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Physiological, Biochemical Responses and Apoptosis-Related Genes Expressions of Hypoxia and Re-Oxygenation Stresses in an Economically Important Mariculture Fish, the Chinese Sea Bass (*Lateolabrax maculatus*)

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Key words: *Lateolabrax maculatus*; hypoxia; re-oxygenation; apoptosis; cardiomyocyte

Abstract

The Chinese sea bass (*Lateolabrax maculatus*) is one of the most important marine aquaculture fish in China. Hypoxia represents a growing threat to *L. maculatus* in intensive mariculture. Here, effects of hypoxia and re-oxygenation conditions on physiological, biochemical responses and apoptosis-related genes expressions in *L. maculatus* were investigated. The results showed that the asphyxiation point and critical non-equilibrium point of adult *L. maculatus* were 0.39 mg/L and 1.17 mg/L dissolved oxygen, respectively. The concentrations of four inorganic ions (Na^+, K^+, Cl^-, Ca^{2+}) in serum and four red blood cell parameters levels (HCT, MCV, MCHC, RDW-CV) were significantly changed in hypoxic and re-oxygenated fish. The concentration of hepatic glycogen was decreased dramatically in re-oxygenated fish compared with control and hypoxic fish, which is contrary to the result of glucose, total cholesterol and triglyceride in serum. Moreover, the activities of certain myocardial enzymes (LDH, CK, CK-MB) were significantly increased in the serum occurred in cardiomyocytes during re-oxygenated condition, speculating that hypoxia/re-oxygenation injury might be occurred in cardiomyocytes. The result of six apoptosis-related genes expressions in heart of *L. maculatus* showed that pro-apoptotic genes (Cytc, Caspase3, Bax, Stat3) were significantly up-regulated in re-oxygenated condition whereas anti-apoptotic genes were (TET1, SGK1) expressed lower in re-oxygenated condition than that in normal. The experimental results suggested that hypoxia and re-oxygenation had a serious impact on *L. maculatus*, such as ion balance destroying, innate immune decreasing, oxygen carrying capacity changing, cardiomyocyte apoptosis. Especially, cardiomyocyte injury caused by hypoxia and re-oxygenation after hypoxia is very severely. These findings might be helpful for breeding management and future hypoxia stress study in *L. maculatus*.

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Introduction

As with the rapid growth throughout the world, China's mariculture has undergone unprecedented development in recent decades (Liu and Su 2017). The Chinese sea bass *L. maculatus* is one of the most economically important and productive mariculture fish in China. It yields about 156.6 thousand tons in 2018, accounting for 11% of the total mariculture fish production. Increasingly, the intensive model of mariculture is being used for *L. maculatus* culture. The stocking density of *L. maculatus* in China has exerted tremendous pressures on the environment, causing hypoxia. *L. maculatus* is a hypoxia-sensitive fish species, which is easily affected by anoxia.

Hypoxia (low O_2 availability) occurs commonly in aquatic environments (Borowiec et al. 2018), especially in intensive mariculture. Studies have investigated the effects of this on fish suggest that short-term hypoxia can have many impacts, such as reduced swimming speed (Herbert and Steffensen 2005), polycythemia (Kupittayanant and Kinchareon 2011), decreased immune competency (Welker et al. 2007), cellular apoptosis (Yuan et al. 2016) and even death. In the marine medaka (*Oryzias melastigma*), long-term hypoxia leads to reproductive impairment (Lai et al. 2016) and transgenerational reproductive impairments (Tse et al. 2016). The 20,000 extant species of fish vary greatly in terms of hypoxia tolerance and adaptive strategies (Xiao 2015). As hypoxia increases in prevalence (Hrycik et al. 2017), it will be vital to understand how hypoxia affects important aquatic species, such as *L. maculatus*, one of the leading marine aquaculture fish in China (Tian et al. 2019). However, no study has yet been conducted to identify physiology and biochemistry impairments of *L. maculatus* under hypoxic and re-oxygenated treatments.

Hypoxia often occurs in the middle and late stages of *L. maculatus* in mariculture, when juvenile fish have grown into adults. Therefore, adult *L. maculatus* were used in our experiment. This paper presents the first measurements of the asphyxiation point, non-equilibrium point, and critical non-equilibrium point of adult *L. maculatus* in order to determine their hypoxia tolerance. Once the dissolved oxygen (DO) concentration of the critical non-equilibrium point was determined, this value was used in the hypoxia treatment. Parameters of blood physiology and serum biochemistry were tested under various oxygen conditions (normoxic, hypoxic and re-oxygenated), in order to understand the effects of hypoxia and re-oxygenation on *L. maculatus*. Then, the expressions of apoptosis-related genes, four pro-apoptotic and two anti-apoptotic genes, were tested to help us to understand the mechanisms of hypoxia-reoxygenation injury of *L. maculatus*.

Materials and Methods

Ethics statement

L. maculatus is not an endangered or protected species, and there is no requirement for permission to undertake experiments involving this species in China.

Experimental animals

Chinese sea bass (average body weight: 597.14 g ± 33 g, about one year old) were collected from a local fish farm in Fenzhou village, Lianzhou town, Zhuhai city, Guangdong province. Fish were randomly assigned to 25 aquaria tanks, which were each 0.5 m3 in volume and continuously aerated. The water temperature was 22.8 ± 0.5 ºC and the pH was kept between 7 and 8. The salinity was maintained at 1.1 ± 0.3 ppt, which is consistent with the aquaculture environment on the fish farm. DO level varied from 7.0 to 7.5 mg/L. The fish in the aquaria tanks were acclimated for one week before exposure to hypoxia. Fish were hand-fed twice daily (8:30 a.m. and 3:30 p.m.) with a commercial floating pellet diet (Tongwei, Sichuan, China) until the day before the experiment. During the acclimatization period, half of the water was changed every day at 10:00 a.m. local time. During the hypoxia treatment, no water was changed. Since of the fish in this experiment were not mature, it was not possible to distinguish between female or male and therefore, the effect of the fish sex was not taken under consideration.

Challenge experiment and sample collection

Forty-eight healthy fish were randomly distributed between six transparent tanks (30 cm × 35 cm × 80 cm) full of sea water, denoted as A, B, C, D, E and F. Fish in tanks A, B, and C were used to measure the asphyxiation point of *L. maculatus*, and fish in tanks D, E, and F were used to measure the critical non-equilibrium point (DO when the first fish lost equilibrium) and non-equilibrium point (DO when half of the fish lost equilibrium) of *L. maculatus*. YSI dissolved oxygen monitors were put into the six transparent tanks, which were covered with transparent plastic films to isolate air. Oxygen in the sea water was consumed by experimental fish, reducing DO values in the sealed-off tanks. DO values and times were recorded when every fish died in tanks A, B and C and when each fish lost equilibrium in tanks D, E and F. When the first fish lost equilibrium (tanks D, E and F), the DO values were calculated and the average was regarded as the critical non-equilibrium point. The temperature, pH and salinity of the seawater during the threshold determinations were the same as during the acclimatization process.

The critical non-equilibrium point of *L. maculatus* (1.17 mg/L of DO) was determined via the above experiment. A total of 280 healthy fish were randomly distributed between 14 aquaria tanks (0.5 m3 per aquaria tank) named tank 1 through tank 14. All tanks were full of sea water with the same temperature, pH and salinity as during the acclimatization process. Tanks 1 to 11 were used for the hypoxia and subsequent re-oxygenation treatments, while tanks 12 to 14 were used as the control groups. DO in the control tanks was maintained between 7.0 and 7.5 mg/L by continuous aeration. 1.17 mg DO L-1 was obtained by bubbling N2 directly into the seawater. Eventually, a hypoxic environment with an oxygen concentration of 1.17 ± 0.3 mg/L was achieved. This environment was maintained for 12 hours to induce hypoxic stress. DO values were recorded approximately every hour. (**Table 1**). The DO values of tank 2, 4, 5, 6 were more stable than those of the other tanks according to the records (see **Table 1**). After 12 hours of exposure to hypoxic conditions, blood was sampled from ten random fish (hypoxia group) from tanks 2, 4, 5 and 6 (two fish from tank 2, three fish from tank 4, two fish from tank 5, three fish from tank 6), which had stable DO values of 1.17 ± 0.3 mg/L. After sampling, the re-oxygenation treatment commenced and the DO in tanks 2, 4, 5 and 6 was increased by continuous aeration for 12 hours. After 12 hours re-oxygenation, blood was sampled from twelve random fish from tanks 2, 4, 5 and 6 (re-oxygenation group). Heart and liver tissues were sampled from three fish in hypoxia and three fish in re-oxygenation conditions, and frozen in liquid nitrogen until later biochemical and molecular analysis.

Table 1 Records of DO of hypoxic treatment (mg/L).*

Tank name	*1h*	*2h*	*3h*	*4h*	*5h*	*6h*	*7h*	*8h*	*9h*	*10h*	*11h*	*12h*
Tank 1	2.5	1.2	1.1	1.1	1.0	1.1	1.1	1.0	0.9	1.1	1.3	1.3
Tank 2	1.1	1.2	1.2	1.1	1.1	1.1	1.1	0.9	0.9	1.0	1.1	1.0
Tank 3	2.1	1.2	1.4	0.9	1.2	1.2	1.1	1.0	1.1	1.1	1.2	1.4
Tank 4	1.0	1.2	1.1	1.1	1.2	1.1	1.2	1.1	1.2	1.3	1.2	1.3
Tank 5	1.2	1.4	1.0	1.1	1.1	1.0	1.1	1.2	1.3	1.4	1.4	1.2
Tank 6	1.1	1.3	1.1	1.1	1.1	1.1	1.2	1.1	1.1	1.1	1.3	1.3
Tank 7	1.4	1.4	1.1	1.0	1.2	1.1	1.1	1.1	1.8	0.9	1.3	1.2
Tank 8	1.6	1.2	1.3	1.2	1.2	1.2	1.1	1.2	1.2	1.2	1.2	1.2
Tank 9	1.8	1.4	1.0	1.6	1.2	0.9	1.0	1.3	1.1	1.1	1.2	1.3
Tank 10	2.1	1.2	1.3	1.3	1.2	1.3	1.2	1.1	1.1	1.1	1.1	1.3
Tank 11	1.3	1.4	0.7	1.3	0.9	1.1	1.2	1.0	1.2	1.2	1.3	1.2

* Tank names with bold type indicate that the DO values of these tanks were stable at 1.17 ± 0.3 mg/L.

After each treatment, 2 mL blood samples (n=17; 5 fish from the hypoxic experiment, 6 fish from the re-oxygenation experiment, 6 fish from the control group) were immediately

collected from the caudal vessel of half of the Chinese sea bass from each group. These fish were anesthetized with an overdose of MS-222 (90 mg/L) using a 2 mL syringe containing 4 mg EDTA-K2 and kept on ice. 5 mL blood samples (n=17) were collected from the caudal vessel of the remaining fish to obtain their serum for further analysis.

Physiological and biochemical study

Routine blood examination was performed using a BC-5500 auto-hemocyte analysis instrument (Mindray, Shenzhen, China). A *Hitachi 7020 Automatic Biochemical Analyzer* (Hitachi Limited, Hitachi, japan) was used to determine the biochemical index of the serum samples. Lactic dehydrogenase (LDH), creatine kinase (CK), creatine kinase isoenzyme (CK-MB) activities in serum were assayed using lactate dehydrogenase assay kit, creatine kinase assay kit and creatine kinase MB isoenzyme assay kit, respectively. These instruments were purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China. The contact of CRE (creatinine), glucose (GLU), total cholesterol (CHO) and Triglyceride (TG) in serum were measured by assay kits for: 1) creatinine, 2) glucose, 3) total cholesterol, and 4) triglycerides. Liver glycogen was tested using an additional assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Sodium (Na^+), potassium (K^+), chlorine (Cl^-), and calcium (Ca^{2+}) were determined using a mixture of galactosidase, Pyruvate Kinase, mercury thiocyanate and azo III, respectively, following the manufacturer's protocols from Hua Zhi Tai Ke, Beijing, China.

Genes expression

Six apoptosis-relative genes, cytochrome C (Cytc), caspase 3, Signal transducers and activators of transcription (STAT3), BCL2 associated X (Bax), TET (ten-eleven translocation 1), Serum/Glucocorticoid Regulated Kinase 1 (SGK1) were selected to test the expression levels by qRT-PCR. Total RNAs of heart in control, hypoxic and re-oxygenated group were obtained using TRIzol™ 1 Reagent (Thermo Scientific, USA). First-strand cDNA was synthesized using a PrimeScript™ RT Reagent kit (TaKaRa Biotechnology, Japan). cDNA-specific primers were designed using AlleleID 6.01 (see **Table 2**). The reference gene was β-actin. qRT-PCR was undertaken using a LightCycler® 480 system (Roche Diagnostics Corporation, USA) with the same method of our previously published paper (annealing temperature in this study was 60 ºC, Chen et al. 2018).

Table 2 Primer sequences for gene expression

Gene	***Gene full name***	***Primer sequence (5'-3')***
Cytc	Cytochrome *c*	**F:**GTATGGCACTGGGCACATTTCTG
		R:CTGGACGAGAGGAAGCACATTTG
Caspase3	Caspase3	**F:**GCCTCATTCGTCTGTGTTCTGTTG
		R:TGCCATCTTCTCCACTGTCTGC
TET1	Ten-eleven translocation protein 1	**F:**AGTGAGGTGAATCTGGAGGTTGAG
		R:GCAGTGATGGTAAGCAGGATGATG
SGK1	Serum and glucocorticoid-induced protein kinase 1	**F:**ACTGCTGCTAGGTGAGAAAGAGAG
		R:GCCGCCGTTACATTATTGTGAGG
Bax	Bcl2-associated X	**F:**TTCATCCGTCTGCTCTTCACAAAC
		R:GGTGGCTGGGAGGGTATTCG
Stat3	signal transducer and activator of transcription 3	**F:**TCTGCTGCTTCTCTGTCACTACTG
		R:CAATAAGGAGTCACACGCCACAC
β-actin	β-actin	**F:**CAACTGGGATGACATGGAGAAG
		R:TTGGCTTTGGGGTTCAGG

Statistical analysis

Data throughout this paper were presented as mean ± standard deviation (M ± SD; control group n=6, hypoxic group n=5, re-oxygenation group n=6). SPSS (version 16.0, IBM Corporation, USA) was used for statistical analysis. For analysis among groups, one-way ANOVAs were performed followed by least significant difference tests if homogeneity of variances were met, and rank sum tests with Mann-Whitney U if not. A significance level of 0.05 was used in all tests ($p < 0.05$).

Results

Fish hypoxia thresholds

The asphyxiation point, with the DO value at which half of the of hypoxic *L. maculatus* hypoxic died, was 0.39 ± 0.03 mg/L and occurred 107.33 ± 8.50 min after aeration of water flow stopped entering the tanks. The average body weight of the fish was 597.14 g ± 33 g. The non-equilibrium point, the DO value at which half of the *L. maculatus* lost equilibrium, was 0.69 ± 0.02 mg/L and occurred 55.00 ± 0.02 min after aeration stopped. The DO value at which the first fish lost equilibrium (critical non-equilibrium point) was 1.17 mg/L (see **Table3**). This value was used in the hypoxia treatment.

Table 3 Measurement of asphyxiation and non-equilibrium point*

Number	***time (min)***	***DO (mg/L)***
Critical non-equilibrium point and non-equilibrium point		
1	43.66±7.09	1.17±0.06
2	49.33±8.33	0.97±0.27
3	52.67±5.51	0.80±0.13
4	55.00±4.58	0.69±0.02
5	59.00±4.58	0.65±0.02
6	63.33±4.73	0.62±0.01
7	68.00±4.58	0.59±0.01
8	71.00±3.46	0.55±0.05
Asphyxiation point		
1	86.67±4.73	0.44±0.01
2	92.00±0	0.43±0.01
3	104.33±12.06	0.40±0.03
4	107.33±8.50	0.39±0.03
5	114.67±6.81	0.35±0.02
6	114.67±6.81	0.35±0.02
7	121.67±8.50	0.32±0.01
8	121.67±8.50	0.32±0.01

* Values are mean ± S.D, N = 3.

Blood physiological indicators

The reference values used in the present study were generated from normoxic fish. The differences between experimental and current reference values are showed in **Table 4**.

Table 4 Routine blood analysis of Chinese sea bass*

Parameters	*Control*	*Hypoxia*	*Re-oxygenation*
WBC (10^9/L)	114.45±3.77	109.36±16.13	111.18±8.97
Neu# (10^9/L)	30.6±6.84^{a}	22.74±6.29^{ab}	18.88±5.39^{b}
Lym# (10^9/L)	73.38±7.21	76.32±10.25	83.5±13.32
Mon# (10^9/L)	9.07±3.49	9.04±2.01	7.67±2.98
Eos# (10^9/L)	0.95±0.27	0.98±0.36	0.75±0.43
Bas# (10^9/L)	0.45±0.18	0.28±0.13	0.38±0.18
Neu (%)	26.78±6.23^{a}	20.48±2.75^{ab}	17.18±5.43^{b}
Lym (%)	64.05±4.74	69.94±2.74	74.73±6.52
Mon (%)	7.95±3.11	8.42±2.35	7.03±3.15
Eos (%)	0.82±0.23	0.88±0.29	0.68±0.44
Bas (%)	0.4±0.14	0.28±0.13	0.37±0.16
HGB (g/L)	119.83±9.66	136.4±24.54	116.33±11.71
RBC (10^{12}/L)	3.42±0.17	3.99±0.72	3.44±0.44
HCT (%)	52.05±2.42^{a}	55.12±8.43^{a}	41.63±3.75^{b}
MCV (fL)	152.33±5.6^{a}	138.86±5.7^{a}	121.62±5.32^{b}
MCH (pg)	34.97±1.92	34.14±0.42	33.92±2.7
MCHC (g/L)	229.5±11.95^{b}	246.2±8.23^{b}	279±16.96^{a}
RDW-CV (%)	18.52±2.11^{a}	14.36±1.3^{b}	14.28±2.58^{b}
RDW-SD (fL)	76.78±8.26	77.3±5.22	62±18.84
PLT (10^9/L)	55.83±30.33	24.8±14.36	70.83±99.76
MPV (fL)	7.48±0.92	7.62±0.7	7±0.63
PDW	17.83±0.46^{a}	17.56±0.59^{ab}	17.07±0.29^{b}
PCT (%)	0.04±0.02	0.02±0.01	0.05±0.06

* Values are mean ± S.D. Units are shown in parentheses. Different letters (a, b, c) represent a significant difference ($P < 0.05$); values with the same letter are not significantly different.

Serum biochemical parameters

The activities of myocardial enzymes (LDH, CK, CK-MB) affected by DO in *L. maculatus* are shown in **Figure 1**. The concentrations of CRE, GLU, CHO and TG in serum and hepatic glycogen in liver were tested in normoxic, hypoxic and re-oxygenated *L. maculatus* (**Figure 2**). The concentrations inorganic ions (Na+, K+, Cl^-, Ca2+) in the serum of *L. maculatus* were significantly affected by hypoxia and re-oxygenation (**Figure 3**).

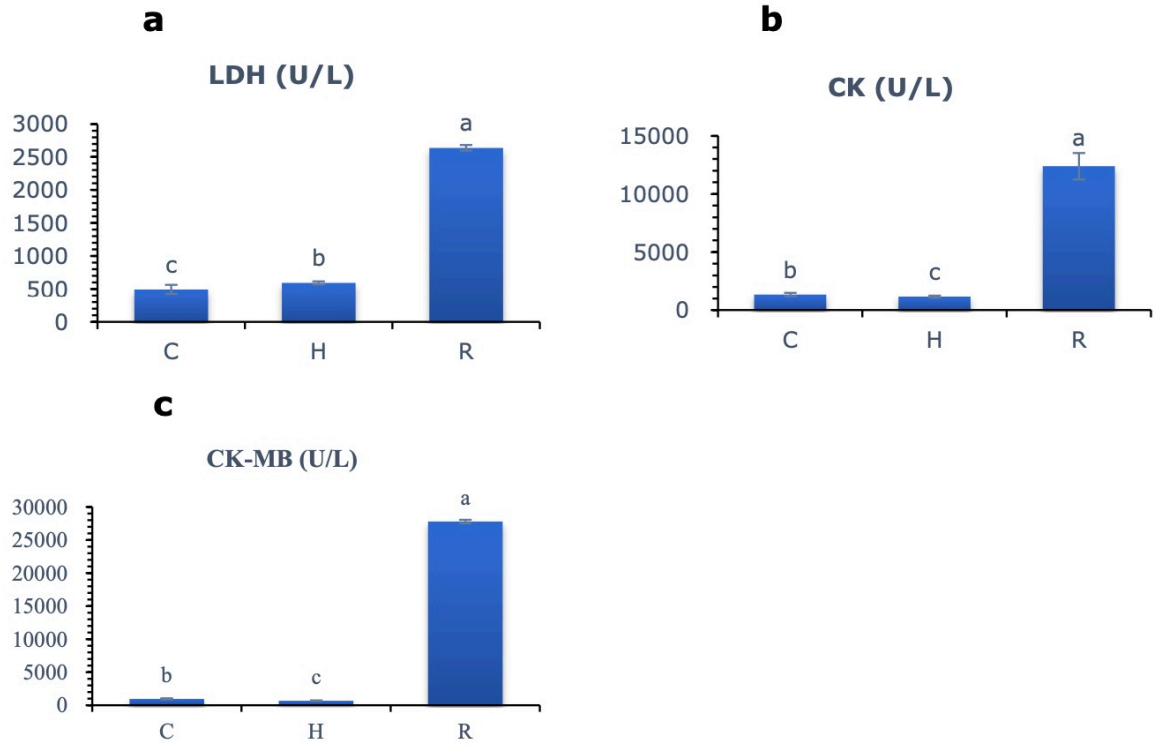

Fig. 1. LDH, CK, CK-MB activities in serum of *L. maculatus* under hypoxic and re-oxygenated conditions. C, means control group; H means hypoxic group; R means re-oxygenated group. Different letters above bars represent significant differences ($P < 0.05$), and the same letters above bars indicate no significant difference. Values are mean ± S.D. N=5 for hypoxia group, N=6 for control group and re-oxygenation group

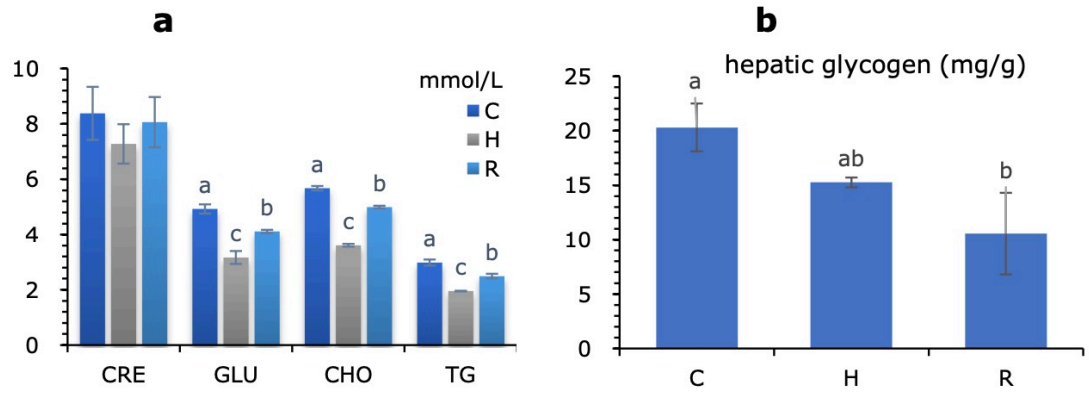

Fig. 2. Creatinine, glucose, cholesterol, triglyceride and blood urea nitrogen levels in serum of *L. maculatus* under hypoxic and re-oxygenated conditions. C, means control group; H means hypoxic group; R means re-oxygenated group. Different letters above bars represent significant differences ($P < 0.05$), and the same letters above bars indicate no significant difference. Values are mean ± S.D. N=5 for hypoxia group, N=6 for control group and re-oxygenation group.

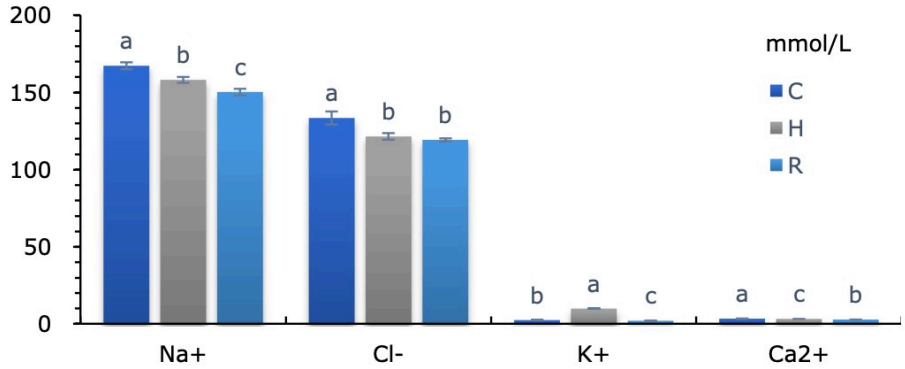

Fig. 3.Four inorganic ions levels in serum of L. maculatus under hypoxic and re-oxygenated conditions. C, means control group; H means hypoxic group; R means re-oxygenated group. Different letters above bars represent significant differences ($P < 0.05$), and the same letters above bars indicate no significant difference. Values are mean ± S.D. N=5 for hypoxia group, N=6 for control group and re-oxygenation group.

The expression of apoptotic-genes

Apoptosis-related genes expressions changes were significantly in hypoxic and re-oxygenated conditions (**Figure 4**). The expression of pro-apoptotic gene Stat3 was significantly up-regulated in both hypoxia and re-oxygenation. The other pro-apoptotic genes (Cytc, Caspase3, Bax) were markedly increased in re-oxygenation. Additionally, the expressions of TET1 and SGK1, the anti-apoptotic gene, were both showed a significant down-regulation of re-oxygenation.

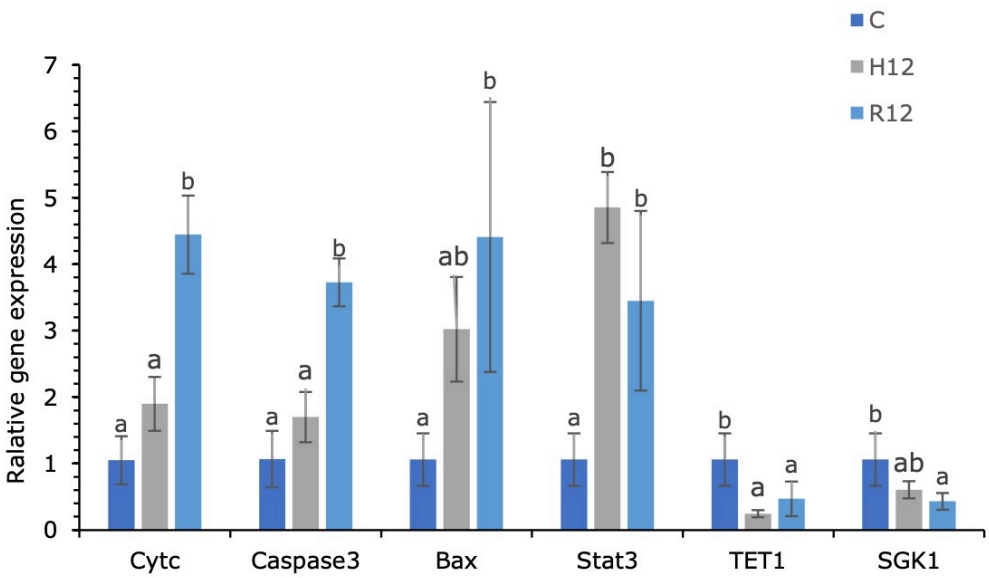

Fig. 4. The expression of apoptosis-relative genes in liver of *L. maculatus* under hypoxic and re-oxygenated conditions. Different letters above bars represent significant differences ($P < 0.05$), and the same letters above bars indicate no significant difference. Values are mean ± S.D. N=3 for every group.

Discussion

The asphyxiation point, which is an indication of the oxygen tolerance of a fish, is important in breeding management. In order to develop and implement management strategies to

avoid mortality events, it is necessary to establish the hypoxia tolerance thresholds of fish. Studies were found evidence of relationships between asphyxiation point and body size in *L. maculatus*: asphyxiation point was negatively correlated with body weight and length, and the ability of the *L. maculatus* to tolerate hypoxia improved with increasing body size (Zhao et al. 2001, Cui et al. 2018). This indicates that adult *L. maculatus* are more tolerant to hypoxia stress than juvenile fish. Compared to other cultured fishes, adult *L. maculatus*, which have an asphyxiation point of 0.39 ± 0.03 mg/L DO, are more tolerant of hypoxia than adult *Megalobrama amblycephala* (with an asphyxiation point of 0.64 mg/L), adult rainbow trout (1.40-2.84 mg/L) and adult *Oreochromis niloticus* (1.54-1.70 mg/L) (Mu et al. 2005, Jin et al. 2009), but less tolerant than adult carp (0.21-0.25 mg/L) and adult *Carassius auratus* (about 0.11 mg/L) at room temperature (20ºC - 26ºC) (Liu et al. 2000, Chen et al. 2007). Hypoxia is a major cause of death during the *L. maculatus* culture process, causing major economic losses; however, there are few reports about specific cases of death or injuries due to hypoxia in *L. maculatus*. Thus, continued research into the physiological, biochemical and apoptosis-related genes' effects of hypoxia in *L. maculatus* is needed.

The physiological function of the entire body depends on correct ion concentrations (Kamil FijoreK et al. 2014), with cell damage and organ dysfunction occurring if the ion balance is destroyed (Sun 2017). Therefore, four inorganic ions (Na+, K+, Cl-, Ca2+) in the serum of *L. maculatus* were measure, analyzed and found to be significantly impacted by hypoxia/re-oxygenation stress. It was speculated that cell damage might happen to *L. maculatus* in hypoxia/re-oxygenation stress.

To determine which aspects were affected by hypoxia/re-oxygenation, blood routine and serum biochemical parameters were initially tested. The results showed that the number of neutrophils significantly decreased in the re-oxygenation group and four red blood cell parameters (HCT, MCV, MCHC, RDW-CV) were significantly altered due to hypoxia/re-oxygenation stress. It is well known that neutrophils, the most abundant type of granulocytes, form an essential part of the innate immune system. It is inferred that hypoxia/re-oxygenation may decrease the capacity of the immune system and change the oxygen-carrying capacity of red blood cells in *L. maculatus*. However, more evidence is needed to demonstrate the mechanism behind it.

The concentrations of the glucose, total cholesterol and triglyceride in serum were initially decreased in hypoxia and subsequently increased in re-oxygenation. However, other fish, such as *Megalobrama amblycephala* (Chen et al. 2017), Rainbow trout (Raaij et al. 1996), and *Colossoma macropomum* (Affonso et al. 2002) could maintain or increase blood glucose and blood lipid homeostasis under hypoxic stress, to continue brain function or sustain survival. Thus, the ability of *L. maculatus* to maintain blood glucose level and blood lipid homeostasis might be severely impaired when hypoxic stress. Hepatic glycogen and lipid (such as cholesterol and triglyceride) are the primary sources of glucose, cholesterol and triglyceride (Zeng 2007, Chen, Wu et al. 2017). In hypoxic animals, glycogenolysis increases and hepatic glucose and energy are utilized via anaerobic glycolysis to maintain adequate basal metabolism for survival (Xue-Qun et al. 2007, Chen, Wu et al. 2017). In *L. maculatus*, hepatic glycogen contents were decreased in hypoxia ($p>0.5$) and re-oxygenation ($p<0.5$), inferring that hepatic glycogen would be massively utilized in hypoxic and re-oxygenated stress for *L. maculatus*, and glycogenolysis was increased during the whole experiment. Nonetheless, the concentration of serum glucose was still maintained at a lower level. It might happen because that hepatic glycogen in *L. maculatus* is unable to sustain the stability of serum glucose under hypoxic stress.

Additionally, LDH, CK, CK-MB are expressed extensively in tissue cells and are particularly concentrated in muscular tissue, such as heart muscles. They are often used as markers to test for cardiac injury, or heart failure (Alhadi and Fox 2004). In our study, these myocardial enzymes were significantly increased in the re-oxygenation group, indicating that cardiomyocytes might be injured during re-oxygenation. This phenomenon is similar to the ischemia-reperfusion injury (or hypoxia/re-oxygenation injury) in mammalian organs (Zhang et al. 2019). In aerobic cells, particularly cardiomyocytes, re-oxygenation can lead to cellular damage due to the production of reactive oxygen species and induce apoptosis (Li and Jackson 2002, Pi et al. 2007). Hypoxia and re-oxygenation

induce apoptotic cell death through up-regulation of the proapoptotic genes and down-regulation of anti-apoptotic genes. In our previous transcriptomic analysis of *L. maculatus*, differentially expressed genes in the heart during re-oxygenation were enriched in an apoptosis pathway (unpublished paper). Therefore, four pro-apoptotic genes (Cytc, Caspase3, Bax, Stat3) and two anti-apoptotic genes (TET1, SGK1) were selected to test their expression in hypoxia and re-oxygenation. Compared with control group, the four pro-apoptotic genes significantly increased and the two anti-apoptotic genes significantly decreased in re-oxygenation. The results of cardiomyocyte apoptosis in *L. maculatus* were similar with those found in zebrafish adults when challenged by hypoxia/re-oxygenation (Parente et al. 2013). Therefore, hypoxia/re-oxygenation injury causes major damage in mammals and mariculture animals, which are frequently exposed to an hypoxic/re-oxygenated environment. Aquatic animals' hypoxia/re-oxygenation injury require attention during breeding management and future fish research.

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