Accepted refereed manuscript of: Luo J, Zhu T, Wang X, Chen X, Yuan Y, Jin M, Betancor M, Tocher D & Zhou Q (2020) Toxicological mechanism of excessive copper supplementation: Effects on coloration, copper bioaccumulation and oxidation resistance in mud crab Scylla paramamosain. Journal of Hazardous Materials, 395, Art. No.: 122600. DOI: https://doi.org/10.1016/j.jhazmat.2020.122600

Toxicological mechanism of excessive copper supplementation: Effects on coloration, copper bioaccumulation and oxidation resistance in mud crab *Scylla paramamosain*

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Keywords: Copper, Coloration, Oxidation resistance, Mud crab *Scylla paramamosain.*

Abstract

Copper is a widespread pollutant in marine environments, and marine animals can ingest large amounts of copper through the food chain. Here, an 8-week feeding trial was designed to investigate the effects of different dietary copper levels on coloration,

copper bioaccumulation, stress response and oxidation resistance of juvenile mud crab *Scylla paramamosain*. The results indicated that crabs fed the diet with 162 mg/kg copper exhibited a dark-blue carapace and hemolymph. The accumulation of copper in tissues was positively correlated with the level of copper in feed. High/excess dietary copper (162 mg/kg) up-regulated the expression of stress response related genes, and reduced the expression/activities of anti-oxidation genes/enzymes. The activity of phenoloxidase decreased significantly when dietary copper level was 86-162 mg/kg, and the expression of *hemocyanin* was up-regulated in crab fed the diets with 28-162 mg/kg copper. Overall, the results of the present study indicated that high dietary copper led to parachrea in carapace and hemolymph of mud crab, and caused copper deposition abnormality in carapace and hepatopancreas. The data suggested that the toxic effects of dietary copper were concentration-dependent such that, excess dietary copper (162 mg/kg) had adverse impacts on oxidation resistance.

1. Introduction

Copper (Cu) is a common contaminant in water due to human activities and elevated concentrations are a threat to aquatic ecosystems, with potential toxicity to aquatic animals attracting significant attention [1, 2]. At high concentrations, copper becomes one of the most toxic contaminants producing various adverse effects on aquatic organisms, such as reduced growth performance in *Macrobrachium nipponense* [3], increased larval deformity rate in *Carassius auratus* [4], olfactory dysfunction in *Orconectes rusticus* [5], impaired reproduction in *Poecilia vivipara* [6], and decreased oxidation resistance and innate immunity response in *Epinephelus coioides* [7]. However, at the same time copper is a crucial trace mineral component, with key roles in the regulation of a range of physiological processes in cellular energy metabolism, innate immunity, and antioxidant protection via scavengers of reactive oxygen species (ROS) [8, 9]. Therefore, with increasing copper contaminants in the environment caused by the industrial and agricultural activities, there is a pressing need to understand the toxicity and benefit of copper in aquatic organisms [10].

Color changes involve alterations in the amount and distribution of pigment, regulated by the endocrine system of crustaceans [11]. A previous study revealed that cadmium chloride led to physiological color changes of *Uca pugilator* [12]. Moreover, several previous studies showed that tissue copper concentrations were sensitive indicators of copper bioaccumulation in crustaceans, and exhibited positive correlations with dietary copper concentrations up to requirement level [3, 13-16]. Furthermore, some copper-containing proteins such as ceruloplasmin (CP), hemocyanin (HC) and phendoxidase (PO) were identified as suitable bioindicators of copper bioaccumulation [17]. Metallothionein (MT) and heat shock protein (HSP) have also been considered as sensitive indicators of stress in aquatic organisms [18], and previous studies have

indicated that the expression profiles of genes encoding for MTs and HSPs might be altered by copper stress [19-21]. The expression profiles of these stress-response genes provided, not only information about the copper stress response in mud crabs, but also identified potential biomarkers for future ecotoxicological studies. The toxicity of excessive copper is mainly associated with reactive oxygen species (ROS) generation, inducing oxidative stress. Antioxidant enzymes such as copper/zinc superoxide dismutase (CuZn-SOD), catalase (CAT), and glutathione peroxidase (GPx) act to mitigate the impact of metal-induced oxidative stress [22]. Dietary supplementation with excess copper reduced antioxidant capacity, as reported in *Eriocheir sinensis* [23]. Similar alteration in antioxidant enzymes was observed in *C. auratus* and *Cyprinus carpiovar Jian* exposed to waterborne copper [22, 24].

Crabs are an important part of aquatic ecosystems and, therefore, the toxicity of waterborne copper exposure in crab has been studied extensively by many workers [25-30]. However, until now, little information was available on the effects of dietary copper on marine crabs, and the metabolic mechanisms underpinning impacts of copper were unclear [31, 32]. Dietary intake of metals is one of the main causes of long-term contamination in wild organisms, and metal bioaccumulation efficiency is affected by the biochemistry of the metal in the prey/food and the physiology of the predator [33-35]. Fish and shellfish accumulate copper from diet, water and sediments, and it is then distributed in their tissues [36]. Shellfish show strong bioaccumulation of metals and are recognized as effective indicators to evaluate the environmental contamination by copper [37], and are also important components of the diet of crabs [38]. Previous studies have reported that copper concentrations in oyster *Crassostrea talienwhanensis* were up to 109.6 mg/kg (wet weight) in the metal polluted coastal areas of China [39], and a positive correlation between waterborne copper exposure and copper

bioaccumulation had been reported in some shellfish species [40-42]. Thus, due to biomagnification impacts, crabs can intake large amounts of copper through the food chain potentially leading to high accumulation of copper in the body. Therefore, investigation of the impacts of dietary copper exposure in crab are necessary to understand the toxicological effects of metals as these effects have been rarely investigated.

Mud crab, *Scylla paramamosain*, is a typical marine carnivorous crab species widely distributed in tropical, subtropical and temperate zones of Southeast Asia [38]. Due to its delicious flavor and valuable nutrition, it has been becoming a mainstay of fishery economies in many countries [38, 43-45]. Regarding aquaculture, the effects of dietary copper on physiological metabolic regulation is worthy of study. From a food safety perspective, it is essential to understand copper bioaccumulation in aquatic products in response to dietary or waterborne copper exposure. Hence, we hypothesize that dietary copper may be responsible for various abnormalities in crab such as disruptions of coloration, stress response and oxidation resistance, based on its potential endocrine-disrupting effects in crustaceans [12, 16, 20, 23]. We further hypothesize that different copper levels exert distinct effects on crab similar to other contaminants. In the present study, we used 10, 20, 28 and 47 mg/kg copper to reflect natural environmental copper concentrations, and 86 and 162 mg/kg copper to investigate the potential effects of high/excess dietary copper on mud crabs. These concentrations were selected based on the copper concentrations of the main prey of mud crab in the natural environment [46]. Overall, the aim of the present study was to clarify the impacts of dietary copper supplementation on coloration, copper deposition, stress response, antioxidant capacity and innate immunity of juvenile mud crab, and to explore the perspectives of toxicity and nutrients modulation in mud crab.

2. Material and methods

2.1 Experimental diets

Six isonitrogenous and isolipidic experimental diets (45 % crude protein, 7 % crude lipid) were formulated to contain different copper levels ($CuSO_4 \cdot 5H_2O$ as the copper source), with the analyzed copper concentrations in the diets being 10, 20, 28, 47, 86 and 162 mg/kg, respectively. The formulation and proximate composition of the diets are shown in Supplementary Table S1. Fishmeal, casein, krill meal and soybean meal were used as protein sources, with fish oil and soy lecithin as the main lipid sources. Feeds were manufactured according to the method described in detail previously [47]. Briefly, all dry ingredients were ground to fine powder with particle size < 177 μm, microcomponents such as minerals and vitamins premix were added followed by lipid and distilled water (35 %, w/w). This was then mixed in a Hobart-type mixer and cold-extruded pellets produced (F-26, Machine factory of South China University of Technology) with pellet strands cut into uniform sizes (diameter 3 mm and 5 mm) (G-250, Machine factory of South China University of Technology). Pellets were steamed for 30 min at 90 °C, and then airdried to approximately 9 % moisture, sealed in vacuum-packed bags and stored at −20 °C until used in the feeding trial.

2.2 Culture crab

Juvenile mud crab were obtained from the fish breeding base of Ningbo Ocean and Fishery Science and Technology Innovation Center, at Xiangshan Bay, Ningbo, China. Prior to the feeding trial, the crabs were reared individually in single cell units (33 x 22.5 x 25 cm), with in a continuous flow-through water system and fed a commercial diet (approximately 45 % crude protein, 10 % crude lipid, 31.10 mg/kg copper concentration) for 2 weeks for acclimation to the experimental conditions. Immediately before the feeding trial, healthy, actively feeding individuals with intact appendages

were selected as the experimental crabs. A total of 270 crabs (initial weight 6.88 ± 0.39 g, initial carapace width 3.23 ± 0.19 cm, initial carapace length 2.10 ± 0.11 cm) were distributed randomly into 270 individual cells, and each experimental diet was randomly assigned to three replicates with fifteen mud crabs in each replicate. Crab were hand-fed once daily at 18: 00 pm over eight weeks with a daily ration of 3–6 % of crab weight, adjusted daily according to consumption and residual feed in order to maintain a level of apparent satiation. Faeces and uneaten feed were removed, and 50 % of seawater were renewed to maintain water quality. During the experimental period, water temperature was 25.1 - 29.0 ℃, pH was 7.3 - 7.9, salinity was 25.8 - 28.4 mg/L, dissolved oxygen 4.5 - 6.5 mg/L, ammonia nitrogen was lower than 0.05 mg/L, and water copper concentration was 2.26 - 2.55 μg/L.

2.3 Sample collection

At the end of the feeding trial, crabs were counted and mildly anaesthetized with 0.02 % MS-222 (Shanghai Reagent Corp., Shanghai, China). Hemolymph samples from six crabs in each replicate were taken from the pericardial cavity using a 1 ml syringe, placed into 1.5 ml centrifuge tubes and left to stand for 24h at 4℃ before centrifugation (956 x *g*, 10 min). The supernatant was collected and stored at -80 °C prior to analysis of color, enzymes activities and hematological characteristics. Hepatopancreas was removed from nine crabs in each replicate and placed in 1.5 ml centrifuge tubes, frozen in liquid nitrogen and stored at -80℃ for analysis of gene expression (pools of 6 crabs per replicate, n = 3) and enzymatic activities (pools of 3 crabs per replicate, n = 3). Finally, muscle, carapace and hepatopancreas samples were collected and stored at -80℃ until further analysis of copper concentration (3 crabs per replicate). The protocols for animal care and handling used in the present study were approved by the Institutional Animal Care and Use Committee of Ningbo University.

2.4 Copper concentration analysis

The copper concentration in seawater and diets, as well as the tissues of mud crab were determined by complete acid digestion and analysis by ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometer, PE2100DV, Perkin Elmer, USA).

2.5 Hemolymph color analysis

Color values (L*, a* and b*) were assessed using a colorimeter (WSC-S, Shenguang, China) standardized under D65 illumination. Six points on the cuvette (5 mm, filled with hemolymph) were selected to measure the values of L*, a* and b*, with results presented as average values.

2.6 Oxidation protection and copper-containing protein parameters

Hepatopancreas samples were homogenized in nine volumes (w/v) of pre-cooled (4 ^{o}C) physiological saline (0·89 %, w/v) in 10 ml centrifuge tubes embedded in a beaker filled with ice-water mixture to keep the temperature low during homogenization. Samples were centrifuged at 956 x *g* for 15 min at 4℃, and supernatant were collected for enzyme and metabolite assays. Total anti-oxidant capacity (T-AOC), and activities of glutathione peroxidase (GPx), manganese superoxide dismutase (Mn-SOD), catalase (CAT) and copper/zinc superoxide dismutase (CuZn-SOD), and contents of reduced glutathione (GSH) and malondialdehyde (MDA) in hepatopancreas, as well as the activity of ceruloplasmin (CP) in hemolymph, were analyzed by respective assay kits (Nanjing Jiancheng Bioengineering Institute, China). The activity of phenoloxidase (PO) in hemolymph was estimated spectrophotometrically using a specific ELISA kit for mud crab (Nanjing Jiancheng Bioengineering Institute, China).

2.7 Gene expression

Gene expression was determined by reverse-transcriptase quantitative PCR (qPCR) as follows. RNA extraction, reverse transcription and real-time quantitative PCR were

carried according to methods described in detail previously [48]. Briefly, RNA was extracted from hepatopancreas samples (50 mg) using TRIzol Reagent (Takara, Japan) and following manufacturer's instructions. Quality and concentration of the extracted RNA were assessed by 1.2 % agarose gel electrophoresis and ultra-micro spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, USA), respectively. RNA samples were reverse transcribed to cDNA using the PrimeScript® RT reagent kit (TaKaRa Co., Japan) according to the manufacturer's protocol.

The specific primers used for RT-qPCR were designed based on cDNA sequences of the corresponding genes in the NCBI database using Primer Premier 5.0 software. Calibration curves were prepared from six individual dilution concentration gradients of cDNA samples. Amplification efficiency was measured as follows: E = 10(–1/Slope)-1, and the amplification efficiencies of all genes were approximately equivalent, ranging from 93 to 102 %. PCR amplification was performed using a quantitative thermal cycler (Lightcycler 96, Roche, Switzerland), with each reaction containing 2 μL of cDNA, 1.0 μL of each primer, 10μL of 2 × conc SYBR Green I Master (Roche, Switzerland) and 6 μL DEPC-water. The quantitative PCR procedure contained an initial activation step at 95 °C for 2 min, followed by 45 cycles of 95 °C for 10 s and 58 °C for 10 s, and 72 °C for 20 s. Relate expression levels of the objective genes were computed by way of 2–ΔΔCt. The *ef1α* gene was used as the house-keeping gene, and the 28 mg/kg treatment group was used as the control/reference group.

2.8 Statistical analysis

Data were presented as means ± S.E.M of three replicates (n = 3). Data were checked for normality and homogeneity of variances, and were normalized when appropriate. The relative gene expression results (qPCR analyses) were expressed as mean normalized ratios (± S.E.M) corresponding to the ratio between the copy numbers of

the target genes and the copy numbers of the reference gene, *ef1a*. The homogeneity of variances between means values were compared by one-way analysis of variance (ANOVA) followed by Tukey's multiple-range test. All statistical analyses were conducted using SPSS 16.0 for Windows (Chicago, IL, USA).

3. Results

3.1 Color parameters

The effect of dietary copper supplementation on carapace color is presented in Fig. 1. The normal dark-green color of carapace was observed in crab fed diets containing 10 - 86 mg/kg copper. However, crabs fed the diet with the highest copper level of 162 mg/kg showed an unusual blueness in the carapace. The color comparison and color values in hemolymph of mud crab fed different levels of dietary copper are shown in Fig. 2 and Table 1, respectively. The color of hemolymph gradually deepened with increased dietary copper level, with the darkest blue observed in hemolymph of crab fed 162 mg/kg copper, although the light blue-green hemolymph were observed in crab fed diets containing 10 - 47 mg/kg copper. The color values of hemolymph were consistent with this result. The L* value represents lightness on a scale of 0 (pure black) to 100 (pure white) and it was significantly lower in crabs fed 162 mg/kg copper, indicating a darker color. A negative a* value represents color towards green and a negative b* value represents color towards blue. The b* value of hemolymph showed an increasing trend and the a* values decreased significantly as dietary copper levels increased from 10 to 162 mg/kg.

Insert Fig. 1 here

Insert Fig. 2 here

Insert Table 1 here

3.2 Copper bioaccumulation

Copper concentrations in the muscle, carapace and hepatopancreas are shown in Fig. 3. Overall, the concentrations of copper in muscle, carapace and hepatopancreas increased significantly in a graded manner with increased dietary copper level, with highest values observed in crab fed the diet with 162 mg/kg copper. Specifically, crab fed 162 mg/kg copper had nearly quadruple and triple contents of copper concentration in the carapace and hepatopancreas compared to crab fed the diet with 10 mg/kg copper.

Gene expression levels and enzyme activities of copper-containing proteins are shown in Fig. 4. The activity of ceruloplasmin (CP) in hemolymph increased significantly as dietary copper levels increased reaching a peak value in crabs fed 86 mg/kg copper. The activity of phenoloxidase (PO) in hemolymph also increased with dietary copper level but peaked at 47 mg/kg rather than 86 mg/kg. The relative expression levels of *hemocyanin 1* (*hc1*) and *hemocyanin 2* (*hc2*) were significantly up-regulated in crabs fed diets containing copper levels higher than 28 mg/kg. Dietary copper had no significant effect on the mRNA expression level of *prophenoloxidase* (*proPO)* in crab hepatopancreas.

Insert Fig. 3 here

Insert Fig. 4 here

3.3 Stress response

The expression of genes related to stress responses including *metallothionein* (*mt)* and *heat shock proteins* (*hsp)* in hepatopancreas were influenced significantly by dietary copper level (Fig. 5). The expression of *mt1* was significantly up-regulated in crabs fed diets containing 28 - 162 mg/kg copper compared to diets with lower copper content. Significantly higher expression of *mt2* was observed in crab fed diets containing 20 - 86 mg/kg copper compared to the diets with lowest and highest copper level. Expression levels of *hsp60, hsp70* and *hsp90* genes were all highest in crabs fed the

highest (162 mg/kg) level of dietary copper compared to the other diets.

Insert Fig. 5 here

3.4 Oxidation and antioxidant parameters

The values of oxidation protection parameters in hepatopancreas of mud crab fed diets containing different copper levels are presented in Fig. 6. The activity of copper/zinc superoxide dismutase (Cu/Zn-SOD) was generally lower in crabs fed the highest level of dietary copper, while the activities of manganese superoxide dismutase (Mn-SOD), catalase (CAT) and glutathione peroxidase (GPx) were unaffected by dietary copper (Fig. 6A). Crab fed diets with high copper (86 - 162 mg/kg) showed significantly lower reduced glutathione (GSH) compared to crab fed the 28 mg/kg copper diet. Crab fed diets with 10 and 162 mg/kg copper had significantly higher contents of malondialdehyde (MDA) compared to crab fed 47 mg/kg copper. The expression of *Cu/Zn sod* was significantly up-regulated with increasing dietary copper up to 86 mg/kg, and down-regulated in crabs fed the highest dietary copper level of 162 mg/kg (Fig. 6B). However, dietary copper level did not significantly influence the expression levels of mitochondrial manganese superoxide dismutase (*mitMn sod)* or cytosolic manganese superoxide dismutase precursor (*cytMn sod pre)*. The mRNA expression levels of *cat* and *gpx* were significantly down-regulated in crab fed 162 mg/kg copper compared with crabs fed 28 mg/kg copper. High dietary copper (86 - 162 mg/kg) significantly down-regulated the expression of thioredoxin (*trx)* and a similar, but non-significant, trend was observed for the expression of thioredoxin peroxidase (*prx*).

Insert Fig. 6 here

4. Discussion

Previous studies in crab have focused largely on waterborne copper exposure and revealed impacts on metabolism, toxicity, metal bioaccumulation, osmoregulation,

organelle metabolism and ionic interactions [25-30, 49]. However, few studies have evaluated the potential effects of dietary copper on the metabolism and toxicity in crab [31, 32]. A typical prey of mud crab is clam, which shows higher copper bioaccumulation in contaminated waters. Taking the Chinese Bohai Sea for example, the copper concentration of short-necked clam *Ruditapes philippinarum* was 4.37 mg/kg (wet weight) on the coast of Qinghuangdao with high industry pollution, three times the copper concentration in *R. philippinarum* from the relatively clean coast of Dalian [50]. Additionally, a 30-day waterborne copper exposure study revealed a positive correlation in copper concentration between levels in water and levels in clams. Thus, as waterborne copper exposure levels increasing from 0.015 to 0.115 mg/L, the copper concentration of *R. philippinarum* increased significantly from 1.50 to 21.00 mg/kg (wet weight) [51]. Therefore, due to its position in the marine ecological system, mud crab has a potentially high-risk of ingesting large amounts of copper through the food chain [38, 52].

The unusual blueness of carapace observed in mud crab fed the diet containing the highest level of dietary copper, 162 mg/kg, was consistent with the carapace in these crabs having the highest bioaccumulation of copper. The changes of carapace color are attributed mainly to variation in the numbers and quality of integumental pigment cells, and a series of color change hormones are involved in the regulation of pigment cells [11, 53]. Several studies revealed that copper could disrupt the endocrine-regulated processes of crustaceans [54]. Perhaps excess dietary copper interfered with hormone secretion that stimulated integumental color change, but the mechanism needs further investigation. This is the first report of blue carapaces of crabs in relation to scientific studies. Previously, it had been reported that fishermen caught some “blue” swimming crabs *P. trituberculatus* in the Chinese Bohai Sea [55, 56], which is one of the sea areas

of China most seriously polluted by metals, with copper concentrations as high as 2755 μg/L in seawater [57, 58]. Therefore, the appearance of "blue" swimming crabs in the Bohai Sea may be related to the very high copper levels in the seawater. In the present study, high levels of dietary copper also led to an unusual dark blue color of hemolymph. It is well known that the blue color of hemolymph in crustaceans is mainly due to the impact of copper in hemocyanin [59], and so high dietary copper probably deepens the color of hemolymph in mud crab by promoting the biosynthesis of hemocyanin. Consistent with this, mRNA expression levels of hemocyanin (*hc1* and *hc2*) were higher in crabs fed the high copper treatments.

Previously, it had been shown that copper bioaccumulation in tissues strongly reflected dietary copper levels [15]. In the present study, a positive correlation between dietary copper level and tissue copper bioaccumulation was observed, and these data also indicated that mud crab could accumulate excessive copper in the body. This result was similar to that reported in a studies in grass shrimp *Penaeus monodon* and freshwater prawn *Macrobrachium rosenbergii* [13, 14]. Among the three tissues investigated in mud crab, copper deposition was greatest in hepatopancreas followed by with much lower accumulation in muscle. Previously, it was demonstrated that hepatopancreas was the main organ for deposition, metabolism and storage of mineral substance in crustaceans [60]. Furthermore, Davis, Lawrence and Gatlin Iii [61] reported that hepatopancreas was the copper storage organ in marine crustaceans. The present study confirmed that hepatopancreas was most sensitive to dietary copper and was likely key in the metabolism of copper in mud crab.

Ceruloplasmin (CP) is a copper protein and the primary extracellular (plasma/hemolymph) copper transport protein [17]. The results of the present study showed that hemolymph CP activity was increased in crab fed intermediate levels of

dietary copper (47 - 86 mg/k) compared to crabs fed low copper, which revealed a positive correlation between dietary copper level and CP activity. Dietary copper deficiency resulted in insufficient copper provision for the biosynthesis of CP [62]. In addition, hemocyanin is a multi-subunit copper-containing protein, which is the main oxygen transport protein in crustaceans [63, 64]. An early study indicated that approximately 40 % of the whole-body copper load in *Carcinus maenas* was found in hemocyanin, thereby indicating the importance of copper in crustaceans [65]. In the present study, the expression levels of *hc1* and *hc2* were significantly up-regulated when dietary copper levels were higher than 28 mg/kg, which suggested dietary copper supplementation could induce *hc* mRNA expression in mud crab, in agreement with a previous study in *E. sinensis* [66]. Phenoloxidase (PO) is regarded as a sensitive indicator of immune status of invertebrates [67]. The close relationship of the proPO system with dietary copper level had been reported previously in *E. sinensis*, in which high dietary levels of copper significantly decreased PO activity [23]. These results are somewhat in agreement with the present study in mud crab where significantly lower activities of hemolymph PO activity were recorded in crabs fed the two highest levels of copper. This may be due to copper being a key component of the PO enzyme, with excessive dietary copper producing a toxic impact inducing toxic stress [68]. Moreover, ProPO is also a copper-containing proenzyme and a key component of the phenoloxidase proenzyme system [68]. In the present study, a numerically lower transcript level of *proPO* was found in mud crab fed the diet with the highest level of dietary copper, albeit not statistically significant. However, this trend was consistent with the impact of high dietary copper on PO activity, supporting the view that excessive levels of dietary copper could reduce the efficacy of the proPO system in mud crab.

Most heat shock proteins are molecular chaperones, salvaging damaged proteins [18]. In the present study, all the *hsp* genes were significantly up-regulated in response to high dietary copper, demonstrating that excess dietary copper induced a stress reaction, with heat shock proteins mitigating damaging stress responses by refolding proteins to prevent the aggregation of damaged proteins [18]. Metallothionein genes have been considered as sensitive indicators of stress in both vertebrates and invertebrates [18]. In the present study, the expression levels of *mt1* were up-regulated in hepatopancreas in crab fed the diet with higher copper (28 - 162 mg/kg), consistent with a previous study in other crustacean species [3]. The up-regulation of *mt1* demonstrated to some extent that dietary copper could lead to stress in mud crab. However, excess dietary copper (162 mg/kg) significantly down-regulated the expression level of *mt2* in hepatopancreas of mud crab, suggesting that hepatopancreatic *mt2* mRNA expression was more sensitive than *mt1* to the level of dietary copper. This confirmed a previous study that indicated that hepatopancreatic *mt2* expression was down-regulated in mud crabs exposed to 40.0 mg/L cadmium compared to 10.0 mg/L cadmium exposure [18]. Overall, these results demonstrated that excess copper concentration could restrict the transcriptional activation of *mt2* in mud crab, although further studies are required.

To date, few studies of copper have examined the potential effects of dietary copper levels on hepatopancreas oxidation protection metabolism in crustaceans [14]. Copper is involved in the antioxidant defense system as an integral component of Cu/Zn-SOD (superoxide dismutase), which catalyzes the dismutation of superoxide radicals to hydrogen peroxide (H_2O_2), which can then be degraded by catalase (CAT) and glutathione peroxidase (GPx). As a copper-dependent enzyme, Cu/Zn SOD had been verified as a good indicator of copper status in crustaceans including Chinese

mitten crab *Eriocheir sinensis* [23] and white shrimp *Litopenaeus vannamei* [47]. In the present study, crab fed the diet with the highest level of copper (162 mg/kg) had lower activities of CuZn-SOD, with peak activity at only 28 mg/kg. However, the expression of the *Cu/Zn sod* gene was upregulated in a graded manner by increasing dietary copper up to a level of 86 mg/kg but expression was then reduced by the highest level of dietary copper. Therefore, it appears that the expression and activity of Cu/Zn SOD is increased by increasing dietary copper and, consequently hepatopancreas copper contents, up to certain levels (lower for activity than expression) after which further increases in dietary copper first inhibit activity and eventually suppress gene expression. In contrast, dietary copper did not influence the activity of Mn-SOD, or the expression levels of *mitMn sod* and *cytMn sod pre*, which suggested that dietary copper influenced Cu/Zn SOD directly through being a component part of the enzyme rather than having an indirect influence that would have affected Mn SOD as well.

The production of MDA results from the peroxidation of membrane polyunsaturated fatty acids, influencing cell membrane fluidity as well as the integrity of biomolecules, and is an important indicator of peroxidation [69]. In the present study, the content of MDA decreased as dietary copper levels increased, with the lowest value in crabs fed 47 mg/kg copper. This demonstrated that MDA content responded to dietary copper in a dose-dependent fashion, and suggested that 47 mg/kg copper supplementation was the optimal requirement level for reducing oxidative stress and decreasing lipid peroxidation in mud crab. In other words, inadequate (10 mg/kg copper) or excessive (162 mg/kg copper) copper supplementation increased MDA contents compared to compared to crabs fed 47 mg/kg copper. Moreover, CAT and GPx as the other main antioxidant enzymes, catalyze ROS to less reactive species [70]. The results of the present study indicated that high/excess dietary copper reduced the

expression of *cat* and *gpx*. Other than CAT and GPx, defense against ROS can also involve other antioxidant proteins including peroxiredoxin (PRX) and thioredoxin (TRX) [71]. In the present study, the mRNA expression levels of *prx* and *trx* were decreased in crab fed the diet with the highest (excess) level of copper, 162 mg/kg. This may suggest that excessive dietary copper would lower the level of these antioxidant proteins and thus reduce antioxidant efficiency, findings that were also observed in other aquatic animals [72, 73]. Thus, high dietary copper may induce oxidative stress resulting in the production of hydroxyl free radicals through the Fenton reaction, causing protein and lipid peroxidation, and DNA damage [74]. Therefore, excessive dietary copper may result in overproduction of ROS [75] and oxidative stress [76], which will result in consumption of these antioxidant proteins [73] while at the same time suppressing their replacement through decreasing expression.

In the present study, excessive dietary copper could decrease the oxidation resistance capacities of mud crab and, consequently, result in impaired health condition when compared to the other treatments. However, no significant differences in survival were observed among the treatments (Fig. S1). This could indicate that mud crab has a strong tolerance capacity to copper within limits. On one hand, mud crab benefits from a series of stress response mechanisms to eliminate the metal stress, such as HSP and MT [18]. On the other hand, mud crab could accumulate excess copper in tissues to avoid the worst adverse effects. However, the impacts of excessive copper accumulation in mud crab on the food chain and human food safety are inevitable. In the wild, some octopus, carnivorous fish and seabirds feed on crabs, so the higher concentrations of copper could be accumulated in their bodies. Copper can be further accumulated and transmitted up the food chain, finally resulting in an irreversible impact on the entire ecosystem [77]. Furthermore, mud crab are popular aquatic

products in the human food basket, with consumption of muscle, hepatopancreas and gonad [38]. In the present study, the copper concentration in hepatopancreas increased from 110.00 to 304.44 mg/kg with increased dietary copper levels. The limit of copper in aquatic products is 50 mg/kg in the Chinese national food safety standard [78]. Obviously, humans would face a health risk by consuming mud crab containing high concentrations of copper in hepatopancreas [79].

5. Conclusions

In summary, this study is the first to show that the coloration of carapace and hemolymph of mud crab were influenced by dietary copper supplementation. A positive correlation between dietary copper levels and tissue copper deposition was also demonstrated, and particularly that high/excess levels of dietary copper induced abnormal copper bioaccumulation in carapace and hepatopancreas. Moreover, dietary copper as a crucial micronutrient component participates in the biosynthesis and metabolic regulation of hemocyanin, ceruloplasmin and phenoloxidase system. Furthermore, all the stress response genes investigated were up-regulated in response to high/excess dietary copper, activities and transcriptional levels of antioxidant key enzymes were decreased, restricting the antioxidant defense mechanism of mud crab. Overall, the copper bioaccumulation patterns in response to dietary ingestion could provide further insight to toxic contaminant mechanisms in marine crustaceans.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This study was supported by the National Key R & D Program of China (2018YFD0900400), China Agriculture Research System-48 (CARS-48), the Nature Science Foundation of Zhejiang Province (LY17C190002), the Key Research Program

of Zhejiang Province of China (2018C02037), and the Major Agriculture Program of Ningbo (2017C110007). This research was also sponsored by the K. C. Wong Magna Fund in Ningbo University. The authors were thankful to all the members in the laboratory for their assistance.

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Fig. 1. Carapace color comparison of mud crab fed diets with different levels of copper supplementation.

Fig. 2. Hemolymph color comparison of mud crab fed diets with different levels of copper supplementation.

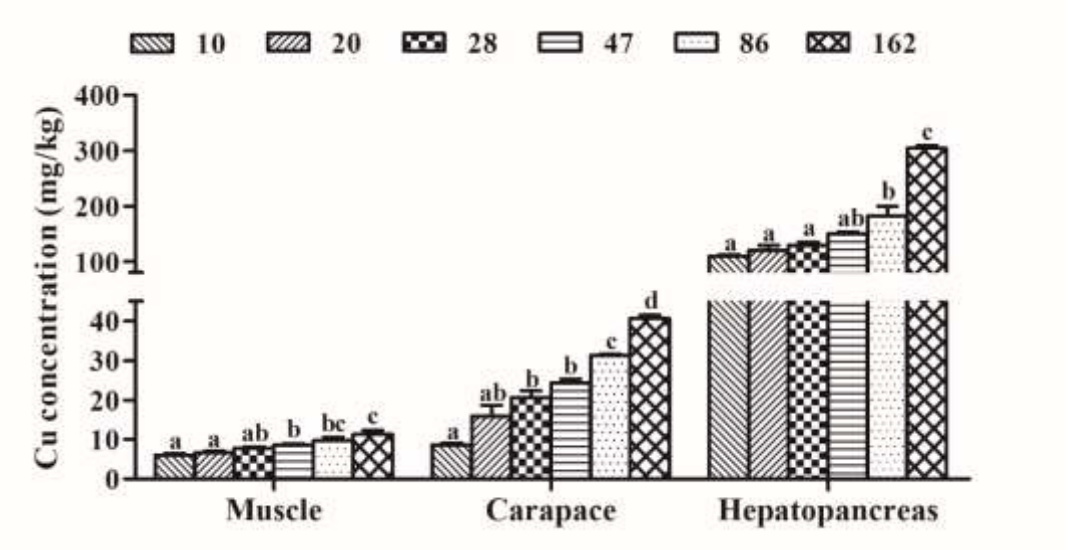


Fig. 3. Copper concentrations (mg/kg dry matter) in muscle, carapace and hepatopancreas of mud crab fed diets with different levels of copper supplementation. Values are means (n = 3), with standard errors represented by vertical bars. Mean values for the same tissue with different superscript letters were significantly different as determined by ANOVA and Tukey's test ($P < 0.05$).

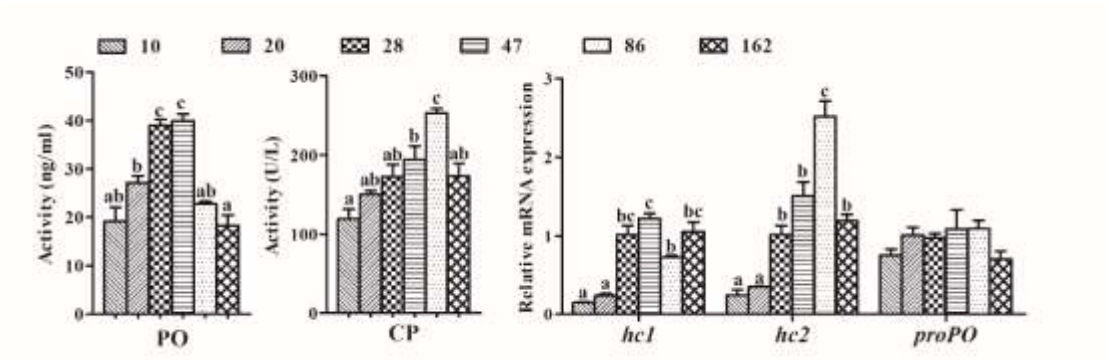


Fig. 4. Effects of dietary copper level on copper-containing proteins including activities of PO and CP in hemolymph, and mRNA expression levels of *hc1*, *hc2* and *proPO* in hepatopancreas of mud crab. Values are means (n = 3), with standard errors represented by vertical bars. Mean values for the same parameter with different superscript letters were significantly different as determined by ANOVA and Tukey's test. CP, ceruloplasmin; *hc1*, hemocyanin 1*; hc2*, hemocyanin 2*; proPO,* Prophenoloxidase; PO, phenoloxidase.

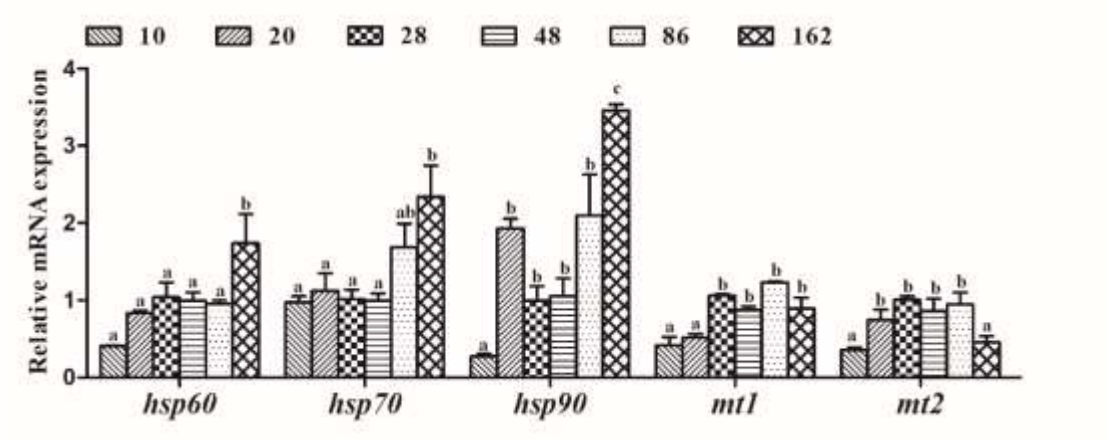


Fig. 5. Relative expression levels of genes involved in stress response in hepatopancreas of mud crab fed diets with different levels of copper supplementation. Values are means (n = 3), with standard errors represented by vertical bars. Mean values for the same gene with different superscript letters were significantly different as determined by Tukey's test ($P < 0.05$). *hsp60*, heat stress protein 60; *hsp70*, heat stress protein 70; *hsp90*, heat stress protein 90; *mt1*, metallothionein 1; *mt2*, metallothionein 2.

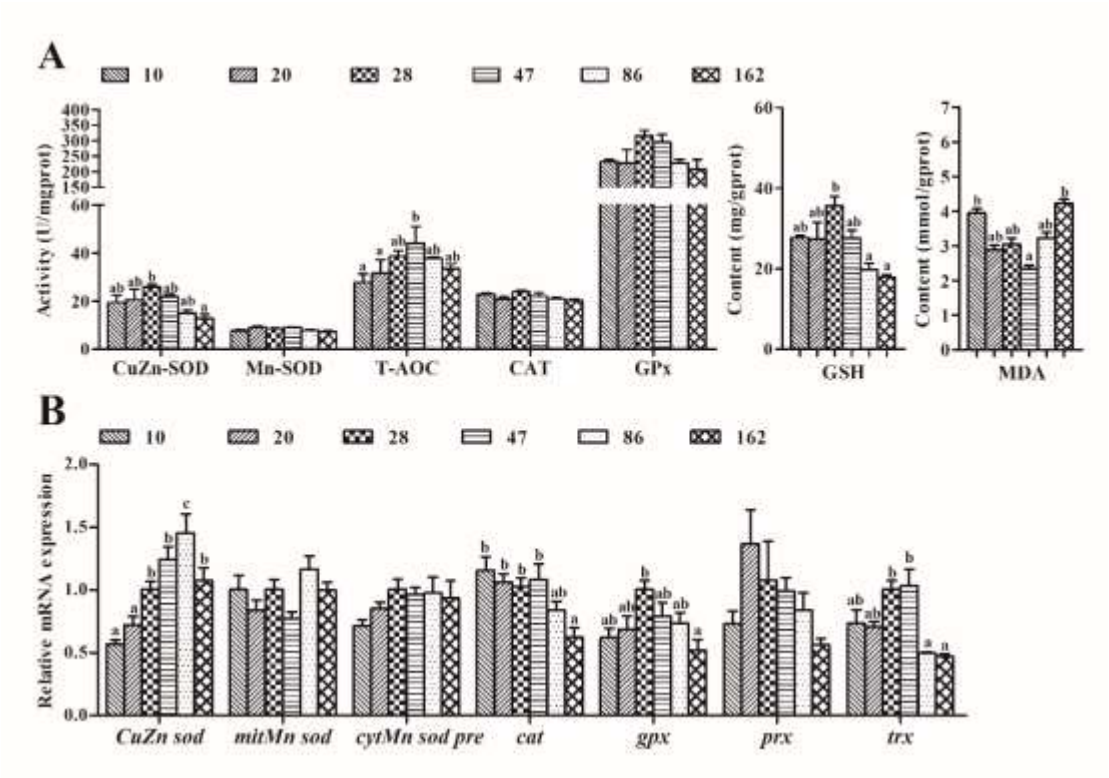


Fig. 6. Effects of dietary copper level on oxidation and antioxidant parameters in hepatopancreas of mud crab. A, antioxidant enzyme activities and metabolite levels; B, relative mRNA expression levels of antioxidant genes. Values are means (n = 3), with standard errors represented by vertical bars. Mean values for the same parameter with different superscript letters were significantly different as determined by ANOVA and Tukey's test ($P < 0.05$). CAT/*cat*, catalase; CuZn-SOD/*CuZn sod*, copper/zinc superoxide dismutase; *cytMn sod pre*, cytosolic manganese superoxide dismutase precursor; GPx/*gpx*, glutathione peroxidase; GSH, reduced glutathione; MDA, malondialdehyde; Mn-SOD/*mitMn sod*, mitochondrial manganese superoxide dismutase; *prx*, thioredoxin peroxidase; T-AOC, total anti-oxidant capacity; *trx*, thioredoxin.

Table 1. Color values in hemolymph of mud crab fed diets with different levels of copper supplementation.

Values	Dietary copper level (mg/kg)					
	10	20	28	47	86	162
L*	68.57±0.30^{d}	65.48±0.55^{c}	61.89±0.39^{b}	61.05±0.41^{b}	60.42±0.48^{b}	57.91±0.53^{a}
a*	-1.15±0.02^{b}	-1.28±0.03^{b}	-1.46±0.03^{ab}	-1.35±0.02^{ab}	-1.55±0.03^{a}	-1.50±0.03^{a}
b*	-1.74±0.02^{a}	-1.76±0.03^{a}	-1.73±0.05^{a}	-1.35±0.06^{b}	-1.39±0.04^{b}	-0.90±0.02^{c}

Data are presented as means ± SEM (n = 3). Values in the same row with different superscript letters are significantly different ($P < 0.05$) as determined by ANOVA and Tukey's test. The L* value represents lightness, negative a* values represent greenness, and negative b* values represent blueness.

Highlights

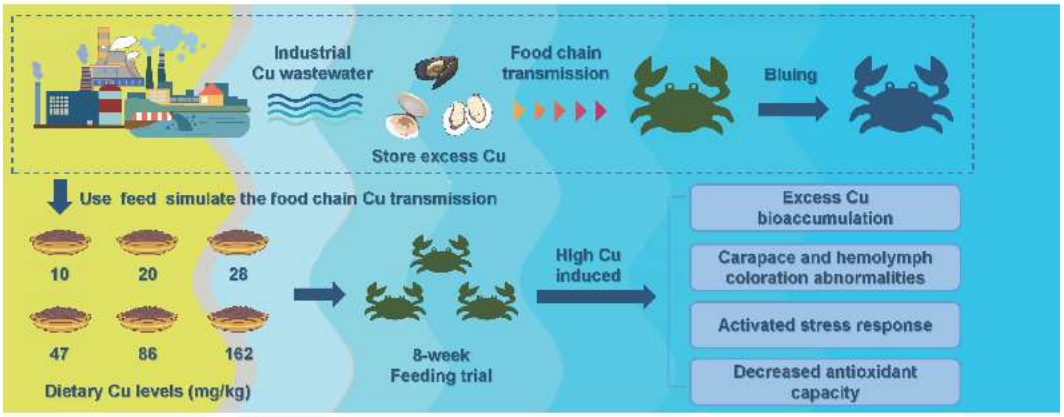
• Excessive dietary copper led to coloration abnormalities in mud crab.

• Main tissues of copper bioaccumulation are the carapace and hepatopancreas, not the muscle.

• Dietary copper supplementation influenced oxidation resistance.

• Dietary copper regulated the metabolism of copper-containing proteins.

• Excessive dietary copper in mud crab could be a potential ecological and human health risk.



Graphical abstract

Supplementary Material

Title:

Toxicological mechanism of excessive copper supplementation: Effects on coloration, copper bioaccumulation and oxidation resistance in mud crab *Scylla paramamosain*

Contents:

Table S1.

Formulation and proximate composition of experimental diets (dry matter).

Ingredients (g/kg)	Dietary copper levels (mg/kg)					
	10.0	20	28	47	86	162
Peruvian fishmeal	252.00	252.00	252.00	252.00	252.00	252.00
Casein	60.00	60.00	60.00	60.00	60.00	60.00
Soybean meal	250.00	250.00	250.00	250.00	250.00	250.00
Krill meal	70.00	70.00	70.00	70.00	70.00	70.00
Wheat flour	260.00	259.96	259.92	259.84	259.69	259.37
Fish oil	10.50	10.50	10.50	10.50	10.50	10.50
Soybean lecithin	40.00	40.00	40.00	40.00	40.00	40.00
Vitamin premix[1]	10.00	10.00	10.00	10.00	10.00	10.00
Mineral premix (copper-free)[2]	15.00	15.00	15.00	15.00	15.00	15.00
$CuSO_4 \cdot 5H_20$ (mg/kg)	0	39.29	78.59	157.17	314.34	628.68
$Ca(H_2PO_4)_2$	20.00	20.00	20.00	20.00	20.00	20.00
Choline chloride	2.00	2.00	2.00	2.00	2.00	2.00
Sodium alginate	10.50	10.50	10.50	10.50	10.50	10.50
Proximate composition						
Crude protein	455.61	458.50	458.73	456.36	453.67	457.60
Crude lipid	72.60	75.90	75.50	73.20	76.20	74.10
Moisture	89.30	92.70	88.60	81.20	84.10	84.10
Ash	104.90	105.30	103.40	103.90	101.30	102.60
Analyzed copper (mg/kg)	10.00	19.50	27.70	47.40	85.60	161.70

[1]Vitamin premix were based on Yuan, Wang, Jin, Sun and Zhou [1]

[2] Mineral premix (per kg mineral premix): $FeC_6H_5O_7$, 4.57 g; $ZnSO_4 \cdot 7H_2O$, 9.43 g; $MnSO_4 \cdot H_2O$ (99 %), 4.14 g; $MgSO_4 \cdot 7H_2O$ (99 %), 238.97 g; KH_2PO_4, 233.2 g; NaH_2PO_4, 137.03 g; $C_6H_{10}CaO_6 \cdot 5H_2O$ (98 %), 34.09 g; $CoCl_2 \cdot 6H_2O$(99 %), 1.36 g; K_2O_3Se, 0.0044 g; KIO_3, 0.0013 g.

Table S2.

Primers for real-time quantitative PCR of mud crab

Gene	Nucleotide sequence (5' –3')	Size (bp)	GenBank reference or Publication
hsp60	F: AGGACGACACGCTGCTACT R: CGGTCCTTCTTCTCGTTG	205	JX262230.1
hsp70	F: AAGGACAAGGTGAGCGAAG R: TGGTGATGATGGGGTTACA	151	EU754021.1
hsp90	F: AAGACTCACTTCATTGCCG R: TCTGTGCGTGTGCCATAC	104	JX987068.1
mt1	F: GCAAATGTGCCAACAAGGA R: TGTGACGGTGGGGAATGA	106	FJ774671
mt2	F: AAGTGTGCCAACAAGGAAGA R: AGCAAGGCTTAGAGCAGGT	149	KJ728655
Cu/Zn sod	F: ATCACCCCAACCTCAACAA R: ATCATCCACAACTCCCCAC	209	FJ774661
mitMn sod	F: TGCACATCTGACCAGCCTTA R: GCTGGTAAGTTACTGCTGGC	188	JX133232.1
cytMn sod pre	F: CATGAATGCACTCCTTCCGG R: TGTCATCCTTGGGGCAGTAG	233	GU213434.1
cat	F: ACAACACTCCCATCTTCTT R: GGACGCAGGGTGATAAAAT	132	FJ774660.1
gpx	F: AAGTTTGGTGACAATCTCG R: ACATCTCCATCTTGGGCTC	139	JN565286.1
prx	F: TGGGGACAAGATTCGTATG R: AGCAAGGGAGCAAGACAGA	184	JX133230.1
trx	F: AGGAAGACTTCAGGAACCGG R: CGAACTTGTCCACCACCTTG	246	JQ863320.1
hc1	F: TGCCGATGTCCTCTCCGAA R: GTGCTGCCACCTCTCTATG	172	KM276088
hc2	F: AACTGTACGTATTTTTGCCTGG R: GCGTGTGATGTGGTTGGAT	136	KM276085
proPO	F: GCTCATCGGGAGAACCTT R: TCTTCTGACCCTGGCTCTC	196	KP710954
ef1a	F: CTACAAGATTGGCGGCAT R: GGGGGCAAAGTTCACGAC	108	JQ824130.1

cat, catalase; *Cu/Zn sod*, copper/zinc superoxide dismutase; *cytMn sod pre*, cytosolic manganese superoxide dismutase precursor; *ef1a*, elongation factor 1a; *gpx*, glutathione peroxidase; *hsp60*, heat shock protein 60; *hsp70*, heat shock protein 70; *hsp90*, heat shock protein 90; *hc1*, hemocyanin 1; *hc2*, hemocyanin 2; *mitMn sod*, mitochondrial manganese superoxide dismutase; *mt1*, metallothionein 1; *mt2*, metallothionein 2; *proPO*, prophrnoloxodase; *prx*, thioredoxin perxidase; *trx*, thioredoxin.

Table S3.

Hematological and biochemical characteristics of mud crab regulation by different levels of dietary copper supplementation.

Parameter	Dietary copper level (mg/kg)					
	10	20	28	47	86	162
ALT (U/L)	66.77±11.46	76.3±6.96	79.66±7.8	75.91±2.45	67.45±2.74	56.37±2.29
AST (U/L)	131.89±30.29	180.96±18.58	151.27±15.05	129.77±13.6	119.55±9.26	108.73±5.84
GGT (U/L)	8.80±0.04	8.34±0.19	8.27±0.09	8.59±0.21	8.90±0.51	8.96±0.27
TP (g/L)	64.27±5.14	64.81±7.98	69.22±10.44	70.49±3.46	76.26±5.07	72.06±3.67
ALB (g/L)	$7.46±0.29^{ab}$	$7.10±0.52^{ab}$	$6.87±0.28^{b}$	$7.29±0.11^{ab}$	$7.78±0.17^{ab}$	$8.48±0.20^{a}$
GLU (mmol/L)	1.66±0.35	1.39±0.28	1.09±0.29	1.39±0.09	1.43±0.13	1.23±0.17

Data are presented as means ± SEM (n = 3). Values in the same row with different superscript letters are significantly different as determined by ANOVA and Tukey's test ($P < 0.05$). ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; GLU, glucose; TP, total protein.

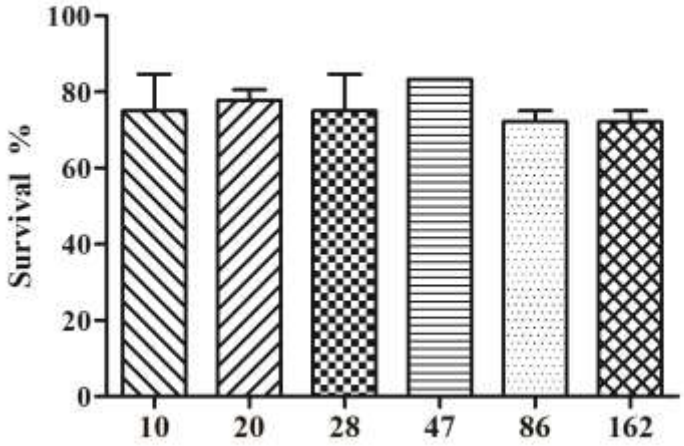


Fig. S1. Survival of mud crab fed diets with different levels of copper supplementation. The values on the X axis represent dietary copper exposure levels (mg/kg). Values are means (n = 3), with standard errors represented by vertical bars.

Proximate composition analysis in experimental diets

Moisture, ash, crude protein and crude lipid contents on diets were analyzed according to standard methods of the Association of Official Analytical Chemists [2]. Moisture content was determined at 105 °C atmospheric pressure overnight and ash content determined at 550 °C in a muffle furnace for 8 h. Crude protein content was determined by the Dumas combustion method using a fully-automatic protein analyzer (FP-528, Leco, USA). Crude lipid content was determined according to the extraction method using a Soxtec System HT (Soxtec System HT6, Tecator, Sweden).

Hematological and biochemical characteristics

The serum samples were obtained as described in the main text. The activities ot levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), total protein (TP), albumin (ALB) and glucose (GLU) were determined using the automatic biochemistry analyzer (VITALAB SELECTRA Junior Pros, Netherlands), and reagent kits from Biosino Bio-Technology and Science Inc. (Beijing, China).

References

[1] Y. Yuan, X. Wang, M. Jin, P. Sun, Q. Zhou, Influence of different lipid sources on growth performance, oxidation resistance and fatty acid profiles of juvenile swimming crab, *Portunus trituberculatus*, Aquaculture, 508 (2019) 147-158. https://doi.org/10.1016/j.aquaculture.2019.04.068.

[2] A.o.O.A.C. AOAC, Official Methods of Analysis of Ofcial Analytical Chemists International, eighteenth ed.,, Association of Official Analytical Chemists, Arlington, VA,, 2006.