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Comparative study of hypoxic tolerance between the hybrids of white crucian carp (♀) × red crucian carp (♂) and its parents

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ABSTRACT

The hybrid of female white crucian carp (*Carassius auratus cuvieri*, WCC) × male red crucian carp (*Carassius auratus* red var. RCC) (WR), combines desirable characteristics of both parents, including tender meat, rapid growth rate, and strong resistance. To test the hypoxia tolerance of WR, an experiment of exposure to hypoxic conditions was conducted on WR, RCC, and WCC. The results showed that WR and RCC exhibited stronger hypoxia tolerance capacity compared to WCC. The strong resistance to hypoxia of WR and RCC was manifested by the absence of damage to the hepatopancreas after hypoxia, and the fish can adapt to the hypoxic environment by enhancing the hematopoietic function of hematopoietic tissue, remodeling gill morphology, and activating the antioxidant system. By contrast, after hypoxia, the number of red blood cells and the concentration of hemoglobin in the blood of WCC decreased, and the hepatopancreas was seriously damaged, indicating that the WCC is not as tolerant to low oxygen conditions as RCC and WR.

1. Introduction

An adequate dissolved oxygen (DO) concentration is a necessary condition for fish to survive. Hypoxia can activate several signaling pathways in the body to disrupt cellular energy metabolism, and the abnormal energy metabolism can cause significant adverse effects in fish. Fish usually have the ability to adapt to fluctuations in dissolved oxygen [1], but long-term hypoxia can lead to changes in physiological status and organ morphological structure, causing damage and even death [2–4].

Crucian carp (*Carassius carassius*) is an extreme anoxia-tolerant species. Most research reported that European crucian carp (inhabits small lakes and ponds in Northern Europe) and goldfish (*Carassius auratus*, a variety of crucian carp, as a domesticated ornamental fish with kinds of body color and shape) can survive in an oxygen-free environment for a long time [5,6]. There were a lot of studies on the mechanism of anoxia-tolerant in these crucian carp. One of the limiting factors for crucian carp to survive in extremely low oxygen conditions is the depletion of the hepatic glycogen reserves [7,8]. Crucian carp can upregulate the glycolytic pathway through anaerobic respiration in the hypoxic state to produce the final metabolite ethanol that is excreted through the gill tissue to avoid the cytotoxic effects of lactic acid [9–11]. Crucian carp's strong tolerance to low oxygen is also manifested as a strong oxygen uptake capacity. The hemoglobin and myoglobin of goldfish show great affinity for oxygen [12], and hypoxia induces an increase in the number of red blood cells in crucian carp [13]. Hypoxia also induces interlamellar cell mass (ILCM) shedding to enlarge the respiratory surface area of gill lamella [14,15]. During hypoxia, the crucian carp's brain maintains a moderate amount of protein synthesis [16], temporarily closing the visual [17] and auditory nerves [18] and maintaining ATP levels to keep the fish in an active state rather than in a coma, thereby allowing active escape from a low-oxygen environment [19,20]. In addition, goldfish can also regulate the antioxidant system to reduce the oxidative damage caused by oxidative stress [21].

Hypoxia inducible factor-1 (*hif-1*) and nuclear factor erythroid 2-related factor 2 (*nrf2*) are two important transcription factors involved in oxidative stress reactions, and they play central roles in the HIF-1 signaling pathway [22] and the Keap1-Nrf2 signaling pathway [23]. *hif-1* is a transcriptionally active heterodimer composed of two subunits, *hif-1α* and *hif-1β*, with a molecular weight of 120 kD for *hif-1α* and 91–94

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kD for *hif-1β* [24]. *hif* performs its function in the absence of oxygen but is quickly degraded via the ubiquitin proteasome pathway in the normal oxygen state [25,26]. *hif-1* specifically binds the promoter region of target genes through the hypoxia-responsive element (HRE) to activate downstream genes [27–29]. In response to a decrease in oxygen concentration, prolyl hydroxylation is inhibited, and stable *hif-1α* is transferred to the nucleus and forms *hif-1* dimers with *hif-1β,* which then bind to HRE in the target gene expression regulatory region [30,31]. *hif-1* transcription factors can regulate the expression of erythropoietin (*epo*), promote the proliferation and differentiation of erythrocytes [32,33], and can also regulate the expression of vascular endothelial growth factor (*vegf*) to promote angiogenesis [34,35], thereby ameliorating the peripheral blood oxygen deficiency. The acute hypoxia study in spotted sea bass indicated HIF signaling related genes were up-regulated [36].

The Keap1-Nrf2-ARE signaling pathway is one of the most important mechanisms by which fish defend against oxidative stress damage [37,38]. Elevated reactive oxide species (ROS) content in oxidative stress reactions leads to dissociation of *nrf2* from endogenous inhibitor *keap1* [39], followed by transferring to the nucleus and binding to the chaperone protein of *maf* to form heterodimers that are recruited in the order of anti-oxidant response element (ARE) enhancers of the target gene and regulate the expression of antioxidant-related genes [40,41]. Increased ROS levels induce the occurrence of many intracellular events, including cell proliferation, gene activation, cell cycle cessation, and apoptosis. Mitochondria are the organelles with the most redox activity and also an essential part of the cell for initiation or inhibition of apoptosis, and thus mitochondrion is a central focus in the field of apoptosis and redox research. Cytochrome C, released into the cytoplasm by mitochondria, is one of the central mechanisms by which *caspase-3* activates and induces apoptosis [42,43], while *bcl-2* inhibits apoptosis by preventing the release of cytochrome C [44]. Therefore, the oxidative stress response induced by hypoxia can also regulate cell proliferation, differentiation, and signal transduction through signaling pathways such as PI3K-Akt, NF-κB, JAK-STAT, and MAPK to ensure that organisms adapt to low-oxygen environments.

Hybridization is one of the most widely employed technologies used in fish breeding [45], as it can significantly improve the trait characteristics of hybrid offspring in terms of growth, reproduction, and stress resistance. WCC (*Carassius auratus cuvieri*) is considered an independent species, *Carassius cuvieri* [46,47], and thus the hybridization of WCC with RCC is considered a distant hybridization. WCC grows rapidly but has poor meat quality; RCC has tender meat and strong resistance but does not grow as fast as WCC. The red color of RCC limits its consumption. However, the hybrid WR effectively inherited the advantages of rapid growth and gray body color from the maternal WCC and the characteristics of tender meat and strong resistance from the paternal RCC. WR exhibited hybrid traits in morphology and variation in genetic composition showing essential difference with its parents. In addition, WR has been approved as a new national aquaculture variety (registration number: GS-02-001-2016). In this study, the hypoxia tolerance of three different strains of crucian carp was explored by comparing the changes in tissue morphology, antioxidant indexes, and expression of genes related to hypoxia in white crucian carp (*Carassius auratus cuvieri*, WCC), red crucian carp (*Carassius auratus* red var. RCC), and their hybrid WR to determine whether the WR had hybrid advantage in terms of hypoxia tolerance.

2. Materials and methods

2.1. Animals

WR, RCC, and WCC were obtained from the State Key Laboratory of Developmental Biology of Freshwater Fish and cultured in ponds (Wangcheng, Changsha, China) with 1333.4 m^2 × 2 m. The experimental fish were healthy and uniform in size, with an average body weight of 50 ± 5 g. The experimental WR was an F_1 generation of female WCC crossed with male RCC, while experimental WCC and RCC were self-crossed progeny. In experiment, the volume of the breeding barrel was 50 L, and all fish were acclimated for one week before the formal experiment. The water used for the experiment was pumped with air for 30 min and then stored for subsequent use. The water temperature was 18 ± 1 °C, and the dissolved oxygen level was 7.0 ± 0.1 mg/L. About one-third of the water was changed every day, and the fish were fed daily. Fish were fed at the same fixed rate with a feed of 4–8% of body weight. Feeding was performed twice a day at 8:00 a.m. and 5:00 p.m. during the experiment. All fish were fed with artificial synthetic feed.

2.2. Hypoxia exposure for WR, RCC, and WCC

WR, RCC, and WCC were divided into normoxic and hypoxia groups, with 15 fish in each group, 5 WR, 5 RCC, and 5 WCC. Five parallel experiments were performed both in the normoxia group and the hypoxia group, each containing three fish, one RCC, one WCC, and one WR. The DO of the water was measured with a dissolved oxygen meter (HACH HQ1130, USA), and the normoxic group was pumped with air to keep the DO in the water at 7.0 ± 0.1 mg/L, while the hypoxia group was pumped with nitrogen for a DO of 0.5 ± 0.01 mg/L [48]. The experiment lasted 7 days, and the feeding was stopped 2 days before experimental fish dissection.

2.3. Sample collection

Before anesthesia, blood was drawn from the caudal vein using 2 mL syringes, and 200 μL of blood was immediately transferred to a single-use vacuum blood collection tube (EDTA-K2) and mixed upside down to prevent clotting. And 1 mL of blood was directly stored overnight at 4 °C for serum extraction. Then, all fish from each group were anesthetized in 100 mg/L MS-222 (Sigma-Aldrich, USA) for about 5 min and dissected the fish to obtain other experimental materials. Tissues of the left side of the first gill arch with five gill filaments, 1 cm^3 head kidney and 1 cm^3 hepatopancreas were fixed in 4 % PFA overnight and then embedded in paraffin for histological analysis. The second gill arch on the left side of the fish with a width of about three gill filaments were fixed in 4 % PFA overnight for apoptosis analysis. The tissues of gill filaments, the head kidney, and hepatopancreas were quickly frozen in liquid nitrogen and stored at −80 °C for RNA extraction, and the hepatopancreas tissues were also used for biochemical indices and enzyme activity analysis.

2.4. Detection of blood composition

The red blood cell count, white blood cell count, and hemoglobin concentration of 200 μL blood samples were detected using a Myriad Veterinary Automatic Hematology Analyzer (BC-2800vet, CN).

2.5. Assay of serum and hepatopancreas biochemical indices and enzyme activity

Serum preparation: The stored 1 mL blood samples were centrifuged at 3000 rpm for 15 min at 4 °C to separate the serum and diluted to an appropriate concentration with 1 × PBS within 24 h for biochemical indices and enzyme activity.

Preparation of hepatopancreas tissue homogenate: The hepatopancreas samples were quickly frozen with liquid nitrogen and stored at −80 °C before being accurately weighed to prepare 10 % or 0.2 % homogenate tissue with 1 × PBS buffer solution for biochemical indices and enzyme activity.

The total superoxide dismutase (T-SOD) activity and malondialdehyde (MDA) content in serum (T-SOD with 50 % serum, MDA with 100 % serum) and hepatopancreas tissue homogenate (T-SOD with 0.2 % homogenate, MDA with 10 % homogenate) were determined according to the instructions of a total protein quantitative assay kit (A045-3), a

Total Superoxide Dismutase (T-SOD) assay kit (Hydroxylamine method, A001-1), and a Malondialdehyde (MDA) assay kit (TBA method, A003-1) from Nanjing Jiancheng Bioengineering Institute, China. The absorbance values (OD) were measured by a Synergy™ Neo2 Multi-Mode Microplate Reader (BioTek, USA). MDA content and T-SOD activity were calculated according to the formulae in the instructions. The relevant calculation formulas are as follows:

$$\text{Total protein concentration} = \frac{\text{measured OD value} - \text{blank OD value}}{\text{standard a OD value} - \text{blank OD value}} \times \text{concentration of standard}(\mu g/ml) \times \text{dilution multiplier before sample testing}$$

$$\text{Total SOD vitality in serum}(U/\text{mL}) = \frac{\text{control OD value} - \text{measured OD value}}{\text{control OD value}} \div 50\% \times \text{dilution multiplier of the reaction system} \times \text{dilution multiplier before sample testing}$$

$$\text{Total SOD vitality in tissues}(U/\text{mg prot}) = \frac{\text{control OD value} - \text{measured OD value}}{\text{control OD value}} \div 50\% \times \frac{\text{total volume of reaction solution(ml)}}{\text{sample volume}(ml)} \div \text{the protein content at the same homogenate concentration } (mgprot/mL)$$

$$\text{MDA content in serum}(nmol/mL) = \frac{\text{measured OD value} - \text{control OD value}}{\text{standard OD value} - \text{blank OD value}} \times \text{concentration of standard}(10nmol/mL) \times \text{dilution multiplier before sample testing}$$

$$\text{MDA content in tissues}(nmol/mL) = \frac{\text{measured OD value} - \text{control OD value}}{\text{standard OD value} - \text{blank OD value}} \times \text{concentration of standard}(10nmol/mL) \times \text{the protein concentration of the sample to be measured}(mg\,prot/mL)$$

2.6. Histological analysis of gill, head kidney, and hepatopancreas in WR, RCC, and WCC

The fresh samples of gill, head kidney, and hepatopancreas tissues were collected into 4 % paraformaldehyde (PFA) fluid before production of paraffin sections. The fixed tissue samples were sent to Servicebio (Wuhan, China). The paraffin sections (5 μm) were stained with hematoxylin and eosin (H&E), and images were acquired under a microscope (OLYMPUS DP73, Tokyo, Japan).

2.7. Detection of apoptotic cells in gill tissues

Fresh gill tissue was fixed in 4 % PFA for 4 h, dehydrated in 30 % sucrose solution, and embedded in OCT. The embedded gill tissue was frozen by liquid nitrogen to prepare frozen sections (8 μm), then dried at room temperature and stored at −20 °C. According to the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick End Labeling method (TUNEL), an In Situ Cell Death Detection Kit, TMR red (Roche, Switzerland) was used, and images were acquired under a fluorescence microscope (OLYMPUS DP73, Japan) with an excitation light wavelength of 590 nm, using detection in the range of 460–550 nm (red).

2.8. Total RNA extraction, cDNA synthesis, and real-time PCR

Total RNA including gills, head kidney and hepatopancreas of RCC, WR, and WCC under normoxic and hypoxic condition were isolated by phenol-chloroform extraction with TRIzol reagent (Invitrogen, USA). Then, 1 % agarose electrophoresis was used to detect the integrity of RNA, and the concentration of RNA was detected using a Synergy™ Neo2 Multi-Mode Microplate Reader (BioTek, USA). One microgram of RNA was reverse transcribed according to the instructions of a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). A quantitative reverse transcription PCR (RT-qPCR) assay was used to detect the expression level of genes. Specific primers were designed by Primer 5 based on the cDNA sequences of Trinity-assembled transcriptome data of WR head kidney (BioSample accession No. SAMN20775157). The primers are shown in Table 1. The reaction mixture (10 μL) contained 1 μL cDNA (200 ng/μL), 5 μL PowerUp SYBR Green Master Mix, 0.5 μL specific forward primers, 0.5 μL reverse primers, and 3 μL water. The amplification conditions were as follows: 50 °C for 2 min; 95 °C for 10 min; and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Gene expression was calculated using the $2^{-\triangle\triangle Ct}$ method [49] and normalized to *β-actin*. A melting curve analysis was performed to validate the specific amplification of the expected products.

2.9. Data analysis

Data are expressed as mean ± standard error of the mean (S.E.M., n

Table 1
Primers in this study.

	Gene	Primer (5′-3′)	Product length
EPOa	Erythropoietin a	F: ACCTCTCTTCCTCCATTCAC R: TCCTTCTTCCTGTTCACC	240
HIF-1α	Hypoxia inducible factor-1α	F: TCGTTCTCAGTGGCATTGTG R: CCTCATCTTCTACCTCCTTGTTC	105
VEGF	Vascular endothelial growth factor	F: ACGCAGGTGCTTCGGTC R: CCTTCTTTGGGTATGTGGG	233
PCNA	Proliferating cell nuclear antigen	F: GAGTTTGCCCGTATCTGTAGG R: AATCGTCACCGCTTCATCC	165
Sod2	Superoxide dismutase 2	F: ACACTGCCTGACCTCCCA R: GGCCACCACCGTTAAATT	203
Nrf2	Nuclear factor erythroid-2p45-related factor2	F: CCTTGAACGGAGACGGAT R: AGGGAGGTTTGGAGTGGC	228
Bcl2	B-cell lymphoma-2	F: ATCTGAGCACCGAGGCG R: GCACAGGGAAACACGAAAT	191
Bax	Bcl2-associated X protein	F: CCCAAGCATTCAACGACC R: ATTCCTGCCAGCAACCAC	160
Casp3a	Caspase 3a	F: CAGCAGAATCATCCATCCG R: TGGGCTTTAGCATCAACCT	252
p53	Tumor suppressor gene 53	F: GGGCTTCCCGCAATCT R: CGCCATCTGGGGTTCTC	224
β-actin	β-actin	F: GATGATGCCCCTCGTGC R: CCTGTTGGCTTTGGGATTG	279

Table 2
Analysis of RBC (red blood cell count),WBC(white blood cell count) and HGB (hemoglobin) concentration in WR, RCC and WCC blood cells in the normoxia group and hypoxia group.

Species	RBC (10^{12}/L)		WBC (10^{9}/L)		HGB (g/L)	
	Normoxia group	Hypoxia group	Normoxia group	Hypoxia group	Normoxia group	Hypoxia group
WR	1.2 ± 0.3	1.5 ± 0.3*	264.2 ± 11.2	254.7 ± 6.8	127.0 ± 27.0	130.6 ± 22.4
RCC	0.7 ± 0.2	1.0 ± 0.4*	255.0 ± 7.6	255.3 ± 11.6	115.8 ± 36.2	120.6 ± 12.6
WCC	1.0 ± 0.3	0.7 ± 0.3*	252.7 ± 32.4	253.9 ± 14.0	137.0 ± 24.0	83.4 ± 13.6*

Annotation: Asterisks indicate significant differences between hypoxic and normoxic groups.

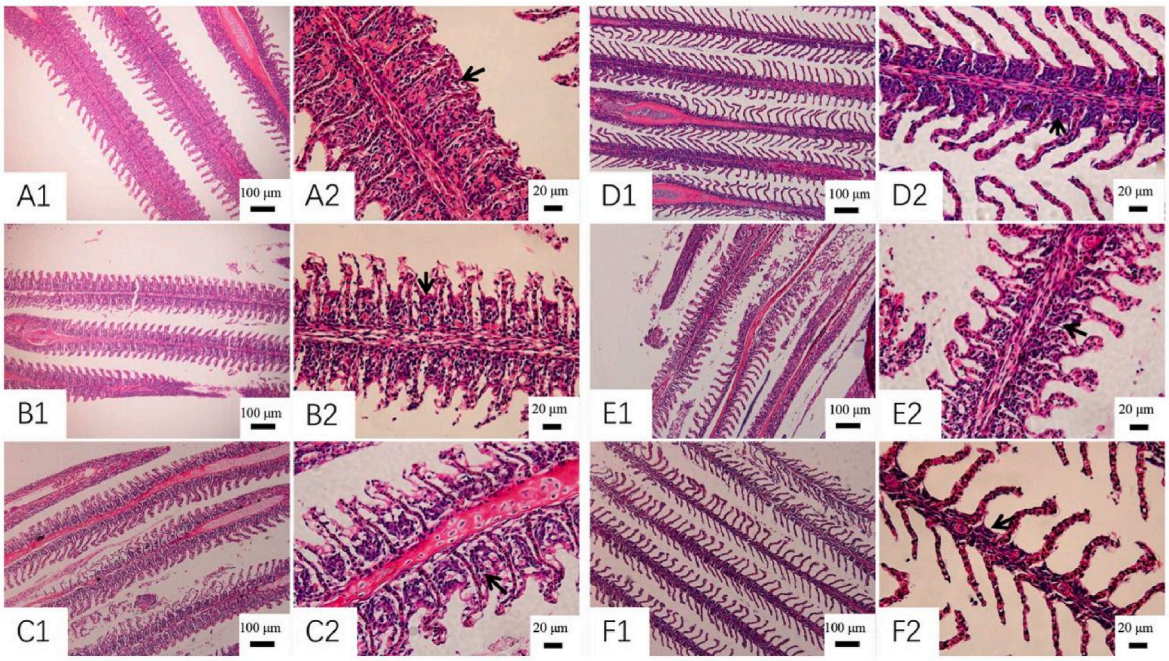

Fig. 1. Morphological changes of RCC, WR, and WCC gills after hypoxia exposure. A1-2, B1-2, and C1-2 represent the gill tissues of the normoxia group RCC, WR, and WCC, respectively; D1-2, E1-2, and F1-2 represent the gill tissues of the hypoxia group of RCC, WR, and WCC, respectively. The arrow "→" refers to the interlamellar cell mass (ILCM).

= 5). Independent-sample Student's t-tests were performed in GraphPad Prism 7.04 (San Diego, CA) to identify significant differences between samples. Differences between multiple groups were analyzed by two-way analysis of variance (ANOVA). We considered $P < 0.05$ as statistically significant.

3. Results

3.1. Blood cell analysis

The changes in blood cell components between hypoxia and normoxia groups in WR, RCC, and WCC are shown in Table 2. In WR and RCC, the number of red blood cells (RBCs) in the hypoxia group was significantly greater than that of the normoxic groups, while in the hypoxic WCC group, the RBC count was significantly less than in the normoxic WCC. There was no significant difference in white blood cells (WBCs) between the hypoxia and normoxia groups in WR, RCC, or WCC. After hypoxia treatment, the hemoglobin (HGB) content of WCC decreased, but there was no significant difference between WR and RCC.

3.2. Effect of hypoxia exposure on histological morphological changes of gill, head kidney, and hepatopancreas

The morphological changes of WR, WCC, and RCC gills under hypoxia exposure are presented in Fig. 1. The gills of the normoxia group (Fig. 1. A1-C1, Fig. 1. A2-C2) showed slightly protruding lamellae; the space between the lamellae was filled by an interlamellar cell mass (ILCM). After exposure to hypoxia, the ILCM of RCC (Fig. 1. D1-D2) and WR (Fig. 1. E1-E2) were decreased significantly, being completely lost in WCC (Fig. 1. F1–F2).

The morphological changes of WR, WCC, and RCC head kidney after hypoxia exposure are presented in Fig. 2. The exposure to hypoxia produced no significant difference in WR (Fig. 2. B1–B2, E1-E2) and RCC (Fig. 2. A1-A2, D1-D2) head kidney, while the lymphocyte and melanocyte macrophage count in the head kidney of hypoxia WCC (Fig. 2. F1–F2) were significantly increased compared with that of normoxia WCC (Fig. 2. C1–C2).

The morphological changes of WR, WCC, and RCC hepatopancreas after hypoxia exposure are presented in Fig. 3. The shapes of hepatocytes were regular, and cells were radially and evenly arranged along the periphery of blood vessels; the boundaries between cells were clearly apparent in the normoxia groups for RCC (Fig. 3. A1-A2), WR (Fig. 3. B1═B2), and WCC (Fig. 3. C1–C2). After exposure to hypoxia, the hepatocytes of the WR (Fig. 3. E1-E2) and RCC (Fig. 3. D1-D2) groups were slightly enlarged; the hepatocytes of WCC (Fig. 3. F1–F2) were damaged, with large necrotic hepatocytes; the boundaries between cells were unclear; the tissue fibers in the cells were deposited, and inflammatory cell infiltration had occurred.

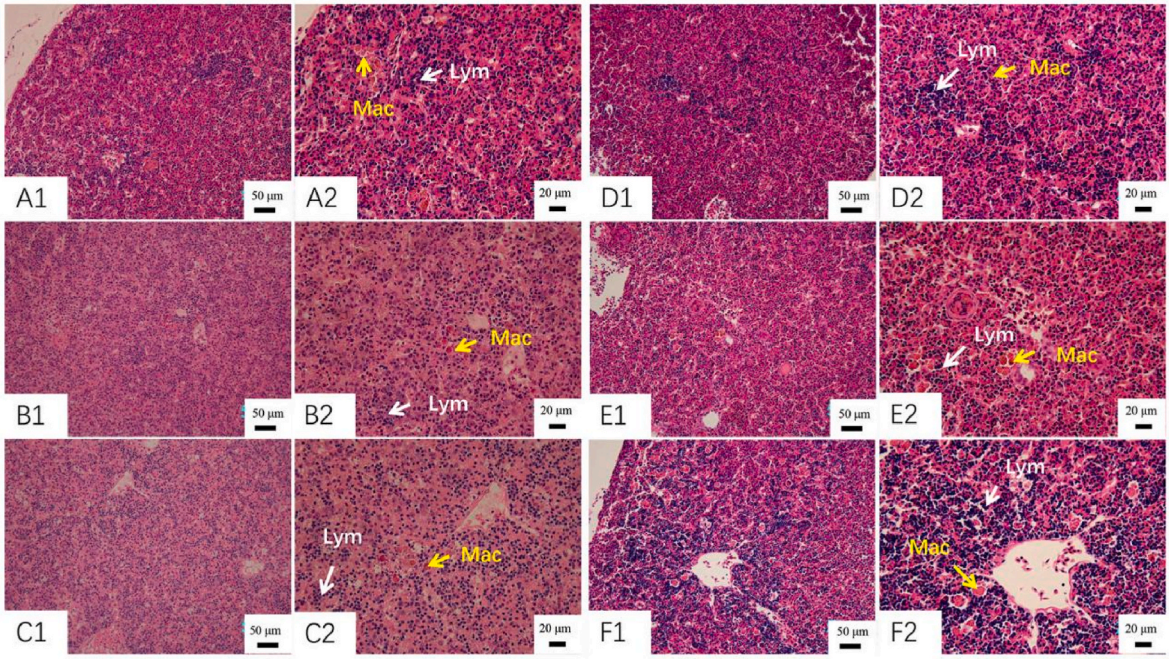

Fig. 2. Morphological changes of RCC, WR and WCC head kidney after hypoxia exposure. A1-2, B1-2, and C1-2 represent the head kidney of the normoxia group RCC, WR, and WCC, respectively; D1-2, E1-2, and F1-2 represent the head kidney of the hypoxic group of RCC, WR, and WCC, respectively. Mac: Melanocyte macrophage; Lym: lymphocyte.

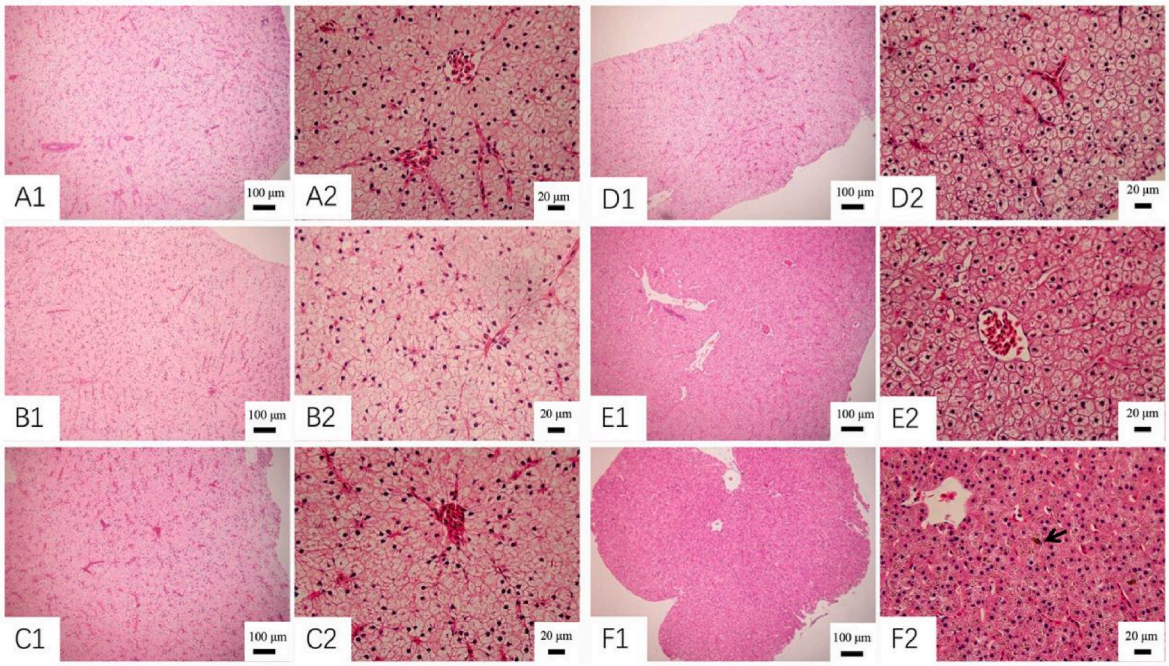

Fig. 3. Morphological changes of RCC, WR, and WCC hepatopancreas after hypoxia exposure. A1-2, B1-2, and C1-2 represent the hepatopancreas of the normoxia group of RCC, WR, and WCC, respectively; D1-2, E1-2, and F1-2 represent the hepatopancreas of the hypoxic group of RCC, WR, and WCC, respectively. The arrow "→" refers to the infiltration of inflammatory cells.

3.3. Cells apoptosis in gills

The TUNEL analysis of RCC, WR, and WCC gills identified the presence of apoptotic cells produced during hypoxia. The results showed that hypoxia exposure had different effects on the gills of the three different strains of crucian carp. Fig. 4 shows that in RCC the ILCM is not completely shed and there is no apoptotic signal. It was probably because RCC had already adapted to the hypoxic environment, and thus its gill tissue no longer needed to initiate cell apoptosis and shed to increase the respiratory surface area. Fig. 5 shows that in WR the ILCM is not completely shed and there are obvious apoptotic signals. We speculate that WR needed to further adapt to the hypoxic environment by shedding the ILCM to increase its respiratory surface area. Fig. 6 shows that in WCC the ILCM is completely shed and there was no apoptotic

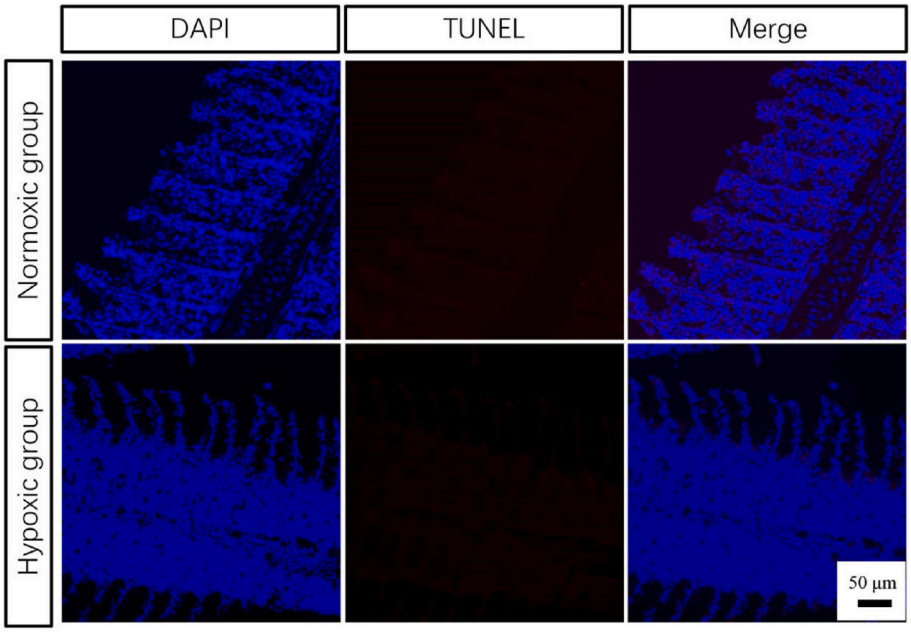

Fig. 4. TUNEL analysis of RCC gill apoptotic cells during hypoxia.

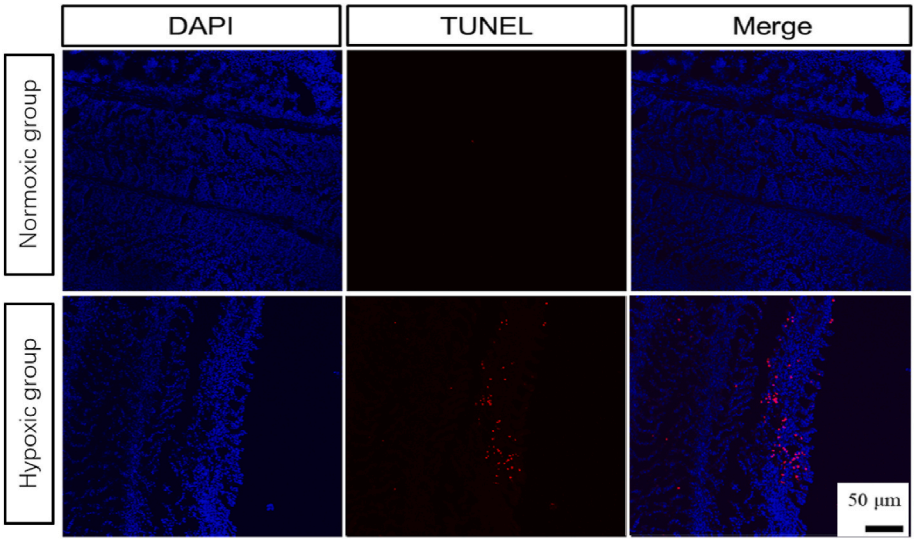

Fig. 5. TUNEL analysis of WR gill apoptotic cells during hypoxia.

signal. It is inferred that the ILCM of WCC had been completely shed and was unable to further increase the respiratory surface area of the gill by apoptosis. In conclusion, RCC had the strongest tolerance to hypoxia, followed by WR, and WCC had the least tolerance to hypoxia.

3.4. Oxidative and antioxidative parameters in serum and hepatopancreas

The effects of hypoxia stress on MDA content in serum and hepatopancreas of WR, WCC, and RCC are presented in Fig. 7. After hypoxia exposure, MDA content in serum and hepatopancreas of WCC increased significantly, but not in RCC and WR. In the hypoxic environment, the activities of T-SOD were significantly increased in hepatopancreas and serum of WCC, serum of WR, while no significant increase in hepatopancreas or serum of RCC (Fig. 8).

3.5. Expression changes of genes after exposure to hypoxia

The expression changes of apoptosis-related genes in RCC, WR, and WCC gills during hypoxia exposure are presented in Fig. 9. The expression of *hif-1α* increased significantly in both hypoxic WR and WCC, but there was no significant change in hypoxic RCC; the expression levels of *p53*, *bax*, *casp3a*, and *bcl2* in the gills of hypoxic WR were significantly upregulated, but there was no significant difference in hypoxic RCC or WCC.

The expression changes of proliferation-related genes in RCC, WR, and WCC head kidney during hypoxia exposure are presented in Fig. 10. The expression levels of hif-1α, epoa, and vegf in the head kidney of RCC, WR, and WCC have significantly increased after hypoxia exposure. However, pcna, an index of cell proliferation [50], has only significantly increased in WR and RCC, and slightly increased in WCC."

For the effects of hypoxia exposure on the expression of antioxidant related genes in RCC, WR, and WCC hepatopancreas, the results showed

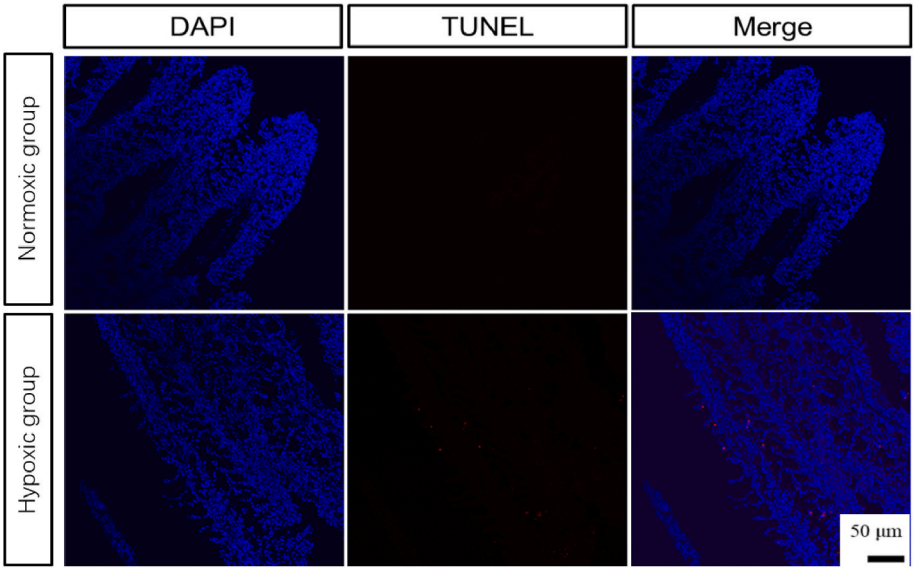

Fig. 6. TUNEL analysis of WCC gill apoptotic cells during hypoxia.

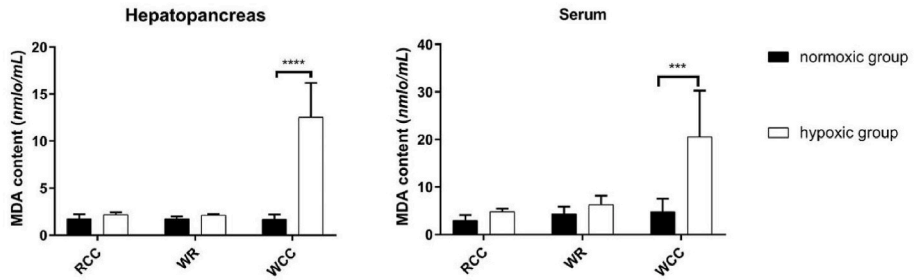

Fig. 7. Effects of hypoxia stress on MDA content in hepatopancreas and serum of WR, RCC, and WCC. There are 6 groups and each group has 5 samples. "*" means a significant difference between the normoxic group and the hypoxic group (***$P < 0.001$, ****$P < 0.0001$).

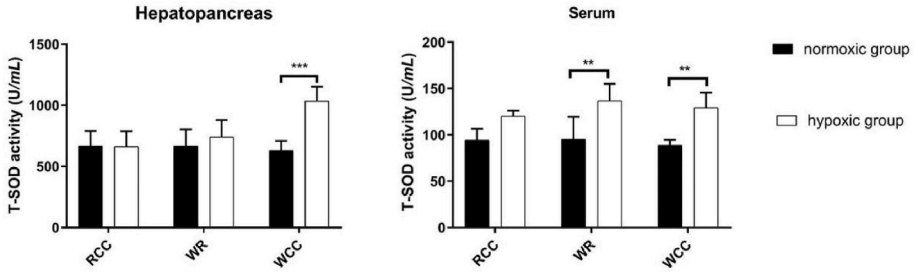

Fig. 8. Effects of hypoxia stress on T-SOD activity in hepatopancreas and serum of WR, RCC, and WCC. There are 6 groups and each group has 5 samples. "*" means a significant difference between the normoxic group and the hypoxic group (**$P < 0.01$, ****$P < 0.0001$).

that the expression of *nrf2* and *sod2* increased significantly in the hepatopancreas of WCC after hypoxia exposure, while there was no significant difference in WR or RCC (Fig. 11).

4. Discussion

In this study, the variation in hypoxia tolerance among WR, RCC, and WCC was analyzed. The results showed that the strong hypoxia tolerance of the hybrid WR was manifested in the following two aspects: WR can increase the red blood cell count after exposure to hypoxia; the histological morphology of organs in WR, such as the gill, head kidney, and hepatopancreas, were less affected by hypoxia. The hypoxic tolerance of WR was close to that of RCC, but greater than that of WCC.

Hypoxia induces gill remodeling and increases the respiratory surface area of gill lamellae to adapt to the hypoxic environment via shedding and apoptosis of ILCM [14,51,52]. After 7 days of exposure to hypoxic conditions, the gill tissues of WR, RCC, and WCC all had significant changes; the ILCM between gill lamellae of RCC and WR were significantly reduced, and the ILCM of WCC totally disappeared. In the TUNEL assay, there was no clear apoptotic signal in the gill tissue of RCC or WCC, while there was a strong apoptotic signal in WR gill. The reason for such results may be that the RCC had already adapted to the low oxygen conditions and had met their oxygen uptake demand by the increased respiratory surface area of the gill lamellae, while WR needed to further increase the respiratory surface area of the gill lamellae, and thus the apoptosis in the ILCM was still occurring. However, in the case

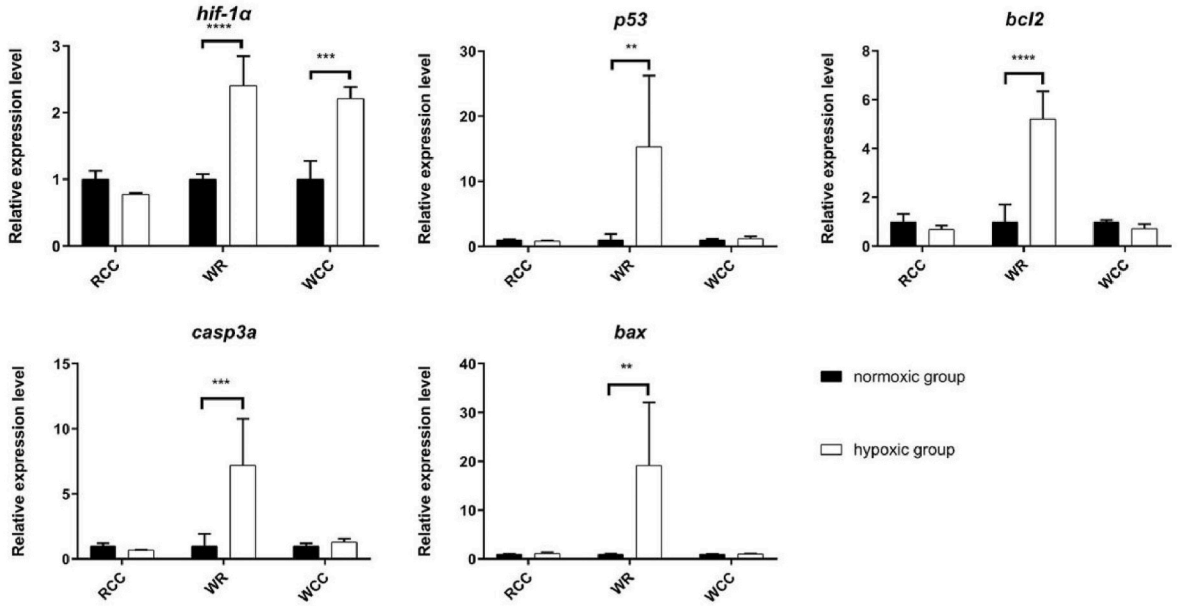

Fig. 9. Effects of hypoxia exposure on the relative expression of apoptosis-related genes in the gills of WR, RCC, and WCC. There are 6 groups and each group has 5 samples. "*" means a significant difference between the normoxic group and the hypoxic group ($^{*}P < 0.05$ to $^{****}P < 0.0001$).

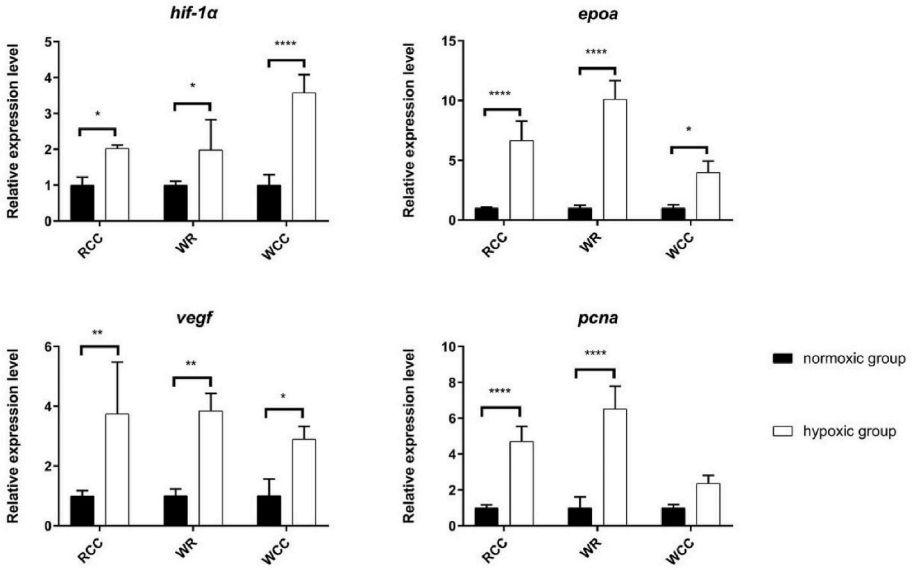

Fig. 10. Effects of hypoxia exposure on the relative expression of proliferation-related genes in head kidney of WR, RCC, and WCC. There are 6 groups and each group has 5 samples. "*" means a significant difference between the normoxic group and the hypoxic group ($^{**}P < 0.01$ to $^{****}P < 0.0001$).

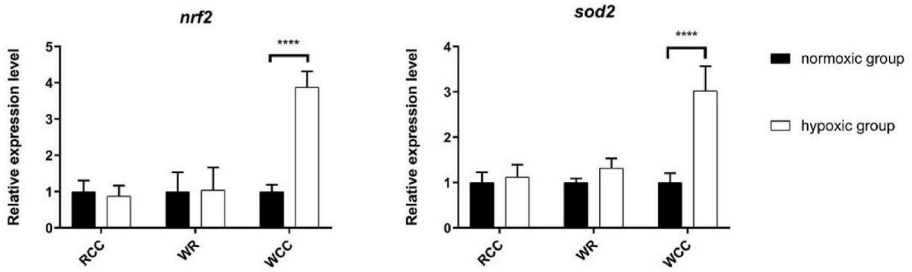

Fig. 11. Effects of hypoxia exposure on the relative expression of antioxidant-related genes in the hepatopancreas of WR, RCC, and WCC. There are 6 groups and each group has 5 samples. "*" means a significant difference between normoxic group and hypoxic group ($^{****}P < 0.0001$).

of WCC, after hypoxia exposure the ILCM was completely shed; only a small amount of apoptotic activity could be detected in lamellar epithelia, indicating that the stress response of WCC can lead to gill damage. The apoptosis-related genes *p53*, *bax*, and *casp3a* and the anti-apoptosis-related gene *bcl2* in gill tissue were also highly expressed only in the hypoxic group of WR, while no differential expression was detected in the hypoxic groups of WCC or RCC. *hif-1α* was highly expressed in the hypoxic group of WR and WCC, indicating that the gill tissues of WR and WCC had an oxidative stress response, while no oxidative stress response was detected in the gills of hypoxic RCC, consistent with the gill histologic and TUNEL assay results.

Head kidney is not only one of the immune organs but also an important hematopoietic organ of fish [53,54]. Hypoxia exposure has been reported to induce an increase in the number of red blood cells in fish [55]. The transcription factor *hif-1* [24,56] initiates the expression of downstream genes such as *epo* and *vegf*, promotes red blood cell proliferation and angiogenesis, and enhances the body's oxygen uptake and delivery. After 7 days of hypoxia exposure, the hematopoietic function of the head kidney of WR and RCC was enhanced, while the head kidney of WCC was damaged. The expression levels of *hif-1α*, *epoa*, *vegf*, and *pcna* genes in the head kidney of hypoxic WR, RCC, and WCC were increased. The head kidney histologic results showed that the lymphocytes and melanocytes in the hypoxic group of WCC were increased. In the blood component, the number of red blood cells and the concentration of hemoglobin were significantly decreased in hypoxic WCC, suggesting that hypoxia damaged the hemopoietic tissue of WCC, and its hematopoietic function was affected, leading to a decrease in the number of red blood cells and the concentration of hemoglobin in the hypoxic WCC.

One of the restrictions on the survival of crucian carp in a low-oxygen environment is the total exhaustion of its glycogen store [7], and the final metabolite ethanol is released into the water to avoid lactate self-poisoning [57]. The hepatopancreas histologic results showed that the hepatocytes of RCC and WR were slightly swollen under anoxia, but the hepatopancreas of hypoxic WCC was severely damaged. The determination of MDA content in the hepatopancreas also showed that the MDA content was significantly increased in hypoxic WCC, indicating that the lipid peroxidation damage occurred in the hepatopancreas of WCC, and the expression of antioxidant-related genes *nrf2* and *sod2* was also increased, which was consistent with the hepatopancreas histologic results. The hepatocytes of the hypoxic group of RCC and WR only showed slight edema that did not cause irreversible damage. MDA is the product of intracellular lipid peroxidation reaction caused by oxygen free radicals. The content of MDA can reflect the degree of lipid peroxidation in the body and indirectly reflect the degree of cell damage. Excessive oxygen free radicals produced in tissues can elevate the MDA level. Hepatocellular glycogen, however, can inhibit the production of oxygen free radicals and prevent them from liver tissue injuries [58]. Therefore, it can be inferred that the level of hepatocellular glycogen can indirectly inhibit the level of MDA. And according to our results, the hepatocellular glycogen storage in the RCC and the WR was probably sufficient, and the hypoxic stress response of the body could be gradually alleviated by increasing the activity of antioxidant enzymes and enhancing the oxygen uptake capacity.

WR has been applied in aquaculture, and it is favored by farmers and consumers. However, it will inevitably experience a hypoxic environment in the process of breeding and transportation. Therefore, by comparing the hypoxia tolerance of three different strains of crucian carp, this study found that WR has stronger hypoxia tolerance than WCC, being similar to RCC. These results not only enrich the basic research on the hypoxia tolerance mechanism of crucian carp but also demonstrate that WR has strong hypoxia tolerance. Combined with the advantages of fresh meat and rapid growth, it may be a good choice for future aquaculture.

5. Conclusion

Hypoxic stress experiments showed that WR has hybrid dominance in hypoxia tolerance, showing extreme hypoxia tolerance similar to that of RCC. We speculate that crucian carp improve hypoxic tolerance through the HIF signaling pathway and the Nrf2 signaling pathway.

Data availability statement

The data are available from the corresponding author based on reasonable requests.

Ethics approval statement

Fish treatments were conducted according to the regulations for protected wildlife and the Administration of Affairs Concerning Animal Experimentation and approved by the Science and Technology Bureau of China, and the Department of Wildlife Administration.

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CRediT authorship contribution statement

Kaikun Luo: Writing – original draft, Resources, Funding acquisition, Conceptualization. **Yu Deng:** Writing – review & editing, Conceptualization. **Lingmei Han:** Methodology, Investigation. **Shengwei Wang:** Methodology, Investigation. **Yirui Zhang:** Methodology, Investigation. **Zhonghua Pen:** Methodology, Investigation. **Ting Liu:** Methodology, Investigation. **Chang Wang:** Methodology, Investigation. **Chengxi Liu:** Methodology, Investigation. **Min Tao:** Resources, Conceptualization. **Chun Zhang:** Methodology, Conceptualization. **Rurong Zhao:** Methodology, Investigation. **Jing Wang:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

Declaration of competing interest

Chun Zhang and Min Tao are editorial board members for Reproduction and Breeding and they were not involved in the editorial review or the decision to publish this article. All authors declare that they have no conflict of interest.

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