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Pre-hypoxia exposure inhibited copper toxicity by improving energy metabolism, antioxidant defence and mitophagy in the liver of the large yellow croaker *Larimichthys crocea*

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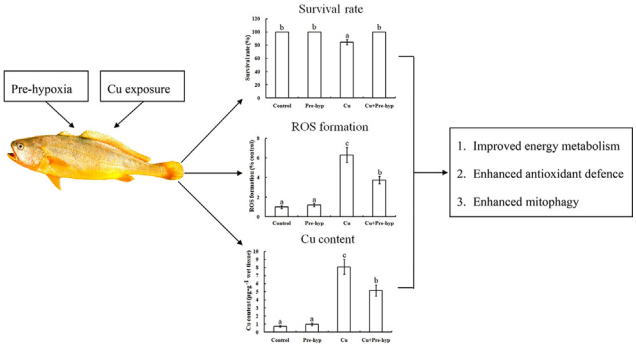
HIGHLIGHTS

- Copper exposure induced oxidative damage and aberrant mitochondrial ultrastructure.
- Pre-hypoxia exposure alleviated Cu-induced toxicological effects.
- ROS were positively correlated with *HIF-1α* and *FoxO3* gene expression.
- MT protein levels were positively associated with Cu content.

GRAPHICAL ABSTRACT



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ABSTRACT

This study investigated the effects of moderate hypoxia pre-exposure on energy metabolism, antioxidant defence and mitophagy in the liver of the large yellow croaker *Larimichthys crocea* exposed to Cu. Fish were pre-exposed to either normoxia or hypoxia (~3.0 mg L^{-1}, 42% O_2 saturation) for 48 h, and subsequently were subjected to either control (without Cu addition) or Cu (168 μg L^{-1}) under normoxic conditions for another 48 h. Copper exposure under normoxia induced Cu toxicity that increased mortality, the production of reactive oxygen species (ROS) and malondialdehyde, and aberrant hepatic mitochondrial ultrastructure. Interestingly, hypoxia pre-exposure improved energy metabolism, antioxidant ability and mitophagy response, and reduced the Cu content to inhibit Cu toxicity, reflecting the enhanced survival rate and reduced oxidative damage. In these processes, hypoxia-inducible factor-1α (*HIF-1α*), transcription factors NFE2-related nuclear factor 2 (*Nrf2*), and forkhead box O-3 (*FoxO3*) mRNA levels were correlated with expression of genes related to energy metabolism, antioxidant defence and mitophagy, respectively, indicating *HIF-1α*, *Nrf2*, and *FoxO3* are required for the induction of their respective target genes. Overall, moderate hypoxia pre-exposure was able to generate adaptive responses to mitigate Cu-induced toxicological effects, underlining a central role of hormesis.

1. Introduction

Dissolved oxygen (DO) is a vital element in the aquaculture environment. DO concentration is especially vulnerable to abiotic and biotic factors, such as water temperature, salinity, tidal cycle,

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eutrophication, and respiration of organisms. Thus, hypoxia is becoming more common in the mariculture industry and natural ecosystems (Howarth et al., 2011). In the natural environment, fish may encounter chemical toxicity and hypoxia. The combined effects of hypoxia and metal exposure on fish have become increasingly popular research fields in recent years (Donnelly et al., 2012; Ransberry et al., 2016). Hypoxic exposure may occur before, after, or simultaneously with metal exposure. Most studies suggest that the interactions between hypoxia and metal stress have synergistic effects that increase oxidative damage in fish (Blewett et al., 2017). However, studies on the effects of pre-hypoxia exposure on metal-induced toxicological responses are commonly neglected. Among these metals, Cu is a widespread global environmental contaminant, mainly owing to the excessive discharge of untreated industrial wastewater and mining activities (Pan and Wang, 2012). Despite the fact that it is essential for various physiological functions, Cu can be toxic to aquatic organisms at excessive concentrations (Bosch et al., 2016). Hypoxia could boost Cu sensitivity of the electron transport system, leading to the increase of Cu uptake (Fitzgerald et al., 2016; Sappal et al., 2016). Thus, it is possible that hypoxia pre-acclimation could affect the toxicological responses of fish exposed to Cu stress, which needs to be further investigated.

Organisms that are pre-exposed to a low concentration of stress may improve tolerance to subsequent higher levels of the same or different stress (defined as priming or conditioning hormesis), which is a common phenomenon in aquatic organisms (Costantini, 2014). For example, mild metal and high temperature pre-acclimations could improve the tolerance of fish to subsequent lethal metal stresses (Driessnack et al., 2017; Vergauwen et al., 2013; Zeng et al., 2017). However, little is known about the effects of hypoxic conditioning hormesis on metal-induced toxicity in fish, especially for essential metals. Dolci et al. (2014, 2017) reported that pre-exposure to hypoxia could enhance mitochondrial viability and reduce Mn-induced toxicity and oxidative damage in the silver catfish *Rhamdia quelen* by changing the activities of CAT and Na^+/K^+-ATPase and the gene expression of prolactin and somatolactin, but the underlying mechanisms are largely unknown.

The mitochondrial electron transport chain and adenosine triphosphate (ATP) synthesis may be inhibited in response to stress, resulting in the overproduction of reactive oxygen species (ROS) (Hosseini et al., 2014; Sappal et al., 2016). Enhanced energy expenditure and oxidative stress are the two important components in stress adaptation, and are closely interrelated (Wang et al., 2019; Zhu et al., 2013). Therefore, energy metabolism and antioxidant defence might participate in the process of hypoxic conditioning hormesis. As a key modulator of the hypoxia response, hypoxia-inducible factor-1α (HIF-1α) can regulate gene expression related to energy metabolism under the condition of hypoxia (Mandic et al., 2018; Stegen et al., 2016). NFE2-related nuclear factor 2 (Nrf2) is a dominant regulator of antioxidant defence by modulating mRNA levels involved in oxidative stress (Dodson et al., 2015). Metallothioneins (MTs), possessing cysteine-rich proteins, are crucial for sustaining cytosolic redox homeostasis by the transportation, sequestration, and detoxification of metals and the scavenging of excessive ROS (Amiard et al., 2006; Figueira et al., 2012). MTs also have an important role for haemostasis of essential trace elements (Kaji et al., 1993). Except for metals, MTs can be activated by other stressors (such as hypoxia and heat), which facilitate our understanding of these protein functions under multiple stressors (Sun et al., 2016).

Mitochondria are regarded as the central site for ROS generation and energy metabolism. Therefore, mitochondria are key intracellular targets for evaluating stress-induced damage. ROS can serve as signalling molecules in the stress-induced mitochondrial autophagy, which is also known as mitophagy. Mitophagy can regulate mitochondrial quantity and quality by selective sequestration of damaged or defunct mitochondria (Coto-Montes et al., 2012). Microtubule-associated protein light chain 3 (LC3α), PTEN-induced putative kinase 1 (PINK1), PARKIN (Parkin), NIP3-like protein X (Nix), and mitofusin2 (Mfn2) take part in the evolutionarily conserved mitophagy process (Bhansali et al., 2017). Forkhead box O-3 (FoxO3) is the master transcription factor regulating gene expression involved in the mitophagy response. FoxO3 also participates in energy metabolism, oxidative stress, and inflammation (Fang et al., 2016). Until now, limited research has been conducted to investigate the regulation of mitophagy in fish under stress.

The large yellow croaker *Larimichthys crocea* is one of the most commercially important marine fish species, the output of which is the highest among the sea cage-cultured fishes in recent years in China. Increasing the number of cages and the density of culture often adopted in large yellow croaker culture to enhance profitability, which easily results in hypoxia and *Cryptocaryon irritans* outbreaks, particularly in the summer (Sun et al., 2017a; Yin et al., 2015). Because of *C. irritans* infection, a high death rate (about 90%) of large yellow croaker occurred in 2016. Copper sulfate ($CuSO_4$) is the best treatment for *C. irritans*, which may lead to Cu pollution in the aquaculture water body. The effects of pre-hypoxia exposure on energy metabolism, antioxidant response, and mitophagy of large yellow croaker exposed to Cu were analysed in the present study. For the first time, our study emphasised the importance of mitochondria in the hormesis response, which provides some novel insights into how hypoxic conditioning hormesis mediates Cu toxicity in fish.

2. Materials and methods

2.1. Experimental treatments

The large yellow croaker specimens were obtained from a local aquaculture farm (Zhejiang, China). Prior to the experiment, fish were maintained in 400 L fiberglass tanks for a 14-day acclimation. During the acclimation period, the fish were fed with a commercial formulated diet (9.60% lipid and 48.80% crude protein) to satiation, twice daily. Salinity, temperature, pH and DO were 26.5 ± 0.76 ppt, 25.6 ± 2.8 °C, 7.73 ± 0.41 and 7.54 ± 0.46 mg L^{-1} (87.82 ± 0.98% O_2 saturation), respectively. Total ammonia nitrogen, nitrite and nitrate were 0.018–0.029 mg L^{-1}, 0.016–0.034 mg L^{-1}, and 0.134–0.218 mg L^{-1}, respectively. After acclimation, 180 uniformly sized fish (mean body weight: 72.3 ± 4.7 g) were assigned to 12 fibreglass tanks, with 15 fish in each tank. First, the fish were exposed to normoxia (continuous bubbling of air) and medium hypoxia (3.0 mg DO L^{-1}, as suggested by Zeng et al. (2016a)) for 48 h, with six replicates per treatment. 3.0 mg DO L^{-1} was obtained as suggested by Zeng et al. (2016a). The tank water surface was covered with plastic wrap to avoid the interchange of oxygen between the air and the water. The DO level of the water was lowered by bubbling a mixture of nitrogen gas (N_2) and air at an appropriate proportion. Mass flow controllers were used to maintain N_2 and air at a constant flow rate. Fifty percent of the seawater volume was renewed daily with the same DO concentration. The DO level was measured using a DO meter (YSI 550A, USA) every 4 h. The actual DO concentrations in the control and hypoxia exposure groups were 7.48 ± 0.34 mg L^{-1} (87.64 ± 0.96% O_2 saturation) and 3.08 ± 0.46 mg L^{-1} (41.92 ± 1.34% O_2 saturation), respectively. Subsequently, the fish were exposed to 0 or 168 μg L^{-1} Cu^{2+} (close to the concentration of Cu^{2+} pollution in aquaculture water when $CuSO_4$ is used to treat *C. irritans* disease) under normoxic conditions for another 48 h, with three replicates for each treatment.

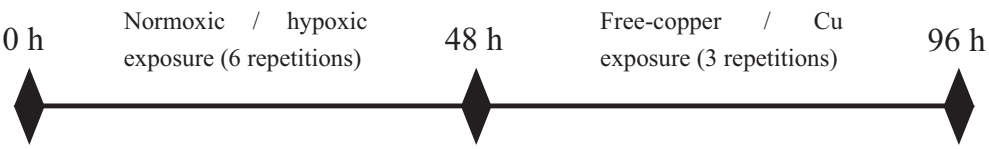

Fig. 1. Experimental design. Fish were exposed to normoxia/hypoxia for 48 h, and were then exposed or not to waterborne Cu (168 μg L^{-1}) for another 48 h.

The $CuSO_4{\cdot}5H_2O$ (AR; Shanghai Sinopharm Group, China) was added into the seawater of the water reservoir as the Cu^{2+} source, and equilibrated for 12 h prior to introduction into the Cu exposure groups (Fig. 1). The water reservoir was pre-washed with 10% HNO_3 (guaranteed reagent; Sinopharm Chemical Reagent Corporation, China) to reduce the effect of contamination to Cu^{2+}. Water was renewed 100% twice daily to maintain the waterborne Cu^{2+} level. The Cu^{2+} concentrations were measured every 12 h. Water samples were acidified with 2% HNO_3, and Cu^{2+} levels were analysed by inductivity coupled plasma mass spectrometry (ICP-MS, Thermo Jarrel Ash Corporation, USA) as described by Liu et al. (2010). The detection limit of Cu^{2+} was 0.04 μg L^{-1}. The actual Cu^{2+} concentrations in the control group, pre-hypoxia exposure group, Cu exposure under normoxia group and Cu plus pre-hypoxia exposure group were 3.18 ± 0.05, 3.17 ± 0.04, 162.21 ± 2.59 and 161.98 ± 2.40 μg L^{-1}, respectively.

At the termination of the exposure trial, fish were anaesthetised in a MS-222 bath (100 mg L^{-1}). The livers of six fish from each tank were removed, the left lobe of the liver samples was fixed in cold 2.5% glutaraldehyde for the ultrastructure study, the right lobe of the liver samples was frozen in liquid nitrogen, and then stored at −80C until analysed.

2.2. Ultrastructural study

Ultrastructural analysis was conducted as described by Abdel-Moneim and Abdel-Mohsen (2010). Briefly, the fixed liver samples were washed with phosphate buffer solution (PBS, pH = 7.2) and were then post-fixed in 1% aqueous osmium tetroxide at 4 °C. The fixed cells were rinsed again with PBS, dehydrated in an ascending ethanol series, and embedded with an epon-araldite mixture. Ultrathin sections were obtained with a diamond knife, and stained with uranyl acetate and lead citrate. Sections were observed with a HITACHI H-600 electron microscope.

2.3. Biochemical analysis

The preparation and extraction of homogenates and supernatants were conducted as described by Zeng et al. (2016b). ROS and malondialdehyde (MDA) were measured as described by LeBel et al. (1992) and Livingstone et al. (1990), respectively. ATP content, lactate levels and cytochrome *c* oxidase (COX) activity were determined using the commercial kits from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). ATP synthase (F-ATPase), succinate dehydrogenase (SDH), malate dehydrogenase (MDH) and pyruvate kinase (PK) activities were assayed as described by Morin et al. (2002), Philip et al. (1995), Luo et al. (2006), and Foster and Moon (1986), respectively. Copper/zinc-superoxide dismutase (Cu/Zn-SOD), glutathione peroxidase (GPx), and catalase (CAT) activities and MT protein levels were spectrophotometrically quantified as described by Nakano et al. (1990), Drotar et al. (1985), Aebi (1984), and Viarengo et al. (1997), respectively. The enzyme activities were expressed as U (units) per mg protein. Protein content was measured according to the Bradford method (1976).

Table 1
Primer sequences used for real-time PCR.

Gene name	Primer sequences (from 5′ to 3′)	Size (bp)	PCR efficiency
F-ATPase	F: TGGTTACTCCGTGTTCGCT R: GGGGCTCGTTCATTTGAC	142	1.02
SDH	F: TACAGGGTCAGGAGAAGAAGC R: AATGGTCAATAACAGGTCGGT	123	0.99
MDH	F: AAGTAGAGTTCCCCGCTGAC R: CACCCTCCTTCCCGTTCA	170	0.98
PK	F: CTGGTTTCCTTATGTGCGAG R: GGTCCTGGATGTCCTTTTCT	256	1.03
HIF-1α	F: AACTGTTCACTCGGGCAATAG R: GAAGTGGCGGTGGTAACG	129	1.03
Cu/Zn-SOD	F: GAGACAATACAAACGGGTGC R: CAATGATGGAAATGGGGC	137	0.97
GPx1a	F: GACTCGTTATTCTGGGTGTTCCCTGTA R: CCATTCCCTGGACGGACATACTTC	103	1.04
GPx1b	F: TCTTGTCCCTGAAGTATGTCCGTCCTG R: GGCATCCTTTCCATTTACATCCACCTT	89	1.02
CAT	F: ATTATGCCATCGGAGACTTG R: GCACCATTTTGCCCACAG	115	0.98
Nrf2	F: CCCTCAAAATCCCTTTCACT R: GCTACCTTGTTCTTGCCGC	90	0.96
LC3α	F: TGGGTCAGAACCACCACAGAACT R: GCTACTACGTGGGCCTGCAATG	82	0.98
PINK1	F: CAGGAGAAACCCGAGCAA R: CACGGCAGACTGGCACA	145	0.97
Parkin	F: ATGGAGGAAGGGTGATGGAGAAGAACA R: CATGGTACGACCTCGAAGGAGAACGT	202	1.02
Nix	F: GAGGACTGCGTGAACAAGTGGAC R: GCCGTGGTGACTGGTAGTTTGAG	148	0.99
Mfn2	F: TTGCTACATCGGCATTGCTACG R: GAGGACACTGTGCCCGAACG	210	1.04
FoxO3	F: GACGAGGTGCCCGATGACGA R: AACCCTGAGGAACCATTTGGAGTG	251	1.01
β-Actin	F: TCGTCGGTCGTCCCAGGCAT R: ATGGCGTGGGGCAGAGCGT	182	1.05
GAPDH	F: GACAACGAGTTCGGATACAGC R: CAGTTGATTGGCTTGTTTGG	89	1.04

2.4. mRNA expression analysis

mRNA expression analysis was conducted according to our previous publications (Zeng et al., 2016b). RNA extraction, cDNA synthesis and quantitative real-time PCR (qPCR) were assayed using the commercial kits from TaKaRa (Dalian, China). qPCR primers were designed based on the genome data of the large yellow croaker in our laboratory (Table 1) (Wu et al., 2014). The relative transcript abundances were calculated using the $2^{-\Delta\Delta Ct}$ method when target genes were normalised to the geometric mean of the best combination of *β-actin* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Pfaffl, 2001).

2.5. Statistical analysis

The results are presented as mean ± standard deviation (SD). Normality of distribution and homogeneity of variances were tested using the Shapiro–Wilk test and Barlett's test, respectively.

Then data were subjected to one-way ANOVA and Duncan's multiple range test ($P < 0.05$). Pearson correlation analysis was used to estimate the relationship between different parameters. Analysis was performed using SPSS 18.0.

3. Results

3.1. Ultrastructural observation

In the control and pre-hypoxia exposure groups, the hepatic ultrastructures were normal, exhibiting cluster formation of mitochondria with clear cristae (Fig. 2A and B). In the Cu exposure under normoxia group, the following changes were observed: the nucleus was swollen and the heterochromatin had disappeared, the mitochondria were atrophic and clustered with regression of the cristae, and the endoplasmic reticula had disappeared (Fig. 2C). In the Cu plus pre-hypoxia exposure group, the following changes were found: the nucleus was dilated, and the mitochondria were atrophic and clustered with regression of the cristae (Fig. 2D). The degree of mitochondrial atrophy and heterochromatin damage in the Cu plus pre-hypoxia exposure group was lower than those in the Cu exposure under normoxia group.

3.2. Survival rate and biochemical indicators

The survival rate was reduced in the Cu exposure under normoxia group (84.45 ± 3.85%) (Fig. 3A). Compared with the control group, in the pre-hypoxia exposure group, ATP content was lower and lactate levels were higher (Fig. 3). ROS, MDA content, Cu content, and lactate levels were increased and ATP content was reduced in the Cu exposure under normoxia and Cu plus pre-hypoxia exposure groups. ROS, MDA content, Cu content and lactate levels were lower, and ATP content was higher in the Cu plus pre-hypoxia exposure group than in the Cu exposure under normoxia group.

The activities of F-ATPase, SDH, and COX were lower, and the activities of Cu/Zn-SOD and GPx were higher in the pre-hypoxia exposure group than in the control group (Figs. 4–6). The activities of F-ATPase, SDH, MDH, GPx, CAT, and COX were reduced and the activities of PK and Cu/Zn-SOD and the MT protein levels were increased in the Cu exposure under normoxia group. The activities of F-ATPase, SDH, MDH, and COX were reduced and the activities of PK, Cu/Zn-SOD, GPx, and CAT and the MT protein levels were increased in the Cu plus pre-hypoxia exposure group. The activities of F-ATPase, SDH, MDH, GPx, CAT, and COX were higher, and the PK activity and MT protein levels were lower in the Cu plus pre-hypoxia exposure group than in the Cu exposure under normoxia group.

3.3. mRNA levels of genes related to energy metabolism

PK mRNA levels were higher and *F-ATPase* and *HIF-1α* mRNA levels were lower in the pre-hypoxia exposure group than in the control group (Fig. 7). *PK* gene expression was increased, and *F-ATPase*, *SDH*, *MDH*, and *HIF-1α* mRNA levels were reduced in the Cu exposure under normoxia and Cu plus pre-hypoxia exposure groups. *F-ATPase*, *MDH*, and *HIF-1α* mRNA levels were higher, and *SDH* and *PK* gene expression was lower in the Cu plus pre-hypoxia exposure group than in the Cu exposure under normoxia group.

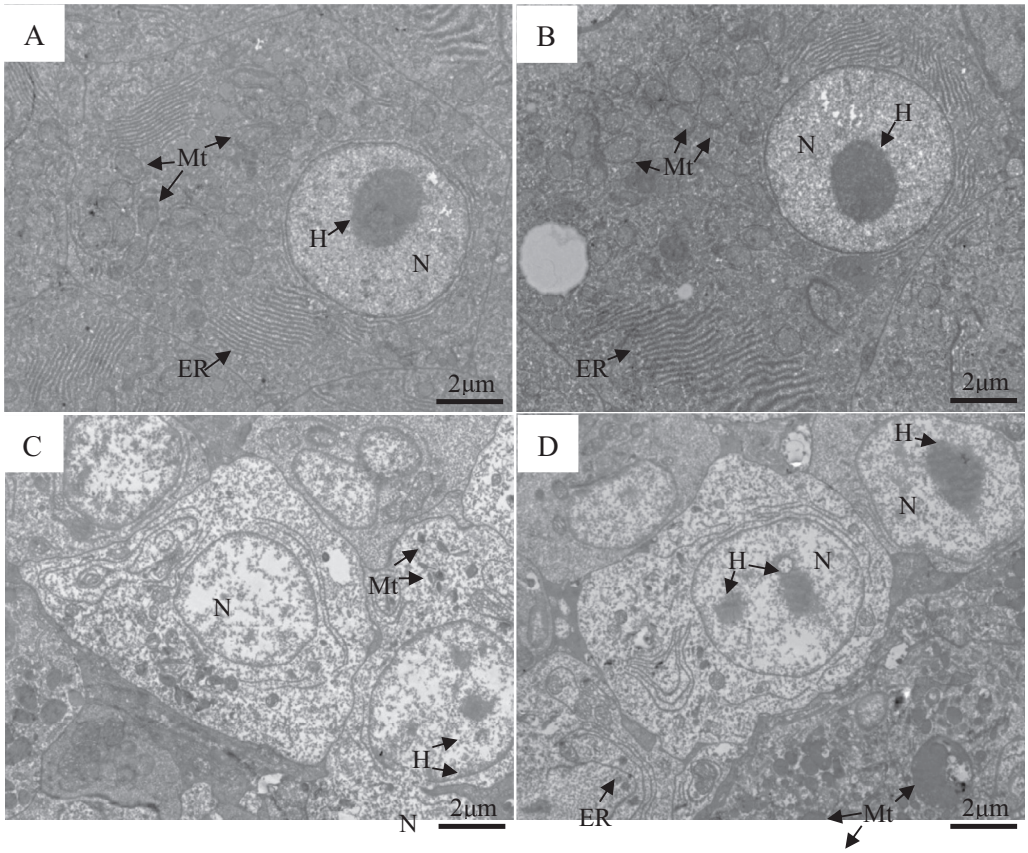

Fig. 2. Ultrastructure of hepatocytes from the large yellow croaker exposed to hypoxia and Cu. A (control group) and B (pre-hypoxia exposure group): the hepatic ultrastructure was normal, showing cluster formation of mitochondria with clear cristae. C (Cu exposure under normoxia group): dilated nucleus, atrophic and clustered mitochondria with cristae regression, and heterochromatin and endoplasmic reticula disappeared. D (Cu plus pre-hypoxia exposure group): dilated nucleus, and atrophic and clustered mitochondria with cristae regression. N, nucleus; H, heterochromatin; Mt, mitochondrial; ER, endoplasmic reticula.

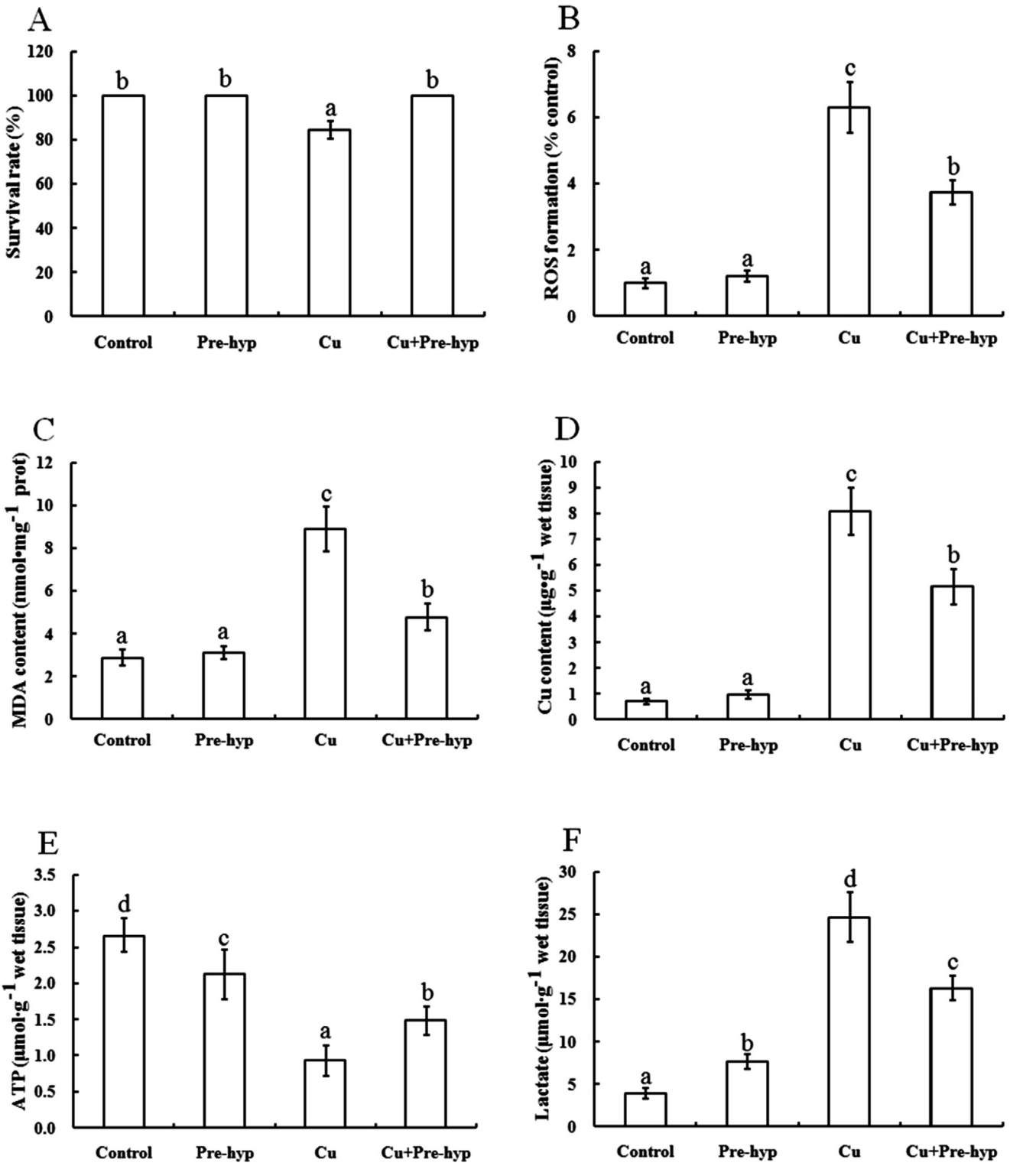

Fig. 3. Changes in the survival rate (A), ROS formation (B), MDA (C), Cu content (D), ATP content (E), and lactate levels (F) in the liver of the large yellow croaker exposed to hypoxia and Cu. Each value represents the mean ± SD. Different letters indicate significant differences ($P < 0.05$).

3.4. mRNA levels of genes related to antioxidant defence

The mRNA levels of *GPx1a*, *GPx1b*, *CAT* and *Nrf2* were higher in the pre-hypoxia exposure group than in the control group (Fig. 8). *Cu/Zn-SOD*, *GPx1a*, and *Nrf2* gene expression was increased and *CAT* mRNA levels were reduced in the Cu exposure under normoxia group. mRNA levels related to antioxidant defence (*Cu/Zn-SOD*, *GPx1a*, *GPx1b*, *CAT* and *Nrf2*) were higher in the Cu plus pre-hypoxia exposure group than in the control and Cu exposure under normoxia groups.

3.5. mRNA levels of genes related to mitophagy

When compared with the control group, *PINK1* gene expression was higher in the pre-hypoxia exposure group than in the control group (Fig. 9). The expression of genes involved in mitophagy

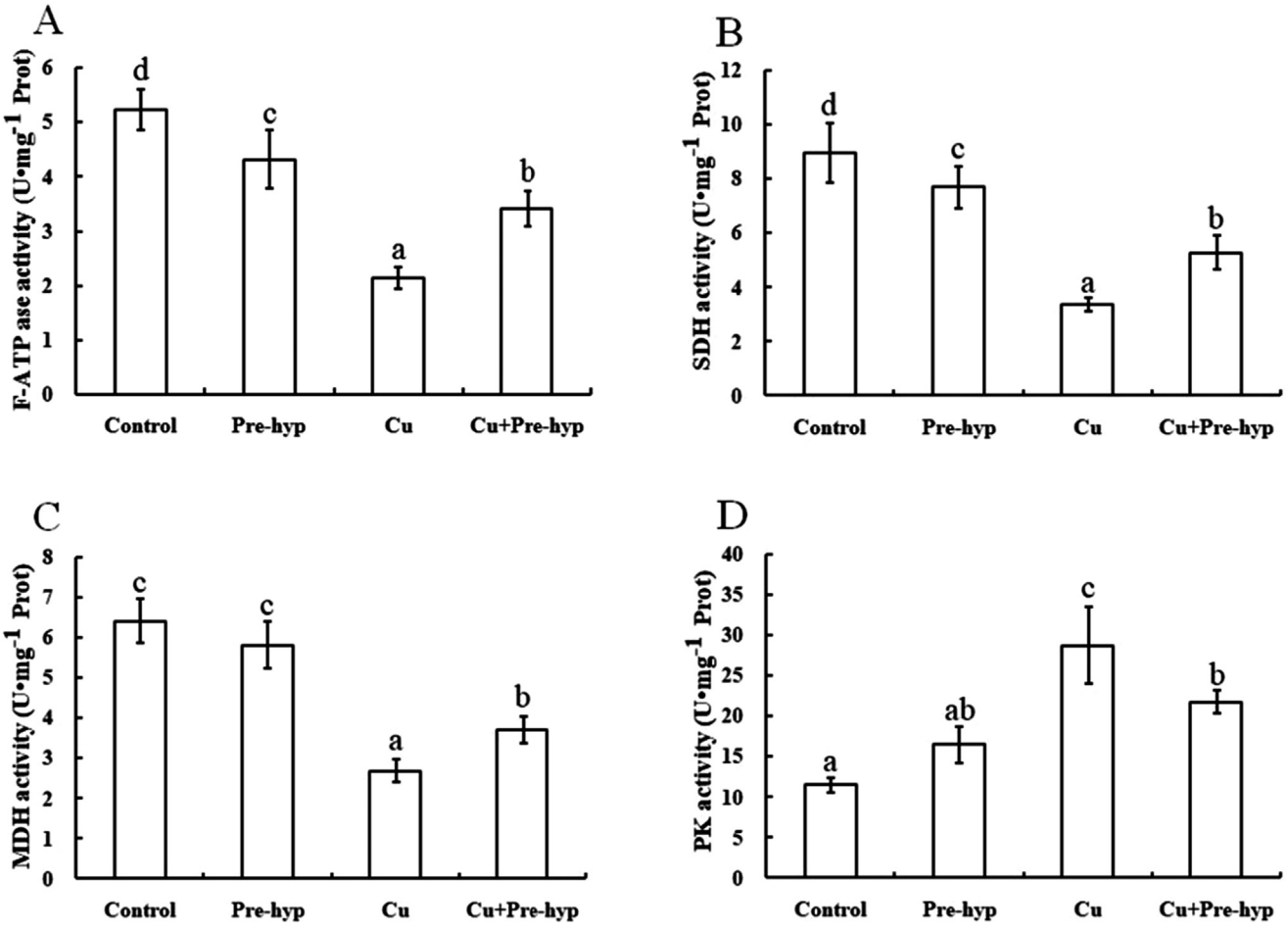

Fig. 4. Changes in the activities of F-ATP (A), MDH (B), SDH (C), and PK (D) in the liver of the large yellow croaker exposed to hypoxia and Cu. Each value represents the mean ± SD. Different letters indicate significant differences ($P < 0.05$).

(*LC3α*, *PINK1*, *Parkin*, *Nix*, *Mfn2*, and *FoxO3*) was up-regulated in the Cu exposure under normoxia and Cu plus pre-hypoxia exposure groups. The mRNA levels of *LC3α*, *PINK1*, *Parkin*, *Nix*, and *FoxO3* were higher in the Cu plus pre-hypoxia exposure group than in the Cu exposure under normoxia group.

3.6. Correlation analysis

Positive correlations were observed between mRNA levels and activities of F-ATPase, MDH, PK, Cu/Zn-SOD and CAT (Table 2). A positive relationship was also observed between *GPx1b* mRNA levels and GPx activity. However, no relationship was observed between *GPx1a* gene expression and GPx activity and between *SDH* mRNA levels and SDH activity. *HIF-1α* gene expression was correlated with the mRNA levels of *F-ATPase*, *MDH*, and *PK*, but no relationship was observed with *SDH* gene expression. *Nrf2* gene expression was positively correlated with the mRNA levels of *GPx1a, GPx1b* and *CAT*, but no relationship was observed with *Cu/Zn-SOD* mRNA levels. *FoxO3* mRNA levels showed positive correlations with the expression of mitophagy genes (*LC3α*, *PINK1*, *Parkin*, *Nix,* and *Mfn2*). ROS production was positively correlated with Cu content and *FoxO3* gene expression and negatively correlated with *HIF-1α* mRNA levels, but no relationship was observed with *Nrf2* mRNA levels. A positive correlation between MT protein levels and Cu content was also observed.

4. Discussion

As a redox-active metal, Cu can participate in ATP synthesis to inhibit ROS formation by the mitochondrial electron transport chain (Horn and Barrientos, 2008; Jomova et al., 2012). However, excessive Cu can impose oxidative stress on aquatic organisms, as reflected by the severe alterations of mitochondrial structure, and the increased ROS, MDA, and mortality in the Cu under normoxia exposure group, highlighting the toxic effects of this metal on fish. Similar observations were reported in *Apistogramma agassizii* and *Paracheirodon axelrodi* (Braz-Mota et al., 2018). It was noteworthy that pre-hypoxia exposure mitigated heterochromatin damage and mortality of the large yellow croaker under Cu stress, reinforcing the notion of priming hormesis. The positive effect of pre-hypoxia exposure on Cu toxicity to fish may be partly related to the reduction of Cu content (Dolci et al., 2014). When compared to the control group, fish in the Cu plus pre-hypoxia exposure and Cu under normoxia exposure groups had increased Cu content of 6.24- and 10.35- fold, respectively. Fish were fully qualified to cope with a certain amount of ROS induced by Cu at a low concentration (Zeng et al., 2017). Most studies suggest that the toxicological effects of Cu on fish occur in a dose-dependent manner (Jiang et al., 2014; Zeng et al., 2019). This notion was supported by the fact that Cu content was positively correlated with ROS formation. The Cu content in the Cu plus pre-hypoxia exposure group was significantly lower than that in the Cu under normoxia exposure group, which is inconsistent with the results of Fitzgerald et al.

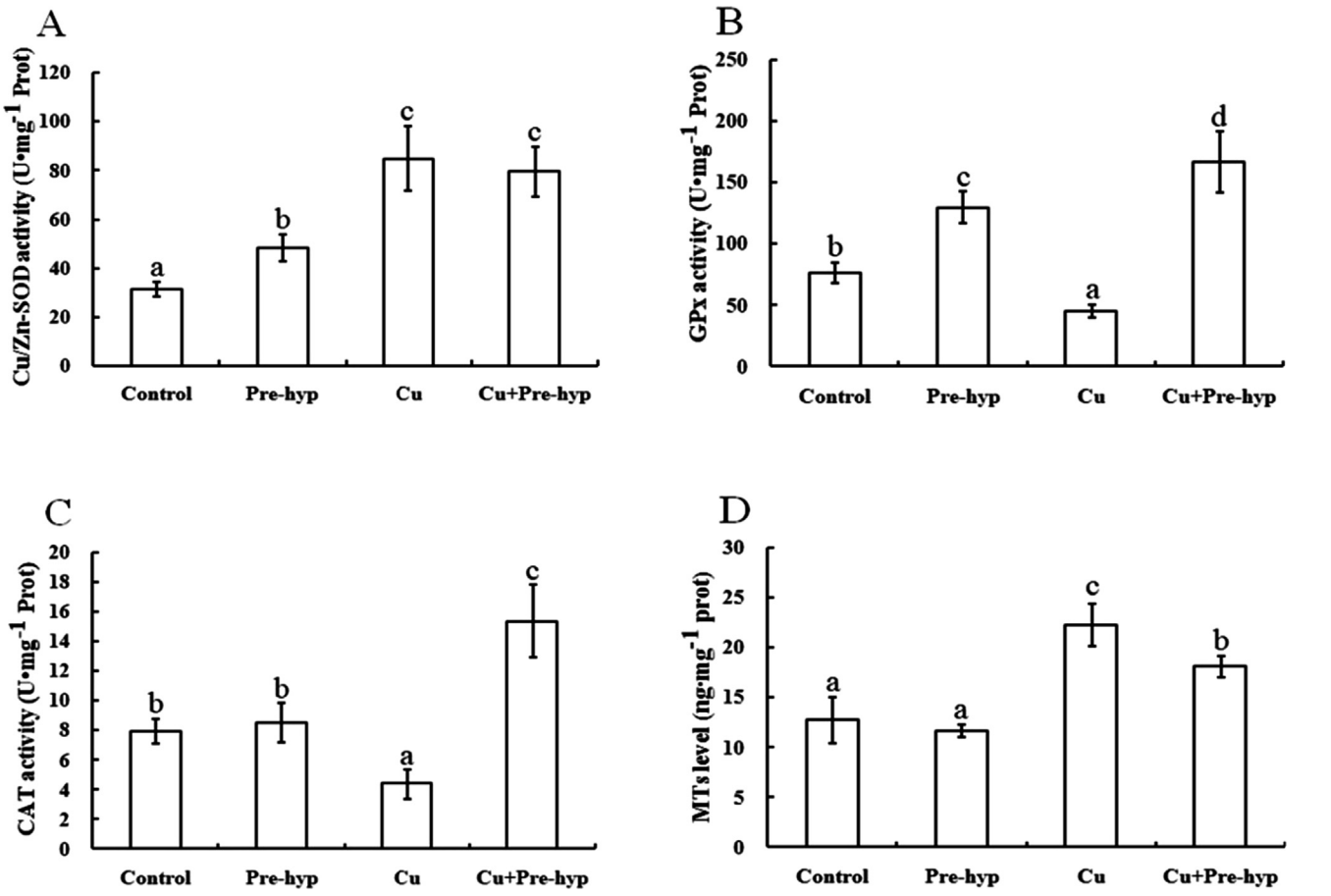

Fig. 5. Changes in the activities of Cu/Zn-SOD (A), GPx (B), CAT (C) and MT protein levels (D) in the liver of the large yellow croaker exposed to hypoxia and Cu. Each value represents the mean ± SD. Different letters indicate significant differences ($P < 0.05$).

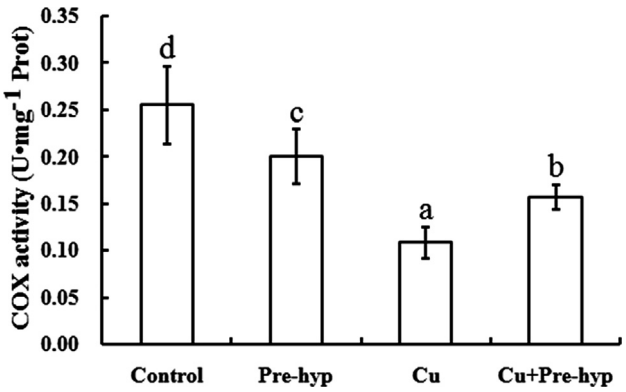

Fig. 6. Change in the COX activity in the liver of the large yellow croaker exposed to hypoxia and Cu. Each value represents the mean ± SD. Different letters indicate significant differences ($P < 0.05$).

(2016) that showed that hypoxic exposure can increase Cu uptake. Possible reasons for this phenomenon were that the effects of hypoxia on Cu content might be related to fish species and size, Cu concentration, exposure route, and duration. Dolci et al. (2017) reported that pre-hypoxia exposure reduced Mn accumulation in the gills of the silver catfish *Rhamdia quelen*. Pre-hypoxia exposure had no effect on ROS, MDA, and survival rate, indicating that large yellow croaker could fully adapt to hypoxia-reoxygenation. Previous studies indicated that liver MDA levels and brain protein carbonyl levels were not affected by hypoxia and normoxic recovery in *Pelteobagrus vachelli* (Zhang et al., 2016). The haematology indexes of the large yellow croaker were not affected by hypoxia (DO at 2.0 mg L^{-1}) until 48 h exposure (Gu and Xu, 2011). Some authors define hypoxia as DO concentrations<3.0 mgL^{-1} (Chen et al., 2007).

Stress tolerance is closely related to energy supply (Lushchak, 2011). F-ATPase is a well-known essential component of mitochondrial energy conversion that produces most of the energy in aerobic cells (Yasuda et al., 1998). SDH inlays the inner mitochondrial membrane that participates in both the electron transport chain and the tricarboxylic acid cycle (Pollard et al., 2005). The mitochondrial MDH is a key component of the tricarboxylic acid cycle (Walsh and Koshland, 1984). Therefore, the three tricarboxylic acid cycle enzymes play important roles in aerobic energy production. In these processes, the mitochondrial membrane generally remains depolarised, which can facilitate the reduction of ROS formation (Korshunov et al., 1997; Sedlic et al. 2010). PK takes part in mitochondrial anaerobic metabolism through the glycolytic pathway (Weber et al., 1966). In the present study, pre-hypoxia exposure inhibited F-ATPase and SDH activities and ATP content and increased PK activity and lactate levels, suggesting a shift from aerobic to anaerobic metabolism when fish were subjected to hypoxia–reoxygenation. Copper exposure under normoxia remarkably reduced the activities of F-ATPase, SDH, and MDH and increased the activity of PK, which might lead to mitochondrial membrane hyperpolarisation and free radical overproduction (Zorov et al., 2006). The excess of ROS would in turn result in increased glycolysis and a reduced tricarboxylic acid cycle to promote ROS oxidation, leading to a decrease in ATP content and an

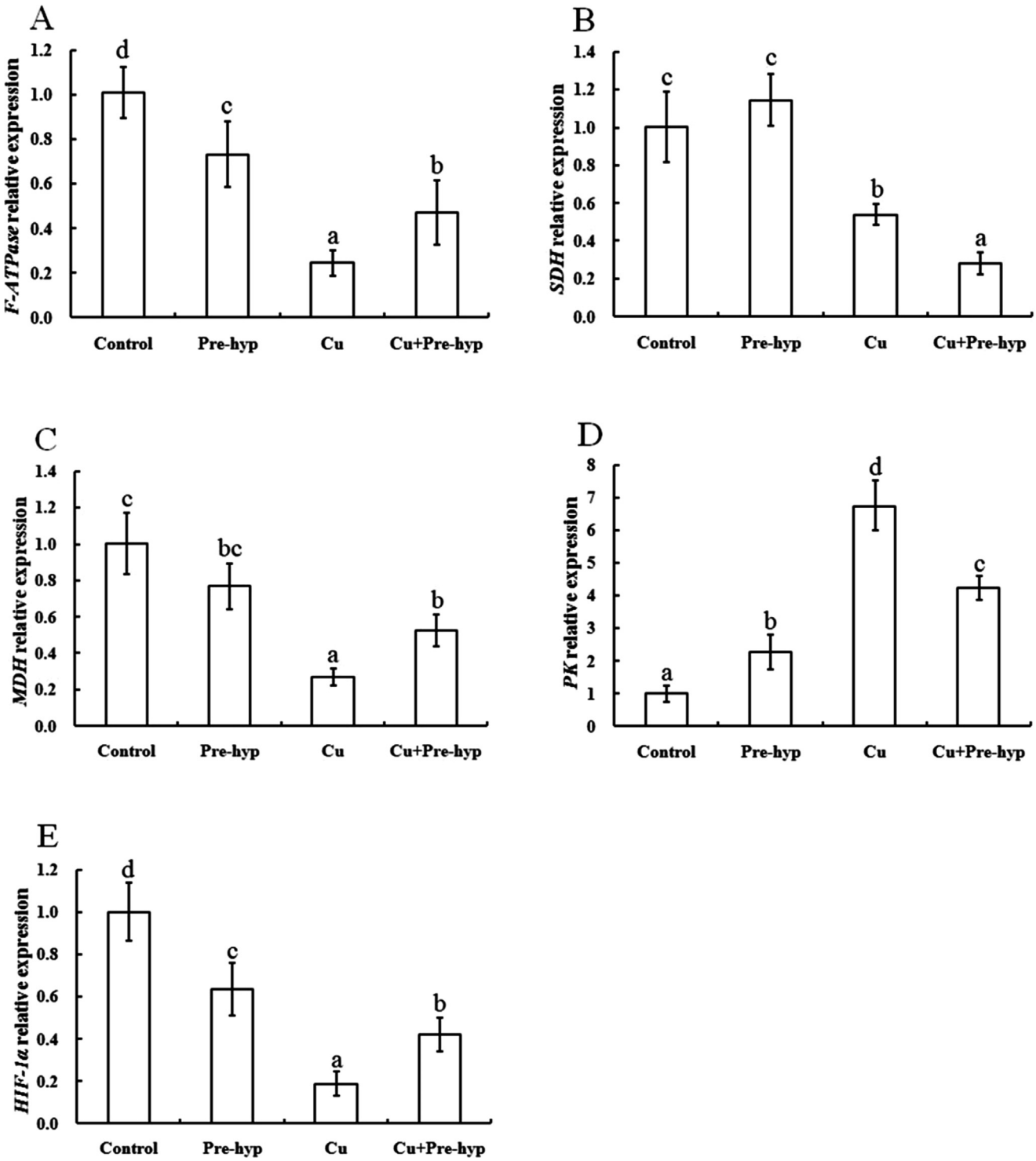

Fig. 7. Changes in the expression of genes involved in energy metabolism in the liver of the large yellow croaker exposed to hypoxia and Cu. Each value represents the mean ± SD. Different letters indicate significant differences ($P < 0.05$).

increase in lactate levels (Martínez-Reyes and Cuezva, 2014). Interestingly, Cu plus pre-hypoxia exposure resulted in remarkably highertricarboxylic acid cycle enzyme activities and reduced PK activity than those in the Cu exposure under normoxia, indicating pre-hypoxia exposure enhanced aerobic metabolism and inhibited anaerobic metabolism of fish exposed to Cu (Gracey et al., 2001; Zeng et al., 2016a). This view was confirmed by the fact that fish in the Cu plus pre-hypoxia exposure group had higher ATP content and lower lactate levels than those in the Cu exposure under normoxia group. In such situations, pre-hypoxia exposure might provide more energy by improving mitochondrial energy metabolism to defend against oxidative damage caused by Cu stress.

The development of antioxidant defence has been considered as a nonspecific and protective adaptation mechanism for Cu toxicity. SOD catalyses O_2^- and H^+ to H_2O_2, which is subsequently converted to H_2O by GPx or CAT. Thus, SOD, GPx, and CAT constitute the mutual antioxidant defence systems. As powerful antioxidants, MTs can protect organisms from oxidative damage (Amiard et al.,

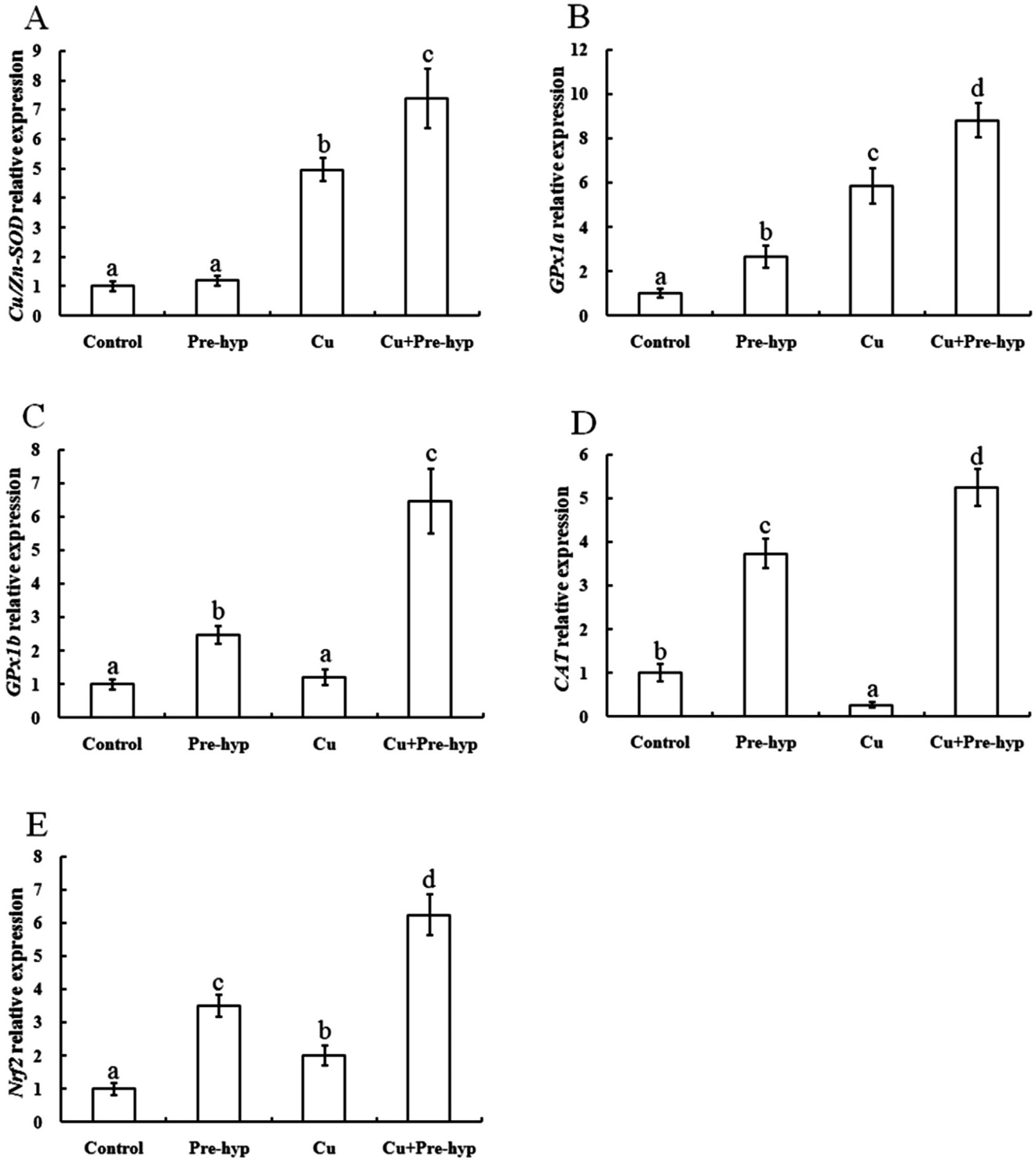

Fig. 8. Changes in the expression of genes involved in antioxidant defense in the liver of the large yellow croaker exposed to hypoxia and Cu. Each value represents the mean ± SD. Different letters indicate significant differences ($P < 0.05$).

2006). In the present study, Cu under normoxia exposure increased Cu/Zn-SOD activity and MT protein levels, but reduced GPx and CAT activities, resulting in the excessive production of ROS, indicating insufficient cellular antioxidant capabilities (Morcillo et al., 2016). Cu plus pre-hypoxia exposure resulted in higher GPx and CAT activities and lower mortality, ROS, and MDA than those in Cu exposure under normoxia, suggesting that pre-hypoxia exposure endowed the fish with protective effects against oxidative stress resulting from Cu stress. However, pre-hypoxia exposure reduced MT protein levels in fish exposed to Cu stress. The main reason for this was that mild hypoxia pre-acclimation might enhance the MT basal concentration in response to Cu stress (Le et al., 2016; Le Croizier et al., 2018). Pre-hypoxia exposure significantly increased Cu/Zn-SOD and GPx activities, which may be an adaptive response to hypoxia-reoxygenation. Although CAT activity was not affected by hypoxia-reoxygenation, the antioxidant capacity of CAT could be compensated by an increase in the GPx activity.

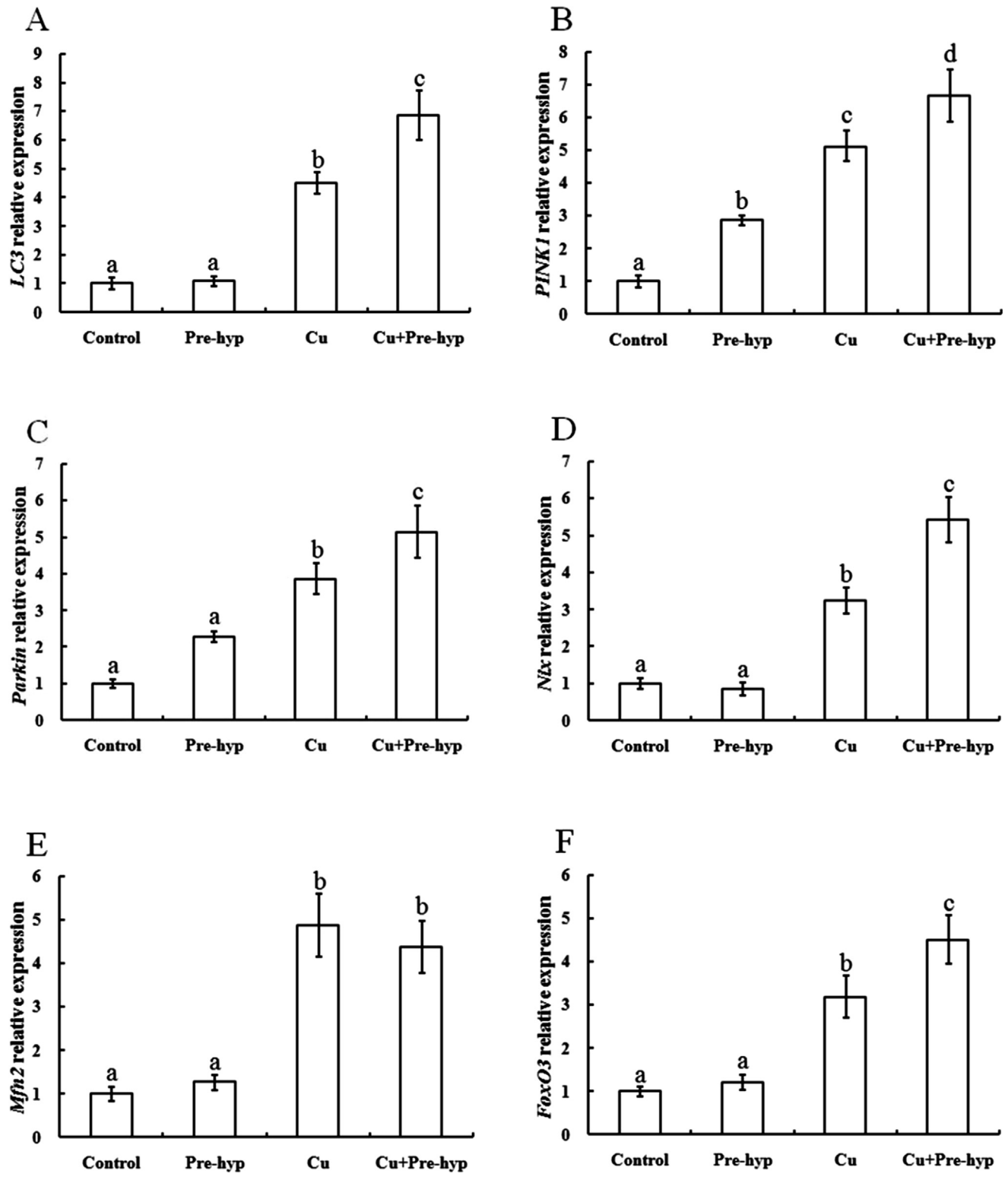

Fig. 9. Changes in the expression of genes involved in mitophagy in the liver of the large yellow croaker exposed to hypoxia and Cu. Each value represents the mean ± SD. Different letters indicate significant differences ($P < 0.05$).

Our study indicated that pre-hypoxia exposure alleviated the Cu-induced toxicological effect of the large yellow croaker by improving energy metabolism and antioxidant defence. In an attempt to further elucidate the mechanisms underlying protective roles of pre-hypoxia exposure against Cu toxicity, we explored the effects of hypoxia and Cu exposures on mitochondrial dysfunction. COX plays a vital role in mitochondrial respiratory function, which is closely associated with ATP synthesis (Porter et al., 2016). Mitophagy is a key mechanism for maintaining mitochondrial homeostasis by removing damaged or defunct mitochondria when organisms are under stressful conditions (Coto-Montes et al., 2012). It is a highly conserved cellular process modulated by

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

Independent parameters	Dependent parameters	Correlation coefficients	*P*
F-ATPase mRNA levels	F-ATPase activities	0.855	<0.001
SDH mRNA levels	SDH activities	0.518	0.192
MDH mRNA levels	MDH activities	0.861	<0.001
PK mRNA levels	PK activities	0.958	<0.001
Cu/Zn-SOD mRNA levels	Cu/Zn-SOD activities	0.817	<0.001
GPx1a mRNA levels	GPx activities	0.298	0.326
GPx1b mRNA levels	GPx activities	0.857	<0.001
CAT mRNA levels	CAT activities	0.824	<0.001
HIF-1α mRNA levels	*F-ATPase* mRNA levels	0.902	<0.001
HIF-1α mRNA levels	*SDH* mRNA levels	0.611	0.079
HIF-1α mRNA levels	*MDH* mRNA levels	0.900	<0.001
HIF-1α mRNA levels	*PK* mRNA levels	−0.911	<0.001
Nrf2 mRNA levels	*Cu/Zn-SOD* mRNA levels	0.560	0.105
Nrf2 mRNA levels	*GPx1a* mRNA levels	0.766	0.001
Nrf2 mRNA levels	*GPx1b* mRNA levels	0.940	<0.001
Nrf2 mRNA levels	*CAT* mRNA levels	0.900	<0.001
FoxO3 mRNA levels	*LC3α* mRNA levels	0.970	<0.001
FoxO3 mRNA levels	*PINK1* mRNA levels	0.906	<0.001
FoxO3 mRNA levels	*Parkin* mRNA levels	0.887	<0.001
FoxO3 mRNA levels	*Nix* mRNA levels	0.950	<0.001
FoxO3 mRNA levels	*Mfn2* mRNA levels	0.881	<0.001
MTs protein levels	Cu contents	0.914	<0.001
ROS formation	Cu contents	0.909	<0.001
ROS formation	*Nrf2* mRNA levels	0.134	0.620
ROS formation	*HIF-1α* mRNA levels	−0.853	<0.001
ROS formation	*FoxO3* mRNA levels	0.701	0.002

PINK1, Parkin, Nix, and Mfn2. LC3α is regarded as the marker of mitophagy (Bhansali et al., 2017). In the present study, Cu exposure under normoxia significantly reduced COX activity and ATP production, indicating the impairment of mitochondrial respiration. Compared to Cu exposure under normoxia, Cu plus pre-hypoxia exposure resulted in higher COX activity, which may contribute to increased ATP content. Although Cu stress under normoxia significantly increased the expression of the *LC3α*, *PINK1*, *Parkin*, *Nix*, and *Mfn2* genes, the triggered mitophagy could not completely protect fish from oxidative damage caused by Cu exposure. This finding was supported by aberrant hepatic mitochondrial ultrastructures. The reason may be that the damaged or defunct mitochondria, as the potential source of ROS production, could not be timely eliminated in the mitophagy process, resulting in the accumulation of lipid peroxidation (MDA), which in turn aggravated oxidative stress and mitochondrial dysfunction that created a vicious cycle. Accompanied with abnormal hepatic histology, cadmium-induced mitophagy has been reported in the zebrafish *Danio rerio* (Pan et al., 2018). Cu plus pre-hypoxia exposure resulted in higher mRNA levels of *LC3α*, *PINK1*, *Parkin*, and *Nix* than those in Cu exposure under normoxia, indicating that pre-hypoxia exposure could increase mitophagy in the liver of the large yellow croaker under Cu stress. The improvement of mitophagy pathways may reflect a compensatory mechanism for protecting organisms against oxidative damage, as suggested by Lu et al. (2016). Previous studies have shown that mild oxidative stress could induce mitophagy pathways to effectively sequester abnormal mitochondria, suppressing the relative increase in ROS accumulation. However, severe oxidative stress may destroy the integrity of the mitochondria, resulting in ROS overproduction or even cell death (Ashrafi and Schwarz, 2013; Di et al., 2015). Pre-hypoxia exposure had no effect on the mRNA levels of *LC3α*, *Parkin*, *Nix*, and *Mfn2*, owing to the activation of mitophagy pathways that depend on ROS overproduction (Diebold and Chandel, 2016). However, pre-hypoxia exposure significantly increased *PINK1* gene expression. The main reason may be that hypoxia-reoxygenation affected mitochondrial respiration and redox state, leading to the enhancement of mitochondrial membrane polarisation (Napolitano et al., 2019), which was confirmed by the reduced COX activity. Additionally, the *PINK1* modulated mitochondrial membrane polarisation might be independent of mitophagy (Rakovic et al., 2018; Scialò et al., 2016).

Modifications of the enzyme activities might be closely associated with the changes in the corresponding gene expression. In the present study, positive correlations were observed between mRNA levels and the activities of F-ATPase, MDH, PK, Cu/Zn-SOD, and CAT, suggesting that changes in the expression of genes would contribute to the modifications in their corresponding enzyme activities. However, there are differential expression patterns between *SDH* gene expression and SDH activity and between *GPx1a* mRNA level and GPx activity in response to hypoxia and Cu exposures, which may be due to RNA stability, a time-lag effect, and/or post-translational modification (Craig et al., 2007; Regoli and Giuliani, 2014; Sadi et al., 2014). Besides, the activity of GPx was codetermined by the two isoenzymes (GPx1a and GPx1b), while each gene only encoded an isoenzyme. This observation is consistent with the results of studies in which fish were subjected to other stresses (Han et al., 2013; Jiang et al., 2014; Zeng et al., 2016b).

Transcription factors *HIF-1α*, *Nrf2*, and *FoxO3* play critical roles in modulating mRNA transcription levels involved in energy metabolism, antioxidant defence and mitophagy, respectively (Dodson et al., 2015; Fang et al., 2016; Kajimura et al., 2006). Our data in this study indicated that *HIF-1α* gene expression was correlated with the mRNA levels of *F-ATPase*, *MDH*, and *PK*, indicating that the change in the expression of these genes was partly dependent on the modifications in *HIF-1α* gene expression. Similarly, studies demonstrated that hypoxia could inhibit Cu toxicity in zebrafish embryos by modulating the *HIF* signalling pathway (Fitzgerald et al., 2016). Hypoxia and reoxygenation also affected energy metabolism via the *HIF-1* pathways in the blunt snout bream *Megalobrama amblycephala* (Sun et al., 2017b). *Nrf2* mRNA levels were positively correlated with the expression of the *GPx1a*, *GPx1b*, and *CAT* genes, suggesting that these antioxidant genes are transcriptionally activated depending on the *Nrf2* signalling pathway. The up-regulated *FoxO3* gene expression was accompanied by increased mRNA levels of *LC3α*, *PINK1*, *Parkin*, *Nix*, and *Mfn2*, indicating mitophagy gene expression might be regulated by *FoxO3*. However, no significant relationships were observed between *Nrf2* and *Cu/Zn-SOD* mRNA levels, and between *HIF-1α* and *SDH* mRNA levels, indicating *Cu/Zn-SOD* and *SDH* gene expression may be modulated by transcription factors at the post-transcriptional levels (Kuschel et al., 2012). *HIF-1α* stabilisation is dependent on mitochondrial ROS (Archer et al., 2008). However, Rissanen et al. (2006) reported that ROS were not essential for the induction of *HIF-1α*. Antioxidant defence and mitophagy can be activated by stresses via ROS/*Nrf2* and ROS/*FoxO3* signalling pathways, respectively (Kovac et al., 2015). In the present study, production of ROS was correlated with *HIF-1α* and *FoxO3* gene expression, indicating that *HIF-1α* and *FoxO3* take part in the modulation of ROS production by energy metabolism and mitophagy, respectively. However, there was no relationship between ROS production and *Nrf2* gene expression. A similar result was observed in cancer cells where *Nrf2* reduced radiation induced DNA damage in a ROS independent manner (Jayakumar et al., 2015). MTs are commonly used as sensitive biomarkers of metal stress, which was confirmed by a positive correlation between MT protein levels and Cu content (Aich et al., 2017).

5. Conclusion

Our present study clearly showed that moderate pre-hypoxia exposure could enhance tolerance to subsequent Cu stress in the liver of the large yellow croaker by improving energy metabolism,

antioxidant defence and mitophagy response. These results contributed to our understanding of the mechanisms underlying hypoxic priming hormesis in response to Cu stress, and provided valuable information for treating *Cryptocaryon irritans* with $CuSO_4$ when fish were pre-exposed to mild hypoxia.

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