British Journal of Nutrition (2020), 124, 681–692 doi:10.1017/S0007114520001531

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

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(Submitted 11 February 2020 – Final revision received 21 April 2020 – Accepted 24 April 2020 – First published online 4 May 2020)

Abstract

The aim of the present study was to investigate the effects of dietary Zn level on growth performance, Zn bioaccumulation, antioxidant capacity and innate immunity in juvenile mud crabs (*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/kg. Dietary Zn level significantly influenced percentage weight gain (PWG), with the highest observed in crabs fed the diet containing 97·3 mg/kg Zn. Tissue Zn concentrations significantly increased as dietary Zn levels increased from 44·5 to 254·7 mg/kg. Retention of Zn in hepatopancreas increased with dietary Zn levels up to 68·5 mg/kg and then significantly decreased. Moreover, inadequate dietary Zn (44·5 and 56·9 mg/kg) reduced antioxidation markers including total superoxide dismutase (SOD) and Cu/Zn SOD activities and total antioxidant level. Crabs fed the diet with 44·5 mg/kg 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/kg Zn. The highest activities of phenoloxidase and alkaline phosphatase were recorded in crabs fed the diets containing 68·5 and 97·3 mg/kg Zn. Expression levels of prophenoloxidase and toll-like receptor 2 were higher in crabs fed the 97·3 mg/kg Zn diet compared with crabs fed the other diets. Based on PWG alone, the optimal dietary Zn level was estimated to be 82·9 mg/kg, with 68·5 to 97·3 mg/kg recommended for maintaining optimal Zn bioaccumulation, oxidation resistance and innate immune response of juvenile mud crabs.

Key words: **Zinc: Mud crabs: *Scylla paramamosain*: Growth performance: Oxidation resistance: Innate immunity**

The 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 flavour and nutritional quality, it is increasingly favoured 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 are 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 and improve immune function and muscle quality of aquatic organisms[13]. Zn is a fundamental microelement that can stabilise cellular membranes and components in organs and tissues[14]. Specifically, it is an essential cofactor for numerous

Abbreviations: AKP, alkaline phosphatase; CAT, catalase; CP, ceruloplasmin; GPx, glutathione peroxidase; MDA, malondialdehyde; PO, phenoloxidase; PWG, percentage weight gain; SOD, superoxide dismutase; ZRR, Zn retention rate.

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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 defence and immune response partly as an integral constituent of Zn-dependent metalloenzymes such as Cu/Zn 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 Se, Cu and Mg and several other metalloenzymes[20]. Therefore, there is an urgent need to understand the nutrition and metabolism of Zn in mud crab.

Although 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, 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 the 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-sulphate) on growth performance and immune function of Pacific white shrimp, showing that Zn-methionine significantly improved survival, percentage weight gain (PWG) and immune-related enzyme activities. Although these studies indicated dietary optimal Zn is vital to crustaceans, comprehensive assessment of dietary Zn level is still relatively limited in crustaceans, especially marine crabs. Therefore, an 8-week nutritional trial was designed to investigate the optimal Zn requirement level for juvenile mud crabs and to evaluate the effects of dietary Zn on growth, tissue Zn bioaccumulation, haemolymph characteristics, antioxidant ability and 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 crabs.

Methods

Ethics statement

The study was performed in strict accordance with the Standard Operating Procedures 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.

Diet preparation

Six isonitrogenous (45 % crude protein) and isolipidic (7·5 % crude lipid) experimental diets were formulated to contain different levels of Zn ($ZnSO_4.7H_2O$ as Zn source), with the analysed Zn concentrations being 44·5, 56·9, 68·5, 97·3, 155·6 and 254·7 mg/kg. Peruvian fishmeal, soyabean meal, krill meal and casein were the main protein sources, with fish oil and soyabean lecithin as the main lipid sources (Table 1). Feeds were manufactured according to the method described in detail previously[33]. Briefly, all dry ingredients were ground

Table 1. Formulation and proximate composition of experimental diets (g/kg DM)

	Dietary Zn levels (mg/kg)					
Ingredients (g/kg)	44·5	56·9	68·5	97·3	155·6	254·7
Peru fishmeal*	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
Soyabean 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
Soyabean 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 (Zn-free)‡	15·00	15·00	15·00	15·00	15·00	15·00
$ZnSO_4.7H_20$ (mg/kg)§	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
Analysed Zn (mg/kg)	44·50	56·90	68·50	97·30	155·60	254·70

* Ingredients were bought from Ningbo Tech-Bank Corp.
† Vitamin premix was 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.

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 was 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 were cut into two uniform sizes (3 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.

Crab rearing and experimental conditions

Juvenile mud crabs were obtained from the breeding base of Ningbo Ocean and Fishery Science and Technology Innovation Center. Prior to the experiment, crabs were acclimated for 2 weeks and fed with a commercial diet (45 % crude protein, 8 % crude lipid and 72 mg/kg Zn; Evergreen Corp.). At the beginning of the experiment, a total of 270 healthy, normally active and similar size juvenile crabs (14·96 (SE 1·11) g, in the ninth molting stage) were randomly allocated into 270 single crab units (33 cm × 22·5 cm × 25 cm), to prevent competition and cannibalism. Each feed was randomly assigned to three experimental replicates with fifteen mud crabs in each replicate. Each single crab unit was mutually independent and supplied with continuous flow-through water[34]. Crabs were fed the allocated experimental diet once daily at 18.00 hours (3–6 % of crab weight), and the daily ration adjusted according to the actual intake (ration – uneaten feed) to ensure feeding to apparent satiation. Faeces 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 24·5–29·0°C, salinity approximately 24·1–28·4 g/l, pH 7·3–8·0, ammonia nitrogen was lower than 0·05 mg/l, dissolved O_2 was not lower than 6·0 mg/l and *Vibrio* concentration in seawater was $<1{\cdot}0 \times 10^5$ colony-forming units/ml.

Sample collection

At the end of the experiment, most crabs were at molting stage 11 and a few were at stage 12. Crabs were anaesthetised with 0·02 % tricaine methane sulphonate (MS-222), and all crabs in each replicate were counted and weighed to determine the survival and PWG. In each replicate, haemolymph was sampled from six crabs and centrifuged at 3500 rpm for 10 min at 4°C (Eppendorf centrifuge 5810 R). The supernatant was collected and stored at –80°C before analyses of biochemical and enzyme activities. Hepatopancreas from the same six crabs was quickly dissected and weighed to calculate hepatosomatic index, and placed in 1·5 ml centrifuge tubes, frozen in liquid N_2 and stored at –80°C prior to the analysis of enzyme activities and gene expression. Muscle, carapace and hepatopancreas from further three crabs were collected and stored at –20°C to determine Zn concentrations and tissue Zn retention rate (ZRR).

Calculations

$$\text{Survival (\%)} = 100 \times ((\text{final number of crabs})/(\text{initial number of crabs}))$$

$$\text{PWG (\%)} = 100 \times ((\text{final body weight} - \text{initial body weight})/\text{initial body weight})$$

$$\text{Hepatosomatic index (\%)} = 100 \times (\text{hepatopancreas weight}/\text{body weight})$$

$$\text{ZRR (\%)} = 100 \times (W_t \times Z_t - W_i \times Z_i)/(W_d \times Z_d)$$

where W_t is the final tissue weight (g), W_i is the initial tissue weight (g), Z_t is the final Zn concentration (mg/kg), Z_i is the initial Zn concentration (mg/kg), W_d is the weight of fed diet (g) and Z_d is the Zn concentration of the diet (mg/kg). 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°C, with the acid solution added dropwise until complete digestion of organic matter. The digested solution was filtered through an aqueous phase syringe filter (SCAA-102, ANPEL Laboratory Technologies Inc.) before tissue Zn concentrations were determined by inductively coupled plasma optical emission spectrometry (PE2100DV, Perkin Elmer).

Haemolymph biochemical analysis

The activities of alanine aminotransferase, aspartate aminotransferase and γ-glutamyl transpeptidase, and the contents of total protein and albumin were analysed in haemolymph supernatant using an automatic biochemistry analyzer (VITALAB SELECTRA Junior Pros), with reagent kits from Biosino Bio-Technology and Science Inc.

Enzyme activity analysis

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

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 R: ATCATCCACAACTCCCCAC	209	FJ774661
mitMn sod	F: TGCACATCTGACCAGCCTTA R: GCTGGTAAGTTACTGCTGGC	188	JX133232.1
Gpx	F: AAGTTTGGTGACAATCTCG R: ACATCTCCATCTTGGGCTC	139	JN565286.1
cat	F: ACAACACTCCCATCTTCTT R: GGACGCAGGGTGATAAAAT	132	FJ774660.1
trx	F: AGGAAGACTTCAGGAACCGG R: CGAACTTGTCCACCACCTTG	246	JQ863320.1
proPO	F: GCTCATCGGGAGAACCTT R: TCTTCTGACCCTGGCTCTC	196	KP710954
clr	F: TGAGAAGGAGGCAGAGGGA R: GATGTTCGGGCAGCGTATT	116	KC902764.1
toll1	F: CCTCCACCACTGTCTTCT R: TACTTAGGCTCTCCGCTC	232	JQ327142.1
toll2	F: GTGAGAAGACCAGTCAGAAT R: AGAGCACACCCAAGAAAC	190	LT835105.1
ef1a	F: CTACAAGATTGGCGGCAT R: GGGGGCAAAGTTCACGAC	108	JQ824130.1

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

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 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), respectively. RNA samples were reverse-converted to complementary DNA (cDNA) using a Prime Script® RT reagent kit (TaKaRa) according to the manufacturer's protocol. Elongation factor 1α (*ef1a*) was chosen as the reference gene (housekeeping gene) after confirmation of its expression stability. Specific primers used for real-time quantitative PCR were designed according to complete cDNA sequences of corresponding genes in the National Center for Biotechnology Information (NCBI) using Primer Premier 5.0 software (Table 2). PCR amplification was conducted by a quantitative thermal cycler (Lightcycler 96, Roche), with reactions containing 2 µl of cDNA, 1·0 µl of each primer, 10 µl of 2× conc SYBR Green I Master (Roche) and 6 µl diethyl pyrocarbonate (DEPC) water. The procedure of quantitative PCR contained an initial activation step at 95°C for 2 min, followed by forty-five 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/kg treatment group. Amplification efficiency was measured as: $E = 10^{(-1/\text{Slope})} - 1$, 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/kg treatment group was used as the control/reference group.

Statistical analysis

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

Data are presented as means and standard errors of three replicates (n 3) and analysed by one-way ANOVA followed by Tukey's multiple-range test. All statistical analyses were conducted using SPSS 16.0 for Windows.

Results

Survival and growth performance

The effects of dietary Zn 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 ($P > 0{\cdot}05$), although numerically lowest survival was observed in crabs fed the diet containing 254·7 mg/kg Zn. Crabs fed the diet containing 254·7 mg/kg Zn had lower PWG than those fed the 68·5 and 97·3 mg/kg Zn diets, and the highest PWG was observed in crabs fed the diet containing 97·3 mg/kg Zn ($P < 0{\cdot}05$). Broken line regression analysis of PWG and dietary Zn level showed $y = 0{\cdot}5232x + 121{\cdot}08$ ($R^2 = 0{\cdot}7261$) and $y = -0{\cdot}3406x + 192{\cdot}76$ ($R^2 = 0{\cdot}9208$), respectively, and the optimum dietary Zn level was estimated to be 82·9 mg/kg for juvenile mud crabs (Fig. 1). Hepatosomatic index (HSI) did not show any statistical differences among dietary treatments ($P > 0{\cdot}05$).

Tissue zinc concentration and retention rate

Zn concentrations in hepatopancreas, muscle and carapace significantly increased as dietary Zn level increased from

British Journal of Nutrition

Table 3. Survival, growth performance and morphology index of mud crabs fed with different dietary zinc levels (Mean values with their standard errors (*n* 3))

	Dietary Zn levels (mg/kg)											
	44·5		56·9		68·5		97·3		155·6		254·7	
Parameters	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
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^{a,b}	7·68	145·54^{a,b}	17·01	167·58^{b}	2·00	168·39^{b}	1·71	129·31^{a,b}	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

IW, initial weight; PWG, percentage weight gain; HSI, hepatopancreas index.
^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$) as determined by ANOVA and Tukey's test.

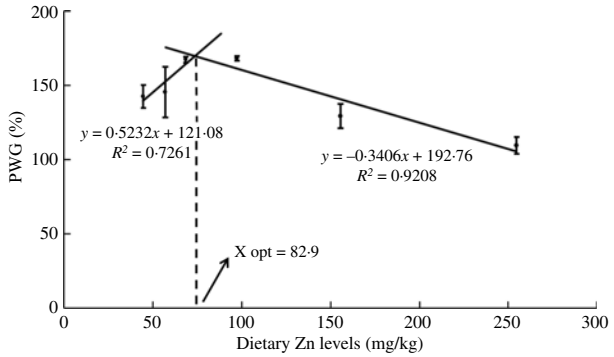

Fig. 1. Relationship between the percentage weight gain (PWG) and dietary zinc level based on two slope broken-line regression analysis, where X opt represents the optimal dietary zinc level for maximum PWG.

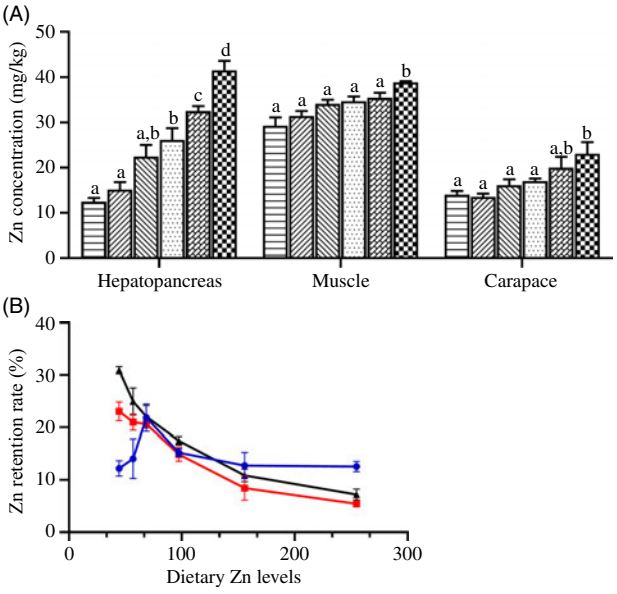

Fig. 2. Zinc concentration (A) and retention rate (B) in hepatopancreas, muscle and carapace of juvenile mud crabs fed diets containing different zinc levels. Values are means (*n* 3), with standard errors represented by vertical bars. ^{a,b,c,d} Mean values for the same tissue with unlike superscript letters were significantly different as determined by ANOVA and Tukey's test ($P < 0·05$). (A) ▭, 44·5; ▨, 56·9; ▧, 68·5; ▭, 97·3; ▨, 155·6; ▩, 254·7; (B) —●—, hepatopancreas; —■—, muscle; —▲—, carapace.

44·5 to 254·7 mg/kg, with highest tissue Zn concentrations observed in crabs fed the diet containing 254·7 mg/kg Zn (Fig. 2(A)) ($P < 0·05$).

The 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 reaching a peak value in crabs fed the diet containing 68·5 mg/kg Zn and then decreased as dietary Zn level increased from 68·5 to 254·7 mg/kg. 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 crabs fed the diet containing 254·7 mg/kg Zn.

Haemolymph biochemical analysis

Biochemical parameters of haemolymph of crabs fed the experimental diets are presented in Table 4. alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transpeptidase, total protein and albumin in haemolymph supernatant were not significantly influenced by dietary Zn level ($P > 0·05$).

Antioxidant enzyme activities and gene expression in haemolymph and hepatopancreas

The values of antioxidant status parameters in haemolymph and hepatopancreas are shown in Fig. 3. Crabs fed the diets containing 44·5 and 56·9 mg/kg Zn exhibited significantly lower total SOD and Cu/Zn-SOD activities in haemolymph than those fed the other diets ($P < 0·05$). Compared with crabs fed the 44·5 and 254·7 mg/kg Zn diets, higher CAT activity and GSH content in haemolymph were observed in crabs fed the diet containing 68·5 mg/kg 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 Zn ($P < 0·05$). Hepatopancreas of crabs fed the diets with 155·6 and 254·7 mg/kg Zn had significantly higher MDA concentrations than those fed the diets with 56·9 and 97·3 mg/kg Zn ($P < 0·05$).

The expression levels in hepatopancreas of antioxidant 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 Zn ($P < 0·05$), while the expression level of *mitMn*

Table 4. Haemolymph characteristics of mud crabs fed with different dietary zinc levels (Mean values with their standard errors (*n* 3))

	Dietary Zn levels (mg/kg)											
	44·5		56·9		68·5		97·3		155·6		254·7	
Parameters	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
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	7·77	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

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

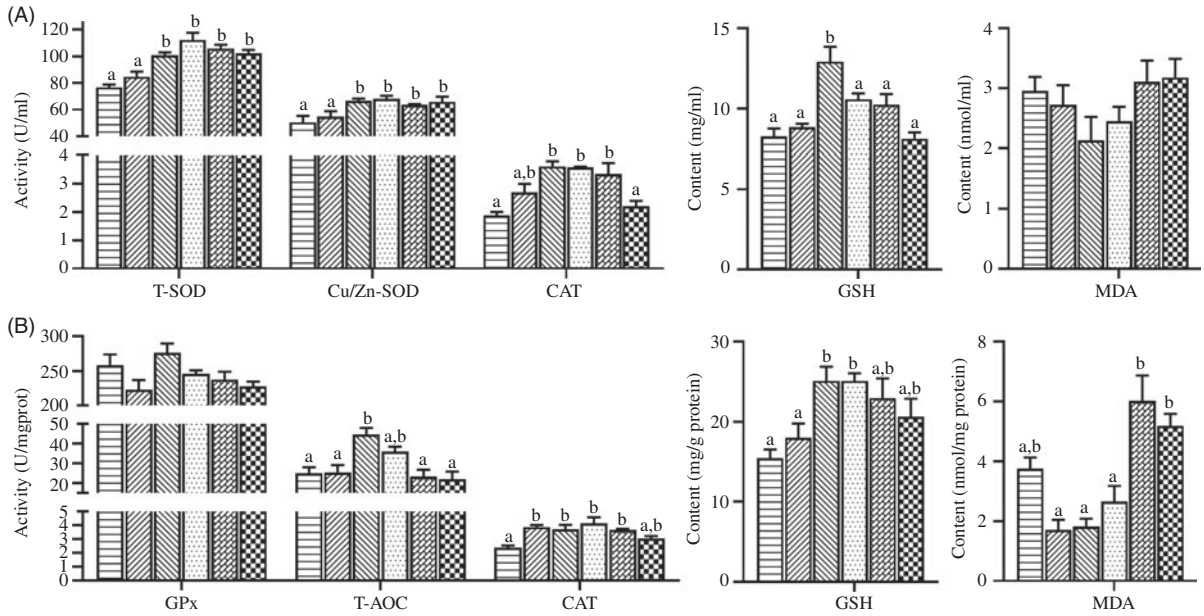

Fig. 3. Antioxidant enzyme activities and antioxidant contents in haemolymph (A) and hepatopancreas (B) of juvenile mud crabs fed diets containing different zinc levels. Values are means (*n* 3), with standard errors represented by vertical bars. [a,b] Mean values for the same tissue with unlike superscript 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; MDA, malondialdehyde; GPx, glutathione peroxidase; T-AOC, total antioxidation capacity. ▭, 44·5; ▨, 56·9; ▧, 68·5; ▭, 97·3; ▨, 155·6; ▩, 254·7.

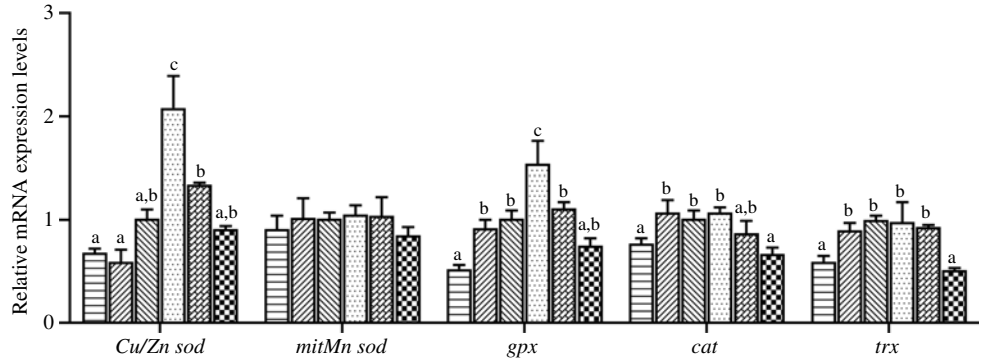

Fig. 4. Effects of dietary zinc level on relative expression of genes involved in oxidation resistance in hepatopancreas of juvenile mud crabs. Values are means (*n* 3), with standard errors represented by vertical bars. [a,b,c] Mean values for the same tissue with unlike superscript letters were significantly 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. ▭, 44·5; ▨, 56·9; ▧, 68·5; ▭, 97·3; ▨, 155·6; ▩, 254·7.

sod was not affected by dietary Zn level ($P > 0{\cdot}05$). The expression level of *gpx* in hepatopancreas was significantly higher in crabs fed the diet with 97·3 mg/kg Zn than in crabs fed the other diets ($P < 0{\cdot}05$). Moreover, crabs fed the 44·5 and 254·7 mg/kg Zn diets had lower relative expression levels of *cat* and *trx* than those fed the 56·9 and 97·3 mg/kg Zn diets ($P < 0{\cdot}05$).

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 and then significantly decreased with further increase of dietary Zn level ($P < 0{\cdot}05$). Crabs fed diets containing 97·3–254·7 mg/kg Zn had significantly lower CP activity compared with crabs fed diets containing 44·5–68·5 mg/kg Zn, with lowest CP activity observed in crabs fed the 254·7 mg/kg Zn diet ($P < 0{\cdot}05$). The activities of AKP and acid phosphatase in haemolymph were significantly higher in crabs fed the diets containing 68·5 and 97·3 mg/kg Zn compared with activities in crabs fed the other diets ($P < 0{\cdot}05$).

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 Zn diet compared with crabs fed the other diets ($P < 0{\cdot}05$). Dietary Zn level had no significant effect on the mRNA expression levels of *clr* and *toll1* in hepatopancreas ($P > 0{\cdot}05$).

Heat map visualisation analyses of the antioxidative and innate immune parameters

Heat map visualisation was conducted to present the macroscopic effects of dietary Zn level on oxidation resistance and innate immunity parameters of mud crabs (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 Zn. The lowest values of peroxidation products were found in crabs fed the diets with 68·5 and 97·3 mg/kg Zn.

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].

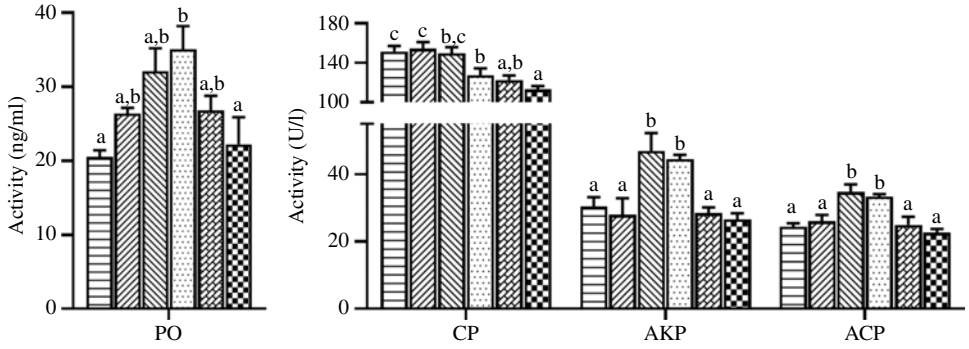

Fig. 5. Activities of innate immunity enzymes in haemolymph of juvenile mud crabs fed diets containing different zinc levels. Values are means (n 3), with standard errors represented by vertical bars. [a,b,c] Mean values for the same tissue with unlike superscript letters were significantly different as determined by ANOVA and Tukey's test ($P < 0{\cdot}05$). PO, phenoloxidase; CP, ceruloplasmin; AKP, alkaline phosphatase; ACP, acid phosphatase. ▭, 44·5; ▨, 56·9; ▧, 68·5; ▭, 97·3; ▨, 155·6; ▩, 254·7.

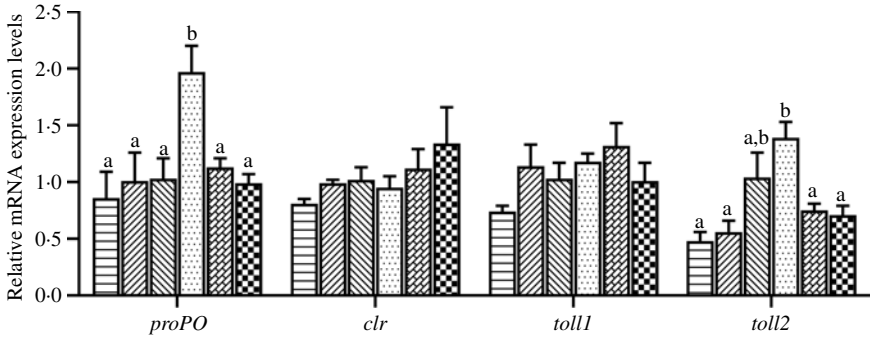

Fig. 6. Effects of dietary zinc level on relative expression of genes involved in innate immunity in hepatopancreas of juvenile mud crabs. Values are means (n 3), with standard errors represented by vertical bars. [a,b] Mean values for the same tissue with unlike superscript letters were significantly different as determined by ANOVA and Tukey's test ($P < 0{\cdot}05$). *proPO*, prophenoloxidase; *clr*, C-type lectin receptor. *toll1*, toll-like receptor 1; *toll2*, toll-like receptor 2. ▭, 44·5; ▨, 56·9; ▧, 68·5; ▭, 97·3; ▨, 155·6; ▩, 254·7.

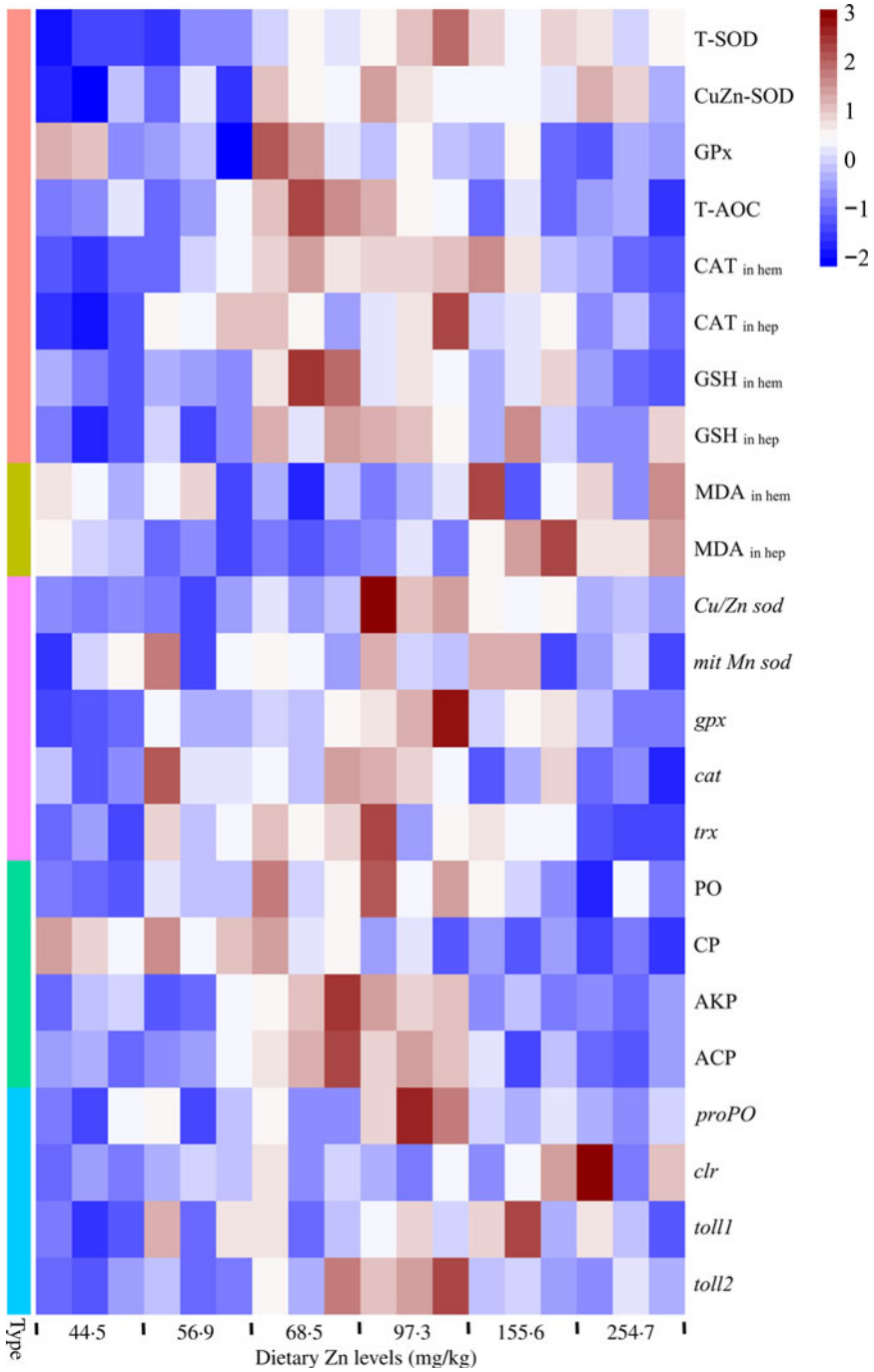

Fig. 7. Heat map visualisation of oxidation resistance and innate immunity parameters in haemolymph and hepatopancreas of mud crabs fed different dietary zinc levels. Before analysis, all data were checked for homogeneity. The colour box for each compound in the heat map indicates the abundance of the compound and represents the fold change according to the scale on the right: red for higher values; blue for lower values. Type: ■, antioxidase and antioxidant; ■, peroxidation products; ■, antioxidation-related genes; ■, innate immunity enzymes; ■, innate immunity-related genes.

Nevertheless, intensive aquaculture can cause physiological and metabolic disorders and hypoimmunity 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 crustacean species[15,23,24,29,31,32,38].

The present study demonstrated that dietary Zn could promote growth performance of juvenile mud crabs, 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 crabs fed the diet without supplementary Zn (44·5 mg/kg 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 crabs in the present study was 82·9 mg/kg diet. This value was similar to other crustaceans, such as Pacific white shrimp (71·48–95·06 mg/kg)[31], but different from Chinese mitten crab (105·34 mg/kg)[24], freshwater prawn (60 mg/kg)[15] and grass shrimp (34·1 mg/kg)[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 basal diet. For instance, in the Zn requirement study in Chinese mitten crab, the initial weight of the crab was only 7·16 (SE 0·48) mg[24], while the initial weight of mud crab in the present study was 14·96 (SE 1·11) g.

Zn concentration in different tissues could reflect Zn utilisation[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* × *Oreochromis aureus*)[40], Indian major carp (*Labeo rohita*) and yellow catfish (*Pelteobagrus fulvidraco*)[20]. Furthermore, the main tissue of Zn 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 Zn was almost 4-fold higher compared with crab fed 5 mg/kg Zn[23]. In grass shrimp, hepatopancreas Zn concentration was highest in shrimp fed the diet containing the highest Zn 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 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, Zn deposition rate in hepatopancreas was lower, possibly reflecting negative feedback regulation.

In general, defence systems against lipid peroxidation consist of antioxidant enzymes, such as SOD, CAT and GPx, as well the nonenzymatic antioxidant GSH[42–44]. SOD catalyses the dismutation of superoxide anion to hydrogen peroxide and oxygen, and Zn is required at the active centre 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 total SOD, Cu/Zn-SOD and CAT activities, and total antioxidation capacity and GSH levels were observed in crabs fed the diets containing 44·5 and 56·9 mg/kg Zn compared with those fed the diets containing 68·5 and 97·3 mg/kg Zn. 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 Zn, which indicated that appropriate levels of dietary Zn contribute 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 (*O. niloticus*)[48], Jian carp (*Cyprinus carpio*)[46] and yellow catfish[25].

In the present study, expression levels of genes involved in antioxidation such as *Cu/Zn sod*, *gpx*, *cat* and *trx* were increased in crabs fed diets containing 68·5 and 97·3 mg/kg Zn compared with 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 signalling 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 Zn shields membrane from Fe-initiated peroxidation of lipids by blocking negatively charged Fe 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 defence[35]. The prophenoloxidase (proPO) system is considered as a constituent of the immune system and probably responsible, at least in part, for the non-self recognition process of the defence mechanism in crustaceans, when transformed to the active form (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 Zn had significantly lower PO activity in haemolymph than those fed the 97·3 mg/kg 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) supplementation in mud crab decreased PO and CP activities in haemolymph. Cu is a key component of PO and CP[35,57], and there is interaction between Cu and Zn[58]. Thus, excessive dietary Zn may disrupt normal Cu 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 Zn compared with crabs fed the other diets, and similar results were found in blunt snout bream (*Megalobrama amblycephala*)[27], grass carp[44], Indian major carp[20], Nile tilapia[60] and Siberian sturgeon (*Acipenser baerii*)[61].

The ProPO and Toll pathways are two major signalling 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 Zn was up-regulated compared with 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 Zn 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 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 significantly 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, c-Jun N-terminal kinase (JNK) and myosin light-chain kinase (MLCK) signalling pathway[66].

The bioinformatic statistical tool 'heat map' has been used to analyse 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 visualise 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 Zn sources (zinc sulphate, Zn amino acid complex, mixed Zn source) showed inconsistent biological effects in Pacific white shrimp, and shrimp fed the mixed Zn source diet showed better growth response and meat quality. In the present study, the heat map clearly showed that crabs fed the diets with 68·5 and 97·3 mg/kg 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 the broken-line regression analysis between PWG and dietary Zn level, the optimal dietary Zn requirement of juvenile mud crabs was estimated to be 82·9 mg/kg. Moreover, the results of the present study demonstrated a positive correlation between tissue Zn bioaccumulation and dietary Zn levels in mud crabs, 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/kg Zn and, therefore, enhanced the antioxidant defence and innate immune responses of mud crabs.

Acknowledgements

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.

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), 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.

J. X. L., M. J. and Q. C. Z. conceived and designed the experiments. J. X. L., T. T. Z., X. X. W., Y. Y., X. C. and J. J. L. performed the experiments. J. X. L. analysed the data. J. X. L. and T. T. Z. contributed reagents/materials/analysis tools. J. X. L., T. T. Z., X. X. W., Y. Y. and X. C. prepared the crab diets. J. X. L. conducted the crab feeding trial. J. X. L., T. T. Z., X. X. W., Y. Y. and X. C. collected the samples. J. X. L., D. R. T. and Q. C. Z. wrote the paper. All authors contributed to and approved the manuscript.

The authors declared that there were no conflicts of interest.

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British Journal of Nutrition

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British Journal of Nutrition