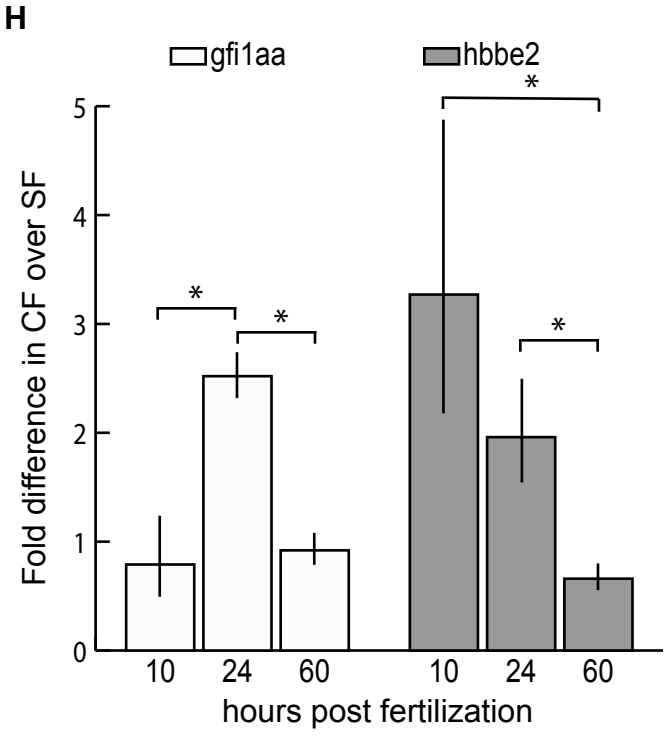

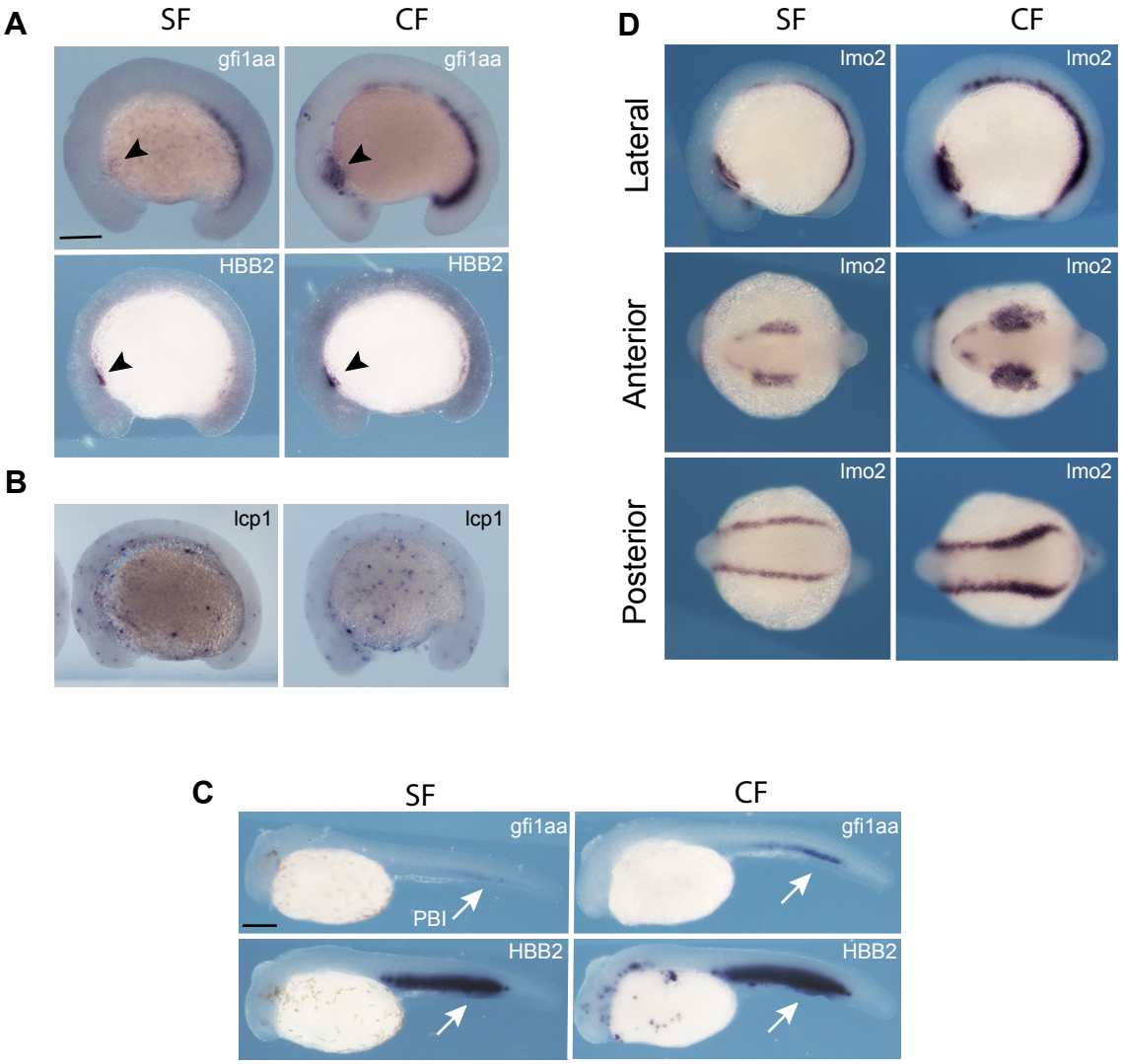

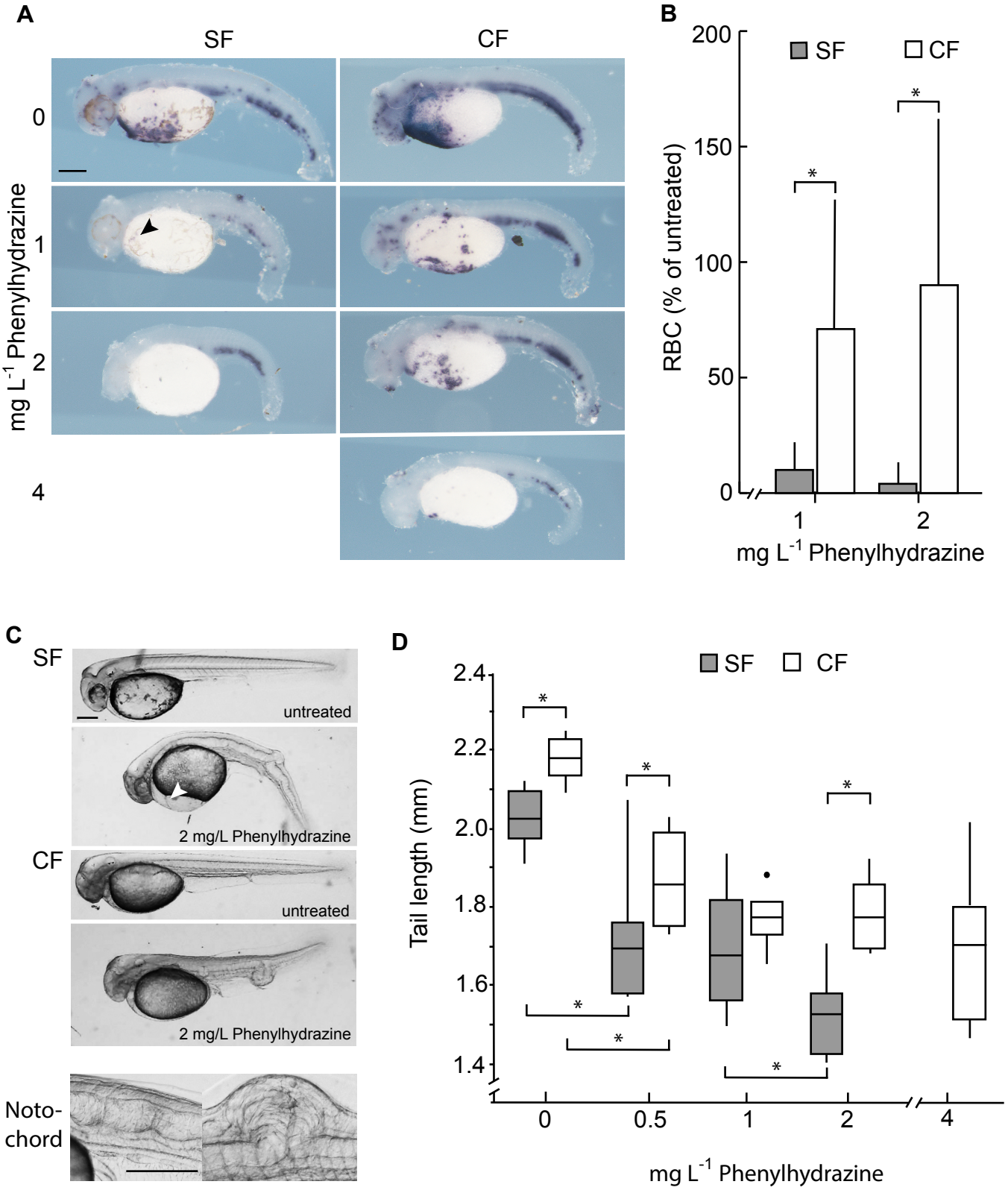

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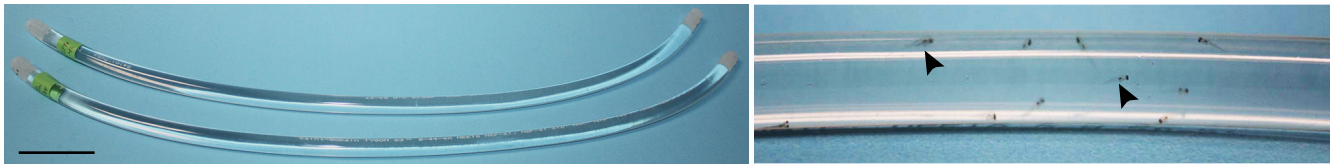

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