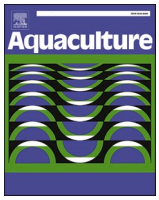
Aquaculture 596 (2025) 741843

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



Changes of hemoglobin resulted by distant hybridization contributed to increased hypoxia tolerance of allotriploid crucian carp

Yuxuan Yang [a,b], Shudong Yang [a,b], Jiao Gong [a,b], Siyao Weng [a,b], Min Tao [a,b], Rong Zhou [a,b,*], Shaojun Liu [a,b,*]

[a] State Key Laboratory of Developmental Biology of Freshwater Fish, Hunan Normal University, Changsha 410081, China
[b] College of Life Sciences, Hunan Normal University, Changsha 410081, China

ARTICLE INFO

Keywords:
Hemoglobin
Hypoxia tolerance ability
Erythrocyte
Distant hybridization
Allotriploid crucian carp

ABSTRACT

Hypoxia tolerance, one of the important goals of fish breeding, is beneficial for aquaculture and the transporting of fish. Based on the bisexual fertile allotetraploid crucian carp hybrid lineage obtained by distant hybridization, we successfully developed the allotriploid crucian carp via interploidy crossing with diploid red crucian carp. This allotriploid fish exhibited obvious hypoxia tolerance with lower values of dissolved oxygen at P_{crit} (critical oxygen press) and 50 % ASR (aquatic surface respiration) than its original parents. Hemoglobin is the main vector of oxygen in vivo. This study examined the changes in amino acid sequences and natural protein conformation of hemoglobin, and found that the Hbα sequences of allotriploid crucian carp displayed obvious hybridity, including recombination and variation, when compared with its original parents. Moreover, the expression of *hbα* gene is higher in the liver and blood of allotriploid crucian carp than in its original parents. After acute anoxia treatment, the expression of *gata1* gene, which served as the general switch for erythroid development, decreased in the blood of all the three kinds of fish. However, only in the allotriploid crucian carp *hbα* gene expression significantly decreased, coupled with the erythrocytes displaying an increased ratio of nuclei/cytoplasm. Taken together, this study suggested that changes in hemoglobin coupled with the erythrocyte morphological alterations under hypoxia increased the hypoxia tolerance ability of allotriploid crucian carp, which would provide a new perspective to guide fish breeding to obtain high-quality fish species with increased hypoxia tolerance.

1. Introduction

Dissolved oxygen (DO) in the aqueous environment, as the primary limiting factor restricting the living state of aquatic animals, is usually changed quickly for the influence of complex factors, such as temperature variation, seasonal change, barometric pressure, and so on (Diaz and Breitburg, 2009). As the aquatic animal, fish should cope with the change of dissolved oxygen to avoid death. Thus, hypoxia tolerance ability is one of the important economic characteristics focused on fish breeding, which is associated with efficient aquaculture and fish transport.

To cope with the challenges caused by a low DO environment, fish exhibit behavior changes observed easily to increasing oxygen intake and reducing oxygen consumption, including but not limited to the floating head phenomenon, slowing down movement (Herbert et al., 2011; Wannamaker and Rice, 2000). The lasted hypoxia conditions even influenced the growth and reproduction of fish (Bera et al., 2020; Claireaux and Chabot, 2016). Moreover, a series of physiological and biochemical reactions occurred in fish to adapt hypoxia environment, including an altered energy metabolism state, disruption of erythrocyte biogenesis, oxidative stress, and decreased immune function (Bickler and Buck, 2007; Wang et al., 2023). The hypoxia-related genes up-regulated or down-regulated their expression to respond to the external low DO concentration (Fang et al., 2010). The HIF signaling pathway was the major pathway in previous hypoxia stress studies (Xiao, 2015).

As the oxygen carrier, the erythrocyte is directly involved in the internal gas transport. The erythrocyte in fish is ellipsoidal nucleated cells of different sizes, which has an inverse relationship with aerobic swimming ability (Lay and Baldwin, 1999; Witeska, 2013). Also, the number of erythrocytes in fish is strongly correlated with environmental DO concentration (Ahmed et al., 2020). Hemoglobin, which is the main

* Corresponding authors at: State Key Laboratory of Developmental Biology of Freshwater Fish, Hunan Normal University, Changsha 410081, China.
E-mail addresses: rongzhou@hunnu.edu.cn (R. Zhou), lsj@hunnu.edu.cn (S. Liu).

https://doi.org/10.1016/j.aquaculture.2024.741843
Received 7 July 2024; Received in revised form 4 October 2024; Accepted 30 October 2024
Available online 3 November 2024

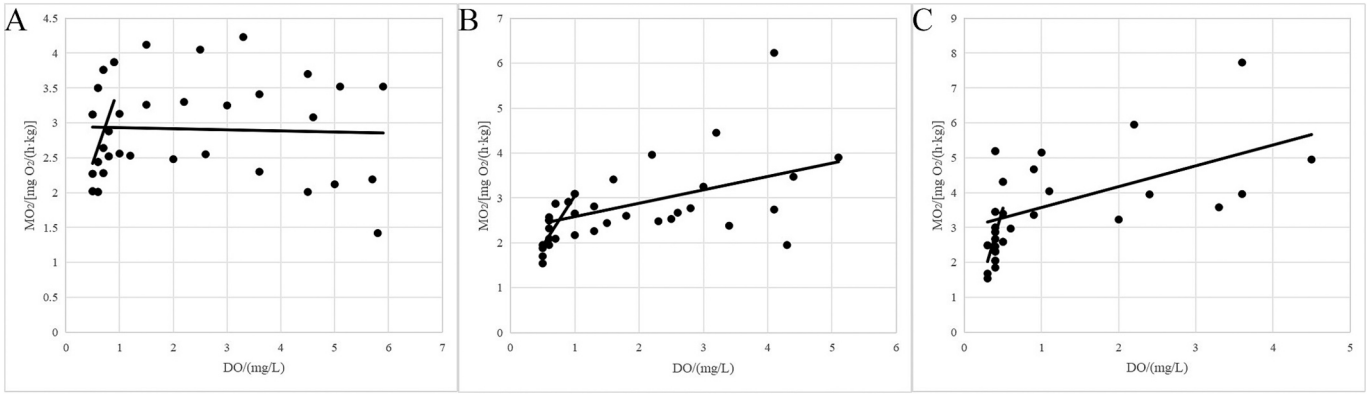


Fig. 1. The value of DO at Pcrit of CC (A), RCC (B) and 3*n* fish (C). CC P_{crit} is 0.77 ± 0.30 mg/L, RCC P_{crit} is 0.83 ± 0.17 mg/L, 3*n* P_{crit} is 0.46 ± 0.03 mg/L.

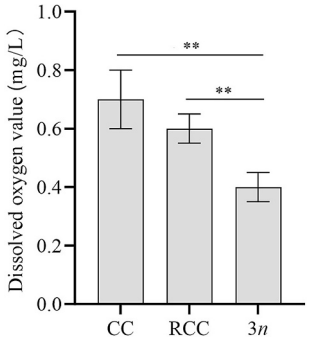


Fig. 2. The value of DO at 50 % ASR of 3*n* fish, RCC and CC. ** means $p < 0.01$.

component of erythrocytes, is a globin heterotetramer composed by two hemoglobin α subunits and two hemoglobin β subunits, in which each subunit includes a protein and a heme (De Souza and Bonilla-Rodriguez, 2007). Thus, as the main vector of oxygen, the spatial conformation and concentration of hemoglobin would influence the oxygen binding affinity and transporting ability (Wells, 2009). Under the survival challenge of low oxygen, the crucian carp increased its erythrocyte counts and improved the oxygen-combination ability of hemoglobin (Sun et al., 2012).

Distant hybridization combines two sets of genomes into one, thus resulting in lots of recombination and variation. The above genetic change would lead to phenotypic change (Chen et al., 2018). Thus, distant hybridization is one of the important fish breeding technology for resulting offspring with obvious advantages (Wang et al., 2019). The first bisexual fertile allotetraploid fish was produced by crossing crucian carp and common carp (abbreviated as CC) (Liu et al., 2001). Based on this hybrid lineage, interploid hybridization between the diploid red crucian carp (abbreviated as RCC) and allotetraploid fish was performed to obtain the allotriploid crucian carp (abbreviated as 3*n* fish) (Chen et al., 2009). This allotriploid fish exhibited a fast growth rate, infertile reproductive characteristics, disease-resistant ability, also obvious hypoxia-tolerance ability in practical aquaculture processes (Liu et al., 2022).

The phenotypic characteristics of erythrocytes of different ploidy fishes have been studied (Liu et al., 2004), while no study reported the relationship between hemoglobin and hypoxia tolerance ability in 3*n* fish. Here, in this study, we first compared the sequence alignment difference and protein natural conformation of hemoglobin in 3*n* fish and its original parents, and found that both displayed obvious recombination and variation. Then, we focused on examining the gene expression of *hbα* and phenotypic characteristics of erythrocytes before and after acute anoxia treatment. The *hbα* gene expressed more in 3*n* fish and exhibited more significant change after acute anoxia. Moreover, the erythrocytes of 3*n* fish displayed a more obvious change in the ratio of nuclei to cytoplasm. Taken together, our results suggested that the multiplicity and expression of hemoglobin and erythrocyte phenotype all might directly contribute to the hypoxia resistance ability of 3*n* fish. This study would provide a new perspective to guide fish breeding to obtain high-quality fish species with increased hypoxia tolerance.

2. Methods and materials

2.1. Ethics statement

The guidelines established by the Administration of Affairs Concerning Animal Experimentation state that approval from the Science and Technology Bureau of China and the Department of Wildlife Administration is not necessary when the fish in question are neither rare nor near extinction (first- or second-class state protection level). Therefore, approval was not required for the experiments conducted in this study.

2.2. Animals

The experimental fishes (RCC, CC, and 3*n* fish) referred to in this study were raised in the Engineering Research Center of Polyploid Fish Breeding and Reproduction of the State Education Ministry at Hunan Normal University. All fishes were deeply anesthetized with 100 mg/L MS-222 (Sigma-Aldrich, St. Louis, MO, USA) before dissection.

2.3. Molecular cloning of *hbα* and *hbβ*

Based on the genomic information of RCC and CC, specific primers were designed to amplify and obtain the open reading frame (ORF) sequences of *hbα* and *hbβ* genes in RCC, CC, and 3*n* fish (Table S1). The blood cDNA of different fishes was used as templates in the experiments of molecular cloning.

2.4. Multiple sequence alignment and phylogenetic analysis

The protein sequences were assembled using the program MEGA, version 11. Multiple sequence alignment analysis was carried out using the program CLUSTAL W. Model-based phylogeny reconstructions were performed with concatenated sequence alignments using the neighbor-joining (NJ), using the program MEGA, version 11. And the neighbor-joining bootstrap analyses consisted of 1000 replicates.

2.5. Real-time PCR (RT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen), and then reverse transcribed with oligo (dT) primers using MMLV reverse

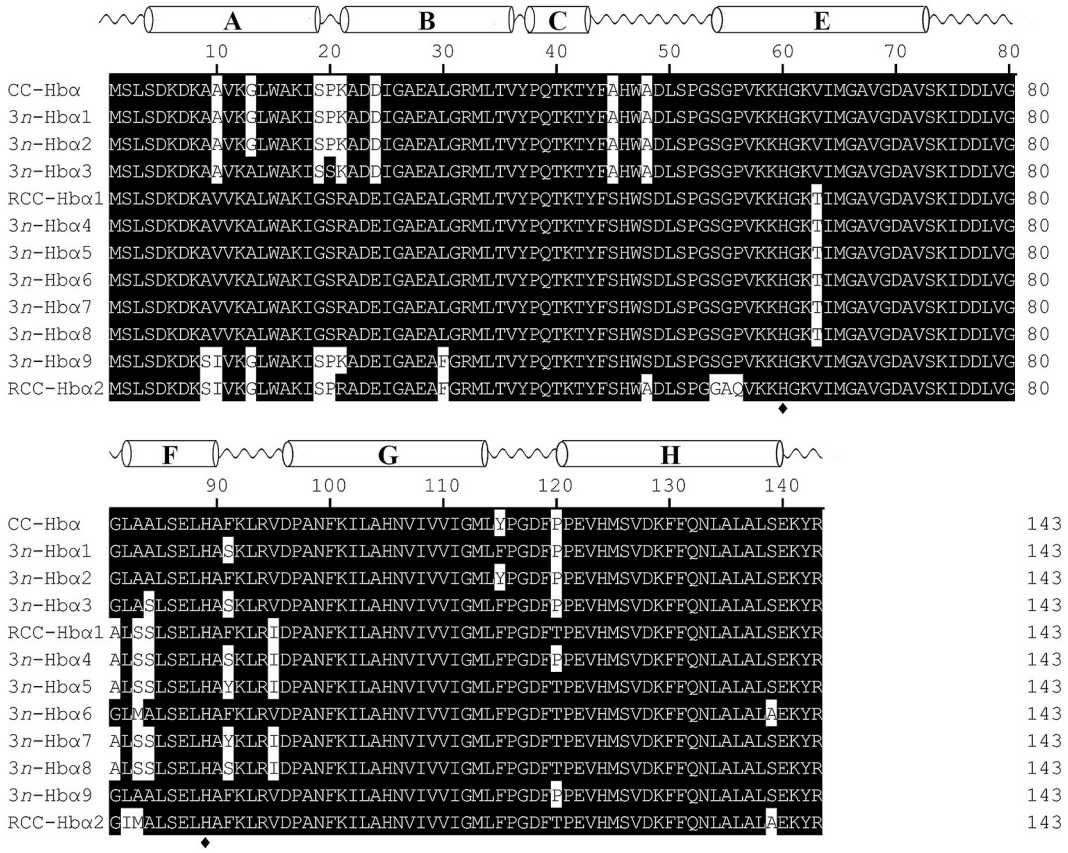


Fig. 3. Alignment of the amino acid sequences of Hbα between allotriploid crucian carp (3*n* fish) and its original parents (RCC and CC). A–H indicate the predicated α-helical contents of the subunits. The solid diamonds represent the heme-oxygen binding sites.

transcriptase (TaKaRa). The RT-PCR was performed using PowerUp™ SYBR Green master Mix(Thermo Scientific™)on QuantStudio™ 5 Real-time PCR instrument (Thermo Fisher Scientific, MA, USA) with gene-specific primers (Table S1). *β-actin* served as the internal reference. The relative mRNA amounts were analyzed by the comparative CT method as described by Schmittgen and Livak (Schmittgen and Livak, 2008).

2.6. Hemoglobin extraction

Hemoglobin was extracted from the blood of CC, RCC, and 3*n* fish, respectively, as previously described (Bao et al., 2013). Briefly, blood, mixed with acid-citrate-dextrose solution (ACD) at a volume ratio of 1:10, was centrifuged at 3000*g* for 10 min at 4 °C. The blood cell precipitate was washed with 0.9 % NaCl solution, and centrifuged. Then, add the same volume of water to the precipitation and vortex. The hemoglobin crude extract was the supernatant obtained after centrifuging at 12, 000*g* for 10 min at 4 °C. The protein concentration was quantified by the Modified Bradford Protein Assay Kit (Sangon Biotech).

2.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The same amounts of hemoglobin extracts from different fishes were mixed with 5 × SDS-loading buffer (Beyotime Biotechnology) and denatured by incubation at 100 °C for 10 min. Then, the denatured protein samples were loaded and separated on 15 % SDS-PAGE gel at a constant current of 20 mA. After electrophoresis, the gel was stained by Coomassie brilliant blue solution.

2.8. Native-polyacrylamide gel electrophoresis (native-PAGE)

The same amounts of hemoglobin extracts from different fishes were mixed with 5 × native loading buffer (Beyotime Biotechnology), and then separated with 4 % stacking gel and 8 % separating gel at a constant voltage of 100 V in the ice bath. Until the tracking dye migrated to the bottom, the electrophoresis stopped, and the protein bands could be observed directly on the gel without staining.

2.9. Hypoxia indexes measurement and acute anoxia treatment

A sealed fish tank (4.2 L), full with 3 L of water. An oxygen electrode (SMART, AR8406, China) was used to monitor [O_2] every 10 min. When a fish showed aquatic surface respiration (ASR), the [O_2] and time were recorded. The experiment was repeated to obtain 50 %*ASR*. The metabolic rate MO_2[mg/(kg·h)] was calculated according to the changes of body weight and [O_2]:

$$MO_2 = \left([O_2]_{t1} - [O_2]_{t2}\right) \times \frac{V}{t} \times \frac{1}{\mathrm{BW}}$$

where $[O_2]_{t1}$ is the dissolved oxygen level at the monitoring point *t1*; $[O_2]_{t2}$ is the dissolved oxygen level at the monitoring point *t2*; *V* is the total volume of the tank minus the volume of the fish; *t* is the interval between time points *t1* and *t2* (10 min); BW is the body weight of the experimental fish (kg). P_{crit} represents the dissolved oxygen level in water when the fish cannot maintain its metabolic rate with the decrease of dissolved oxygen level. This value is inversely proportional to the low oxygen tolerance of the fish. The metabolic rate and dissolved oxygen level were plotted, and the two-segmented straight lines were used to find the significant turning point of MO_2 decline with the decrease of dissolved oxygen level, which was P_{crit} (Rogers et al., 2016).

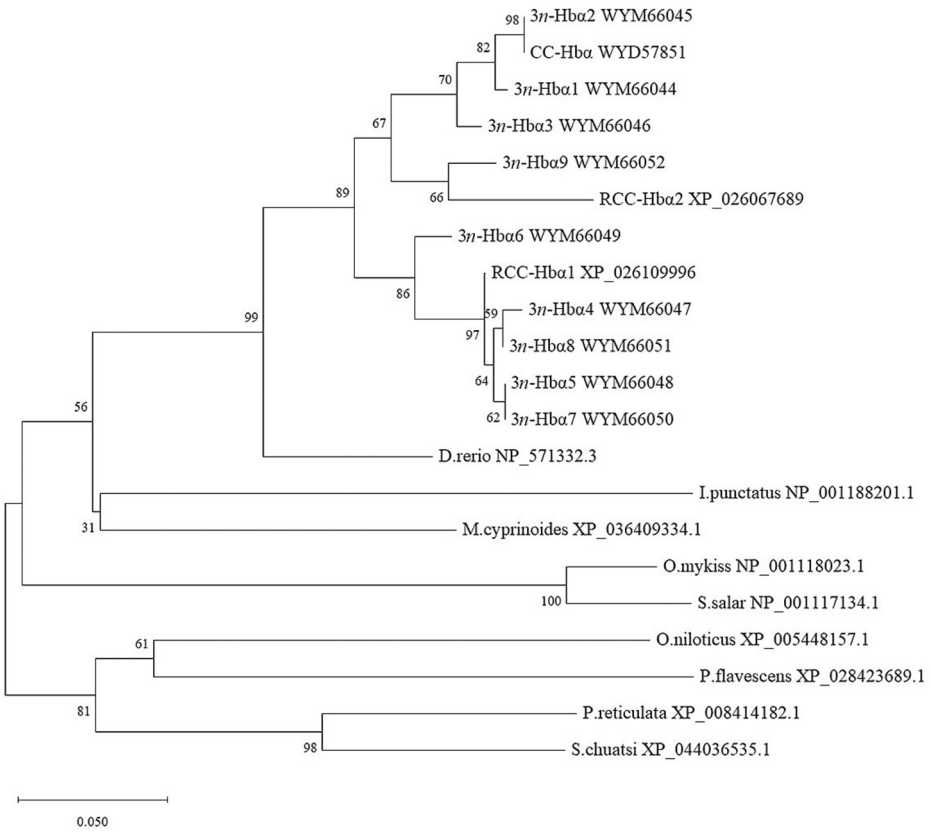


Fig. 4. Phylogenetic analysis of the amino acid sequences of Hbα from multiple species. GenBank accession numbers of the sequences used in this analysis are listed in the figure.

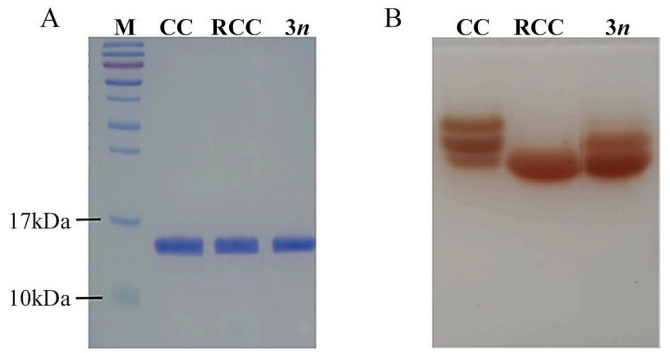


Fig. 5. SDS-PAGE (A) and native-PAGE (B) of total hemoglobin extracted from blood of 3*n* fish and its original parents (RCC and CC). M means marker.

For acute anoxia treatment, a sealed fish tank (3 L), full of water, was injected nitrogen continuously to replace oxygen in the water, which coupled with the DO value change from 6.2 mg/L to 0.2 mg/L in 15 min. Observe the behavior of fish. The blood of asphyxiated fish was extracted without ACD for RNA extraction, and with ACD for the following red blood cell count and blood smear.

2.10. Red blood cell count and blood smear

Red blood cell (RBC) count was determined with a blood cell counting plate according to standard methods (Dacie and Lewis, 1991). Blood smear was performed as previously described (Liu et al., 2004). Briefly, the blood was diluted with 0.9 % NaCl solution at a ratio of 1:4. 10 μL of blood sample was dropped on the slide and quickly smeared evenly and dried. The slides were stained with Wright Stain solution (Solarbio)for 3 min, and then treated with phosphate buffer for 5 min. After staining, wash the slides with water and dry them at room temperature for observation. The morphology of erythrocytes was examined and measured by Image J.

2.11. Statistical analysis

All data, expressed as means ± SD from three independent experiments, were analyzed by one-way ANOVA. Significant differences were confirmed by the Student's *t*-test using SPSS 13.0 software. *P*-values <0.05 (*) indicate statistical difference and $p < 0.01$ (**) means significant difference.

3. Results

3.1. Hypoxia tolerance ability of allotriploid crucian carp

In practical aquaculture, the allotriploid crucian carp exhibited high hypoxia tolerance. Here, we employed the hypoxia indexes, P_{crit} and 50 %ASR, to compare the hypoxia tolerance ability of 3*n* fish with their original parents, RCC and CC. According to the results of two-segmented straight lines, the DO value at P_{crit} of 3*n* fish was 0.46 ± 0.03 mg/L, which was significantly lower than RCC (0.83 ± 0.17 mg/L) and CC (0.77 ± 0.30 mg/L) (Fig. 1). The DO values at 50 %ASR of 3*n* fish displayed similar variation, lower than RCC and CC obviously (Fig. 2). The above two indexes revealed that 3*n* fish exhibited more excellent hypoxia tolerance ability than their original parents.

3.2. Amino acid sequences analysis of Hbα and Hbβ

Based on the genome information and existing transcriptome of RCC and CC, we cloned the complete ORF of *hbα* gene in the two original

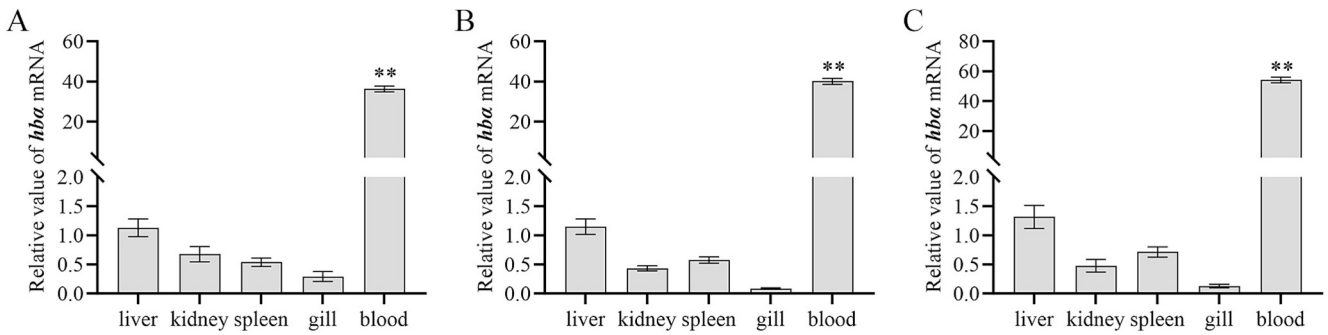


Fig. 6. The relative gene expression of *hbα* detected by real-time PCR in different tissues of CC (A), RCC (B) and 3*n* fish (C). The mRNA amounts were normalized to those of *β-actin* mRNA. Data are represented as mean ± SE of $n = 3$ independent repeats. Statistical analyses of the differences between expression levels in the two pathways were performed by two-tailed, paired Student's *t*-tests. ** represented $p < 0.01$.

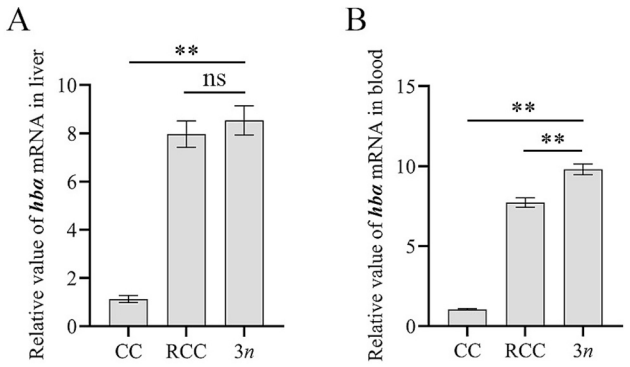


Fig. 7. The relative gene expression of *hbα* in the liver (A) and blood (B) of three kinds of fishes. The mRNA amounts were normalized to those of *β-actin* mRNA. Data are represented as mean ± SE of n = 3 independent repeats. Statistical analyses of the differences between expression levels in the two pathways were performed by two-tailed, paired Student's t-tests. NS represented $p > 0.05$, ** represented $p < 0.01$.

Table 1
The number of erythrocytes before and after asphyxiation.

Species	No. of erythrocytes ($10^{12}L^{-1}$)	
	before	after
CC	2.02 ± 0.32	1.83 ± 0.43
RCC	2.24 ± 0.52	2.16 ± 0.40
3*n* fish	1.12 ± 0.07*	0.91 ± 0.09*

Note: * represented significant difference.

parental fishes. There are two kinds of *hbα* gene sequences in RCC (named RCC- *hbα*1 and RCC- *hbα*2), and one in CC (named CC- *hbα*). All *hbα* genes encoded 143 amino acids. The amino acid sequence similarity between RCC-Hbα1 and CC-Hbα is 89.5 %, while 90.9 % between RCC-Hbα2 and CC-Hbα. We further cloned *hbα* gene sequences in 3*n* fish and obtained 9 sequences (named 3*n*-*hbα*1 to 3*n*-*hbα*9), which all displayed hybridity (Fig. 3). Phylogenetic analysis revealed that 4 Hbα sequences of 3*n* fish clustered with CC-Hbα, 4 sequences clustered with RCC-Hbα1, and 1 sequence clustered with RCC-Hbα2 (Fig. 4). All sequences of crucian carp are closely related to the zebrafish sequence.

Moreover, we cloned ORF sequences of *hbβ* gene in the two original parental fishes, named RCC-*hbβ* and CC-*hbβ*, which both encoded 148 amino acids. The similarity of the above two amino acid sequences is 98.0 %. We obtained 10 sequences (named 3*n*-*hbβ*1 to 3*n*-*hbβ*10) of the *hbβ* gene in 3*n* fish via molecular cloning, which displayed obvious hybridity (Fig. S1). Phylogenetic analysis revealed that 5 Hbβ sequences of 3*n* fish clustered with CC-Hbβ, 5 sequences clustered with RCC-Hbβ (Fig. S2). All Hbβ sequences of crucian carp are closely related to the zebrafish sequence.

3.3. Natural protein conformation of hemoglobin

Protein conformation is the main factor influencing the hemoglobin-oxygen affinity. We further examined the protein characteristics of hemoglobin via SDS-PAGE and non-denature PAGE. According to the amino acid sequences of Hbα and Hbβ, the probable molecular weight of hemoglobin protein was deduced and summarized in Table S2. All hemoglobin protein molecular weight is distributed in a narrow area (15.3 kD to 16.4 kD). The result of SDS-PAGE showed that the hemoglobin extract of all the three kinds of fishes is a broadband concentrated near 16kD (Fig. 5A). However, results of non-denature PAGE showed differences of hemoglobin in different fishes (Fig. 5B). Hemoglobin of CC displayed three protein natural conformation, while RCC had only one conformation. Different from its original parents, the hybrid 3*n* fish showed obvious recombination characteristics, including three bands, among which, the top one was very weak, the medial one was similar to the medial band of CC hemoglobin, and the below one was similar to RCC hemoglobin band.

3.4. Expression patterns of hbα gene

For the significant sequence difference of the *hbα* gene between 3*n* fish and its original parent fishes, we are further mainly concerned with this gene. The tissue expression patterns in one fish and expression differences in different fish were examined by real-time PCR. The results revealed that in the three kinds of fish *hbα* gene displayed similar tissue expression patterns. *Hbα* gene was expressed highest in the blood, followed by the liver, and lowest in the gill (Fig. 6). Moreover, we compared *hbα* gene expression in the blood and liver of the three fishes. In the two tissues, the *hbα* gene expressed highest in 3*n* fish and lowest in CC (Fig. 7).

3.5. Erythrocyte morphological and molecular alterations after hypoxia treatment

To compare the cellular and molecular alterations after hypoxia, the blood of fish was sampled after suffocation by acute anoxia treatment for the following examination. All the three fishes displayed decreased erythrocyte numbers after asphyxia (Table 1). Moreover, the erythrocyte numbers of RCC and CC were similar, about two times those of 3*n* fish. Then, we detected the erythrocyte morphological characteristics of the three fishes before and after hypoxia treatment by blood smear and wright staining (Fig. 8). In 3*n* fish, the proportion of normal erythrocytes decreased from 91.72 % to 89.95 % after asphyxiation. The proportion of dumbbell-shaped erythrocytes increased from 4.85 % to 6.12 %. The proportion of teardrop-shaped erythrocytes increased from 3.43 % to

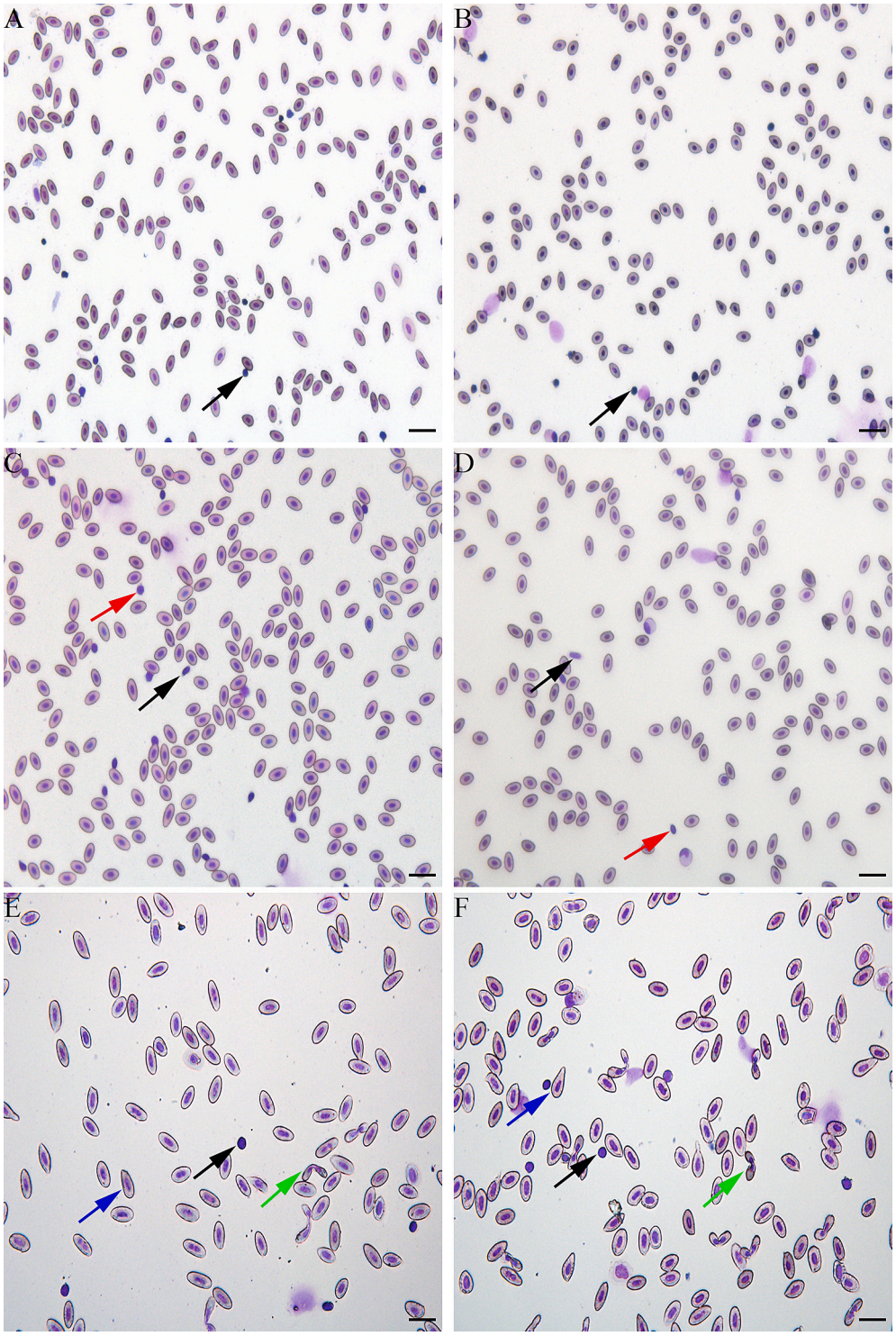


Fig. 8. Blood cell morphology of different fishes observed by microscopy before or after anoxia treatment. (A) blood cell of RCC before anoxia; (B) blood cell of RCC after anoxia; (C) blood cell of CC before anoxia; (D) blood cell of CC after anoxia; (E) blood cell of 3*n* fish before anoxia; (F) blood cell of 3*n* fish after anoxia. Black arrow, thrombus cells; red arrow, small lymphocytes; green arrow, dumbbell-shaped erythrocytes; blue arrow, teardrop-shaped erythrocytes. Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.93 %. The long diameter and short diameter of the erythrocyte cytoplasm and nuclei of the three fishes were measured before and after hypoxia (Table S3). Statistic analysis revealed that the long diameter ratio and the short diameter ratio of erythrocyte cytoplasm to nuclei increased in 3*n* fish, while unchanged in RCC and CC (Table 2). Gene expression of *gata1*, a general switch factor for erythroid development, decreased in the blood of all the three fishes after hypoxia treatment (Fig. 9A). *Hbα* gene exhibited different expression patterns (Fig. 9B). First, the gene expression of *hbα* expressed highest in the blood of 3*n* fish, when compared with its original parents. Second, *hbα* gene expression decreased significantly in the blood of 3*n* fish after hypoxia treatment, while displaying no apparent alterations in RCC and CC.

Table 2
Comparison of red blood cell diameters before and after asphyxiation.

Species	Long diameters ratio(nuclei/cytoplasm)		Short diameters ratio(nuclei/cytoplasm)	
	before	after	before	after
CC	0.401 ± 0.011	0.395 ± 0.01	0.43 ± 0.014	0.425 ± 0.007
RCC	0.463 ± 0.008	0.464 ± 0.013	0.456 ± 0.01	0.438 ± 0.009
3*n* fish	0.462 ± 0.013	0.501 ± 0.009*	0.399 ± 0.008	0.46 ± 0.007*

Note: * represented significant difference.

4. Discussion

Dissolved oxygen is an important factor that influences the growth and survival of aquatic animals. Coupled with the increased demands of aquatic production, an intensive culture model was performed to improve production and save resources. Dissolved oxygen is one of the main restrictive factors. The 3*n* fish obtained by distant hybridization and interploidy hybridization exhibited obvious hypoxia tolerance. The hypoxia indexes, P_{crit} and 50 %ASR, of 3*n* fish were lower than their original parents, RCC and CC. This fish provided a perfect model to study the relationship between hybridization and hypoxia resistance. We explored the mechanism of hypoxia resistance of 3*n* fish from phenotypic and genetic analysis via comparing with its original parental fishes, RCC and CC.

The multiplicity of hemoglobin genes in fish has been reported in the teleost fish via screening genomic information due to the whole genome duplication (Opazo et al., 2013). However, there are not too many isoforms co-expressed in adult fish except rainbow trout (*Oncorhynchus mykiss*) co-expressed nine Hb isoforms (Fago et al., 2001), and adult schizothoracinae fish co-expressed five isoforms in the red blood cells (Lei et al., 2021b). Here, we cloned the hemoglobin gene sequence from the cDNA of adult fish blood. One isoform of *hbα* gene was cloned in CC and two isoforms in RCC, while nine isoforms in 3*n* fish. One isoform of *hbβ* gene was cloned both in CC and RCC, while ten isoforms in 3*n* fish. From the results of sequence alignments, parts of the isoforms in 3*n* fish are the same as their original parents, some showed recombination, and some showed variations, which revealed the genetic and variable properties of the RCC × CC hybrids resulted from distant hybridization leading to genomic shocking (Liu et al., 2016). The multiplicity of hemoglobin genes co-expressed in the adults of 3*n* fish might provide multiple distinct structural and functional hemoglobin to cope with different environments.

The Root effect is a functional property of fish hemoglobin found until now only in teleost species (De Souza and Bonilla-Rodriguez, 2007). Sequence alignments revealed that amino acids involved in heme-oxygen binding, Root effect, and nitric oxide transport were conserved in all the hemoglobin gene variants of 3*n* fish when compared to its original parents (Lei et al., 2021a). However, the multipolicy variants of hemoglobin with amino acid hydrophobic property change at different sites made the variation of heme-pocket surface, volume, depth, and hydrophobicity of hemoglobin combined with different subunits. Especially, there is a mutated site in Hbα (αF91 mutated to S or Y). This mutation site is at the edge of the heme-pocket (Fig. S3). Moreover, the hydrophobicity of mutated amino acids decreased, which might influence the oxygen binding capacity and stability.

Consistent with the change of amino acid sequences of Hb, the natural protein conformation revealed by the natural protein PAGE, included the protein conformation of its original parents, but was not equal to the simple addition of parental protein conformation. The result of electrophoresis displayed that the relative proportion of the three conformations changed, which might be matched with the characteristic of higher hypoxia tolerance ability of 3*n* fish for including more native conformation.

The erythrocytes' shape evolved in teleost fish in response to different environmental selective pressures (Martins et al., 2021). The volume of erythrocytes in allotriploid crucian carp is bigger than its diploid original parents (Liu et al., 2004). Therefore, the number of erythrocytes in equal volume in 3*n* fish was about half of that in RCC and CC. Moreover, the phenotype of erythrocytes in 3*n* fish is more. Except for the usual circle pattern, there are dumbbell-shaped, teardrop patterns, and pear-shaped erythrocytes (Liu et al., 2004). The multiple phenotypes and bigger volume might provide more buffer space for maintaining the combination affinity of hemoglobin and oxygen. Usually, in hypoxic conditions, there is also an increase in cell volume, which results from the deoxygenation of hemoglobin and consequent decrease in its negative charge (Boggs et al., 2022). Furthermore, the change in the ratio of nuclei to cytoplasm suggested the change of cell metabolism and cell proliferation (Balachandra et al., 2022). Here, in response to hypoxia, the 3*n* fish changed the ratio of nuclei to cytoplasm, making it bigger. However, its original parents could not change the phenotype and cell characteristics to respond to hypoxia.

Hb transgenic zebrafish has been produced and found that the transgenic fish exhibited higher hypoxia tolerance when compared to wild-type zebrafish (Guan et al., 2011). Moreover, levels of the *hbα* gene reduced under severe hypoxia in zebrafish (Roesner et al., 2006). In this experiment, the expression of the *hbα* gene in 3*n* fish is higher than its original parents. When hypoxia, the *gata1* gene, which regulated the genesis of erythrocytes, expressed abnormally to regulate the number and development of erythrocytes (Lyons et al., 2002). All the cyprinid fishes in this study showed an abnormal decrease in *gata1* gene expression. The expression of *hbα* decreased in 3*n* fish, while displaying no significant change in RCC and CC. Moreover, the decreased *hbα* expression was still higher in 3*n* fish than in RCC and CC, which might suggest that the higher expression of the *hbα* gene contributed to the

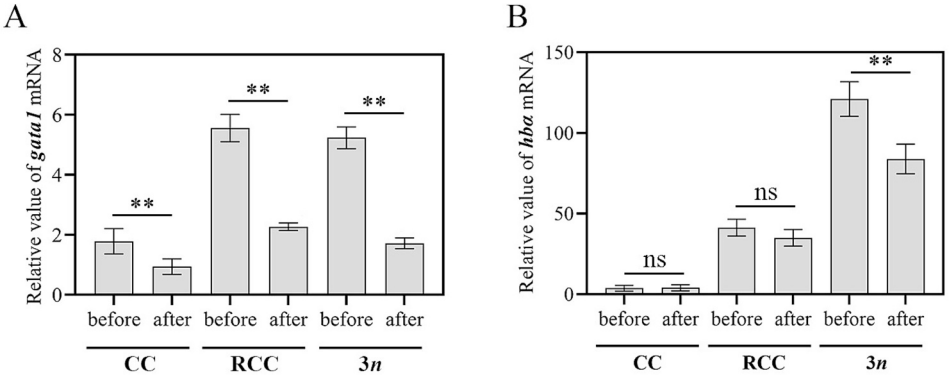


Fig. 9. Gene expression of *gata*1 (A) and *hbα* (B) in the blood of different fishes before and after acute anoxia treatment. The mRNA amounts were normalized to those of *β-actin* mRNA. Data are represented as mean ± SE of n = 3 independent repeats. Statistical analyses of the differences between expression levels in the two pathways were performed by two-tailed, paired Student's t-tests. Ns represented $p > 0.05$, ** represented $p < 0.01$.

hypoxia tolerance ability.

5. Conclusion

Taken together, in this study, we analyzed the genetic and phenotypic changes of hemoglobin genes and erythrocytes, respectively, and found that the hybridization made the hemoglobin exhibit multiple variants and conformation changes to provide a genetic basis for increased hypoxia tolerance ability in 3*n* fish. This study provided a new perspective to guide fish breeding to obtain high-quality fish species with increased hypoxia tolerance.

Author statement

YuxuanYang: performed the experiments, analyzed the data, drafted the manuscript. Shudong Yang: performed the experiments. Jiao Gong: performed the experiments. Siyao Weng: performed the experiments. Min Tao: formal analysis. Rong Zhou: conceived and designed the experiments, analyzed the data, paper revision. Shaojun Liu: conceived and designed the experiments, paper revision.

CRediT authorship contribution statement

Yuxuan Yang: Writing – original draft, Validation, Data curation. **Shudong Yang:** Validation. **Jiao Gong:** Validation. **Siyao Weng:** Validation. **Min Tao:** Formal analysis. **Rong Zhou:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Shaojun Liu:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant No. 32293253, 32293252); National Natural Science Foundation of China (Grant No. 31402297); the earmarked fund for Agriculture Research System of China (Grant No. CARS-45); the Research Foundation of Education Bureau of Hunan Province (Grant No. 23A0094); the Natural Science Foundation of Hunan Province (Grant No. 2022JJ30385).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.741843.

References

Ahmed, I., Reshi, Q.M., Fazio, F., 2020. The influence of the endogenous and exogenous factors on hematological parameters in different fish species: a review. Aquac. Int. 28, 869–899.

Balachandra, S., Sarkar, S., Amodeo, A.A., 2022. The nuclear-to-cytoplasmic ratio: coupling DNA content to cell size, cell cycle, and biosynthetic capacity. Annu. Rev. Genet. 56, 165–185. https://doi.org/10.1146/annurev-genet-080320-030537.

Bao, Y., Yang, T., Wang, S., Wang, C., You, Z., Jiao, H., Lin, Z., 2013. Hemoglobin and its derived peptides from blood clam (Tegillarca granosa) exhibiting an antimicrobial activity. Fish Shellfish Immunol. 34 (1696–1696).

Bera, A., Chadha, N.K., Dasgupta, S., Chakravarty, S., Sawant, P.B., 2020. Hypoxia-mediated inhibition of cholesterol synthesis leads to disruption of nocturnal sex steroidogenesis in the gonad of koi carp, Cyprinus carpio. Fish Physiol. Biochem. 46, 2421–2435.

Bickler, P.E., Buck, L.T., 2007. Hypoxia tolerance in reptiles, amphibians, and fishes: life with variable oxygen availability. Annu. Rev. Physiol. 69, 145–170.

Boggs, T.E., Friedman, J.S., Gross, J.B., 2022. Alterations to cavefish red blood cells provide evidence of adaptation to reduced subterranean oxygen. Sci. Rep. 12, 3735.

Chen, S., Wang, J., Liu, S., Qin, Q., Xiao, J., Duan, W., Luo, K., Liu, J., Liu, Y., 2009. Biological characteristics of an improved triploid crucian carp. Sci China C Life Sci 52, 733–738.

Chen, J., Luo, M., Li, S., Tao, M., Ye, X., Duan, W., Zhang, C., Qin, Q., Xiao, J., Liu, S., 2018. A comparative study of distant hybridization in plants and animals. Sci. China Life Sci. 61, 285–309.

Claireaux, G., Chabot, D., 2016. Responses by fishes to environmental hypoxia: integration through Fry's concept of aerobic metabolic scope. J. Fish Biol. 88, 232–251.

Dacie, J.V., Lewis, S.M., 1991. Practical Haematology, Seventh ed. Churchill Livingstone, London.

De Souza, P., Bonilla-Rodriguez, G.O., 2007. Fish hemoglobins. Braz. J. Med. Biol. Res. 40, 769–778.

Diaz, R.J., Breitburg, D.L., 2009. The hypoxic environment. In: Fish Physiology. Elsevier, pp. 1–23.

Fago, A., Forest, E., Weber, R., 2001. Hemoglobin and subunit multiplicity in the rainbow trout (Oncorhynchus mykiss) hemoglobin system. Fish Physiol. Biochem. 24, 335–342.

Fang, S., Liu, D., Li, Z., Zou, G., Wang, G., Tian, R., 2010. Express and regulation of animal anti-hypoxia stress-related genes. China Biotechnol. 30, 79–85.

Guan, B., Ma, H., Wang, Y., Hu, Y., Lin, Z., Zhu, Z., Hu, W., 2011. Vitreoscilla hemoglobin (VHb) overexpression increases hypoxia tolerance in zebrafish (Danio rerio). Mar. Biotechnol. 13, 336–344.

Herbert, N.A., Skjæraasen, J.E., Nilsen, T., Salvanes, A.G., Steffensen, J.F., 2011. The hypoxia avoidance behaviour of juvenile Atlantic cod (Gadus morhua L.) depends on the provision and pressure level of an O 2 refuge. Mar. Biol. 158, 737–746.

Lay, P.A., Baldwin, J., 1999. What determines the size of teleost erythrocytes? Correlations with oxygen transport and nuclear volume. Fish Physiol. Biochem. 20, 31–35.

Lei, Y., Yang, L., Jiang, H., Chen, J., Sun, N., Lv, W., He, S., 2021a. Recent genome duplications facilitate the phenotypic diversity of Hb repertoire in the Cyprinidae. Sci. China Life Sci. 64, 16.

Lei, Y., Yang, L., Zhou, Y., Wang, C., Lv, W., Li, L., He, S., 2021b. Hb adaptation to hypoxia in high-altitude fishes: fresh evidence from schizothoracinae fishes in the Qinghai-Tibetan plateau. Int. J. Biol. Macromol. 185, 471–484.

Liu, S., Liu, Y., Zhou, G., Zhang, X., Luo, C., Feng, H., He, X., Zhu, G., Yang, H., 2001. The formation of tetraploid stocks of red crucian carp× common carp hybrids as an effect of interspecific hybridization. Aquaculture 192, 171–186.

Liu, Q., Wang, Y., Liu, S., Guo, X., Luo, K., Zhang, C., Liu, Y., 2004. Comparative analysis of blood and blood cell of different ploidy crucian carp X common carp. Prog. Nat. Sci. 14, 1111–1117.

Liu, S., Luo, J., Chai, J., Ren, L., Zhou, Y., Huang, F., Liu, X., Chen, Y., Zhang, C., Tao, M., 2016. Genomic incompatibilities in the diploid and tetraploid offspring of the goldfish× common carp cross. Proc. Natl. Acad. Sci. 113, 1327–1332.

Liu, S., Tao, M., Zhou, Y., Zhou, R., Cui, J., Huang, L., Wang, S., Wang, Q., Zhao, R., Tang, C., 2022. The basic biological characteristics of sterile allotriploid fish. In: Fish Distant Hybridization. Springer, pp. 71–102.

Lyons, S.E., Lawson, N.D., Lei, L., Bennett, P.E., Weinstein, B.M., Liu, P.P., 2002. A nonsense mutation in zebrafish gata1 causes the bloodless phenotype in vlad tepes. In: Proceedings of the National Academy of Sciences of the United States of America, p. 99.

Martins, B.O., Franco-Belussi, L., Siqueira, M.S., Fernandes, C.E., Provete, D.B., 2021. The evolution of red blood cell shape in fishes. J. Evol. Biol. 34.

Opazo, J.C., Butts, G.T., Nery, M.F., Storz, J.F., Hoffmann, F.G., 2013. Whole-genome duplication and the functional diversification of teleost fish hemoglobins. Mol. Biol. Evol. 30, 140–153.

Roesner, A., Hankeln, T., Burmester, T., 2006. Hypoxia induces a complex response of globin expression in zebrafish (Danio rerio). J. Exp. Biol. 209, 2129–2137.

Rogers, N.J., Urbina, M.A., Reardon, E.E., McKenzie, D.J., Wilson, R.W., 2016. A new analysis of hypoxia tolerance in fishes using a database of critical oxygen level (Pcrit). Conserv. Physiol. 4, cow012.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C (T) method. Nat. Protoc. 3 (6), 1101–1108.

Sun, H., Li, J., Tang, L., Yang, Z., 2012. Responses of crucian carp Carassius auratus to long-term exposure to nitrite and low dissolved oxygen levels. Biochem. Syst. Ecol. 44, 224–232.

Wang, S., Tang, C., Tao, M., Qin, Q., Zhang, C., Luo, K., Zhao, R., Wang, J., Ren, L., Xiao, J., 2019. Establishment and application of distant hybridization technology in fish. Sci. China Life Sci. 62, 22–45.

Wang, Z., Pu, D., Zheng, J., Li, P., Lü, H., Wei, X., Li, M., Li, D., Gao, L., 2023. Hypoxia-induced physiological responses in fish: from organism to tissue to molecular levels. Ecotoxicol. Environ. Saf. 267, 115609.

Wannamaker, C.M., Rice, J.A., 2000. Effects of hypoxia on movements and behavior of selected estuarine organisms from the southeastern United States. J. Exp. Mar. Biol. Ecol. 249, 145–163.

Wells, R.M., 2009. Blood-gas transport and hemoglobin function: adaptations for functional and environmental hypoxia. In: Fish Physiology. Elsevier, pp. 255–299.

Witeska, M., 2013. Erythrocytes in teleost fishes: a review. Zool. Ecol. 23, 275–281.

Xiao, W., 2015. The hypoxia signaling pathway and hypoxic adaptation in fishes. Sci. China Life Sci. 58, 148–155.