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Cu pre-exposure alters antioxidant defense and energy metabolism in large yellow croaker *Larimichthys crocea* in response to severe hypoxia

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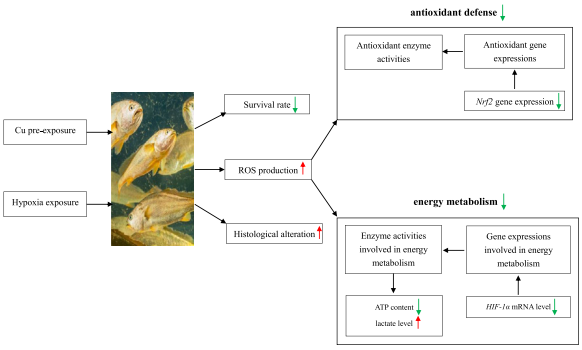
HIGHLIGHTS

- Hypoxia induced ROS and increased hepatic vacuoles.
- Cu pre-exposure had a synergistic effect on hypoxia-induced oxidative damage.
- Nrf2 mRNA level was paralleled with antioxidant gene expressions.
- HIF-1α mRNA level was correlated with gene expressions of energy metabolism.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of the present study was to evaluate the effects of Cu pre-exposure on antioxidant defense and energy metabolism in the liver of the large yellow croaker exposed to severe hypoxia. Fish were pre-acclimated to 0 and 30 μg Cu L^{-1} for 96 h, and subsequently exposed to 7.0 and 1.5 mg DO L^{-1} for another 24 h. Hypoxic stress alone increased reactive oxygen species and hepatic vacuoles. When compared to hypoxic stress alone, hypoxic stress plus Cu pre-exposure increased mortality and ROS production, and worsened histological structure by inhibiting antioxidant defense and aerobic metabolism, and enhancing anaerobic metabolism, suggesting Cu pre-acclimation aggravated hypoxia-induced oxidative damage. NFE2-related nuclear factor 2 and hypoxia-inducible factor-1α might participate in the transcriptional regulation of genes related to antioxidant response and energy metabolism, respectively. In conclusion, Cu pre-acclimation had a synergistic effect on antioxidant response and energy metabolism in fish under severe hypoxia, which contributes to understanding the molecular mechanisms underlying negative effects of Cu pre-acclimation against hypoxic damage in fish.

1. Introduction

Environmental hypoxia is a naturally occurring phenomenon in near-bottom waters of estuaries and coastal zones, which is worsened by global warming and eutrophication (Howarth et al., 2011). The industrial and mining activities have resulted in the severity and prevalence of metal pollutants in the aquatic systems during the past decades. Aquatic organisms are often threatened by hypoxia and metal exposures (Blewett et al., 2017; Fitzgerald et al., 2016). Hypoxic stress may occur before or after metal stress, or the two stresses occur simultaneously. Many studies indicated that simultaneous hypoxic stress could increase toxicological effects caused by metal stresses (Fitzgerald et al., 2017; Ransberry et al., 2016); pre-hypoxia exposure

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could mitigate metal toxicities (Dolci et al., 2014). But few studies have investigated whether metal pre-acclimation has effect on the physiological status of fish under hypoxic stress (Blewett et al., 2017).

The effect of copper (Cu) on fish has been a focus of aquatic toxicology, considering the discharge of industrial effluents containing Cu ions. The concentrations of Cu reached 0.60 and 1.22 mg L^{-1} in Jiaozhou Bay and Jinzhou Bay, China, respectively (Pan and Wang, 2012). Although Cu is one of the essential elements for fish to achieve various biological functions, excessive Cu might be potentially toxic to fish (Birnie-Gauvin et al., 2017; Wang et al., 2015). Previous studies indicated that Cu could alter hypoxic sensitivity in fish (Blewett et al., 2017). Therefore, we speculate that the hypoxic responses of fish are affected by Cu pre-acclimation, which needs to be further investigated.

Hypoxia and metal exposures may inhibit mitochondrial electron transport chain and ATPase activity, resulting in oxidative stress (Hosseini et al., 2014; Sappal et al., 2015; Sappal et al., 2016). Increased energy starvation and reactive oxygen species (ROS) production are the two major components of stress responses, which are intimately related to each other (Zhu et al., 2013). Fish have developed antioxidant defense system to defend against oxidative damage caused by various stresses (Winston and Di Giulio, 1991). NFE2-related nuclear factor 2 (Nrf2) is the key transcription factor modulating the antioxidant response (Dodson et al., 2015). Hypoxia-inducible factor-1α (HIF-1α) is a dominant modulator of the hypoxic responses by activating target gene expressions involved in energetic homeostasis, oxygen transport, and so on (Huang et al., 2004; Mandic et al., 2019). Metallothioneins (MTs) play an important role in the sequestration of metal ions. Besides, MTs have strong antioxidant capacities to eliminate or neutralize the excess of ROS (Amiard et al., 2006). MTs can also be induced by non-metallic stressors including hypoxia, which contributes to understanding the biological functions of these proteins in response to multiple stressors (Sun et al., 2016).

The large yellow croaker *Larimichthys crocea* is a main commercially marine fish, the annual yield of which is the highest among the marine cage-farmed fish species in China. High stocking density of this species is conducted in practice, which often leads to ciliate *Cryptocaryon irritans* (known as white spot disease) outbreaks. As a result, for example, a high mortality (about 90%) occurred in 2016 (Sun et al., 2017; Yin et al., 2015). Copper sulphate ($CuSO_4$) is considered as the most efficient treatment for *Cryptocaryon irritans*, which may result in Cu pollution in cultivation water. In addition, high-density culture also easily causes oxygen-poor "dead zones", especially during the high temperature season. The objective of this study was to evaluate the effects of Cu pre-exposure on the antioxidant defense and energy metabolism in the liver of the large yellow croaker exposed to severe hypoxia, which may facilitate the rational use of $CuSO_4$ in the context of frequent oxygen depletion in intensive aquaculture. The biochemical indicators, including survival rate, histological analysis, Cu content, ROS, ATP, lactate and MTs protein levels, activities of copper/zinc-superoxide dismutase (Cu/Zn-SOD), catalase (CAT), glutathione peroxidase (GPx), ATP synthase (F-ATPase), malate dehydrogenase (MDH), succinate dehydrogenase (SDH), pyruvate kinase (PK) and cytochrome *c* oxidase (COX) were detected. In addition, molecular indicators involved in antioxidant defense (mRNA levels of *Cu/Zn-SOD*, *CAT*, *GPx1a*, *GPx1b* and *Nrf2*) and energy metabolism (mRNA levels of *F-ATPase*, *MDH*, *SDH*, *PK* and *HIF-1α*) were also evaluated.

2. Materials and methods

2.1. Challenge experiment and sample collection

240 healthy juvenile large yellow croaker of similar size (mean body mass 97.2 ± 6.3 g) were procured from a mariculture farm (Wenzhou, China). The fish were maintained in twelve 500-L fiberglass tanks (n = 20 fish per tank) for a two-week acclimation. The ranges of the water quality parameters were as follows: salinity 27.4 ± 0.58, temperature 22.3 ± 3.4 °C, pH 7.78 ± 0.46, DO 7.31 ± 0.48 mg L^{-1}, total ammonia 0.020–0.034 mg L^{-1}, nitrite 0.017–0.036 mg L^{-1} and nitrate 0.127–0.243 mg L^{-1}. Then the large yellow croaker were randomly divided into the control group (six tanks) and treatment group (six tanks). Fish of the control group were exposed to 0 μg Cu L^{-1}, and fish of the treatment group were exposed to 30 μg Cu L^{-1}, the level of which is close to that of Cu contamination when CuSO4 is used to treat *Cryptocaryon irritans* disease, for 96 h. Before Cu exposure experiment, fiberglass tanks were pre-washed with 10% HNO_3 (guaranteed reagent; Sinopharm Chemical Reagent Corporation, China) for 24 h to reduce the impacts of contaminations, then $CuSO_4 \cdot 5H_2O$ (AR; Sinopharm Chemical Reagent Corporation, China) was dissolved into the seawater as the Cu sources, and equilibrated for 24 h prior to use. During Cu exposure period, water was replaced completely twice daily to maintain constant Cu content. Water was acidified with 2% HNO_3, and Cu concentrations were measured by inductivity coupled plasma mass spectrometry (ICP-MS, Thermo Jarrel Ash Corporation, USA) according to Liu et al. (2010). The detection limit of Cu was 0.04 μg L^{-1} in water. The actual Cu concentrations of 0 and 30 μg L^{-1} groups were 3.11 ± 0.46 and 31.54 ± 0.72 μg L^{-1}, respectively. During the two-week acclimation and Cu exposure, the fish were fed two times daily with a commercial formulated diet (50% crude protein and 10% lipid). Subsequently, fish were subjected to dissolved oxygen (DO) concentrations at 7.0 and 1.5 mg L^{-1} for another 24 h, 3 replicates/treatment. 7.0 and 1.5 mg DO L^{-1} were obtained by bubbling air and N_2 directly into the seawater (free of $CuSO_4 \cdot 5H_2O$), respectively, as suggested by Zeng et al. (2016a). The oxygen contents were monitored by a DO meter (YSI, Canada), the actual oxygen concentrations in the control and hypoxic stress groups were 7.24 ± 0.57 and 1.45 ± 0.48 mg DO L^{-1}, respectively.

At the end of the experiment, all fish were anesthetized in a 200 mg L^{-1} solution of MS-222, then 6 fish were randomly selected from each tank and the liver tissues were collected. The left lobes of live sample were fixed for histological study, the remaining samples were frozen in N_2 immediately, and kept at −80 °C until later biochemical and molecular analysis. Animal care and experiments were performed in accordance with approvals obtained from the Animal Care Committee of Zhejiang Ocean University.

2.2. Histological study

Histological analysis was conducted as described in Yuan et al. (2017). Briefly, samples were fixed in 4% paraformaldehyde/phosphate-buffered saline (pH 7.2) for approximately 24 h, and

Table 1
Primer sequences used for real-time PCR.

Gene name	Primer sequences (from 5′ to 3′)	Size (bp)	PCR efficiency
Cu/Zn-SOD	F: GAGACAATACAAACGGGTGC R: CAATGATGGAAATGGGGC	137	0.97
CAT	F: ATTATGCCATCGGAGACTTG R: GCACCATTTTGCCCACAG	115	0.98
GPx1a	F: GACTCGTTATTCTGGGTGTTCCCTGTA R: CCATTCCCTGGACGGACATACTTC	103	1.04
GPx1b	F: TCTTGTCCCTGAAGTATGTCCGTCCTG R: GGCATCCTTTCCATTTACATCCACCTT	89	1.02
Nrf2	F: CCCTCAAAATCCCTTTCACT R: GCTACCTTGTTCTTGCCGC	90	0.96
F-ATPase	F: TGGTTACTCCGTGTTCGCT R: GGGGCTCGTTCATTTGAC	142	1.02
MDH	F: AAGTAGAGTTCCCCGCTGAC R: CACCCTCCTTCCCGTTCA	170	0.98
SDH	F: TACAGGGTCAGGAGAAGAAGC R: AATGGTCAATAACAGGTCGGT	123	0.99
PK	F: CTGGTTTCCTTATGTGCGAG R: GGTCCTGGATGTCCTTTTCT	256	1.03
HIF-1α	F: AACTGTTCACTCGGGCAATAG R: GAAGTGGCGGTGGTAACG	129	1.03
β-Actin	F: TCGTCGGTCGTCCCAGGCAT R: ATGGCGTGGGGCAGAGCGT	182	1.05
GAPDH	F: GACAACGAGTTCGGATACAGC R: CAGTTGATTGGCTTGTTTGG	89	1.04

dehydrated through increasing concentrations of ethanol before embedding in paraffin wax. Sagittal sections (6 μm thickness) were obtained with a diamond knife, and stained with hematoxylin/eosin (H&E). Sections were observed with a light microscopy. For statistics of relative areas (%) for hepatic vacuoles in H&E, we randomly examined 6 microscope fields for each sample, and then the results from individual observation were combined for the overall results.

2.3. Biochemical analysis

The preparation and extraction of homogenates and supernatants were carried out on the basis of the descriptions in our previous study (Zeng et al., 2016b). ROS was assayed according to LeBel et al. (1992). ATP content, lactate level and COX activity were assayed using ATP assay kit (Beyotime, Haimen, China), lactate assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and COX assay kit (Genmed, Shanghai,China), respectively, following the manufacturer's protocols. Cu/Zn-SOD, CAT and GPx activities, and MTs protein levels were determined by the spectrophotometric methods as described by Nakano et al. (1990), Aebi (1984), Drotar et al. (1985) and Viarengo et al. (1997), respectively. F-ATPase, MDH, SDH and PK activities were measured according to the methods described by Morin et al. (2002), Luo et al. (2006), Philip et al. (1995), and Foster and Moon (1986), respectively. Soluble protein content was determined by the Coomassie brilliant blue method (Bradford, 1976). All enzyme activities were expressed as U (units) per mg of soluble protein.

The Cu content in liver was determined by ICP-MS according to the methods described by Liu et al. (2010). Briefly, liver samples were acidified in concentrated HNO_3 for 72 h (110 °C), then diluted to appropriate concentrations for Cu measurement. The detection limit of Cu was 0.8 μg kg^{-1} dry weight, and the recovery of Cu was between 94.00 and 104.00%. The assays were performed in triplicate.

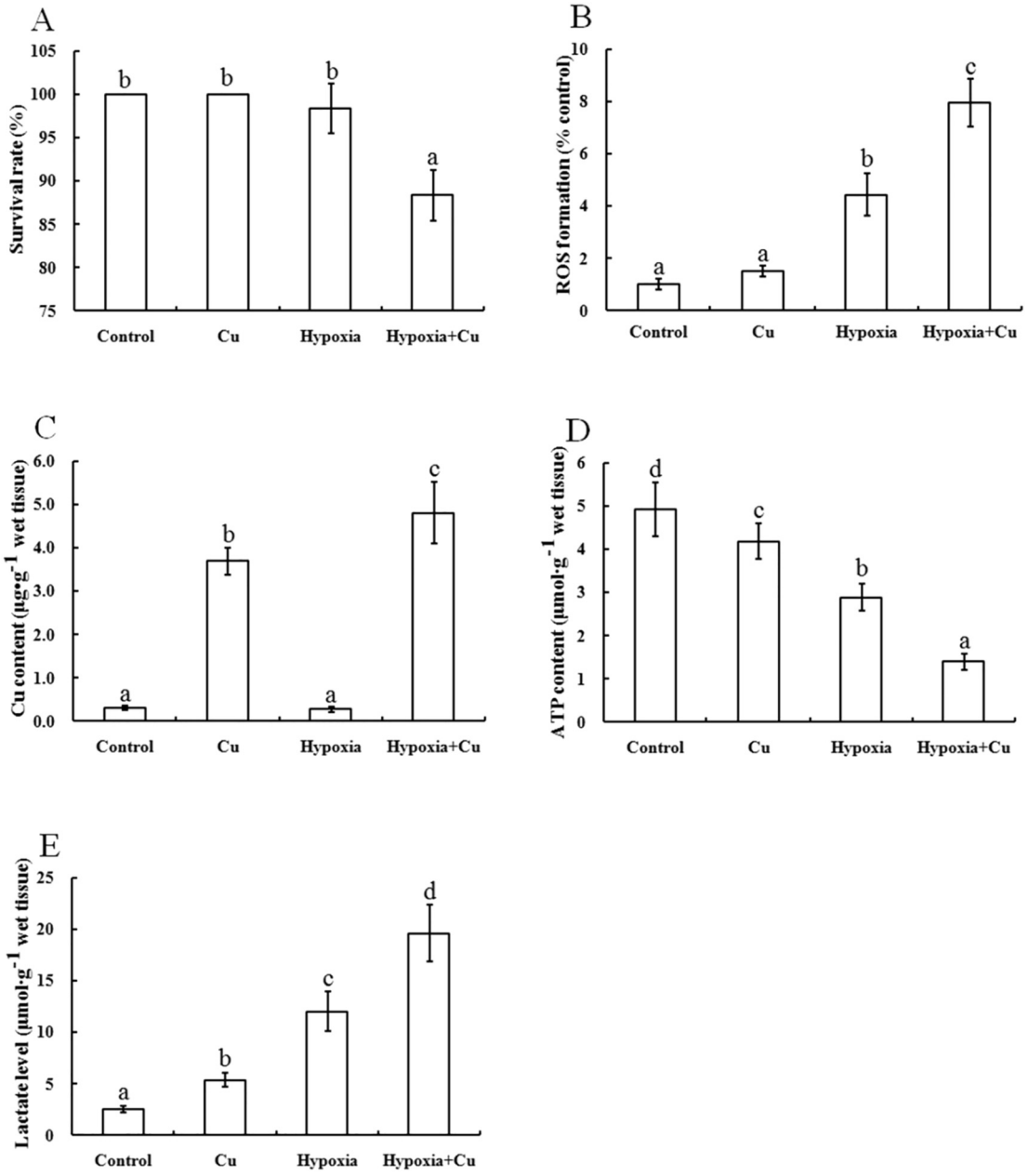

Fig. 1. Changes in the survival rate (A), ROS formation (B), Cu content (C), ATP content (D) and lactate level (E) in the liver of the large yellow croaker exposed to Cu and hypoxia. Each value represents means ± SEM ($n = 6$). Different letters indicate significant differences ($P < 0.05$).

2.4. Gene expression

Gene expression analysis was performed following the methods in our previous study (Zeng et al., 2016b). Briefly, RNAiso Plus (TaKaRa, China), first-strand cDNA synthesis kit (Fermentas) and SYBR® Premix Ex Taq™ Kit (TaKaRa, China) were used for RNA extraction, cDNA synthesis and quantitative real-time PCR (qPCR), respectively. qPCR primers were designed based on the genome data of the large yellow croaker in our laboratory (Table 1) (Wu et al., 2014). As described in our previous study (Zeng et al., 2016a), target genes were normalized to the geometric mean of the best combination of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *β-actin* to calculate relative transcript abundances with the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001).

2.5. Statistical analysis

Data were expressed as means ± SEM ($n = 6$). Normality of distribution and homogeneity of variances were verified by Kolmogornov-Smirnov test and Barlett's test, respectively. Then the data were subjected to two-way ANOVA (hypoxia and Cu exposures as factors) and Duncan's multiple range test to rank significant differences among treatments ($P < 0.05$). The relative areas (%) for the hepatic vacuoles in H&E were analyzed by Image-Pro Plus 6.0. Pearson correlation analysis was used to estimate the relationship between different parameters. Analysis was performed with SPSS 19.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Survival rate and biochemical indicators

The survival rate in the hypoxic stress plus Cu pre-exposure group was significantly lower (88.33 ± 2.98%) than those in the other three groups (Fig. 1A). When compared to the controls, Cu pre-exposure elevated Cu, lactate and MTs levels, Cu/Zn-SOD, CAT and PK activities, had no effects on ROS production, GPx and MDH activities, and inhibited ATP content, F-ATPase, SDH and COX activities (Figs. 1–3). Hypoxic stress alone induced increases in ROS and lactate levels, Cu/Zn-SOD and PK activities, had no effects on Cu content, MTs levels and CAT activity, and induced reductions in ATP content, GPx, F-ATPase, MDH, SDH and COX activities. Hypoxic stress plus Cu pre-exposure enhanced the levels of ROS, Cu, lactate and MTs, and the activities of Cu/Zn-SOD and PK, and inhibited the level of ATP and the activities of CAT, GPx, F-ATPase, MDH, SDH and COX. As compared to hypoxic stress alone, hypoxic stress plus Cu pre-exposure increased ROS, Cu, lactate and MTs levels, Cu/Zn-SOD and PK activities, and reduced ATP content, CAT, GPx, F-ATPase, MDH, SDH and COX activities.

3.2. Gene expressions involved in antioxidant defense

When compared to the controls, Cu pre-exposure up-regulated all of the antioxidant gene expressions (*Cu/Zn-SOD*, *CAT*, *GPx1a*, *GPx1b* and *Nrf2*) (Fig. 4). Increased gene expressions of *Cu/Zn-SOD*, *CAT*, *GPx1b* and *Nrf2*, and reduced mRNA level of *GPx1a* were observed in fish exposed to hypoxic stress alone. Hypoxic stress plus Cu pre-exposure showed higher mRNA levels of *Cu/Zn-SOD* and *GPx1b*, and lower mRNA levels of *CAT*, *GPx1a* and *Nrf2*. As compared to hypoxic stress alone, hypoxic stress plus Cu pre-exposure up-regulated *Cu/Zn-SOD* gene expression, and down-regulated *CAT*, *GPx1a*, *GPx1b* and *Nrf2* gene expressions.

3.3. Gene expressions involved in energy metabolism

When compared to the controls, Cu pre-exposure up-regulated *PK* gene expression, had no effects on *SDH* mRNA level, and down-

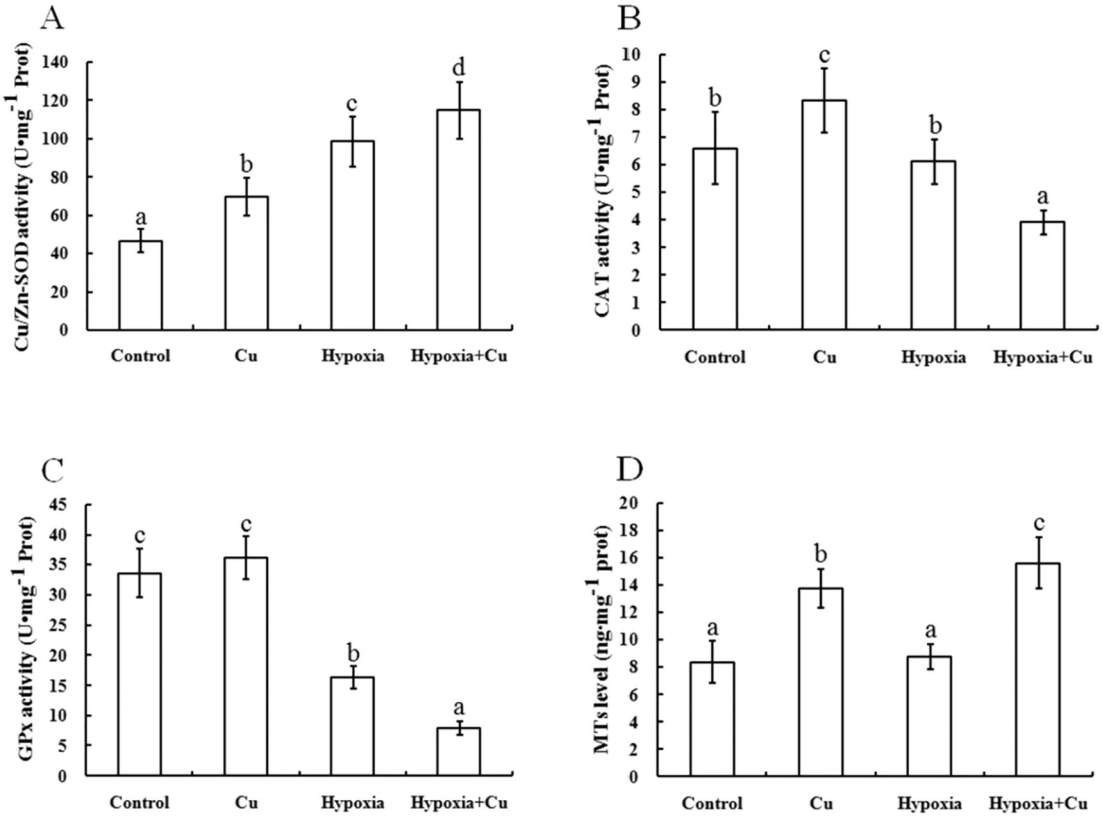

Fig. 2. Changes in the activities of Cu/Zn-SOD (A), CAT (B) and GPx (C) and MTs protein levels (D) in the liver of the large yellow croaker exposed to Cu and hypoxia. Each value represents means ± SEM ($n = 6$). Different letters indicate significant differences ($P < 0.05$).

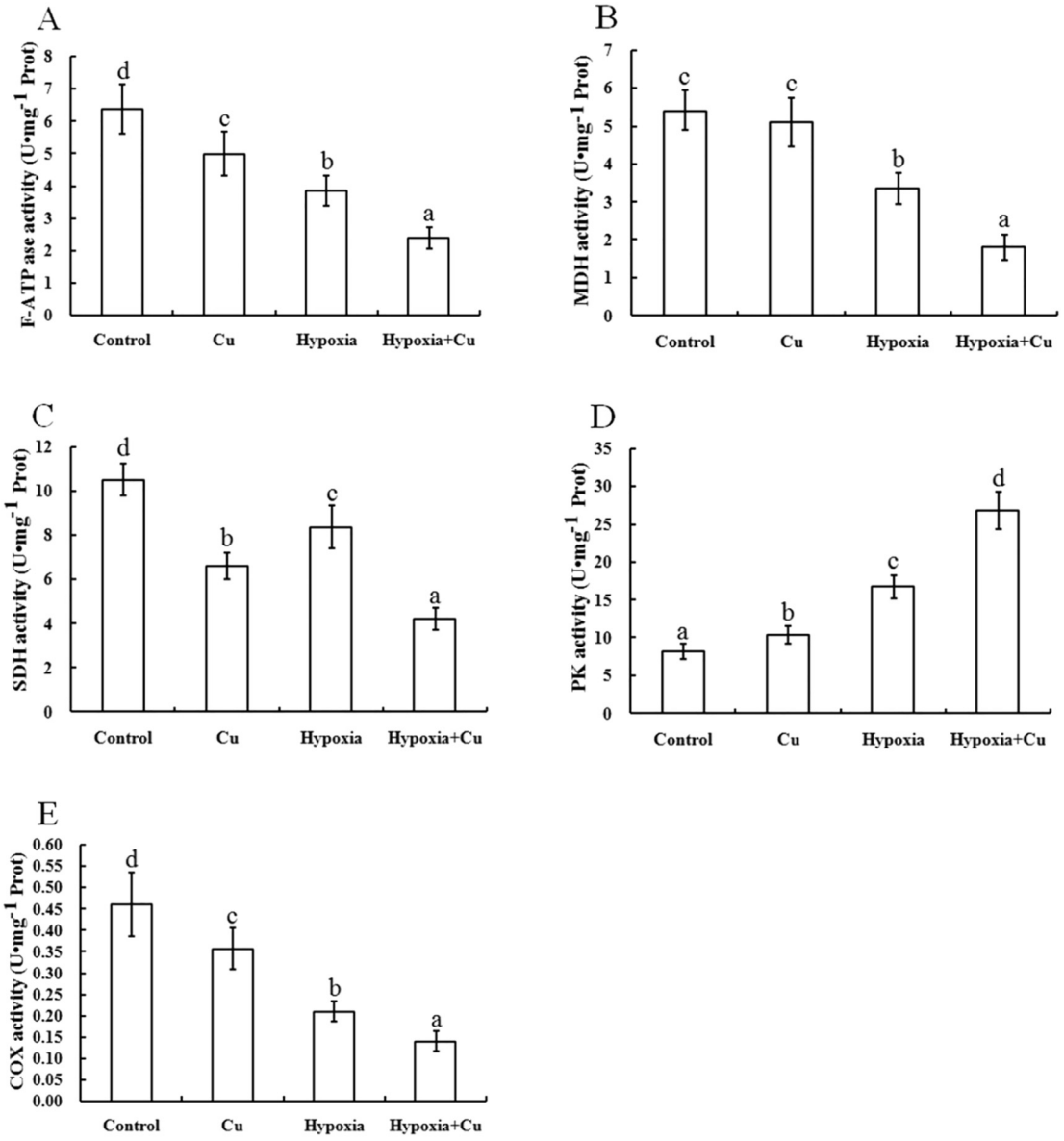

Fig. 3. Changes in the activities of F-ATP (A), MDH (B), SDH (C), PK (D) and COX (E) in the liver of the large yellow croaker exposed to Cu and hypoxia. Each value represents means ± SEM ($n = 6$). Different letters indicate significant differences ($P < 0.05$).

regulated *F-ATPase*, *MDH* and *HIF-1α* gene expressions (Fig. 5). Hypoxic stress alone induced an increase in *SDH* and *PK* gene expressions, and induced decreases in *F-ATPase*, *MDH* and *HIF-1α* gene expressions. Hypoxic stress plus Cu pre-exposure increased mRNA level of *PK*, and reduced gene expressions of *F-ATPase*, *MDH*, *SDH* and *HIF-1α* in comparison to the controls and hypoxic stress alone.

3.4. Correlation analysis

Pearson's correlation coefficient of parameters is shown in Table 2. There were positive relationships between activities and mRNA levels of *Cu/Zn-SOD*, *CAT*, *F-ATPase*, *MDH* and *PK*. A positive correlation between GPx activity and *GPx1a* gene expression also was observed. But there were no relationships between GPx activity and *GPx1b* mRNA level, and between SDH activity and *SDH* gene expression. *Nrf2* mRNA level presented positive correlations with gene expressions of *CAT*, *GPx1a* and *GPx1b*, but no relationship was observed with *Cu/Zn-SOD* gene expression. *HIF-1α* mRNA level was paralleled with gene expressions of *F-ATPase*, *MDH* and *PK*, but no relationship was observed with *SDH* mRNA level. COX activity showed a positive correlation with ATP content. ROS was positively related to PK activity, and negatively correlated with F-ATPase, MDH and SDH activities, and *Nrf2* and *HIF-1α* gene expressions.

3.5. Histological observations

Normal hepatocytes and hepatocyte nucleus were observed in the control group (Fig. 6). A small amount of vacuoles were observed in the Cu pre-exposure group. A large amount of vacuoles were observed in the hypoxic stress alone group. Indistinction between two edges of neighbor cells, and severe pyknotic nuclei and vacuoles were observed in the hypoxic stress plus Cu pre-exposure group.

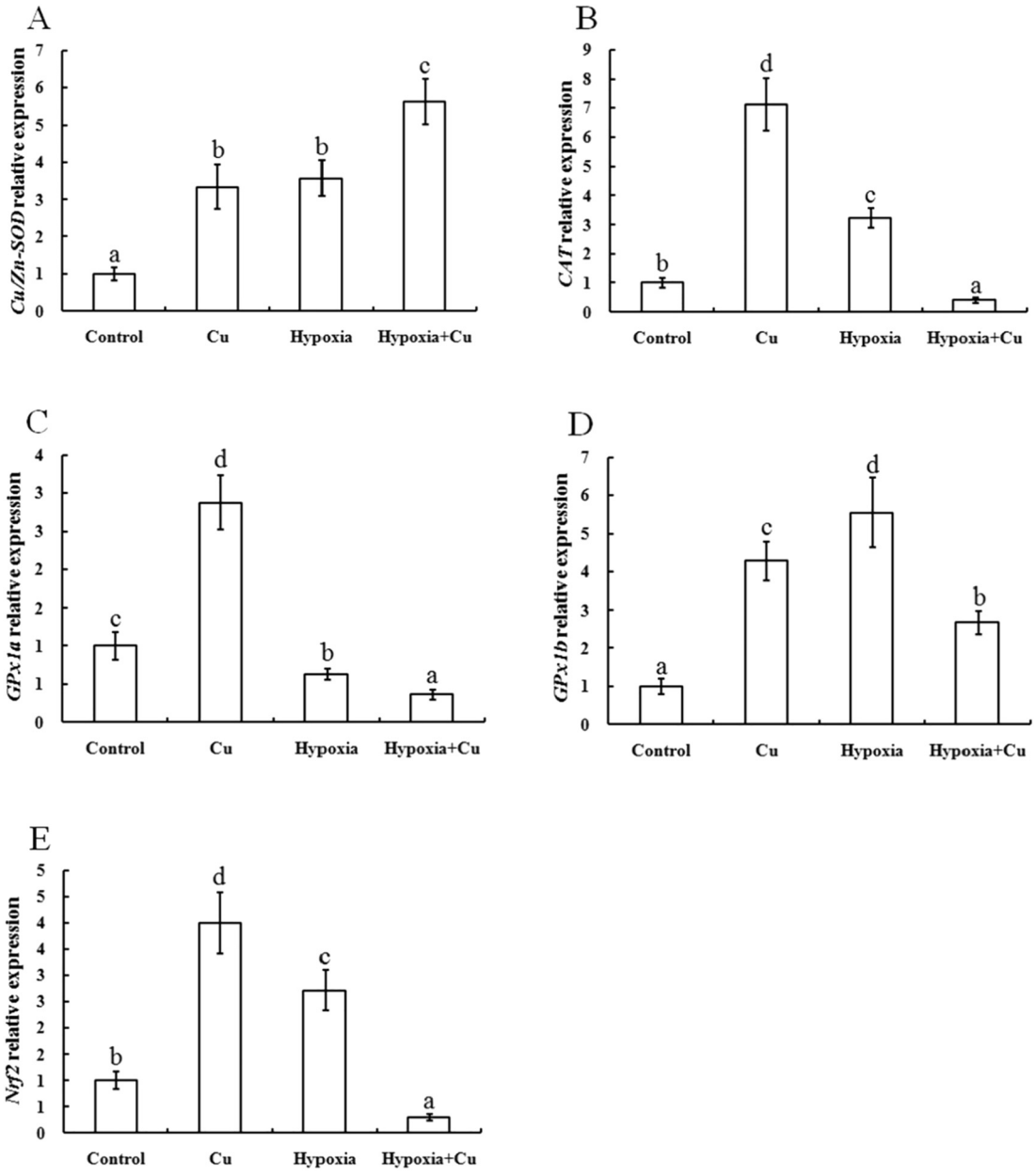

Fig. 4. Changes in the gene expressions involved in antioxidative defense in the liver of the large yellow croaker exposed to Cu and hypoxia. Each value represents means ± SEM ($n = 6$). Different letters indicate significant differences ($P < 0.05$).

4. Discussion

Organisms that are pre-acclimated to a mild concentration of stressor may be able to enhance resistance to future higher level of that (or a different) stressor (Costantini et al., 2012; Costantini, 2014). The phenomenon is termed as priming or conditioning hormesis, which has been observed in various fish species (Dolci et al., 2014; Vergauwen et al., 2013). Our previous study in large yellow croaker has demonstrated that low concentration of Cu pre-acclimation could enhance innate immune defense to mitigate oxidative damage induced by the subsequent sub-lethal Cu exposure (Zeng et al., 2017). However, little information is available regarding the effects of metal pre-acclimation on the adaptation mechanism of fish exposed to non-metallic stresses.

In this study, the increased ROS production was observed in the hypoxic stress group when compared to that of the control group, indicating that 1.5 mg DO L^{-1} may exceed stress tolerance limit of fish. The large yellow croaker are particularly vulnerable to the environmental changes (Ao et al., 2015). Similar result was reported in our previous study (Zeng et al., 2016a). Excessive ROS could induce oxidative damage such as protein carbonylation, lipid peroxidation, or even death, emphasizing negative effects of xenobiotics on fish (Del Rio et al., 2005). This was confirmed by the increased hepatic vacuoles in the hypoxic stress group. Cu pre-exposure did not cause significant impacts on survival rate, ROS generation and histological structure in comparison to the control group, indicating that the large yellow croaker has a strong ability to adapt to waterborne copper exposure (30 μg L^{-1}). However, hypoxic stress plus Cu pre-exposure remarkably increased mortality and ROS production, and worsened histological structure when compared to hypoxic stress alone, suggesting Cu pre-acclimation aggravated hypoxia-induced oxidative damage, which is not consistent with the phenomenon of priming hormesis. Our data showed that Cu pre-

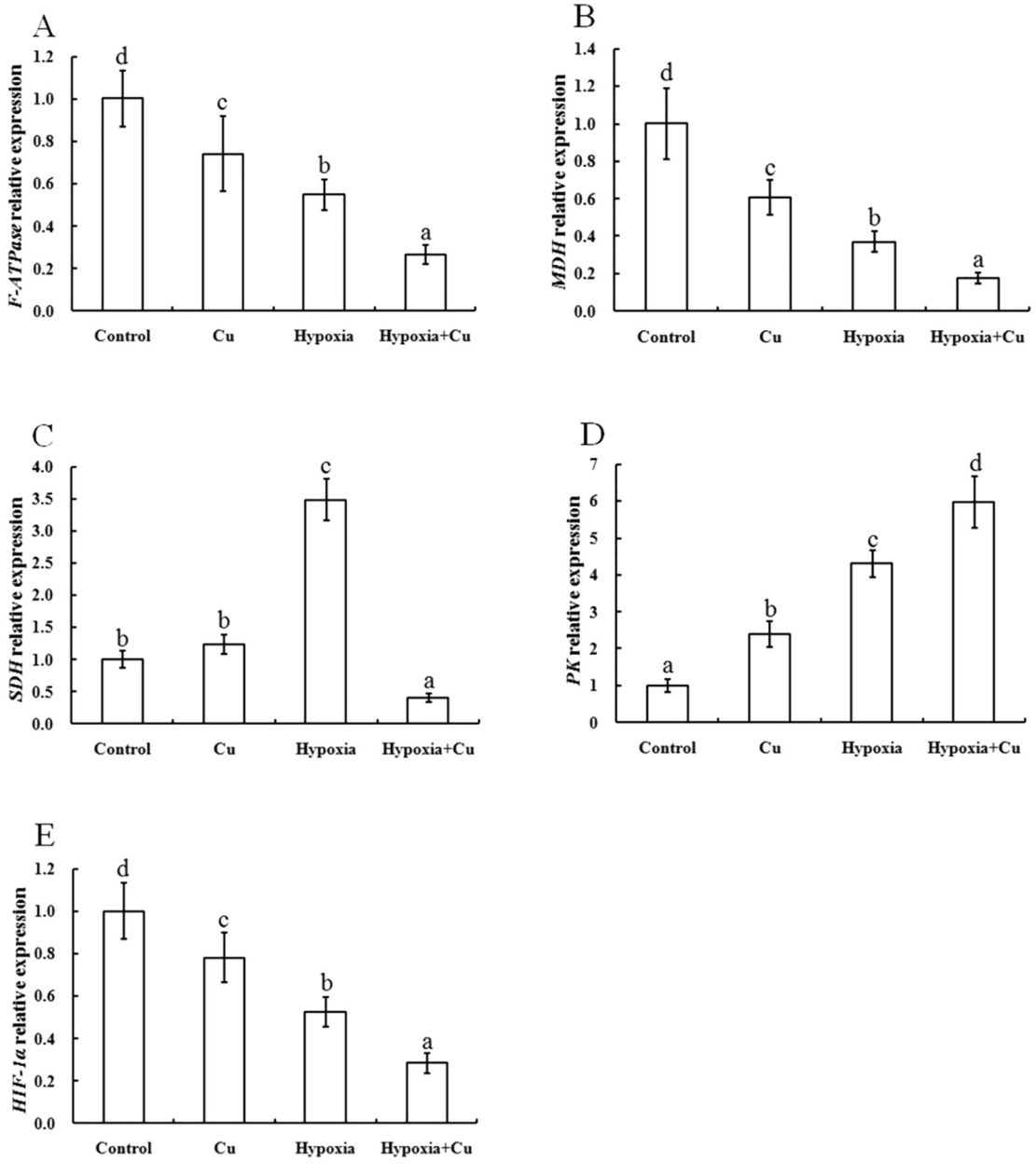

Fig. 5. Changes in the gene expressions involved in energy metabolism in the liver of the large yellow croaker exposed to Cu and hypoxia. Each value represents means ± SEM ($n = 6$). Different letters indicate significant differences ($P < 0.05$).

exposure enhanced Cu content (15.56 times) in response to hypoxia when compared to the same Cu concentration under normoxia (11.94 times). The absence of O_2 as the terminal electron acceptor may damage the mitochondrial electron transport chain, leading to the increased retention of the intracellular Cu (Donnelly et al., 2012; Fitzgerald et al., 2016). The increased Cu content may in turn act additively to some hypoxia responses, including the impairment of mitochondrial function and enhanced ROS production (Heath, 1991; Sappal et al., 2015).

ROS can act as signaling molecules in modulating antioxidative defense and energy metabolism (Archer et al., 2008; Rabinovitch et al., 2017). SOD catalyzes superoxide anion radical (O_2^-) and H^+ to H_2O_2, which is subsequently reduced to non-toxic H_2O by CAT or GPx. Thus, SOD, CAT and GPx constitute the mutual antioxidant defense systems (Winston and Di Giulio, 1991). MTs act as powerful antioxidants to protect organisms against oxidative damage (Figueira et al., 2012), and also participate in the regulation of redox equilibrium by detoxification and metal transport (Amiard et al., 2006). In the present study, hypoxic stress alone remarkably increased Cu/Zn-SOD activity, indicating a protective effect of antioxidant response. But the increased Cu/Zn-SOD could not fully prevent oxidative damage caused by the severe hypoxia. This notion was confirmed by the increment of ROS and the decrement of GPx activity, which might be concerned with the excess of H_2O_2 catalyzed by Cu/Zn-SOD (Winston and Di Giulio, 1991). Although hypoxic stress alone had no impact on CAT activity, dramatically increased transcriptional levels should not be neglected. Possibly, hypoxia aroused an adaptive stress response at the molecular level (Dolci et al., 2014). When compared to the control group, Cu pre-acclimation boosted MTs levels, Cu/Zn-SOD and CAT activities, which contributes to counteracting oxidative stress resulted from Cu exposure. As compared to hypoxic stress alone, hypoxic stress plus Cu pre-exposure increased Cu/Zn-SOD activity and MTs levels. Cu is an essential cofactor required for Cu/Zn-SOD. Cu pre-exposure could increase Cu accumulation,

Table 2
Pearson's correlation coefficient of parameters in the liver of the large yellow croaker.

Independent parameters	Dependent parameters	Correlation coefficients	*P*
Cu/Zn-SOD mRNA levels	Cu/Zn-SOD activities	0.836	0.000
CAT mRNA levels	CAT activities	0.723	0.000
GPx1a mRNA levels	GPx activities	0.763	0.000
GPx1b mRNA levels	GPx activities	−0.219	0.303
F-ATPase mRNA levels	F-ATPase activities	0.824	0.000
MDH mRNA levels	MDH activities	0.810	0.000
SDH mRNA levels	SDH activities	0.348	0.096
PK mRNA levels	PK activities	0.931	0.000
Nrf2 mRNA levels	*Cu/Zn-SOD* mRNA levels	−0.127	0.555
Nrf2 mRNA levels	*CAT* mRNA levels	0.912	0.000
Nrf2 mRNA levels	*GPx1a* mRNA levels	0.754	0.000
Nrf2 mRNA levels	*GPx1b* mRNA levels	0.658	0.000
HIF-1α mRNA levels	*F-ATPase* mRNA levels	0.872	0.000
HIF-1α mRNA levels	*MDH* mRNA levels	0.846	0.000
HIF-1α mRNA levels	*SDH* mRNA levels	−0.036	0.869
HIF-1α mRNA levels	*PK* mRNA levels	−0.918	0.000
COX activities	ATP contents	0.877	0.000
ROS formation	F-ATPase activities	−0.887	0.000
ROS formation	MDH activities	−0.943	0.000
ROS formation	SDH activities	−0.741	0.000
ROS formation	PK activities	0.935	0.000
ROS formation	*Nrf2* mRNA levels	−0.507	0.011
ROS formation	*HIF-1α* mRNA levels	−0.893	0.000

which might in turn stimulate Cu/Zn-SOD activity to enhance antioxidant defense (Lartigue et al., 2015; Turski and Thiele, 2009). The existing studies revealed that most of metal exposures could induce MTs (Abril et al., 2018; Le et al., 2016). However, the increased Cu/Zn-SOD activity and MTs levels in the hypoxic stress plus Cu pre-exposure group were mainly used for reducing Cu toxicity instead of hypoxia-induced oxidative damage, as reflected by the reduced CAT and GPx activities and the deterioration of histological structure. Similar result was reported in pacu *Piaractus mesopotamicus* exposed to the combined Cu and hypoxia exposures (Sampaio et al., 2008).

The tolerance of environmental stress largely depends on energy supply (Lushchak, 2011). Previous studies focused only on a single aspect of energy deprivation or ROS production in response to Cu and hypoxia exposures, the relationship between ROS production and energy metabolism has been neglected. F-ATPase, MDH and SDH participate in mitochondrial aerobic metabolism through tricarboxylic acid cycle. They can effectively utilize respiration-generated proton gradient to drive ATP production. The mitochondrial membrane generally remains depolarized in these processes, which may contribute to the inhibition of ROS generation (Walsh and Koshland, 1984; Yasuda et al., 1998; Pollard et al., 2005). PK is the rate-limiting enzyme of glycolytic pathway that generates energy through anaerobic metabolism (Weber et al., 1966). COX is regarded as a key enzyme involved in respiratory function in the mitochondria, which is closely related to ATP production (Ogunbona et al., 2018). In this study, hypoxic stress alone significantly inhibited F-ATPase, MDH and SDH activities, and enhanced PK activity, which may result in the enhancement of mitochondrial membrane hyperpolarization and the excessive production of free radicals. The excessive ROS would in turn down-regulate tricarboxylic acid cycle and up-regulate glycolysis to accelerate ROS oxidation (Martínez-Reyes and Cuezva, 2014). This notion was supported by a positive relationship between ROS and PK activity, and negative correlations between ROS and activities of tricarboxylic acid cycle enzymes. Hypoxic stress plus Cu pre-exposure further reduced tricarboxylic acid cycle enzyme activities and increased PK activity when compared to hypoxic stress alone, indicating Cu pre-acclimation inhibited aerobic metabolism, enhanced anaerobic metabolism, and aggravated oxidative damage when fish were exposed to severe hypoxia. In such situations, Cu pre-acclimation is more likely to depend on glycolytic ATP production to defeat against hypoxic response.

As a redox-active metal, Cu could participate in electron transport chain to drive ATP synthesis and reduce ROS production (Jomova et al., 2012). However, excessive Cu might destroy the mitochondrial electron transport chain (mitochondrial respiration), especially under hypoxic condition, resulting in boosting anaerobic metabolism to generate energy (Donnelly et al., 2012). This view was confirmed by the decreased COX activity and ATP content, and the increased ROS and lactate levels. However, our previous study indicated that β-glucan could enhance glycolysis and inhibit tricarboxylic acid cycle to ameliorate hypoxia-induced oxidative stress in the liver of the large yellow croaker (Zeng et al., 2016a). This seeming contradiction can be partially explained as follows: β-glucan itself has an adverse effect on the energy metabolism in fish (Sinha et al., 2011). In addition, our recent study has shown that β-glucan could hinder Cu absorption and bioavailability in the large yellow croaker (Zeng et al., 2018). But hypoxia could sensitize the electron transport system to increase Cu uptake (Sappal et al., 2015).

Modifications in the enzyme activities might be involved in the changes in their respective mRNA transcription levels. In this study, the mRNA levels of *Cu/Zn-SOD*, *CAT*, *F-ATPase*, *MDH* and *PK* were positively correlated with their respective enzyme activities, suggesting these enzyme activities might be modulated at the transcriptional level (Kuschel et al., 2012). However, differential expression patterns between GPx activity and *GPx1b* gene expression, and between SDH activity and *SDH* gene expression were observed, which may be closely connected with RNA stability, post-translational modification and/or time-lag effect (Craig et al., 2007; Sadi et al., 2014). In addition, GPx activity was codetermined by the two isoenzymes (GPx1a and GPx1b), while each gene only encoded an isoenzyme. Similar results were reported in fish exposed to metal stresses (Jiang et al., 2014; Zeng et al., 2016b).

Nrf2 and *HIF-1α* take part in modulating transcriptional expression of genes related to antioxidant defense and energy metabolism, respectively (Huang et al., 2004; Sant et al., 2017). Previous studies indicated that hypoxia could restrain Cu-induced toxicological effects of zebrafish embryos by regulating *HIF* signaling pathway (Fitzgerald et al., 2016). In the study, *Nrf2* gene expression was positively correlated with *CAT*, *GPx1a* and *GPx1b* mRNA levels, indicating these antioxidant genes were transcriptionally induced by *Nrf2* signal pathway. *HIF-1α* mRNA level was paralleled with *F-ATPase*, *MDH* and *PK* gene expressions, indicating the changes of gene expressions may refer to the modification in *HIF-1α* mRNA level. However, there were no relationships between *Nrf2* and *Cu/Zn-SOD* gene expressions, and between *HIF-1α* and *SDH* gene expressions, indicating these gene expressions may be regulated by transcription factors at the post-transcriptional levels (Kuschel et al., 2012). Antioxidant defense could be activated by metal through ROS/Nrf2 signaling pathway (Kovac et al., 2015). Mitochondrial ROS are an essential part of HIF-1α stabilization (Archer et al., 2008). However, some studies indicated that HIF-1α could be regulated independently from ROS (Rissanen et al., 2006). In this study, ROS were positively correlated with both *Nrf2* and *HIF-1α*, indicating *Nrf2* and *HIF-1α* signaling pathways participate in the regulation of ROS production by antioxidant response and energy metabolism, respectively.

5. Conclusion

This study demonstrated for the first time that Cu pre-exposure aggravated hypoxia-induced oxidative damage in the liver of the large yellow croaker by inhibiting antioxidant defense and energy metabolism, challenging the phenomenon of priming hormesis. The present results underline the metal pollution history needs to be taken into consideration when the effects of hypoxia on the oxidative stress and energy metabolism of fish are to be evaluated in the future studies, and also contribute to accurately predicting the consequences of the interactions

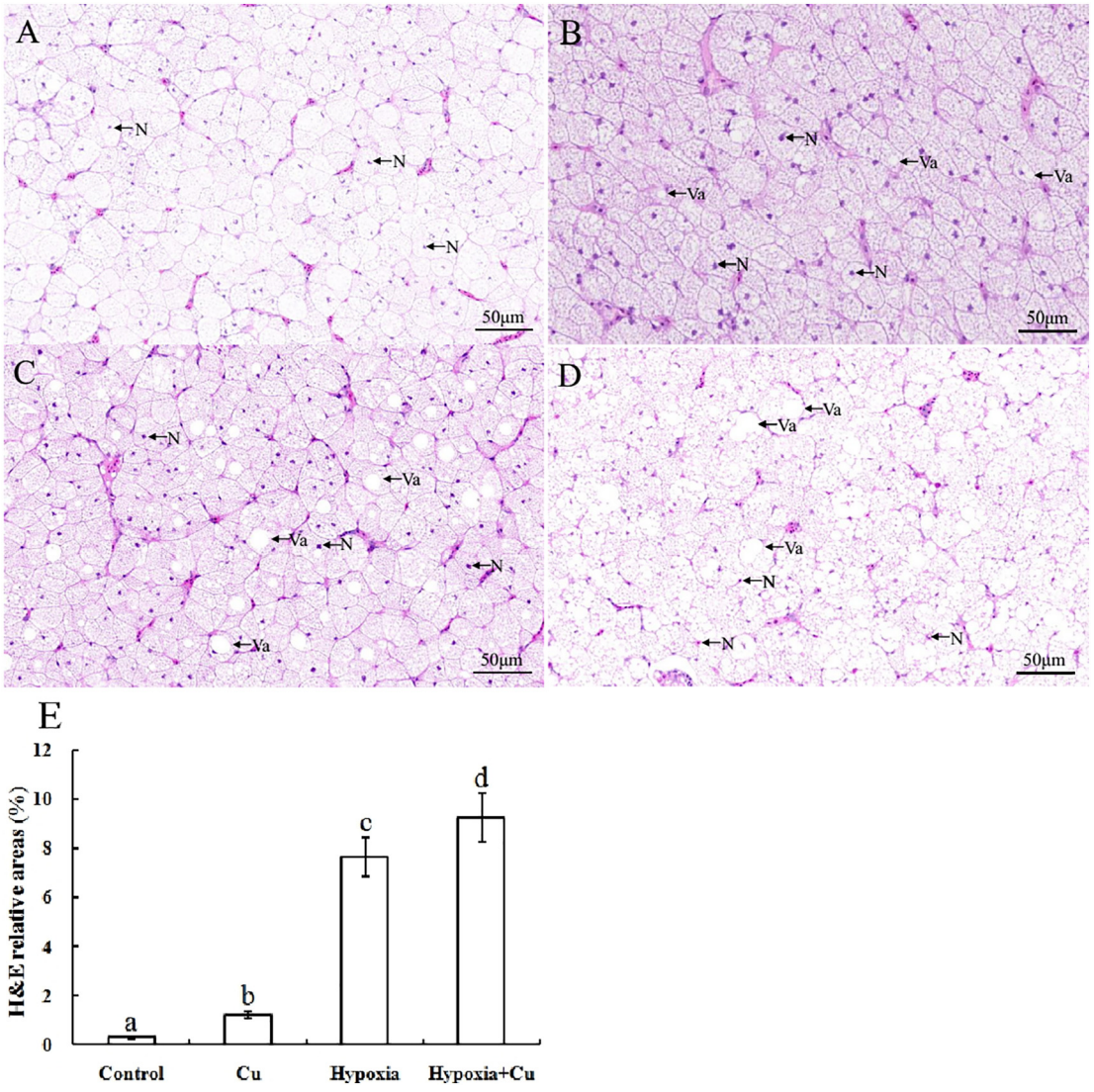

Fig. 6. Liver histology of the large yellow croaker exposed to Cu and hypoxia. A: control group, normal hepatocytes and hepatocyte nucleus. B: Cu pre-exposure group, mild vacuoles appeared. C: hypoxic stress group, severe vacuoles appeared. D: hypoxic stress plus Cu pre-exposure group, histologically indistinct intermembrane boundary, abnormal nuclear chromosome and severe vacuole appeared. Va: vacuole; N: nucleus. Relative areas for hepatic vacuoles in H&E staining (E). Each value represents means ± SEM ($n = 6$) and are normalized to % of field area. Different letters indicate significant differences ($P < 0.05$).

between Cu pre-exposure and hypoxia in the large yellow croaker farming.

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References

Abril, S.I.M., Costa, P.G., Bianchini, A., 2018. Metal accumulation and expression of genes encoding for metallothionein and copper transporters in a chronically exposed wild population of the fish *Hyphessobrycon luetkenii*. Comp. Biochem. Physiol. C 211, 25–31.

Aebi, H., 1984. [13] Catalase in vitro. Methods Enzymol. 105, 121–126.

Amiard, J.C., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P.S., 2006. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. Aquat. Toxicol. 76, 160–202.

Ao, J., Mu, Y., Xiang, L.X., Fan, D., Feng, M., Zhang, S., Nie, L., 2015. Genome sequencing of the perciform fish *Larimichthys crocea* provides insights into molecular and genetic mechanisms of stress adaptation. PLoS Genet. 11, e1005118.

Archer, S.L., Gomberg-Maitland, M., Maitland, M.L., Rich, S., Garcia, J.G., Weir, E.K., 2008. Mitochondrial metabolism, redox signaling, and fusion: a mitochondria-ROS-HIF-1α-Kv1.5 O_2-sensing pathway at the intersection of pulmonary hypertension and cancer. Am. J. Physiol.-Heart C. 294, H570–H578.

Birnie-Gauvin, K., Costantini, D., Cooke, S.J., Willmore, W.G., 2017. A comparative and evolutionary approach to oxidative stress in fish: a review. Fish Fish. 18, 928–942.

Blewett, T.A., Simon, R.A., Turko, A.J., Wright, P.A., 2017. Copper alters hypoxia sensitivity and the behavioural emersion response in the amphibious fish *Kryptolebias marmoratus*. Aquat. Toxicol. 189, 25–30.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Costantini, D., 2014. Does hormesis foster organism resistance to extreme events? Front. Ecol. Environ. 12, 209–210.

Costantini, D., Monaghan, P., Metcalfe, N.B., 2012. Early life experience primes resistance to oxidative stress. J. Exp. Biol. 215, 2820–2826.

Craig, P.M., Wood, C.M., McClelland, G.B., 2007. Oxidative stress response and gene expression with acute copper exposure in zebrafish (*Danio rerio*). Am. J. Physiol.-Reg. I. 293, R1882–R1892.

Del Rio, D., Stewart, A.J., Pellegrini, N., 2005. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. Nutr. Metab. Cardiovasc. 15, 316–328.

Dodson, M., Redmann, M., Rajasekaran, N.S., Darley-Usmar, V., Zhang, J., 2015. KEAP1-NRF2 signalling and autophagy in protection against oxidative and reductive proteotoxicity. Biochem. J. 469, 347–355.

Dolci, G.S., Vey, L.T., Schuster, A.J., Roversi, K., Roversi, K., Dias, V.T., Pase, C.S., Barcelos, R.C.S., Antoniazzi, C.T.D., Glanzner, W.G., 2014. Hypoxia acclimation protects against oxidative damage and changes in prolactin and somatolactin expression in silver catfish (*Rhamdia quelen*) exposed to manganese. Aquat. Toxicol. 157, 175–185.

Donnelly, P.S., Liddell, J.R., Lim, S., Paterson, B.M., Cater, M.A., Savva, M.S., Mot, A.I., James, J.L., Trounce, I.A., White, A.R., Crouch, P.J., 2012. An impaired mitochondrial electron transport chain increases retention of the hypoxia imaging agent diacetylbis (4-methylthiosemicarbazonato) copperII. Proc. Natl. Acad. Sci. U. S. A. 109, 47–52.

Drotar, A., Phelps, P., Fall, R., 1985. Evidence for glutathione peroxidase activities in cultured plant cells. Plant Sci. 42, 35–40.

Figueira, E., Branco, D., Antunes, S.C., Gonçalves, F., Freitas, R., 2012. Are metallothioneins equally good biomarkers of metal and oxidative stress? Ecotoxicol. Environ. Saf. 84, 185–190.

Fitzgerald, J.A., Jameson, H.M., Dewar Fowler, V.H., Bond, G.L., Bickley, L.K., Uren Webster, T.M., Bury, N.R., Wilson, R.W., Santos, E.M., 2016. Hypoxia suppressed copper toxicity during early development in zebrafish embryos in a process mediated by the activation of the HIF signaling pathway. Environ. Sci. Technol. 50, 4502–4512.

Fitzgerald, J.A., Katsiadaki, I., Santos, E.M., 2017. Contrasting effects of hypoxia on copper toxicity during development in the three-spined stickleback (*Gasterosteus aculeatus*). Environ. Pollut. 222, 433–443.

Foster, G.D., Moon, T.W., 1986. Enzyme activities in the Atlantic hagfish, Myxine glutinosa: changes with captivity and food deprivation. Can. J. Zool. 64, 1080–1085.

Heath, A.G., 1991. Effect of water-borne copper on physiological responses of bluegill (*Lepomis macrochirus*) to acute hypoxic stress and subsequent recovery. Comp. Biochem. Physiol. C 100, 559–564.

Hosseini, M.J., Shaki, F., Ghazi-Khansari, M., Pourahmad, J., 2014. Toxicity of copper on isolated liver mitochondria: impairment at complexes I, II, and IV leads to increased ROS production. Cell Biochem. Biophys. 70, 367–381.

Howarth, R., Chan, F., Conley, D.J., Garnier, J., Doney, S., Marino, R., Billen, G., 2011. Coupled biogeochemical cycles: eutrophication and hypoxia in temperate estuaries and coastal marine ecosystems. Front. Ecol. Environ. 9, 18–26.

Huang, Y., Hickey, R.P., Yeh, J.L., Liu, D., Dadak, A., Young, L.H., Johnson, R.S., Giordano, F.J., 2004. Cardiac myocyte-specific HIF-1α deletion alters vascularization, energy availability, calcium flux, and contractility in the normoxic heart. FASEB J. 18, 1138–1140.

Jiang, W.D., Liu, Y., Hu, K., Jiang, J., Li, S.H., Feng, L., Zhou, X.Q., 2014. Copper exposure induces oxidative injury, disturbs the antioxidant system and changes the Nrf2/ARE (CuZnSOD) signaling in the fish brain: protective effects of myo-inositol. Aquat. Toxicol. 155, 301–313.

Jomova, K., Baros, S., Valko, M., 2012. Redox active metal-induced oxidative stress in biological systems. Transit. Met. Chem. 37, 127–134.

Kovac, S., Angelova, P.R., Holmström, K.M., Zhang, Y., Dinkova-Kostova, A.T., Abramov, A.Y., 2015. Nrf2 regulates ROS production by mitochondria and NADPH oxidase. BBA-Gen. Subjects 1850, 794–801.

Kuschel, A., Simon, P., Tug, S., 2012. Functional regulation of HIF-1α under normoxia—is there more than post-translational regulation? J. Cell. Physiol. 227, 514–524.

Lartigue, A., Burlat, B., Coutard, B., Chaspoul, F., Claverie, J.M., Abergel, C., 2015. The megavirus chilensis Cu, Zn-superoxide dismutase: the first viral structure of a typical cellular copper chaperone-independent hyperstable dimeric enzyme. J. Virol. 89, 824–832.

Le, T.Y., Zimmermann, S., Sures, B., 2016. How does the metallothionein induction in bivalves meet the criteria for biomarkers of metal exposure? Environ. Pollut. 212, 257–268.

LeBel, C.P., Ischiropoulos, H., Bondy, S.C., 1992. Evaluation of the probe 2′, 7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem. Res. Toxicol. 5, 227–231.

Liu, X.J., Luo, Z., Xiong, B.X., Liu, X., Zhao, Y.H., Hu, G.F., Lv, G.J., 2010. Effect of waterborne copper exposure on growth, hepatic enzymatic activities and histology in *Synechogobius hasta*. Ecotoxicol. Environ. Saf. 73, 1286–1291.

Luo, C., Wang, X., Long, J., Liu, J., 2006. An NADH-tetrazolium-coupled sensitive assay for malate dehydrogenase in mitochondria and crude tissue homogenates. J. Biochem. Biophys. Methods 68, 101–111.

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30.

Mandic, M., Tzaneva, V., Careau, V., Perry, S.F., 2019. Hif-1α paralogs play a role in the hypoxic ventilatory response of larval and adult zebrafish (*Danio rerio*). J. Exp. Biol. 222 (2) (jeb-195198).

Martínez-Reyes, I., Cuezva, J.M., 2014. The H^+-ATP synthase: a gate to ROS-mediated cell death or cell survival. BBA-Bioenergetics 1837, 1099–1112.

Morin, C., Zini, R., Simon, N., Tillement, J.P., 2002. Dehydroepiandrosterone and α-estradiol limit the functional alterations of rat brain mitochondria submitted to different experimental stresses. Neuroscience 115, 415–424.

Nakano, M., Kimura, H., Hara, M., Kuroiwa, M., Kato, M., Totsune, K., Yoshikawa, T., 1990. A highly sensitive method for determining both Mn-and Cu/Zn superoxide dismutase activities in tissues and blood cells. Anal. Biochem. 187, 277–280.

Ogunbona, O.B., Baile, M.G., Claypool, S.M., 2018. Cardiomyopathy-associated mutation in the ADP/ATP carrier reveals translation-dependent regulation of cytochrome c oxidase activity. Mol. Biol. Cell 29, 1449–1464.

Pan, K., Wang, W.X., 2012. Trace metal contamination in estuarine and coastal environments in China. Sci. Total Environ. 421, 3–16.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45-e45.

Philip, G.H., Reddy, P.M., Sridevi, G., 1995. Cypermethrin-induced in vivo alterations in the carbohydrate metabolism of freshwater fish, *Labeo rohita*. Ecotoxicol. Environ. Saf. 31, 173–178.

Pollard, P.J., Briere, J.J., Alam, N.A., Barwell, J., Barclay, E., Wortham, N.C., Hunt, T., Mitchell, M., Olpin, S., Moat, S.J., Hargreaves, I.P., Heales, S.J., Chung, Y.L., Griffiths, J.R., Dalgleish, A., McGrath, J.A., Gleeson, M.J., Hodgson, S.V., Poulsom, R., Rustin, P., Tomlinson, I.P.M., 2005. Accumulation of Krebs cycle intermediates and over-expression of HIF1α in tumours which result from germline FH and SDH mutations. Hum. Mol. Genet. 14, 2231–2239.

Rabinovitch, R.C., Samborska, B., Faubert, B., Ma, E.H., Gravel, S.P., Andrzejewski, S., Raissi, T.C., Pause, A., Pierre, J.S., Jones, R.G., 2017. AMPK maintains cellular metabolic homeostasis through regulation of mitochondrial reactive oxygen species. Cell Rep. 21, 1–9.

Ransberry, V.E., Blewett, T.A., McClelland, G.B., 2016. The oxidative stress response in freshwater-acclimated killifish (*Fundulus heteroclitus*) to acute copper and hypoxia exposure. Comp. Biochem. Physiol. C 179, 11–18.

Rissanen, E., Tranberg, H.K., Sollid, J., Nilsson, G.E., Nikinmaa, M., 2006. Temperature regulates hypoxia-inducible factor-1 (HIF-1) in a poikilothermic vertebrate, crucian carp (*Carassius carassius*). J. Exp. Biol. 209, 994–1003.

Sadi, G., Bozan, D., Yildiz, H.B., 2014. Redox regulation of antioxidant enzymes: post-translational modulation of catalase and glutathione peroxidase activity by resveratrol in diabetic rat liver. Mol. Cell. Biochem. 393, 111–122.

Sampaio, F.G., de Lima Boijink, C., Oba, E.T., dos Santos, L.R.B., Kalinin, A.L., Rantin, F.T., 2008. Antioxidant defenses and biochemical changes in pacu (*Piaractus mesopotamicus*) in response to single and combined copper and hypoxia exposure. Comp. Biochem. Physiol. C 147, 43–51.

Sant, K.E., Hansen, J.M., Williams, L.M., Tran, N.L., Goldstone, J.V., Stegeman, J.J., Hahn, M.E., Timme-Laragy, A., 2017. The role of Nrf1 and Nrf2 in the regulation of glutathione and redox dynamics in the developing zebrafish embryo. Redox Biol. 13, 207–218.

Sappal, R., MacDougald, M., Fast, M., Stevens, D., Kibenge, F., Siah, A., Kamunde, C., 2015. Alterations in mitochondrial electron transport system activity in response to warm acclimation, hypoxia-reoxygenation and copper in rainbow trout, *Oncorhynchus mykiss*. Aquat. Toxicol. 165, 51–63.

Sappal, R., Fast, M., Purcell, S., MacDonald, N., Stevens, D., Kibenge, F., Siah, A., Kamunde, C., 2016. Copper and hypoxia modulate transcriptional and mitochondrial functional-biochemical responses in warm acclimated rainbow trout (*Oncorhynchus mykiss*). Environ. Pollut. 211, 291–306.

Sinha, A.K., Kumar, V., Makkar, H.P., De Boeck, G., Becker, K., 2011. Non-starch polysaccharides and their role in fish nutrition–a review. Food Chem. 127, 1409–1426.

Sun, S., Gu, Z., Fu, H., Zhu, J., 2016. The metallothionein gene from the oriental river prawn *Macrobrachium nipponense* (De Haan, 1849): characterization and expression in response to hypoxia and reoxygenation. Crustaceana 89, 1083–1097.

Sun, P., Bao, P., Tang, B., 2017. Transcriptome analysis and discovery of genes involved in immune pathways in large yellow croaker (*Larimichthys crocea*) under high stocking density stress. Fish Shellfish Immunol. 68, 332–340.

Turski, M.L., Thiele, D.J., 2009. New roles for copper metabolism in cell proliferation, signaling, and disease. J. Biol. Chem. 284, 717–721.

Vergauwen, L., Knapen, D., Hagenaars, A., Blust, R., 2013. Hypothermal and hyperthermal acclimation differentially modulate cadmium accumulation and toxicity in the zebrafish. Chemosphere 91, 521–529.

Viarengo, A., Ponzano, E., Dondero, F., Fabbri, R., 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs. Mar. Environ. Res. 44, 69–84.

Walsh, K., Koshland, D.E., 1984. Determination of flux through the branch point of two metabolic cycles. The tricarboxylic acid cycle and the glyoxylate shunt. J. Biol. Chem. 259, 9646–9654.

Wang, B., Feng, L., Jiang, W.D., Wu, P., Kuang, S.Y., Jiang, J., Tang, L., Tang, W.N., Zhang, Y.A., Liu, Y., Zhou, X.Q., 2015. Copper-induced tight junction mRNA expression changes, apoptosis and antioxidant responses via NF-κB, TOR and Nrf2 signaling molecules in the gills of fish: preventive role of arginine. Aquat. Toxicol. 158, 125–137.

Weber, G., Convery, H.J.H., Lea, M.A., Stamm, N.B., 1966. Feedback inhibition of key glycolytic enzymes in liver: action of free fatty acids. Science 154, 1357–1360.

Winston, G.W., Di Giulio, R.T., 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. Aquat. Toxicol. 19, 137–161.

Wu, C., Zhang, D., Kan, M., Lv, Z., Zhu, A., Su, Y., Jiang, L., 2014. The draft genome of the large yellow croaker reveals well-developed innate immunity. Nat. Commun. 5, 5227.

Yasuda, R., Noji, H., Kinosita Jr., K., Yoshida, M., 1998. F1-ATPase is a highly efficient molecular motor that rotates with discrete 120 steps. Cell 93, 1117–1124.

Yin, F., Gong, H., Ke, Q., Li, A., 2015. Stress, antioxidant defence and mucosal immune responses of the large yellow croaker *Pseudosciaena crocea* challenged with *Cryptocaryon irritans*. Fish Shellfish Immunol. 47, 344–351.

Yuan, S.S., Lv, Z.M., Zhu, A.Y., Zheng, J.L., Wu, C.W., 2017. Negative effect of chronic cadmium exposure on growth, histology, ultrastructure, antioxidant and innate immune responses in the liver of zebrafish: preventive role of blue light emitting diodes. Ecotoxicol. Environ. Saf. 139, 18–26.

Zeng, L., Wang, Y.H., Ai, C.X., Zheng, J.L., Wu, C.W., Cai, R., 2016a. Effects of β-glucan on ROS production and energy metabolism in yellow croaker (*Pseudosciaena crocea*) under acute hypoxic stress. Fish Physiol. Biochem. 42, 1395–1405.

Zeng, L., Zheng, J.L., Wang, Y.H., Xu, M.Y., Zhu, A.Y., Wu, C.W., 2016b. The role of Nrf2/Keap1 signaling in inorganic mercury induced oxidative stress in the liver of large yellow croaker *Pseudosciaena crocea*. Ecotoxicol. Environ. Saf. 132, 345–352.

Zeng, L., Zhang, J.S., Zheng, J.L., Wu, C.W., 2017. Pre-acclimation to low copper mitigated immunotoxic effects in spleen and head-kidney of large yellow croaker (*Pseudosciaena crocea*) when exposed subsequently to high copper. Ecotoxicol. Environ. Saf. 144, 54–61.

Zeng, L., Wang, Y.H., Ai, C.X., Zhang, J.S., 2018. Differential effects of β-glucan on oxidative stress, inflammation and copper transport in two intestinal regions of large yellow croaker *Larimichthys crocea* under acute copper stress. Ecotoxicol. Environ. Saf. 165, 78–87.

Zhu, C.D., Wang, Z.H., Yan, B., 2013. Strategies for hypoxia adaptation in fish species: a review. J. Comp. Physiol. B. 183, 1005–1013.