View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Stirling Online Research Repository

Accepted manuscript

Title

Influence of dietary zinc on growth, zinc bioaccumulation and expression of genes involved in antioxidant and innate immune in juvenile mud crab (*Scylla paramamosain*)

Authors

Jiaxiang Luo[1], Tingting Zhu[1], Min Jin[1*], Xin Cheng[1], Ye Yuan[1], Xuexi Wang[1], Jingjing Lu[1], Lefei Jiao[1], Douglas R. Tocher[2*], Qicun Zhou[1*]

Affiliations

[1] Laboratory of Fish and Shellfish Nutrition, School of Marine Sciences, Ningbo University, Ningbo 315211, China

[2] Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland, UK

*Corresponding author

Dr. Min Jin: jinmin@nbu.edu.cn (M. Jin)

Dr. Douglas R. Tocher: d.r.tocher@stir.ac.uk (D.R. Tocher)

Dr. Qicun Zhou: zhouqicun@nbu.edu.cn (Q.C. Zhou)

Tel/Fax: +86-574-876-09878

Running Head

Physiological evaluation of dietary zinc in mud crab

Keywords:

Zinc, Mud crab *Scylla paramamosain*, Growth performance, Oxidation resistance, Innate immunity

This peer-reviewed article has been accepted for publication but not yet copyedited or typeset, and so may be subject to change during the production process. The article is considered published and may be cited using its DOI
10.1017/S0007114520001531
The British Journal of Nutrition is published by Cambridge University Press on behalf of The Nutrition Society

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

Abstract

The aim of present study was to investigate the effects of dietary Zn level on growth performance, Zn bioaccumulation, antioxidant capacity and innate immunity in juvenile mud crab (*Scylla paramamosain*). Six semi-purified diets were formulated to contain dietary Zn levels of 44.5, 56.9, 68.5, 97.3, 155.6 or 254.7 $mg \cdot kg^{-1}$, respectively. Dietary Zn level significantly influenced percent weight gain (PWG), with highest observed in crab fed the diet containing 97.3 $mg \cdot kg^{-1}$ Zn. Tissue Zn concentrations significantly increased as dietary Zn levels increased from 44.5 to 254.7 $mg \cdot kg^{-1}$. Retention of Zn in hepatopancreas increased with dietary Zn levels up to 68.5 $mg \cdot kg^{-1}$ and then significantly decreased. Moreover, inadequate dietary Zn (44.5 and 56.9 $mg \cdot kg^{-1}$) reduced anti-oxidation markers including total superoxide dismutase and copper/zinc superoxide dismutase activities and total anti-oxidant level. Crabs fed the diet with 44.5 $mg \cdot kg^{-1}$ Zn also showed significantly lower expression of genes involved in antioxidant status, such as Cu/Zn sod, glutathione peroxidase, catalase and thioredoxin than those fed diets containing 68.5 and 97.3 $mg \cdot kg^{-1}$ Zn. Highest activities of phenoloxidase and alkaline phosphatase were recorded in crab fed the diets containing 68.5 and 97.3 $mg \cdot kg^{-1}$ Zn. Expression levels of prophenoloxidase and toll-like receptor 2 were higher in crab fed the 97.3 $mg \cdot kg^{-1}$ Zn diet compared to crab fed the other diets. Based on PWG alone, the optimal dietary Zn level was estimated to be 82.9 $mg \cdot kg^{-1}$, with 68.5 to 97.3 $mg \cdot kg^{-1}$ recommended for maintaining optimal Zn bioaccumulation, oxidation resistance and innate immune response of juvenile mud crab.

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

Introduction

Mud crab (*Scylla paramamosain)*, a typical marine omnivorous crab species, is widely distributed in tropical, subtropical and temperate zones of Asia[1] and, due to its delicious flavor and nutritional quality, it is increasingly favored by seafood consumers[2]. However, wild populations of mud crab have dramatically declined in recent decades due to over-fishing and environmental deterioration[3]. With the success of larval cultivation, mud crab has become one of the most important aquaculture crustacean species in Asia, especially China [1, 4-6]. Mud crab production in China reached 157,712 tons in 2018, and accounted for 66.5 % of total production[7]. However, with increasing wild fishery and marine environmental protection, the traditional feeds of trash fish and shellfish is insufficient to meet the requirements of crab culture[8]. Thus, studies on the nutritional requirements of mud crabs are important to develop cost-effective, environmentally friendly, and nutritionally balanced formulated feed for mud crab[1]. To date, studies have reported the nutritional requirements of mud crab for protein, lipid and phospholipid [2, 9-11], however, few researchers have focused on trace element requirements of mud crab.

Minerals are essential nutrients for animal life, as cofactors and activators of a variety of enzymes and hormones participating in multiple biochemical processes[12]. Adequate supplementation of mineral elements in the diet can enhance growth performance, improve immune function, and muscle quality of aquatic organisms[13]. Zinc (Zn) is a fundamental microelement that can stabilize cellular membranes and components in organs and tissues[14]. Specifically, it is an essential cofactor for numerous enzymes and proteins involved in many physiological processes including nucleic acid, protein, fatty acid, phospholipid and carbohydrate metabolism that are critical to growth, reproduction and development of aquatic animals [15-17]. In addition, Zn has key roles in antioxidant defense and immune response partly as an integral constituent of Zn-dependent metalloenzymes such as copper/zinc superoxide dismutase (Cu/Zn SOD) and alkaline phosphatase (AKP)[18,19], and so inhibits free radical-induced oxidative damage to cells and tissues, and supports immune functions by interacting with minerals such as selenium, copper and magnesium, and several other metalloenzymes[20]. Therefore, there is an urgent need to understand the nutrition and metabolism of Zn in mud crab.

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

While the mechanisms of dietary Zn functions in fish and shrimp have been studied[21, 22], little information is available on the nutritional role of Zn in crabs and, so far, only Zn requirement of Chinese mitten crab (*Eriocheir sinensis)* has been determined[23, 24]. Deficiency of dietary Zn in fish results in decreased growth performance and survival rate, eye cataracts, skin lesions, and bone malformations and short body dwarfism[12, 20], while excessive dietary Zn is toxic to fish and reduces antioxidant ability and sperm motility[25-28]. In crustaceans, previous studies demonstrated that dietary Zn deficiency reduced growth of Chinese mitten crab[23], freshwater prawn (*Macrobrachium rosenbergii)*[15], grass shrimp (*Penaeus monodon)*[29] and Pacific white shrimp (*Litopenaeus vannamei)*[30, 31], while excessive dietary Zn decreased digestive enzyme activities in freshwater prawn[15]. Lin *et al.* [32] compared the effects of different dietary Zn sources (Zn-methionine, Zn-lysine, Zn-glycine and Zn-sulfate) on growth performance and immune function of Pacific white shrimp, showing that Zn-methionine significantly improved survival, PWG and immune-related enzyme activities. While these studies indicated dietary optimal Zn is vital to crustaceans, comprehensive assessment of dietary Zn level is still relatively limited in crustacean, especially marine crab. Therefore, an 8-week nutritional trial was designed to investigate the optimal Zn requirement level for juvenile mud crab, and to evaluate the effects of dietary Zn on growth, tissue Zn bioaccumulation, haemolymph characteristics, antioxidant ability and the innate immune response. The outcomes of the study will enhance our understanding of the nutritional metabolism of Zn in mud crab, and will also explore strategies to improve disease resistance and antioxidant capacity through nutritional modulation of marine crab.

Methods

Ethics statement

The study was performed in strict accordance with the Standard Operating Procedures (SOPs) of the Guide for Use of Experimental Animals of Ningbo University. The experimental protocol and procedures were approved by the Institutional Animal Care and Use Committee of Ningbo University.

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

Diet preparation

Six isonitrogenous (45% crude protein) and isolipidic (7.5% crude lipid) experimental diets were formulated to contain different levels of Zn ($ZnSO_4 \cdot 7H_2O$ as Zn source), with the analyzed Zn concentrations being 44.5, 56.9, 68.5, 97.3, 155.6 and 254.7 $mg \cdot kg^{-1}$, respectively. Peruvian fishmeal, soybean meal, krill meal and casein were the main protein sources, with fish oil and soybean lecithin as lipid sources (Table 1). Feeds were manufactured according to the method described in detail previously [33]. Briefly, all dry ingredients were ground into fine powder with particle size < 177 μm, and micro components including minerals and vitamin premix were added by the progressive enlargement method. Lipid and distilled water (35 %, w/w) were added to the dry ingredients and the mixture blended until homogenous 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 two uniform sizes (3 mm and 5 mm diameter pellets) (G-250, Machine factory of South China University of Technology). Pellets were heated at 90 °C for 30 min, air-dried to approximately 10% moisture, sealed in vacuum-packed bags and stored at -20 °C until use.

Table 1 insert here

Crab rearing and experimental conditions

Juvenile mud crabs were obtained from the breeding base of Ningbo Ocean and Fishery Science and Technology Innovation Center (Zhejiang, China). Prior to the experiment, crabs were acclimated for 2 weeks and fed with commercial diet (45% crude protein, 8% crude lipid and 72 $mg \cdot kg^{-1}$ Zn; Evergreen Corp., Guangdong, China). At the beginning of the experiment, a total of 270 healthy, normally active and similar size juvenile crabs (14.96 ± 1.11 g, in the ninth molting stage) were randomly allocated into 270 single crab units (SCU, 33cm × 22.5cm × 25 cm), to prevent competition and cannibalism. Each feed was randomly assigned to three experimental replicates with fifteen mud crabs in each replicate. Each SCU was mutual independent and supplied with continuous flow-through water[34]. Crabs were fed the allocated experimental diet once daily at 18:00 h (3 - 6 % of crab weight), and the daily ration adjusted according to actual intake (ration - uneaten feed) to ensure feeding to apparent satiation. Feces and uneaten feed were removed by siphon and spoon-net each morning. During the 10-week experimental period, water temperature in each culture unit was 24.5 -

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

29.0 °C, salinity approximately 24.1 - 28.4 $g \cdot L^{-1}$, pH 7.3 - 8.0, ammonia nitrogen was lower than 0.05 $mg \cdot L^{-1}$, dissolved oxygen was not lower than 6.0 $mg \cdot L^{-1,}$ and *vibrio* concentration in seawater was less than 1.0×10^5 $cfu \cdot mL^{-1}$.

Sample collection

At the end of the experiment, most crabs were at molting stage eleven and a few were at stage twelve. Crabs were anesthetized with 0.02 % tricaine methane sulphonate (MS-222), and all crabs in each replicate were counted and weighed to determine the survival and percent weight gain (PWG). In each replicate, haemolymph was sampled from six crabs and centrifuged at 3500 rpm for 10 min at 4 ℃ (Eppendorf centrifuge 5810R, Germany). The supernatant was collected and stored at - 80 ℃ before analyses of biochemical and enzyme activities. Hepatopancreas from the same six crabs was quickly dissected and weighed to calculate hepatosomatic index (HSI), and placed in 1.5 ml centrifuge tubes, frozen in liquid nitrogen and stored at - 80 ℃ prior to the analysis of enzyme activities and gene expression. Muscle, carapace and hepatopancreas from a further three crabs were collected and stored at - 20 ℃ to determine Zn concentrations and tissue Zn retention rate.

Calculations

Survival (%) = 100 × ((final number of crabs) / (initial number of crabs))

Percent weight gain (PWG, %) = 100 × ((final body weight - initial body weight) / initial body

weight)

Hepatosomatic index (HSI, %) = 100 × (Hepatopancreas weight / body weight)

Zinc retention rate (ZRR, %) = $100 \times(W_t \times Z_t - W_i \times Z_i)/(W_d \times Z_d)$

W_t is the final tissue weight (g), W_i is the initial tissue weight (g), Z_t is the final zinc concentration ($mg \cdot kg^{-1}$), Z_i is the initial zinc concentration ($mg \cdot kg^{-1}$), W_d is the weight of fed diet (g), and Z_d is the zinc concentration of the diet ($mg \cdot kg^{-1}$). The calculation of these parameters was based on three experimental replicates per diet.

Zinc concentration analysis

All collected tissues (hepatopancreas, muscle and carapace) and experimental diets were weighed and freeze-dried before acid digestion, where samples were digested in 70 % HNO_3 solution at 80 ℃, with the acid solution added drop-wise until complete digestion of organic

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

matter. The digested solution was filtered through an aqueous phase syringe filter (SCAA-102, ANPEL Laboratory Technologies Inc, China) before tissue Zn concentrations were determined by inductively coupled plasma optical emission spectrometry (PE2100DV, Perkin Elmer, USA).

Haemolymph biochemical analysis

The activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), and total protein (TP) and albumin (ALB) contents were analyzed in haemolymph supernatant using an automatic biochemistry analyzer (VITALAB SELECTRA Junior Pros, Netherlands), with reagent kits from Biosino Bio-Technology and Science Inc. (Beijing, China).

Enzymes activities analysis

Hepatopancreas samples were homogenized on ice in nine volumes of normal saline solution, centrifuged at 3500 rpm for 15 min at 4 ℃, and the supernatant collected in a PCR tube and stored at - 80 ℃ prior to analysis of enzyme activities. The activities of total superoxide dismutase (T-SOD), copper/zinc superoxide dismutase (Cu/Zn-SOD), catalase (CAT), phenoloxidase (PO), ceruloplasmin (CP), alkaline phosphatase (AKP), and acid phosphatase (ACP) in haemolymph supernatant, and the activities of CAT, glutathione peroxidase (GPx), and total anti-oxidation capacity (T-AOC) in hepatopancreas homogenates, as well as the contents of glutathione (GSH) and malondialdehyde (MDA) in both haemolymph and hepatopancreas were assayed by Multiskan spectrum (Thermo, USA) according to the manufacturer's instructions using commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China)

Total RNA extraction, reverse transcription and real-time PCR

RNA extraction, reverse transcription and real-time quantitative PCR were conducted according to methods described in detail previously[35]. Briefly, RNA was extracted from crab hepatopancreas samples of approximately 50 mg by TRIzol Reagent (Takara, Japan) following the manufacturer's protocol with RNA quality and concentration confirmed by 1.2 % agarose gel electrophoresis and ultra-micro spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, USA), respectively. RNA samples were reverse converted to cDNA using PrimeScript® RT reagent kit (TaKaRa, Japan) according to the manufacturer's protocol.

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

Elongation factor 1 alpha (*ef1a*) was chosen as the reference gene (housekeeping gene) after confirmation of its expression stability. Specific primers used for RT-qPCR were designed according to complete cDNA sequences of corresponding genes in NCBI using Primer Premier 5.0 software (Table 2). PCR amplification was conducted by a quantitative thermal cycler (Lightcycler 96, Roche, Switzerland), with reactions containing 2 μL of cDNA, 1.0 μL of each primer, 10 μL of 2×conc SYBR Green I Master (Roche, Switzerland) and 6 μL DEPC-water. The procedure of quantitative PCR 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. Standard curves were generated using six different dilutions of one cDNA sample of the 68.5 $mg \cdot kg^{-1}$ treatment group. Amplification efficiency was measured as: $E=10^{(-1/Slope)}-1$, and the amplification efficiencies of all genes were approximately equal and ranged from 93 to 102 %. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, and the 68.5 $mg \cdot kg^{-1}$ treatment group was used as the control/reference group

Table 2 insert here

Statistical analysis

For heat map visualization analysis, all data were homogenized and performed using the online program Image GP (http://www.ehbio.com/ImageGP/index.php/).

Data are presented as means ± S.E.M of three replicates (n = 3), and analyzed 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).

Results

Survival and growth performance

The effects of dietary zinc levels on survival and growth performance of mud crabs are presented in Table 3. Survival ranged from 72.2 % to 86.1 %, with no statistical differences among treatments ($P > 0.05$), although numerically lowest survival was observed in crab fed the diet containing 254.7 $mg \cdot kg^{-1}$ Zn. Crabs fed the diet containing 254.7 $mg \cdot kg^{-1}$ Zn had lower PWG than those fed the 68.5 and 97.3 $mg \cdot kg^{-1}$ Zn diets, and the highest PWG was observed in crab fed the diet containing 97.3 $mg \cdot kg^{-1}$ Zn ($P < 0.05$). Broken-line regression analysis of PWG and dietary Zn level showed $y = 0.5232x + 121.08$ ($R^2 = 0.7261$) and y =

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

−0.3406x + 192.76 (R^2 = 0.9208), respectively, and the optimum dietary Zn level was estimated to be 82.9 mg·kg^{-1} for juvenile mud crab (Fig. 1). HSI did not show any statistical differences among dietary treatments ($P > 0.05$).

Table 3 insert here

Fig. 1 insert here

Tissues zinc concentration and retention rate

Zn concentrations in hepatopancreas, muscle and carapace significantly increased as dietary Zn level increased from 44.5 to 254.7 mg kg^{-1}, with highest tissue concentrations observed in crab fed the diet containing 254.7 mg·kg^{-1} Zn (Fig. 2 (A)) ($P < 0.05$).

The Zn retention rate (ZRR) of crab tissues is presented in Fig. 2 (B). The ZRR in hepatopancreas increased as dietary Zn level increased from 44.5 to 68.5 mg·kg^{-1} reaching a peak value in crab fed the diet containing 68.5 mg·kg^{-1} Zn, and then decreased as dietary Zn level increased from 68.5 to 254.7 mg·kg^{-1}. Contrasting results were found in muscle and carapace, where ZRR significantly decreased with increased dietary zinc level, with lowest ZRR in muscle and carapace observed in crab fed the diet containing 254.7 mg·kg^{-1} Zn.

Fig. 2 insert here

Haemolymph biochemical analysis

Biochemical parameters of haemolymph of crab fed the experimental diets are presented in Table 4. ALT, AST, GGT, TP and ALB in haemolymph supernatant were not significantly influenced by dietary zinc level ($P > 0.05$).

Table 4 insert here

Antioxidant enzyme activities and gene expression in haemolymph and hepatopancreas

The values of anti-oxidant status parameters in haemolymph and hepatopancreas are shown in Fig. 3. Crabs fed the diets containing 44.5 and 56.9 mg·kg^{-1} Zn exhibited significantly lower T-SOD and Cu/Zn-SOD activities in haemolymph than those fed the other diets ($P < 0.05$). Compared with crab fed the 44.5 and 254.7 mg·kg^{-1} Zn diets, higher CAT activity and GSH content in haemolymph were observed in crab fed the diet containing 68.5 mg·kg^{-1} Zn ($P < 0.05$), while no significant differences were found in haemolymph MDA contents among treatments ($P > 0.05$). Lowest values of T-AOC, CAT and GSH in hepatopancreas were observed in crabs fed the diet containing 44.5 mg·kg^{-1} Zn ($P < 0.05$). Hepatopancreas of

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

crabs fed the diets with 155.6 and 254.7 mg·kg^{-1} Zn had significantly higher MDA concentrations than those fed the diets with 56.9 and 97.3 mg·kg^{-1} Zn ($P < 0.05$).

Fig. 3 insert here

The expression levels in hepatopancreas of anti-oxidant system genes are shown in Fig. 4. The highest relative expression of *Cu/Zn sod* was observed in crabs fed the diet containing 97.3 mg·kg^{-1} Zn ($P < 0.05$), while the expression level of *mitMn sod* was not affected by dietary Zn level ($P > 0.05$). The expression level of *gpx* in hepatopancreas was significantly higher in crabs fed the diet with 97.3 mg·kg^{-1} Zn than in crabs fed the other diets ($P < 0.05$). Moreover, crabs fed the 44.5 and 254.7 mg·kg^{-1} Zn diets had lower relative expression levels of *cat* and *trx* than those fed the 56.9 and 97.3 mg·kg^{-1} Zn diets ($P < 0.05$).

Fig. 4 insert here

Innate immunity enzyme activities and gene expression

Activities of enzymes of innate immunity in haemolymph of crabs fed different dietary levels of Zn are presented in Fig. 5. The activity of PO in haemolymph significantly increased as dietary Zn level increased from 44.5 to 97.3 mg·kg^{-1}, and then significantly decreased with further increase of dietary Zn level ($P < 0.05$). Crabs fed diets containing 97.3 - 254.7 mg·kg^{-1} Zn had significantly lower CP activity compared to crabs fed diets containing 44.5 - 68.5 mg·kg^{-1} Zn, with lowest CP activity observed in crabs fed the 254.7 mg·kg^{-1} Zn diet ($P < 0.05$). The activities of AKP and ACP in haemolymph were significantly higher in crabs fed the diets containing 68.5 and 97.3 mg·kg^{-1} Zn compared to activities in crabs fed the other diets ($P < 0.05$).

Fig. 5 insert here

The expression levels of genes related to innate immunity in hepatopancreas are shown in Fig. 6. The mRNA expression levels of *proPO* and *toll2* in hepatopancreas were significantly up-regulated in crabs fed the 97.3 mg·kg^{-1} Zn diet compared with crabs fed the other diets ($P < 0.05$). Dietary Zn level had no significant effect on the mRNA expression levels of *clr* and *toll1* in hepatopancreas ($P > 0.05$).

Fig. 6 insert here

Heat map visualization analyze of the antioxidative and innate immune parameters

Heat map visualization was conducted to present the macroscopic effects of dietary Zn

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

level on oxidation resistance and innate immunity parameters of mud crab (Fig. 7). All data were normalised, with red colour representing higher values, and blue colour representing lower values. The results clearly indicated that higher values of activities and gene expression of antioxidation enzymes and parameters, as well innate immunity enzymes and innate immunity-related genes were observed in crabs fed the diets containing 68.5 and 97.3 mg·kg^{-1} Zn. The lowest values of peroxidation products were found in crabs fed the diets with 68.5 and 97.3 mg·kg^{-1} Zn.

Fig. 7 insert here

Discussion

Due to the limited availability of land culture resources and the high-demand of the consumer market, intensive aquaculture has become the main mode of culture in crustaceans[1, 33, 36]. Nevertheless, intensive aquaculture can cause physiological and metabolic disorders and hypo-immunity, that can lead to increased disease [37]. Zn is an essential trace element and acts as a cofactor in numerous proteins and enzymes involved in many biological processes[12, 22]. Recently, a number of studies have demonstrated that dietary Zn could promote growth, improve anti-stress responses and improve disease resistance in several crustaceans species[15, 23, 24, 29, 31, 32, 38].

The present study demonstrated that dietary Zn could promote growth performance of juvenile mud crab, and crabs fed excessive Zn diet had poor PWG, similar to results found in freshwater prawn and Pacific white shrimp[15, 30]. Although, there were no statistically significant differences in PWG between mud crab fed the diet without supplementary Zn (44.5 mg·kg^{-1} Zn) and those with graded Zn, the regression design indicated that dietary Zn supplementation could improve growth performance of mud crab. Based on PWG, the optimal level of dietary Zn for juvenile mud crab in the present study was 82.9 mg·kg^{-1} diet. This value was similar to other crustaceans, such as Pacific white shrimp (71.48 - 95.06 mg·kg^{-1})[31], but different from Chinese mitten crab (105.34 mg·kg^{-1})[24], freshwater prawn (60 mg·kg^{-1})[15] and grass shrimp (34.1 mg·kg^{-1})[29]. These discrepancies between Zn requirements among crustaceans may be due to differences between the species themselves but may also reflect differences in the studies including growth stage, culture conditions and

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

basal diet. For instance, in the Zn requirement study in Chinese mitten crab, the initial weight of the crab was only 7.16 ± 0.48 mg[24], while the initial weight of mud crab in the present study was 14.96 ± 1.11 g.

Zn concentration in different tissues could reflect Zn utilization[29]. In the present study, Zn concentrations in tissues increased with increased dietary Zn level, in agreement with previous studies in Chinese mitten crab[23], grass shrimp[29], grouper (*Epinephelus malabaricus*)[39], hybrid tilapia (*Oreochromis niloticus* × *O. aureus*)[40], Indian major carp (*Labeo rohita)* and yellow catfish (*Pelteobagrus fulvidraco)*[20]. Furthermore, the main tissue of zinc deposition was hepatopancreas, followed by muscle, and then carapace, which was similar to Chinese mitten crab where Zn concentration in hepatopancreas of crabs fed 85 mg·kg^{-1} Zn was amost 4-fold higher compared to crab fed 5 mg·kg^{-1} Zn[23]. In grass shrimp, hepatopancreas Zn concentration was highest in shrimp fed the diet containing the highest zinc level, and lowest in shrimp fed the basal, unsupplemented diet[29]. These data reflect the role of the hepatopancreas as the main site of nutrient metabolism and mineral bioaccumulation in crustacean species[41]. In the present study, ZRR of muscle and carapace of mud crab reduced with increased dietary Zn level, similar to Zn retention in whole body of tilapia fed graded Zn in a soya bean meal-based diet[40]. However, ZRR in hepatopancreas of mud crab showed a different trend to the other tissues and reached a peak value in crabs fed 68.5 mg·kg^{-1} Zn diet, and then decreased as dietary Zn level increased further. This indicated that when dietary Zn level was higher than 68.5 mg·kg^{-1}, Zn deposition rate in hepatopancreas was lower, possibly reflecting negative feedback regulation.

In general, defense systems against lipid peroxidation consist of antioxidant enzymes, such as SOD, CAT and GPx, as well the nonenzymatic antioxidant GSH[42-44]. SOD catalyzes the dismutation of superoxide anion to hydrogen peroxide and oxygen, and Zn is required at the active center of Cu/Zn-SOD[45]. CAT and GPx are responsible for scavenging radicals and are involved in protective mechanisms within tissues following oxidative process and phagocytosis[46]. GSH is a scavenger in the body that can remove free radicals such as superoxide ions (O_2^-) and hydroxyl groups (OH^-)[47]. In the present study, lowest T-SOD, Cu/Zn-SOD and CAT activities, and T-AOC and GSH levels were observed in crabs fed the diets containing 44.5 and 56.9 mg·kg^{-1} Zn compared with those fed the diets containing 68.5 and 97.3 mg·kg^{-1} Zn.

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

Deficiency or excessive dietary Zn decreasing biological efficiency of antioxidation enzymes and antioxidants, has been reported previously in other aquatic animals[15, 20, 25, 26, 46, 48]. Production of MDA results from the peroxidation of PUFA, influencing cell membrane fluidity as well as the integrity of biomolecules and is an important indicator of peroxidation[49] reflecting the antioxidant status of aquatic animals[27]. In the present study, the lowest MDA concentrations in haemolymph and hepatopancreas were observed in crabs fed the diets containing 68.5 and 97.3 mg·kg^{-1} Zn, which indicated that appropriate levels of dietary Zn contributes to the reduction of peroxidation products largely due to the actions of the antioxidant enzymes[46]. Similar results were obtained in grass carp (*Ctenopharyngodon idella)*[26], Indian major carp [20], Nile tilapia (*Oreochromis niloticus)*[48], Jian carp (*Cyprinus carpio)*[46] and yellow catfish [25].

In the present study, expression levels of genes involved into antioxidant such as *Cu/Zn sod*, *gpx*, *cat* and *trx* were increased in crabs fed diets containing 68.5 and 97.3 mg kg^{-1} Zn compared to those fed the other diets. The expression data were consistent with the higher activities of Cu/Zn-SOD and CAT at these dietary Zn levels, which demonstrated a positive correlation between gene expression and enzymatic activity. Dietary Zn could directly interact with intracellular transcription factors to regulate gene transcription and expression[50], or indirectly regulate gene expression by stimulating various signaling pathways, hormones, cytokines and other intermediate regulatory substances[51]. Overall, the results indicated that optimum dietary Zn level could enhance oxidation resistance capabilities of mud crab, partly due to its role in maintaining activities of Cu/Zn-SOD and other antioxidant enzymes [52]. Meanwhile, Zn is also a free radical scavenger and can inhibit the synthesis of free radicals[53], and it is thought that zinc shields membrane from iron-initiated peroxidation of lipids by blocking negatively charged iron binding sites[54]. Thus, the synergistic actions of Zn with water-soluble or lipid-soluble antioxidants can prevent lipid peroxidation[20].

Immunological responses are important indicators of health status in organisms[15]. Since crustaceans have no adaptive immunity memory cells to produce immunoglobulins, they mainly depend on innate immune systems for host defense[35]. The prophenoloxidase (proPO) system is considered as a constituent of the immune system and probably responsible, at least

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

in part, for the nonself recognition process of the defense mechanism in crustaceans, when transformed to the active form (phenoloxidase, PO) by metal ions[55], with PO being one of the most important enzymes involved in the innate immune system of invertebrates[56]. In the present study, crabs fed the diet with 44.5 mg·kg^{-1} Zn had significantly lower PO activity in haemolymph than those fed the 97.3 mg·kg^{-1} Zn diet, indicating that the appropriate dietary Zn level could activate PO activity and promote immune responses. Furthermore, excessive dietary Zn (254.7 mg·kg^{-1}) supplementation in mud crab decreased PO and CP activities in haemolymph. Copper is a key component of PO and CP[35; 57], and there is interaction between copper and zinc[58]. Thus, excessive dietary Zn may disrupt normal copper metabolism, and thus restrain the activities of PO and CP in mud crab. There often exists interaction between Zn and other cations, including competitive inhibition during gastrointestinal absorption, due to similarities in the physiochemical attributes of these cations[20]. Another Zn-dependent metalloenzyme whose activity could be regulated by dietary Zn level is AKP[12]. All highly purified AKP have been shown to be Zn (II) metalloenzymes, whose role was related to the saturation of Zn (II) binding sites[59]. Thus, AKP can be used as an important indicator to evaluate Zn status in aquatic animals[27]. In the present study, AKP activity in haemolymph was higher in crabs fed the diets containing 68.5 and 97.3 mg·kg^{-1} Zn compared to crabs fed the other diets, and similar results were found in blunt snout bream (*Megalobrama amblycephala)*[27], grass crap[44], Indian major carp[20], Nile tilapia[60] and Siberian sturgeon (*Acipenser baerii)*[61].

The ProPO and Toll pathways are two major signaling pathways of crustaceans that are essential for inducing immune-related genes during innate immune response[62]. In the present study, expression of *proPO* and *toll2* in crabs fed the diet containing 97.3 mg·kg^{-1} Zn were up-regulated compared to those fed the other diets. Higher expression levels of *toll* were found in hepatopancreas of Pacific white shrimp when the level of Zn in feed was appropriate[38]. Inhibitory zinc finger protein (A20) is an important protein involved in the Toll receptor, and so up-regulation of toll in the optimal dietary Zn group might be related to the mediation of A20[63]. Overall, this may indicate that optimal dietary Zn levels could enhance the innate immunity response of crustaceans via impacts on AKP, ProPO and Toll systems. However, other than the consistent effects on AKP, dietary Zn affects the immune

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

regulation of fish in different ways. In basa catfish (*Pangasius hypophthalmus)*, dietary Zn supplement resulted in higher contents of total protein and globulin in serum[64] and, in rainbow trout (*Oncorhynchus mykiss*), dietary supplement with Zn-enriched yeast significant increased serum lysozyme activity, complement activity and total immunoglobulin[65]. A study on grass carp also found that dietary Zn deficiency decreased fish intestinal immune barrier function through regulation of NF-κB, TOR, Nrf2, JNK and MLCK signaling pathway[66].

The bioinformatic statistical tool "heat map" has been used to analyze multi-level and complex data and provide integrative analysis of large data sets in multiple treatment groups, and data sets in different measure units, analytical levels and tissues[67]. Heat map is thus a useful way to visualize complex and diversified data with colour coded arrays indicating the intensity (or amount) of the dependent variable[68]. Using heat map and multivariate correlation analysis, Yuan et al.[18] clearly demonstrated that different dietary zinc sources (zinc sulfate, zinc amino acid complex, mixed zinc source) showed inconsistent biological effects in Pacific white shrimp, and shrimp fed the mixed zinc source diet showed better growth response and meat quality. In the present study, the heat map clearly showed that crab fed the diets with 68.5 and 97.3 $mg \cdot kg^{-1}$ Zn up-regulated the expression of genes and enhanced the activities of antioxidant and innate immunity enzymes, and decreased the contents of peroxidation products.

Conclusion

In summary, based on broken-line regression analysis between PWG and dietary Zn level, the optimal dietary Zn requirement of juvenile mud crab was estimated to be 82.9 $mg \cdot kg^{-1}$. Moreover, the results of the present study demonstrated a positive correlation between tissue Zn bioaccumulation and dietary zinc levels in mud crab, particularly in hepatopancreas. Furthermore, the expression levels and activities of antioxidant and innate immune enzymes were increased in crabs fed the diets containing 68.5 and 97.3 $mg \cdot kg^{-1}$ Zn and, therefore, enhanced the antioxidant defense and innate immune responses of mud crab.

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

Acknowledgments

The authors were thankful to all the members in the FSN lab (Laboratory of Fish and Shellfish Nutrition, Ningbo University) for their assistance. The authors also thank Ningbo Institute of Materials Technology and Engineering, Chinese Academy of Sciences (NIMTE, CAS) for use of the ICP-OES and Zhu L.H. for valuable help during the determination of mineral concentration.

Financial Support

This study was supported by National Key R & D Program of China (2018YFD0900400), China Agriculture Research System-48 (CARS-48), the National Natural Science Foundation of China (41476125), and the Nature Science Foundation of Zhejiang Province (LY17C190002), Key Research Program of Zhejiang Province of China (2018C02037), and Major Agriculture Program of Ningbo (2017C110007). This research was also sponsored by K. C. Wong Magna Fund in Ningbo University.

Conflict of Interest

The authors declared that there were no conflicts of interest.

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

References

1. Li Y, Ai C, Liu L (2018) Mud Crab, *Scylla paramamosain* China's leading maricultured crab. In *Aquaculture in China*, pp. 226-233. https://doi.org/https://doi.org/10.1002/9781119120759.ch3_4

2. Zhao J, Wen X, Li S *et al.* (2015) Effects of dietary lipid levels on growth, feed utilization, body composition and antioxidants of juvenile mud crab *Scylla paramamosain (Estampador)*. *Aquaculture* **435**, 200-206. https://doi.org/10.1016/j.aquaculture.2014.09.018

3. Ye H, Tao Y, Wang G *et al.* (2011) Experimental nursery culture of the mud crab *Scylla paramamosain (Estampador)* in China. *Aquacult Int* **19**, 313-321. https://doi.org/10.1007/s10499-010-9399-3

4. Marichamy R, Rajapackiam S (2001) The aquaculture of *Scylla* species in India. *Asian Fisheries Science* **14**, 231-238.

5. Keenan CP (1999) Aquaculture of the mud crab, genus Scylla-past, present and future. *Aciar Proceedings*, 9-13.

6. Le Vay L (2001) Ecology and management of mud crab *Scylla spp*. *Asian FIsheries Science* **14**, 101-111.

7. FCAD FBoCAD (2019) *China Fishery Statistical Yearbook.* Beijing, China: China Agriculture Press.

8. Jin M, Zhou Q, Zhang W *et al.* (2013) Dietary protein requirements of the juvenile swimming crab, *Portunus trituberculatus*. *Aquaculture* **414-415**, 303-308.

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

https://doi.org/10.1016/j.aquaculture.2013.08.028

9. Dong L, Tong T, Zhang Q *et al.* (2017) Effect of dietary protein level on growth performance, body composition, and digestive enzyme activities in green mud crab (*Scylla paramamosain*) juveniles. *Journal of Fishery Sciences of China* **24**. https://doi.org/10.3724/sp.J.1118.2017.16289

10. Kader MA, Bulbul M, Asaduzzaman M *et al.* (2017) Effect of phospholipid supplements to fishmeal replacements on growth performance, feed utilization and fatty acid composition of mud crab, *Scylla paramamosain (Estampador 1949). J Sustain Sci Manag* **2017**, 47-61.

11. Xu H, Wang J, Han T *et al.* (2019) Effects of dietary phospholipids levels on growth performance, lipid metabolism, and antioxidant capacity of the early juvenile green mud crab, *Scylla paramamosain (Estampador)*. *Aquac Res* **50**, 513-520. https://doi.org/10.1111/are.13922

12. NRC NRC (2011) *Nutrient requirements of fish and shrimp*: National academies press.

13. Chitturi R, Baddam VR, Prasad L *et al.* (2015) A review on role of essential trace elements in health and disease. *Journal of Dr NTR University of Health Sciences* **4**. https://doi.org/10.4103/2277-8632.158577

14. Salgueiro MJ, Zubillaga M, Lysionek A *et al.* (2000) Zinc as an essential micronutrient: A review. *Nutr Res* **20**, 737-755. https://doi.org/https://doi.org/10.1016/S0271-5317(00)00163-9

15. Muralisankar T, Bhavan PS, Radhakrishnan S *et al.* (2014) Dietary Supplementation of

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

Zinc Nanoparticles and Its Influence on Biology, Physiology and Immune Responses of the Freshwater Prawn, *Macrobrachium rosenbergii*. *Biol Trace Elem Res* **160**, 56-66. https://doi.org/10.1007/s12011-014-0026-4

16. Tan B, Mai K (2001) Zinc methionine and zinc sulfate as sources of dietary zinc for juvenile abalone, *Haliotis discus hannai Ino*. *Aquaculture* **192**, 67-84. https://doi.org/https://doi.org/10.1016/S0044-8486(00)00435-X

17. Wu K, Luo Z, Hogstrand C *et al.* (2018) Zn stimulates the phospholipids biosynthesis via the pathways of oxidative and endoplasmic reticulum stress in the intestine of freshwater teleost yellow catfish. *Environ Sci Technol* **52**, 9206-9214. https://doi.org/10.1021/acs.est.8b02967

18. Yuan Y, Luo J, Zhu T *et al.* (2020) Alteration of growth performance, meat quality, antioxidant and immune capacity of juvenile *Litopenaeus vannamei* in response to different dietary dosage forms of zinc: Comparative advantages of zinc amino acid complex. *Aquaculture* **522**, 735120. https://doi.org/https://doi.org/10.1016/j.aquaculture.2020.735120

19. Rink L (2000) Zinc and the immune system. *Proceedings of the Nutrition Society* **59**, 541-552. https://doi.org/http://doi.org/10.1017/S0029665100000781

20. Musharraf M, Khan MA (2019) Dietary zinc requirement of fingerling Indian major carp, *Labeo rohita (Hamilton)*. *Aquaculture* **503**, 489-498. https://doi.org/10.1016/j.aquaculture.2019.01.039

21. Davis DA, Gatlin DM (1996) Dietary mineral requirements of fish and marine

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

crustaceans. *Rev Fish Sci* **4**, 75-99. https://doi.org/10.1080/10641269609388579

22. Watanabe T, Kiron V, Satoh S (1997) Trace minerals in fish nutrition. *Aquaculture* **151**, 185-207. https://doi.org/https://doi.org/10.1016/S0044-8486(96)01503-7

23. Li W, Gong Y, Jin X *et al.* (2010) The effect of dietary zinc supplementation on the growth, hepatopancreas fatty acid composition and gene expression in the Chinese mitten crab, *Eriocheir sinensis (H. Milne-Edwards*) (Decapoda: Grapsidae). *Aquac Res* **41**, e828-e837. https://doi.org/10.1111/j.1365-2109.2010.02603.x

24. Sun S, Chen M, Chen LQ *et al.* (2011) Optimal dietary copper and zinc requirements for juvenile Chinese mitten crab, *Eriocheir sinensis*. *J Isr J Aquacult Bamid* **63**, 580-587.

25. Luo Z, Tan X, Zheng J *et al.* (2011) Quantitative dietary zinc requirement of juvenile yellow catfish *Pelteobagrus fulvidraco*, and effects on hepatic intermediary metabolism and antioxidant responses. *Aquaculture* **319**, 150-155. https://doi.org/10.1016/j.aquaculture.2011.06.047

26. Wu Y, Feng L, Jiang W *et al.* (2015) Influence of dietary zinc on muscle composition, flesh quality and muscle antioxidant status of young grass carp *(Ctenopharyngodon idellaVal.). Nutr Res* **46**, 2360-2373. https://doi.org/10.1111/are.12392

27. Jiang M, Wu F, Huang F *et al.* (2016) Effects of dietary Zn on growth performance, antioxidant responses, and sperm motility of adult blunt snout bream, *Megalobrama amblycephala*. *Aquaculture* **464**, 121-128. https://doi.org/10.1016/j.aquaculture.2016.06.025

28. Dekani L, Johari SA, Joo HS (2019) Comparative toxicity of organic, inorganic and

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

nanoparticulate zinc following dietary exposure to common carp (*Cyprinus carpio*). *Sci Total Environ* **656**, 1191-1198. https://doi.org/https://doi.org/10.1016/j.scitotenv.2018.11.474

29. Shiau S, Jiang L (2006) Dietary zinc requirements of grass shrimp, *Penaeus monodon*, and effects on immune responses. *Aquaculture* **254**, 476-482. https://doi.org/https://doi.org/10.1016/j.aquaculture.2005.10.033

30. Davis DA, Lawrence AL, Gatlin Iii DM (1993) Evaluation of the Dietary Zinc Requirement of *Penaeus vannamei* and Effects of Phytic Acid on Zinc and Phosphorus Bioavailability. *J World Aquacult Soc* **24**, 40-47. https://doi.org/10.1111/j.1749-7345.1993.tb00148.x

31. Zhang H (2015) Effects of Zn sources and dietary Zn levels on growth performance, biochemical and immunity indices and tissue Zn content for *Litopenaeus vannamei*. Master, Guangdong Ocean University.

32. Lin S, Lin X, Yang Y *et al.* (2013) Comparison of chelated zinc and zinc sulfate as zinc sources for growth and immune response of shrimp *(Litopenaeus vannamei)*. *Aquaculture* **406-407**, 79-84. https://doi.org/10.1016/j.aquaculture.2013.04.026

33. Sun P, Jin M, Jiao L *et al.* (2020) Effects of dietary lipid level on growth, fatty acid profiles, antioxidant capacity and expression of genes involved in lipid metabolism in juvenile swimming crab, *Portunus trituberculatus*. *Brit J Nutr* **123**, 149-160. https://doi.org/10.1017/S0007114519002563

34. Department FFaA (2011) *Mud crab aquaculture, Grow-out design options and*

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

construction.

35. Yuan Y, Jin M, Xiong J *et al.* (2019) Effects of dietary dosage forms of copper supplementation on growth, antioxidant capacity, innate immunity enzyme activities and gene expressions for juvenile *Litopenaeus vannamei*. *Fish Shellfish Immunol* **84**, 1059-1067. https://doi.org/https://doi.org/10.1016/j.fsi.2018.10.075

36. Sun S, Qin J, Yu N *et al.* (2013) Effect of dietary copper on the growth performance, non-specific immunity and resistance to Aeromonas hydrophila of juvenile Chinese mitten crab, *Eriocheir sinensis*. *Fish Shellfish Immunol* **34**, 1195-1201. https://doi.org/https://doi.org/10.1016/j.fsi.2013.01.021

37. Lin Z, Qiao J, Zhang Y *et al.* (2012) Cloning and characterisation of the SpToll gene from green mud crab, *Scylla paramamosain*. *Dev Comp Immunol* **37**, 164-175. https://doi.org/https://doi.org/10.1016/j.dci.2011.09.003

38. Guo T, Huang X, Su M *et al.* (2011) Effects of zinc supplementation in diet on the immunity，Vibrio-resistant ability，lysozyme mRNA and Toll receptor mRNA expressions in the white shrimp (*Litopenaeus vannamei*). *Journal of Fishery Sciences of China* **35**, 1081-2089.

39. Houng-Yung C, Yu-Chun C, Li-Chi H *et al.* (2014) Dietary zinc requirements of juvenile grouper, *Epinephelus malabaricus*. *Aquaculture* **432**, 360-364. https://doi.org/https://doi.org/10.1016/j.aquaculture.2014.05.020

40. Li MR, Huang CH (2016) Effect of dietary zinc level on growth, enzyme activity and body trace elements of hybrid tilapia, *Oreochromis niloticus* × *O. aureus*, fed soya

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

bean meal-based diets. *Aquacult Nutr* **22**, 1320-1327. https://doi.org/10.1111/anu.12332

41. Dall W, Moriarty DJ (1983) Functional aspects of nutrition and digestion. *The biology of Crustacea* **5**, 215-261.

42. Fang Y, Yang S, Wu G (2002) Free radicals, antioxidants, and nutrition. *Nutrition* **18**, 872-879. https://doi.org/https://doi.org/10.1016/S0899-9007(02)00916-4

43. Fattman CL, Schaefer LM, Oury TD (2003) Extracellular superoxide dismutase in biology and medicine. *Free Radical Bio Med* **35**, 236-256. https://doi.org/https://doi.org/10.1016/S0891-5849(03)00275-2

44. Tang Q, Feng L, Jiang W *et al.* (2013) Effects of dietary copper on growth, digestive, and brush border enzyme activities and antioxidant defense of hepatopancreas and intestine for young grass carp (*Ctenopharyngodon idella*). *Biol Trace Elem Res* **155**, 370-380. https://doi.org/https://doi.org/10.1007/s12011-013-9785-6

45. Nozik-Grayck E, Suliman HB, Piantadosi CA (2005) Extracellular superoxide dismutase. *Int J Biochem Cell B* **37**, 2466-2471. https://doi.org/https://doi.org/10.1016/j.biocel.2005.06.012

46. Feng L, Tan LN, Liu Y *et al.* (2011) Influence of dietary zinc on lipid peroxidation, protein oxidation and antioxidant defence of juvenile Jian carp (*Cyprinus carpio var. Jian*). *Aquacult Nutr* **17**, e875-e882. https://doi.org/10.1111/j.1365-2095.2011.00858.x

47. Rodríguez-Ariza A, Peinado J, Pueyo C *et al.* (1993) Biochemical indicators of oxidative stress in fish from polluted littoral areas. *Can J Fish Aquat Sci* **50**, 2568-2573.

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

https://doi.org/10.1139/f93-280

48. Huang F, Jiang M, Wen H *et al.* (2015) Dietary zinc requirement of adult Nile tilapia (*Oreochromis niloticus*) fed semi-purified diets, and effects on tissue mineral composition and antioxidant responses. *Aquaculture* **439**, 53-59. https://doi.org/10.1016/j.aquaculture.2015.01.018

49. Devasena T, Lalitha S, Padma K (2001) Lipid peroxidation, osmotic fragility and antioxidant status in children with acute post-streptococcal glomerulonephritis. *Clin Chim Acta* **308**, 155-161. https://doi.org/https://doi.org/10.1016/S0009-8981(01)00482-X

50. Bonaventura P, Benedetti G, Albarède F *et al.* (2015) Zinc and its role in immunity and inflammation. *Autoimmunity Reviews* **14**, 277-285. https://doi.org/https://doi.org/10.1016/j.autrev.2014.11.008

51. Murakami M, Hirano T (2008) Intracellular zinc homeostasis and zinc signaling. *Cancer Science* **99**, 1515-1522. https://doi.org/10.1111/j.1349-7006.2008.00854.x

52. Bagchi D, Carryl OR, Tran MX *et al.* (1997) Protection against chemically-induced oxidative gastrointestinal tissue injury in rats by bismuth salts. *Digest Dis Sci* **42**, 1890-1900. https://doi.org/10.1023/A:1018811210175

53. Bray TM, Bettger WJ (1990) The physiological role of zinc as an antioxidant. *Free Radical Bio Med* **8**, 281-291. https://doi.org/https://doi.org/10.1016/0891-5849(90)90076-U

54. Zago MP, Oteiza PI (2001) The antioxidant properties of zinc: interactions with iron and

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

antioxidants. *Free Radical Bio Med* **31**, 266-274. https://doi.org/https://doi.org/10.1016/S0891-5849(01)00583-4

55. Jiravanichpaisal P, Lee BL, Söderhäll K (2006) Cell-mediated immunity in arthropods: Hematopoiesis, coagulation, melanization and opsonization. *Immunobiology* **211**, 213-236. https://doi.org/https://doi.org/10.1016/j.imbio.2005.10.015

56. Wang J, Jiang K, Zhang FY *et al.* (2015) Characterization and expression analysis of the prophenoloxidase activating factor from the mud crab *Scylla paramamosain*. *Genet Mol Res* **14**, 8847-8860. https://doi.org/https://doi.org/10.4238/2015.August.3.8

57. Cao J, Miao X, Xu W *et al.* (2014) Dietary copper requirements of juvenile large yellow croaker *Larimichthys croceus*. *Aquaculture* **432**, 346-350. https://doi.org/https://doi.org/10.1016/j.aquaculture.2014.05.032

58. Cousins RJ (1985) Absorption, transport, and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin. *Physiol Rev* **65**, 238-309. https://doi.org/10.1152/physrev.1985.65.2.238

59. Coleman JE (1992) Structure and Mechanism of Alkaline Phosphatase. *Annual Review of Biophysics and Biomolecular Structure* **21**, 441-483. https://doi.org/10.1146/annurev.bb.21.060192.002301

60. do Carmo e Sá MVc, Pezzato LE, Ferreira Lima MMB *et al.* (2004) Optimum zinc supplementation level in Nile tilapia *Oreochromis niloticus* juveniles diets. *Aquaculture* **238**, 385-401. https://doi.org/10.1016/j.aquaculture.2004.06.011

61. Moazenzadeh K, Rajabi Islami H, Zamini A *et al.* (2018) Effects of dietary zinc level on

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

performance, zinc status, tissue composition and enzyme activities of juvenile *Siberian sturgeon, Acipenser baerii (Brandt 1869)*. *Aquacult Nutr* **24**, 1330-1339. https://doi.org/10.1111/anu.12670

62. Li F, Xiang J (2013) Signaling pathways regulating innate immune responses in shrimp. *Fish Shellfish Immunol* **34**, 973-980. https://doi.org/https://doi.org/10.1016/j.fsi.2012.08.023

63. Blander JM, Medzhitov R (2004) Regulation of phagosome maturation by signals from Toll-like receptors. *Science* **304**, 1014-1018. https://doi.org/10.1126/science.1096158

64. Kumar N, Krishnani KK, Kumar P *et al.* (2017) Dietary zinc promotes immuno-biochemical plasticity and protects fish against multiple stresses. *Fish Shellfish Immunol* **62**, 184-194. https://doi.org/https://doi.org/10.1016/j.fsi.2017.01.017

65. Gharekhani A, Azari Takami G, Tukmechi A *et al.* (2015) Effect of dietary supplementation with zinc enriched yeast (*Saccharomyces cerevisiae*) on immunity of rainbow trout (*Oncorhynchus mykiss*). *Iran J Vet Res* **16**, 278-282.

66. Song Z-X, Jiang W-D, Liu Y *et al.* (2017) Dietary zinc deficiency reduced growth performance, intestinal immune and physical barrier functions related to NF-κB, TOR, Nrf2, JNK and MLCK signaling pathway of young grass carp (*Ctenopharyngodon idella*). *Fish Shellfish Immunol* **66**, 497-523. https://doi.org/https://doi.org/10.1016/j.fsi.2017.05.048

67. Auman JT, Boorman GA, Wilson RE *et al.* (2007) Heat map visualization of high-density clinical chemistry data. *Physiol Genomics* **31**, 352-356. https://doi.org/10.1152/physiolgenomics.00276.2006

68. Pleil JD, Stiegel MA, Madden MC *et al.* (2011) Heat map visualization of complex environmental and biomarker measurements. *Chemosphere* **84**, 716-723. https://doi.org/10.1016/j.chemosphere.2011.03.017

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

Table 1. Formulation and proximate composition of experimental diets (g·kg^{-1} dry matter).

Ingredients (g·kg^{-1})	Dietary zinc levels (mg·kg^{-1})					
	44.5	56.9	68.5	97.3	155.6	254.7
Peru fish meal*	220.00	220.00	220.00	220.00	220.00	220.00
Casein*	60.00	60.00	60.00	60.00	60.00	60.00
Soybean meal*	287.00	287.00	287.00	287.00	287.00	287.00
Krill meal*	80.00	80.00	80.00	80.00	80.00	80.00
Wheat flour*	240.00	240.00	240.00	240.00	240.00	240.00
Fish oil*	12.00	12.00	12.00	12.00	12.00	12.00
Soybean lecithin*	40.00	40.00	40.00	40.00	40.00	40.00
Vitamin premix†	10.00	10.00	10.00	10.00	10.00	10.00
Mineral premix (zinc-free)‡	15.00	15.00	15.00	15.00	15.00	15.00
$ZnSO_4·7H_20$ (mg·kg^{-1})§	0.00	65.96	131.93	263.85	527.70	1055.41
$Ca(H_2PO_4)_2$§	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*	14.00	14.00	14.00	14.00	14.00	14.00
Proximate composition						
Crude protein	451.64	454.98	453.73	452.61	454.93	451.76
Crude lipid	75.00	74.10	76.40	75.90	74.80	75.20
Moisture	96.00	108.50	104.00	106.40	106.10	96.20
Ash	104.30	104.80	102.10	100.90	103.50	106.40
Analyzed zinc (mg·kg^{-1})	44.50	56.90	68.50	97.30	155.60	254.70

*Ingredients were bought from Ningbo Tech-Bank Corp., Ningbo, China.

†Vitamin premix were based on Sun *et al.* [33]

‡Mineral premix (per kg mineral premix): $FeC_6H_5O_7$, 4.57 g; $CuSO_4·5H_2O$(99%), 6.61 g; $MnSO_4·H_2O$ (99%), 4.14 g; $MgSO_4·7H_2O$ (99%), 238.97 g; KH_2PO_4, 233.2 g; NaH_2PO_4, 137.03 g; $C_6H_{10}CaO_6·5H_2O$ (98%), 34.09 g; $CoCl_2·6H_2O$ (99%), 1.36 g; K_2O_3Se, 0.0044 g; KIO_3, 0.0013 g。

§Ingredients were provided by Sinopharm Chemical Reagent Co., Ltd (Shanghai, China)

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

Table 2. Primers for real-time quantitative PCR of mud crab

Gene	Nucleotide sequence (5' – 3')	Size (bp)	GenBank NO
Cu/Zn sod	F: ATCACCCCAACCTCAACAA	209	FJ774661
	R: ATCATCCACAACTCCCCAC		
mitMn sod	F: TGCACATCTGACCAGCCTTA	188	JX133232.1
	R: GCTGGTAAGTTACTGCTGGC		
Gpx	F: AAGTTTGGTGACAATCTCG	139	JN565286.1
	R: ACATCTCCATCTTGGGCTC		
cat	F: ACAACACTCCCATCTTCTT	132	FJ774660.1
	R: GGACGCAGGGTGATAAAAT		
trx	F: AGGAAGACTTCAGGAACCGG	246	JQ863320.1
	R: CGAACTTGTCCACCACCTTG		
proPO	F:GCTCATCGGGAGAACCTT	196	KP710954
	R: TCTTCTGACCCTGGCTCTC		
clr	F: TGAGAAGGAGGCAGAGGGA	116	KC902764.1
	R: GATGTTCGGGCAGCGTATT		
toll1	F:CCTCCACCACTGTCTTCT	232	JQ327142.1
	R: TACTTAGGCTCTCCGCTC		
toll2	F:GTGAGAAGACCAGTCAGAAT	190	LT835105.1
	R: AGAGCACACCCAAGAAAC		
ef1a	F: CTACAAGATTGGCGGCAT	108	JQ824130.1
	R: GGGGGCAAAGTTCACGAC		

Cu/Zn sod, copper/zinc superoxide dismutase; *mitMn sod*, mitochondrial manganese superoxide dismutase; *gpx*, glutathione peroxidase; *cat*, catalase; *trx*, thioredoxin; *proPO*, prophrnoloxodase; *clr*, C-type lectin receptor; *toll1*, toll-like receptor 1; *toll2*, toll-like receptor 2; *ef1a*, elongation factor 1a.

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

Table 3. Survival, growth performance and morphology index of mud crab fed with different dietary zinc levels.

Parameters	Dietary Zn levels (mg·kg^{-1})											
	44.5		56.9		68.5		97.3		155.6		254.7	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Survival (%)	76.11	5.80	86.11	2.78	75.00	8.33	83.33	9.62	86.11	2.78	72.22	5.55
IW (g)	14.97	2.35	14.83	0.78	14.66	1.58	14.84	0.54	14.73	1.08	15.78	2.07
PWG (%)	142.61^{ab}	7.68	145.54^{ab}	17.01	167.58^{b}	2.00	168.39^{b}	1.71	129.31^{ab}	8.10	109.55^{a}	5.54
HSI (%)	5.96	0.28	6.49	0.16	6.20	0.32	6.26	0.75	6.66	0..37	6.36	0.39

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.

IW, initial weight; PWG, percent weight gain; HSI, hepatopancreas index.

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

Table 4. Haemolymph characteristics of mud crab fed with different dietary zinc levels.

Parameters	Dietary Zn levels (mg·kg^{-1})											
	44.5		56.9		68.5		97.3		155.6		254.7	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
ALT (U/L)	147.87	5.85	178.82	35.28	147.43	8.18	137.78	21.62	178.32	13.32	169.46	18.55
AST (U/L)	231.22	10.45	215.02	31.01	231.75	22.32	210.79	10.20	236.26	7.7	213.27	11.60
GGT (U/L)	8.77	0.34	8.12	0.34	8.08	0.21	8.45	0.55	8.25	0.04	8.24	0.58
TP (g/L)	63.36	3.97	61.83	3.82	56.50	5.78	61.24	5.46	59.18	3.99	55.97	5.98
ALB (g/L)	8.64	1.07	9.15	1.01	7.85	0.58	6.42	0.82	7.67	0.42	7.52	0.50

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.

ALT, alanine aminotransferase. AST, aspartate aminotransferase. GGT, gamma-glutamyl transpeptidase. TP, total protein. ALB, albumin.

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

Figure legends

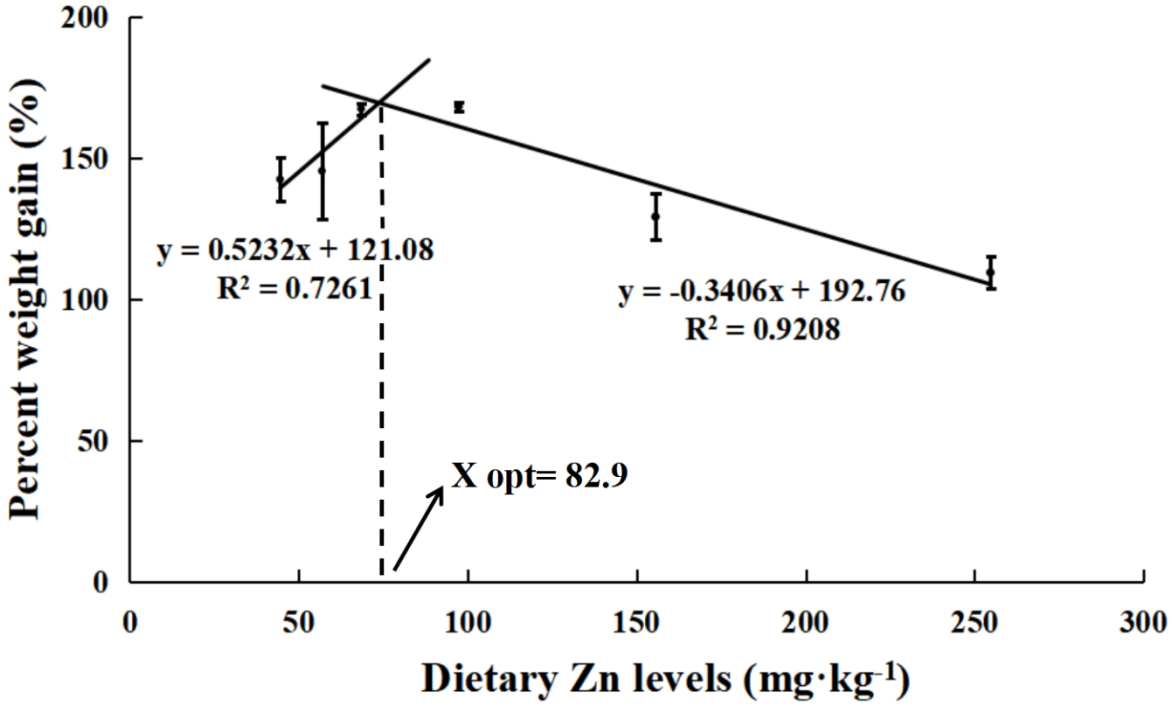

Fig. 1. Relationship between the percent weight gain (PWG) and dietary Zn level based on two slope broken-line regression analysis, where Xopt represents the optimal dietary Zn level for maximum PWG.

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

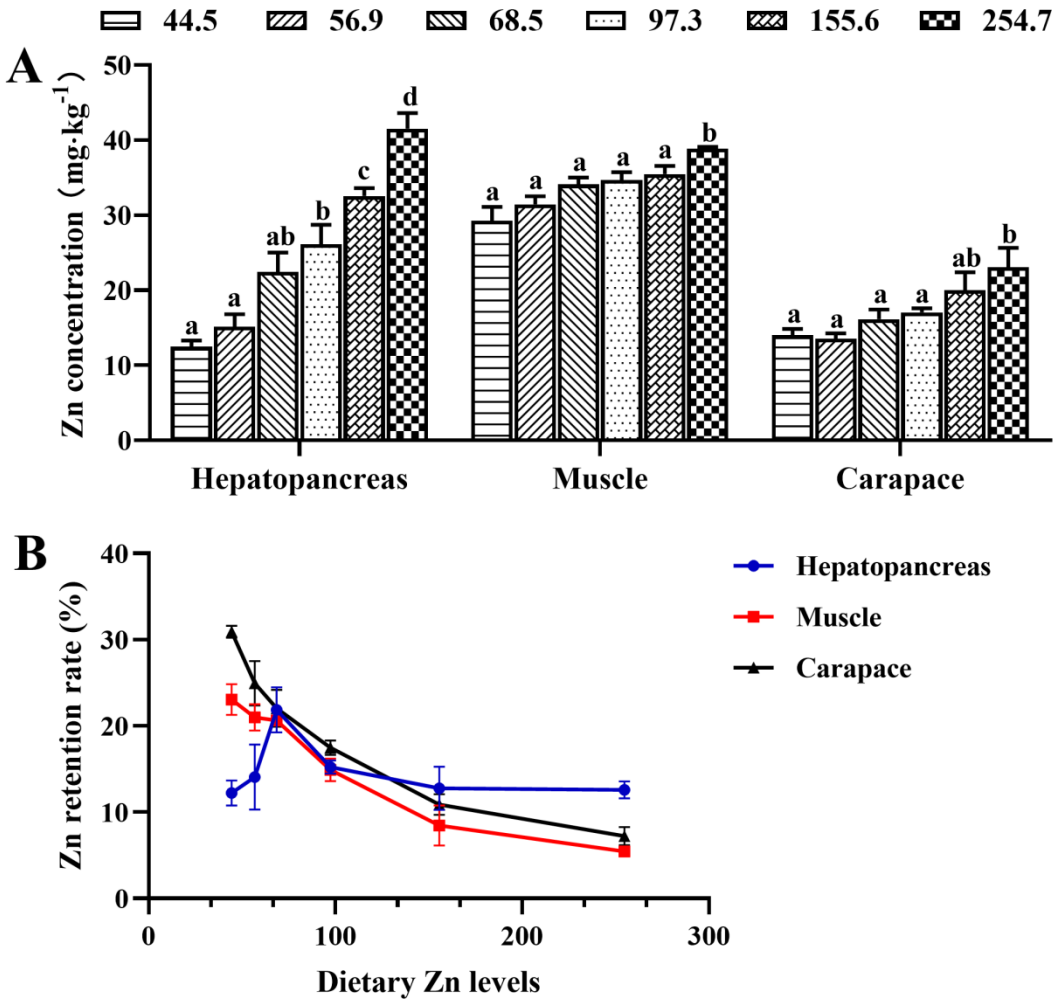

Fig. 2. Zn concentration and retention rate in hepatopancreas, muscle and carapace of juvenile mud crab fed diets containing different Zn levels. Values are means (n = 3), with standard errors represented by vertical bars. Mean values for the same tissue with different superscript letters were significantly different as determined by ANOVA and Tukey's test ($P < 0.05$).

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

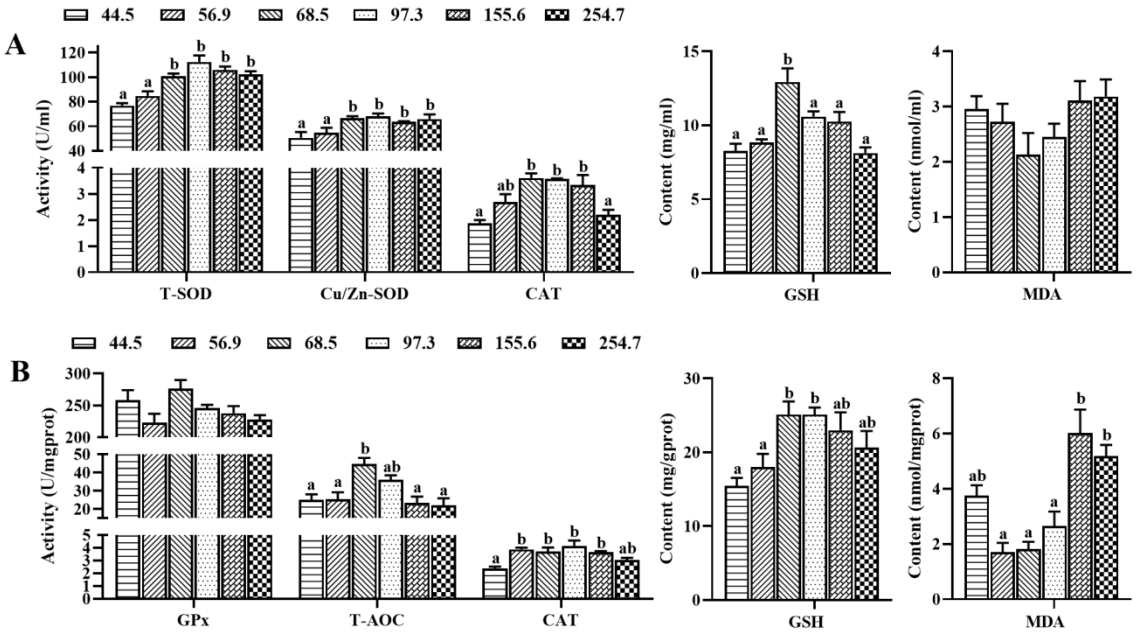

Fig. 3. Antioxidant enzyme activities and antioxidant contents in haemolymph (A) and hepatopancreas (B) of juvenile mud crab fed diets containing different Zn levels. Values are means (n = 3), with standard errors represented by vertical bars. Mean values for the same tissue with different superscript letters were significantly different as determined by ANOVA and Tukey's test ($P < 0.05$). Cu/Zn-SOD, copper/zinc superoxide dismutase; T-SOD, total superoxide dismutase; CAT, catalase; GSH, glutathione; MDA, malondialdehyde; GPx, glutathione peroxidase; T-AOC, total anti-oxidation.

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

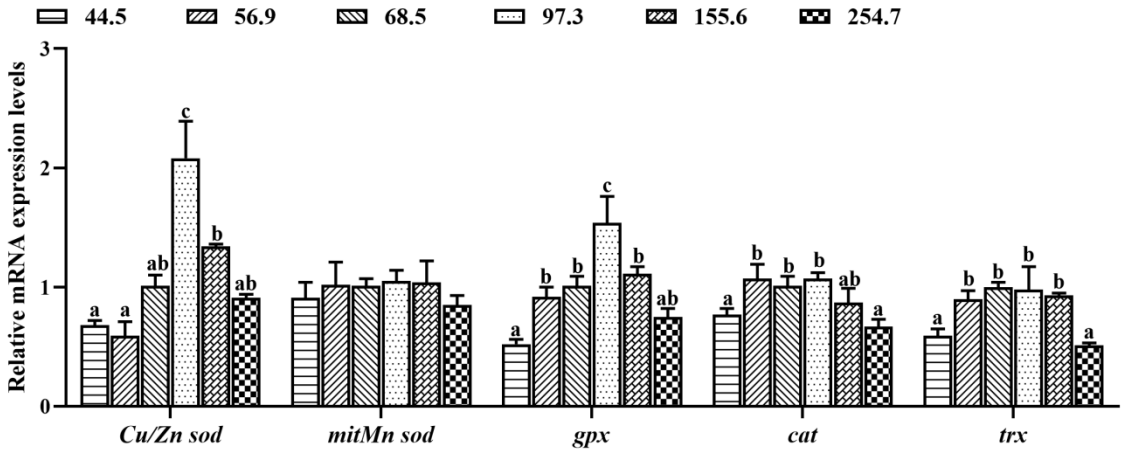

Fig. 4. Effects of dietary Zn level on relative expression of genes involved in oxidation resistance in hepatopancreas of juvenile mud crab. Values are means (n = 3), with standard errors represented by vertical bars. Mean values for the same tissue with different superscript letters were significantly different as determined by ANOVA and Tukey's test ($P < 0.05$). *Cu/Zn sod*, copper/zinc superoxide dismutase; *mitMn sod*, mitochondrial manganese superoxide dismutase; *gpx*, glutathione peroxidase; *cat*, catalase; *trx*, thioredoxin.

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

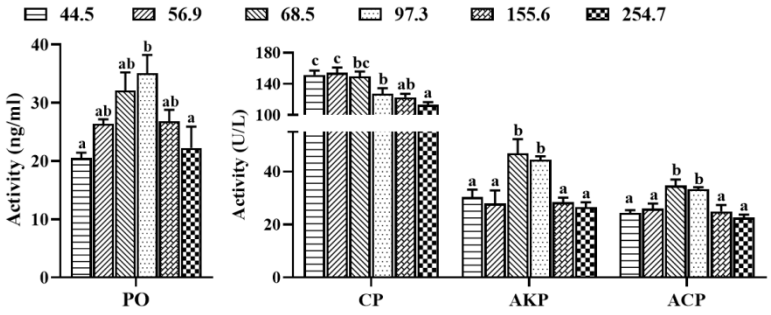

Fig. 5. Activities of innate immunity enzymes in haemolymph of juvenile mud crab fed diets containing different Zn levels. Values are means (n = 3), with standard errors represented by vertical bars. Mean values for the same tissue with different superscript letters were significantly different as determined by ANOVA and Tukey's test ($P < 0.05$).PO, phenoloxidase; CP, ceruloplasmin; AKP, alkaline phosphatase; ACP, acid phosphatase.

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

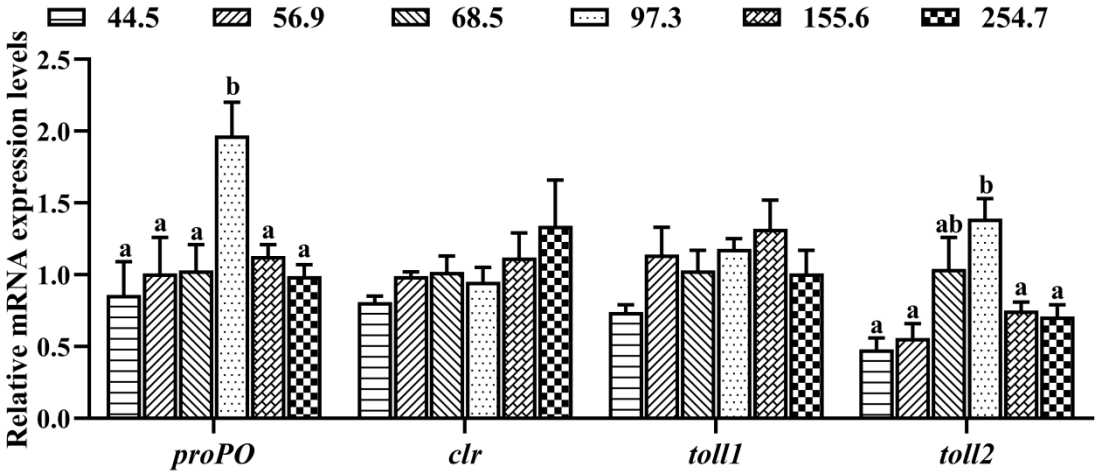

Fig. 6. Effects of dietary Zn level on relative expression of genes involved in innate immunity in hepatopancreas of juvenile mud crab. Values are means (n = 3), with standard errors represented by vertical bars. Mean values for the same tissue with different superscript letters were significantly different as determined by ANOVA and Tukey's test ($P < 0.05$). *proPO,* Prophenoloxidase*; clr,* C-type lectin receptor. *toll1*, toll-like receptor 1*; toll2*, toll-like receptor 2.

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531

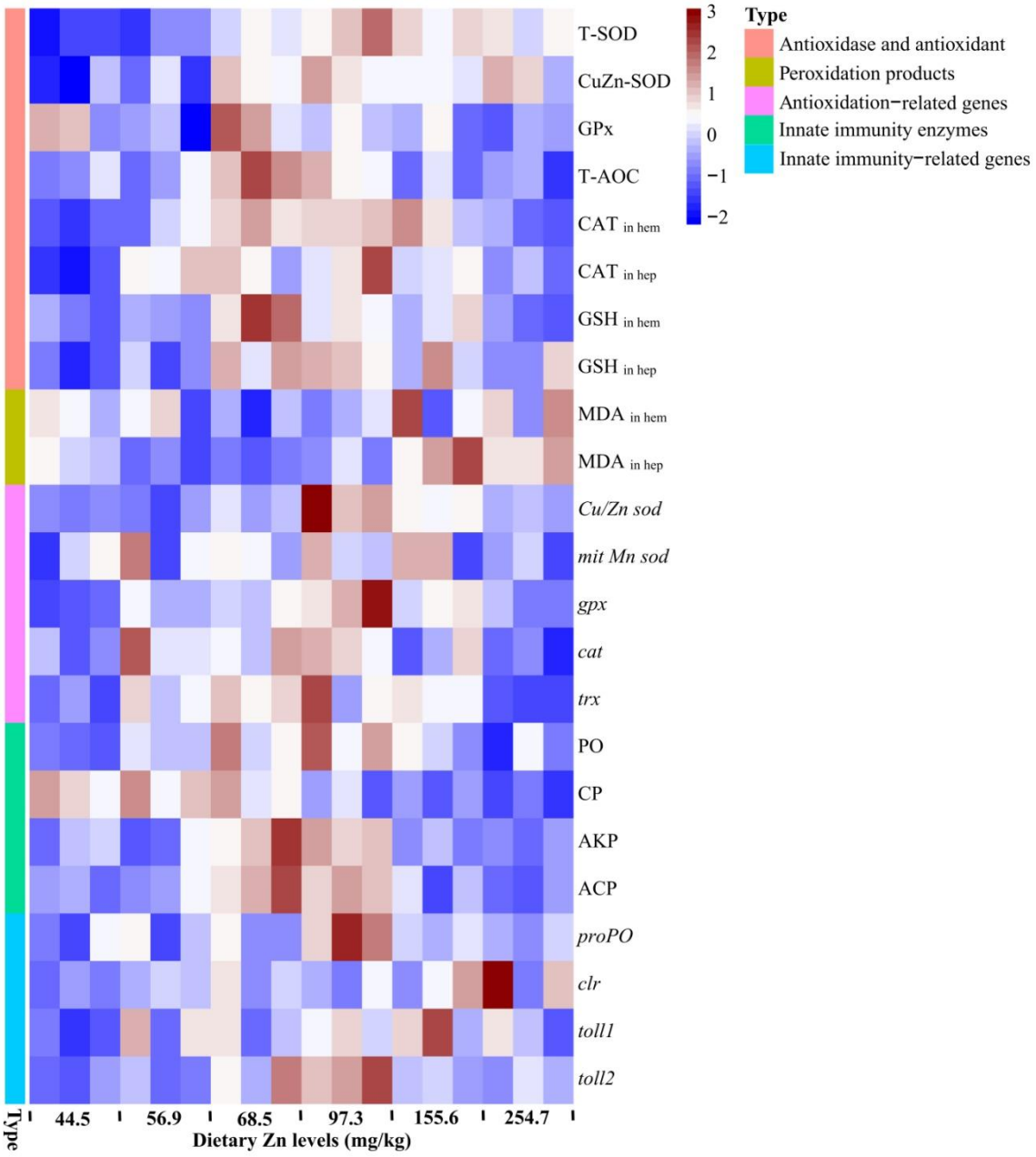

Fig. 7. Heat map visualization of oxidation resistance and innate immunity parameters in haemolymph and hepatopancreas of mud crab fed different dietary Zn levels. Before analysis, all data were checked for homogeneity. The color box for each compound in the heatmap indicates the abundance of the compound and represents the fold-change according to the scale on the right: red for higher values; blue for lower values.

Downloaded from https://www.cambridge.org/core. IP address: 86.134.108.12, on 12 May 2020 at 08:53:19, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1017/S0007114520001531