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# Dietary organic zinc promotes growth, immune response and antioxidant capacity by modulating zinc signaling in juvenile Pacific white shrimp (*Litopenaeus vannamei*)

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ABSTRACT

An 8-week feeding trial was conducted to evaluate effects of dietary organic zinc (zinc amino acid chelate) on growth performance, mineral bioaccumulation in whole body, hepatopancreas and carapace, innate immune response and antioxidant capacity of juvenile Pacific white shrimp *Litopenaeus vannamei*. Five isonitrogenous and isolipidic diets were formulated to contain different zinc levels of 46.4 (basal diet), 65.5, 85.9, 108.4 and 130.6 mg $kg^{-1}$. Dietary zinc level significantly influenced growth and feed utilization, with the lowest weight gain and highest feed conversion ratio observed in shrimp fed the basal diet. The optimal dietary zinc requirement was estimated to be 104.8 mg $kg^{-1}$ for juvenile Pacific white shrimp. Shrimp fed the diet containing 130.6 mg $kg^{-1}$ Zn had the highest zinc concentration in hepatopancreas and carapace, but there were no significant differences in calcium or phosphorus concentration in tissues. Dietary Zn increased the activities of lysozyme, alkaline phosphatase and polyphenol oxidase in hepatopancreas. Shrimp fed the diets supplemented with zinc had significantly higher activity of Cu/Zn SOD and lower content of malondialdehyde in hepatopancreas. The expression levels of *toll*, *imd*, *lzm*, *proPO* and *alp* involved in immunity and *Cu/Zn sod* related to oxidation resistance were up-regulated. Zinc also promoted the expression levels of *mt* and *mtf-1*, and up-regulated the expression of SLC39 family genes (*zip3*, *zip9*, *zip11*, *zip14*) in hepatopancreas. These data provided novel insights in the potential mechanism of organic zinc-induced enhancement of immunity and antioxidant capacity in Pacific white shrimp.

## 1. Introduction

Zinc has been extensively studied since Todd et al. (1933) first reported the necessity of zinc for growth and development in rats. Further in-depth studies have provided novel insights into the relationship between dietary zinc and bone growth, inflammation, oxidative stress and lipid metabolism. Zinc is an integral constituent or active central ion of zinc-dependent metalloenzymes (including alkaline phosphatase, superoxide dismutase, alcohol dehydrogenase and carbonic anhydrase) and insulin (Rink and Gabriel, 2000; Miranda and Dey, 2004; Hara et al., 2017). Zinc can prevent oxidative damage to tissues caused by free radicals, which is confirmed by the physiological concentration of zinc inhibiting the production of superoxide anion and hydrogen peroxide (Salgueiro et al., 2000; Ogawa et al., 2011). Zinc directly affects the activity of antioxidant enzymes by being a cofactor of Cu/Zn superoxide dismutase (Cu/Zn SOD), or indirectly regulates metallothionein (MT) promoting antioxidant effects (Chalaux et al., 1999; Li et al., 2010). Studies have reported that zinc improved immunity and oxidation resistance by increasing the activities of phenol oxidase, ALP and SOD in Pacific white shrimp (Lin et al., 2013) and grass shrimp *Penaeus*

*Abbreviations:* ACP, acid phosphatase; ALP, alkaline phosphatase; CAT, catalase; CP, ceruloplasmin; Cu/Zn SOD, Cu/Zn superoxide dismutase; *imd*, immune deficiency protein; LZM, lysozyme; MDA, malondialdehyde; *mtf-1*, metal regulatory transcription factor-1; *mt*, metallothionein; PPO, polyphenol oxidase; *proPO*, prophenoloxidase; *toll*, toll protein; *zip3*, solute carrier family 39 member 3; *zip9*, solute carrier family 39 member 9; *zip11*, solute carrier family 39 member 11; *zip14*, solute carrier family 39 member 14.

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*monodon* (Shiau and Jiang, 2006). Therefore, zinc may be a promising candidate additive in shrimp feed to improve immunity and antioxidant capacity.

Zinc signaling plays a critical role in the body, and is strictly regulated by zinc transporters and Zn-binding proteins (Fukada et al., 2011). The intracellular and extracellular concentration and distribution of zinc is regulated by metallothionein (MT) and solute carrier family 39 (SLC39/ZIP) by controlling the cytosolic $Zn^{2+}$ level (Fukada et al., 2011). MT is a cytoplasmic protein in the liver that functions in metal storage, transport and detoxification by binding and releasing $Zn^{2+}$ (Cherian, 1994). When zinc enters a cell, it can be bound by MT and carried to another part of the cell where it is released to other organelles or proteins (Cherian, 1994). Zinc signaling is also mediated by zinc transporters of SLC39 family that carry $Zn^{2+}$ across the biological membranes to increase intracellular $Zn^{2+}$ level. Therefore, studying Zn signaling and its role in physiological processes will help reveal the mechanism of zinc homeostasis in the body.

The bioavailability of trace elements in diet is influenced by their chemical forms and dietary anti-nutritional factors (Jezierny et al., 2010). In commercial shrimp diet, soybeans or other plant protein raw materials were used as protein sources, these feed ingredients contain phytate that is unavailable for absorption (Shiau and Jiang, 2006). Phytic acid and phytate have strong binding affinity to dietary minerals including zinc, inhibiting it absorption (Jezierny et al., 2010). Therefore, low bioavailability of inorganic microelements has led to the overfortification of diet consequently resulting in growth restriction, damaged immune system and increasing losses into the environment and thus, chelated minerals have been viewed as a positive alternative to inorganic sources (Clearwater et al., 2002; National Research Council (NRC, 2011). Typically, they are more stable and can combine with amino acids, peptides or proteins to form complexes in the digestive tract to facilitate intestinal mucosal transport and reduce the binding of phytic and Zn (Apines et al., 2001; Formigoni et al., 2011). There have been numerous studies in animals demonstrating greater bioavailability of organic Zn sources (Lin et al., 2013; Apines et al., 2001; Paripatananont and Lovell, 1995; Li and Robinson, 1996; Tan and Mai, 2001; Ma et al., 2014), and several studies have shown the beneficial effects of dietary organic minerals on immunity and oxidation resistance in channel catfish *Ictalurus punctatus* (Li and Robinson, 1996), rainbow trout *Oncorhynchus mykiss* (Apines-Amar et al., 2004) and Pacific white shrimp (Yuan et al., 2018, 2020).

Pacific white shrimp are important cultured shrimp worldwide, accounting for 80 % of total penaeid shrimp production due to its great economic value and rapid growth rate (National Research Council (NRC, 2011). With rapid development of intensive culture, disease is the most important factor restricting shrimp breeding (Lun et al., 2014; Tang and Lightner, 2014). Therefore, the objective of the present study was to precisely quantify the dietary requirement of zinc in commercial feed, and provide the scientific basis for organic zinc improving immunity of Pacific white shrimp. The results of this study will provide further insight into the physiological function of zinc in shrimp.

## 2. Materials and methods

### 2.1. Experimental diets

Five experimental diets were formulated with zinc amino acid chelate (Zn content of 12 %, amino acids of 21 %, and the rest is corncob powder and zeolite (as carrier for zinc); Zinpro Corp., USA) as Zn source, the analyzed values of dietary Zn being 46.4 (basal diet), 65.5, 85.9, 108.4 and 130.6 mg $kg^{-1}$ diet, respectively (**Supplementary Table S1**). The diets were prepared following the protocol described in detail previously (Shi et al., 2021a).

### 2.2. Shrimp rearing and experimental conditions

Pacific white shrimp juveniles were obtained from Chia-Tai Ningbo Company (Ningbo, China). Prior to the feeding trial, shrimp were reared in cement pools and fed a commercial feed (40 % protein, 8 % lipid; Yue-Hai Aquafeed Corp., Jiaxing, China) for two weeks to acclimate the experimental conditions. The feeding trial was conducted at the breeding base of Ningbo Ocean and Fishery Science and Technology Innovation Center (Zhejiang, China). Juveniles (initial weight 1.33 ± 0.01 g) were randomly distributed into 300 L cylindrical fiber-glass tanks filled with 250 L of seawater at a stocking density of 30 shrimp per tank, with each experimental diet was randomly assigned to five replicate tanks. The daily management procedure of the feeding trial was described in detail previously (Shi et al., 2020). Tanks aeration was provided through air stones, with dissolved oxygen level maintained at no less than 6.0 mg $L^{-1}$. During the 8-week feeding trial, seawater conditions including temperature (28−32 °C), salinity (23−27 g $L^{-1}$), pH (7.4–7.6), dissolved oxygen level (not less than 6.0 mg $L^{-1}$) and ammonia nitrogen (lower than 0.05 mg $L^{-1}$) were measured by YSI Proplus (YSI, Yellow Springs, Ohio, USA).

### 2.3. Sample collection

One hundred juvenile Pacific white shrimp were randomly sampled at the initial of the feeding trial for determining initial whole body zinc concentration. At the termination of the experiment, shrimp were fasted for 24 h and anesthetized with 10 mg $L^{-1}$ eugenol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) before sampling. All shrimp from each tank were counted and weighed to calculate growth performance and feed utilization related parameters (SR, WG, SGR, FCR, FI). Furthermore, body length, whole body and hepatopancreas weight from four shrimp in each tank were taken to calculate morphological parameters (CF, HSI). Five shrimp from each tank were used to analyze the Zn concentration in tissues. Hemolymph samples from five shrimp in each tank were taken from the pericardial cavity using a 1-ml syringe, placed in 1.5-ml microfuge tubes and centrifuged at 4 °C, 850×g for 10 min (Eppendorf centrifuge 5810R, Germany). Supernatant was collected and stored at −80 °C until analysis of hematological characteristics. Ten hepatopancreas samples were also collected and stored at −80 °C until analysis of immunity and antioxidant capacity related parameters and gene expressions.

### 2.4. Chemical analyses

Crude protein, crude lipid, ash and moisture contents in diets were analyzed by standard methods and described in our previous study (AOAC, 2006; Shi et al., 2020). Amino acid profiles of diets were determined using a High-speed Amino Acid Analyzer (L-8900, Hitachi High-Technologies Co., Tokyo, Japan) based on the method described previously (Shi et al., 2021b) (**Supplementary Table S2**). Zinc, calcium and phosphorus concentration in tissues (whole body, hepatopancreas and carapace) and experimental diets were measured using ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometer, PE 2100DV, Perkin Elmer, USA) at the Ningbo Institute of Materials Technology and Engineering, Chinese Academy of Sciences (Ningbo, China).

### 2.5. Analysis of hemolymph and hepatopancreas parameters

Hepatopancreas samples were homogenized on ice in 9 volumes (w: v) of ice-cold physiological saline 8.9 g $ml^{-1}$, then centrifugated at 850×g for 10 min at 4 °C. The resultant supernatant was collected and aliquots stored at −80 °C until analysis. Activities of immune enzymes including LZM, polyphenol oxidase (PPO), ALP and ACP were determined. ALP and ACP activities in hemolymph were determined by an automatic chemistry analyzer (Hitachi 7600-110, Tokyo, Japan), and

activities of other immune enzymes were determined using the relevant diagnostic reagent kits (Nanjing Jiancheng Co., Nanjing, China) according to the manufacturer's instructions. Activities of antioxidant enzymes including Cu/Zn SOD, ceruloplasmin (CP), and catalase (CAT), and content of malondialdehyde (MDA) were determined using the relevant diagnostic reagent kits (Nanjing Jiancheng Co., Nanjing, China).

### 2.6. Total RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from hepatopancreas with Trizol Reagent (Vazyme, China) and the quantity and quality of total RNA assessed using a NanoDrop spectrophotometer (Thermo Scientific NanoDrop 2000, USA) and 1.2 % denaturing agarose gel electrophoresis. The cDNA was synthesized for quantitative reverse-transcriptase polymerase chain reaction (qPCR) using the HiScript® RT SuperMix Reagent kit (Vazyme, China) according to the manufacturer's instructions. The gene-specific primers used for mRNA quantification by RT-PCR were designed by Primer Premier 5.0 and are shown in **Supplementary Table S3**. The qPCR was carried out in a quantitative thermal cycler system (Light cycler96, Roche, Switzerland), with 20 μl reaction volume containing 2×ChamQ SYBR qPCR Green Master Mix (Vazyme, China), 0.4 μl each primer, 8.4 μl DEPC treated water and 0.8 μl of 1/4 diluted cDNA. The real-time PCR program was 95 °C for 2 min, followed by 45 cycles of 95 °C for 10 s, 58 °C for 10 s and 72 °C for 20 s. Standard curves were made with six different dilutions (in triplicate) of cDNA samples and amplification efficiency was analyzed according to the following equation $E = 10^{(-1/slope)}$-1. During analysis, each sample was run in triplicate and the E-values ranged from 94.3–104.1 %. The data were optimized using the comparative Ct ($2^{-\Delta\Delta Ct}$) value method as described by Livak and Schmittgen (2001), and the basal diet was used as the control/reference group.

### 2.7. Calculations

Parameters were calculated as follows:

Weight gain (WG, %) = 100 × [final body weight (g) - initial body weight (g)] / initial body weight (g);

Specific growth rate (SGR, %/day) = 100 × [Ln (final body weight) - Ln (initial body weight)] /days;

Survival (SR, %) = 100 × (final number of shrimp) / (initial number of shrimp);

Feed conversion rate (FCR) = feed consumption (g) / [final body weight (g) - initial body weight (g)];

Feed intake (FI, %bw/days) = 100 × feed consumption / [(initial body weight + final body weight) / 2] / days;

Hepatosomatic index (HSI, %) = 100 × [hepatopancreas wet weight (g)] / [body wet weight (g)];

Condition factor (CF, g/cm$^3$) = 100 × [body weight (g) / body length$^3$ (cm$^3$)].

Deposition rate of zinc (%) = 100 × (final body weight (g) × final whole shrimp of zinc (mg kg$^{-1}$) - initial body weight (g) × initial whole shrimp of zinc (mg kg$^{-1}$)) / (feed consumption (g) × feed zinc content (mg kg$^{-1}$)).

### 2.8. Statistical analysis

Results are presented as the means ± SEM of five replicates (n = 5). All data were checked for normality and homogeneity of variances, and were normalized when appropriate. Data were analyzed using one-way analysis of variance ANOVA to investigate differences among treatments with group means further compared by Duncan's multiple tests. Differences were considered to be statistically significant at $P < 0.05$. Quadratic regression analysis was conducted to analyze WG in response to dietary zinc level (Fig. 1). All statistical analyses were performed using SPSS 20.0 (SPSS, IBM, USA).

## 3. Results

### 3.1. Growth performance, feed utilization and morphometric parameters

The effects of dietary Zn level on growth performance, feed utilization and morphometric parameters of Pacific white shrimp are presented in Table 1. Survival ranged from 89.2 % to 94.7 %, with on statistical differences among all treatments. Dietary Zn level significantly affected WG, SGR, FI, FCR and CF of Pacific white shrimp. Shrimp fed the basal diet containing 46.4 mg kg$^{-1}$ zinc had the lowest WG, SGR and CF, and highest FI and FCR among all treatments. Quadratic regression analysis of WG against dietary zinc level showed that the optimal dietary zinc requirement was 104.8 mg kg$^{-1}$ (Fig. 1).

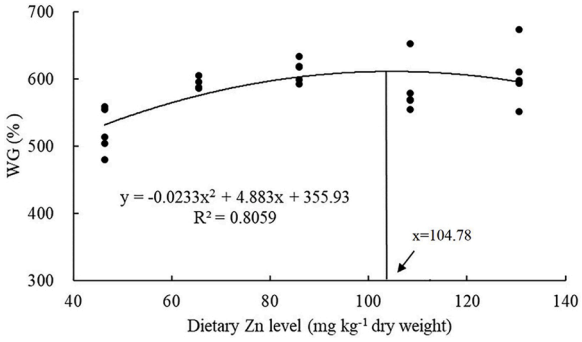


**Fig. 1.** Relationship between WG (%) and dietary zinc level (mg kg$^{-1}$, dry weight) based on quadratic regression analysis, where x represents optimal dietary Zn for the maximum WG of juvenile *L. vannamei*.

**Table 1**
Effects of different dietary zinc levels on growth performance, feed utilization and morphologic index in juvenile L. *vannamei*.

| Items | Dietary zinc level (mg kg$^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | 46.4 | 65.5 | 85.9 | 108.4 | 130.6 | ANOVA *P* |
| IBW (g) | 1.34 ± 0.00 | 1.34 ± 0.01 | 1.33 ± 0.01 | 1.33 ± 0.01 | 1.34 ± 0.01 | 0.200 |
| WG (%) | 522.40 ± 15.21$^a$ | 592.45 ± 3.63$^b$ | 608.37 ± 7.94$^b$ | 589.71 ± 26.20$^b$ | 605.84 ± 19.69$^b$ | 0.009 |
| Survival (%) | 94.67 ± 1.33 | 94.67 ± 2.00 | 91.33 ± 2.71 | 89.17 ± 2.10 | 90.67 ± 1.63 | 0.253 |
| SGR (%/day$^{-1}$) | 3.36 ± 0.03$^a$ | 3.55 ± 0.03$^b$ | 3.66 ± 0.05$^b$ | 3.53 ± 0.03$^b$ | 3.66 ± 0.05$^b$ | 0.000 |
| FI (%/body weight day) | 3.97 ± 0.08$^b$ | 3.61 ± 0.04$^a$ | 3.51 ± 0.03$^a$ | 3.65 ± 0.10$^a$ | 3.51 ± 0.09$^a$ | 0.002 |
| FCR | 1.54 ± 0.05$^b$ | 1.35 ± 0.02$^a$ | 1.30 ± 0.01$^a$ | 1.37 ± 0.05$^a$ | 1.31 ± 0.04$^a$ | 0.003 |
| HSI (%) | 4.79 ± 0.27 | 5.21 ± 0.12 | 5.11 ± 0.26 | 4.93 ± 0.17 | 5.18 ± 0.20 | 0.586 |
| CF (g/cm$^3$) | 0.58 ± 0.03$^a$ | 0.62 ± 0.01$^b$ | 0.63 ± 0.02$^b$ | 0.65 ± 0.01$^b$ | 0.63 ± 0.02$^b$ | 0.020 |

Data are presented as means ± S.E.M. (n = 5). Values in the same column with different superscripts are significantly different ($P < 0.05$). CF, condition factor; FCR, feed conversion ratio; FI, feed intake; HSI, hepatosomatic index; IBW, initial body weight; SGR, specific growth rate; WG, weight gain.

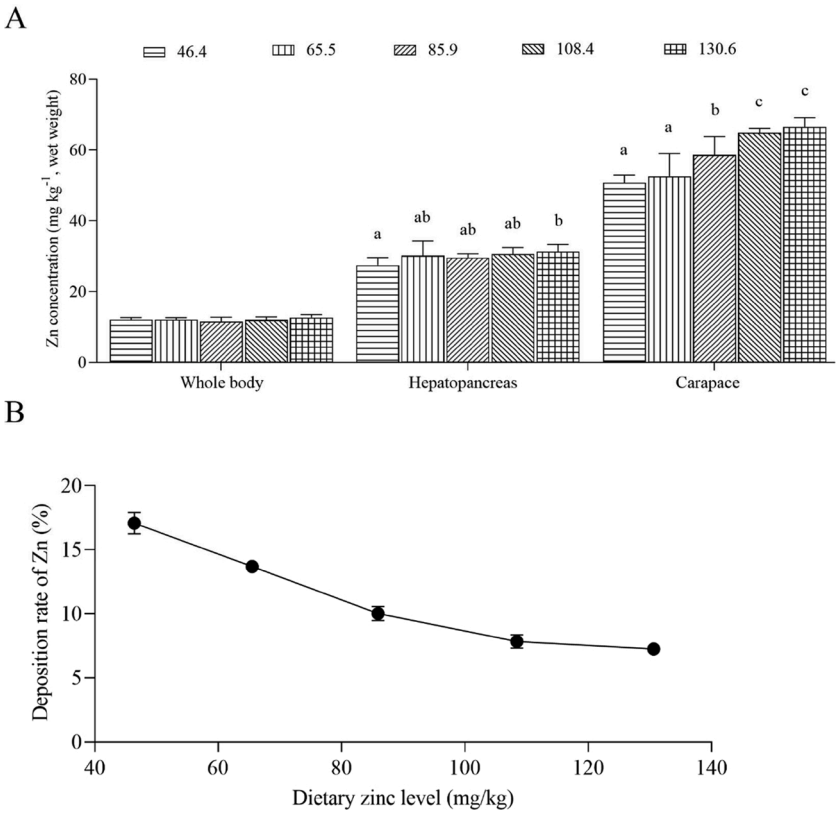


**Fig. 2.** Zn concentration in tissues (**A**) and Zn deposition rate (**B**) of juvenile *L. vannamei* fed diets containing different Zn levels. Values are means (n = 5), with standard errors represented by vertical bars. Mean values with different superscript letters were significantly different as determined by ANOVA and Duncan's multiple−range test ($P < 0.05$).

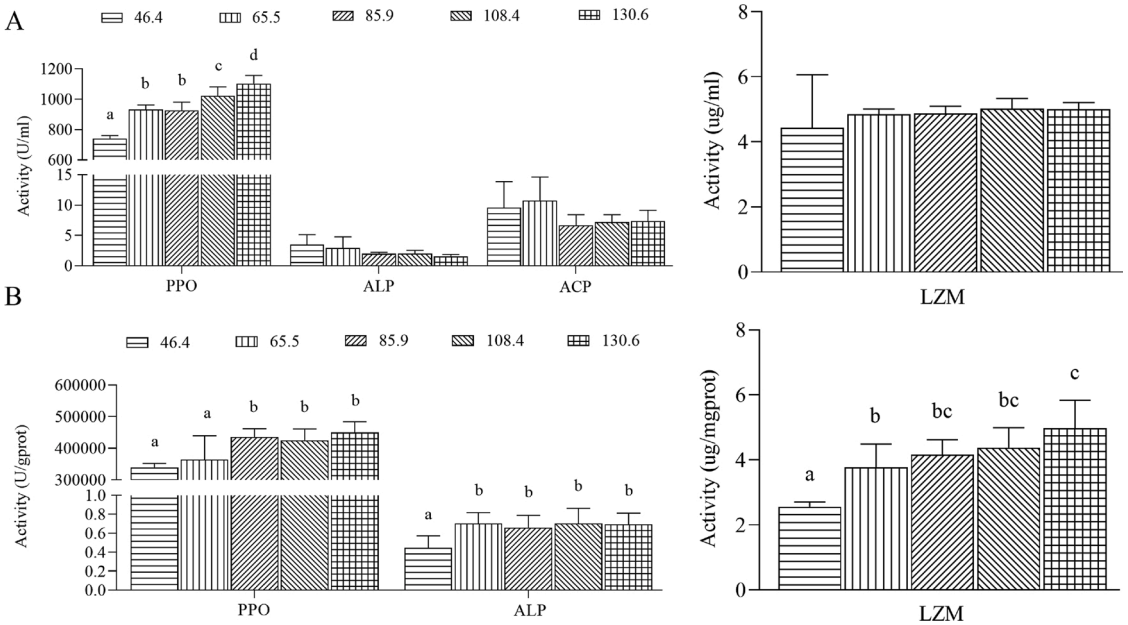


**Fig. 3.** Activities of innate immunity enzymes in hemolymph (**A**) and hepatopancreas (**B**) of juvenile *L. vannamei* fed the diets containing different Zn levels. Values are means (n = 5), with standard errors represented by vertical bars. Mean values with different superscript letters were significantly different as determined by ANOVA and Duncan's multiple−range test ($P < 0.05$).

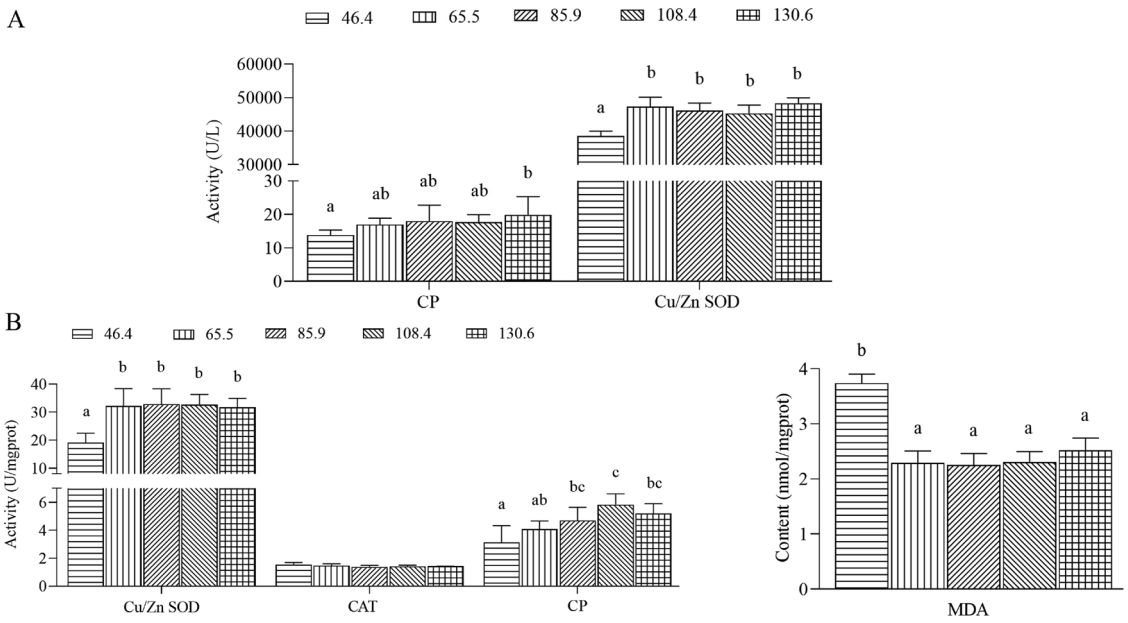


**Fig. 4.** Antioxidant indexes in hemolymph (**A**) and hepatopancreas (**B**) of juvenile *L. vannamei* fed the diets containing different Zn levels. Values are means (n = 5), with standard errors represented by vertical bars. Mean values with different superscript letters were significantly different as determined by ANOVA and Duncan's multiple−range test ($P < 0.05$).

### 3.2. Zinc (Zn) concentration in tissues and zinc deposition rate

Zn concentration in tissues (whole body, hepatopancreas and carapace) and zinc deposition rate are presented in Fig. 2. Dietary zinc level significantly affected zinc concentration in hepatopancreas and carapace. Shrimp fed the diet containing 130.6 mg $kg^{-1}$ Zn had higher zinc concentration in hepatopancreas than those fed the basal diet. Similarly, shrimp fed the diets containing 108.4 and 130.6 mg $kg^{-1}$ Zn had higher Zn concentration in carapace than those fed the other diets. Deposition rate of Zn significantly decreased with increasing dietary Zn level.

### 3.3. Innate immunity responses

As shown in Fig. 3, the activities of LZM, ALP and PPO in hepatopancreas were affected by dietary zinc level. Shrimp fed the basal diet had the lowest activities of LZM, ALP and PPO in hepatopancreas, and the highest LZM activity was observed in shrimp fed the diet containing 130.6 mg $kg^{-1}$ Zn. Shrimp fed the diets supplemented with zinc had significantly higher PPO activity in hemolymph than those fed the basal diet, the highest hemolymph PPO activity was occurred at shrimp fed the diet with 130.6 mg $kg^{-1}$ Zn. No significant differences were observed in the activities of LZM, ALP or ACP in hemolymph.

### 3.4. Antioxidant ability

The antioxidant parameters in hemolymph and hepatopancreas are shown in Fig. 4. The activities of Cu/Zn SOD in hemolymph and hepatopancreas were significantly higher in shrimp fed the diets supplemented with zinc than those fed the basal diet. Furthermore, MDA level in hepatopancreas was significantly lower in shrimp fed the Zn supplementation diets. Shrimp fed the diet containing 130.6 mg $kg^{-1}$ Zn had higher CP content in hemolymph than those fed the basal diet, and highest CP content in hepatopancreas was recorded in shrimp fed the 108.4 mg $kg^{-1}$ Zn diet. No difference was observed in the activity of hepatopancreas CAT among the treatments.

### 3.5. Gene expression

Transcript levels of immune-related genes in hepatopancreas are shown in Fig. 5**A**. The expression levels of *toll*, *imd*, *lzm*, *proPO* and *alp* were up-regulated as dietary zinc level increased, the lowest expression levels were occurred at shrimp fed the basal diet. Shrimp fed the diet containing 130.6 mg $kg^{-1}$ Zn had higher expression levels of *toll* and *lzm* than those fed the other diets. Similar results were also found for expression of *imd*, shrimp fed the diets containing 108.4 and 130.6 mg $kg^{-1}$ Zn had higher expression level of *imd* than those fed the other diets.

Fig. 5**B** shows expression levels of genes involved in oxidation resistance (*Cu/Zn sod*, *cp*). Shrimp fed the diet containing 130.6 mg $kg^{-1}$ Zn had higher expression level of *Cu/Zn sod* in hepatopancreas than those fed the basal diet. There was no significant difference in the expression of *cp* among the treatments.

Dietary Zn level also affected the expression of genes involved in Zn homeostasis (*mtf-1*, *mt*) and transport (*zip3*, *zip9*, *zip11*, *zip14*) (Fig. 6). The expression levels of *mtf-1*, *mt* and SLC39 family genes (*zip3*, *zip9*, *zip11*, *zip14*) in hepatopancreas were significantly up-regulated as dietary Zn level increased. Shrimp fed the diets containing 85.9, 108.4 and 130.6 mg $kg^{-1}$ Zn had higher expression levels of *mtf-1* and *mt* than those fed the basal diet. The lowest expression levels of *zip3*, *zip9*, *zip11* and *zip14* were recorded in shrimp fed the basal diet, and the highest level were found in shrimp fed the diet containing 130.6 mg $kg^{-1}$ Zn.

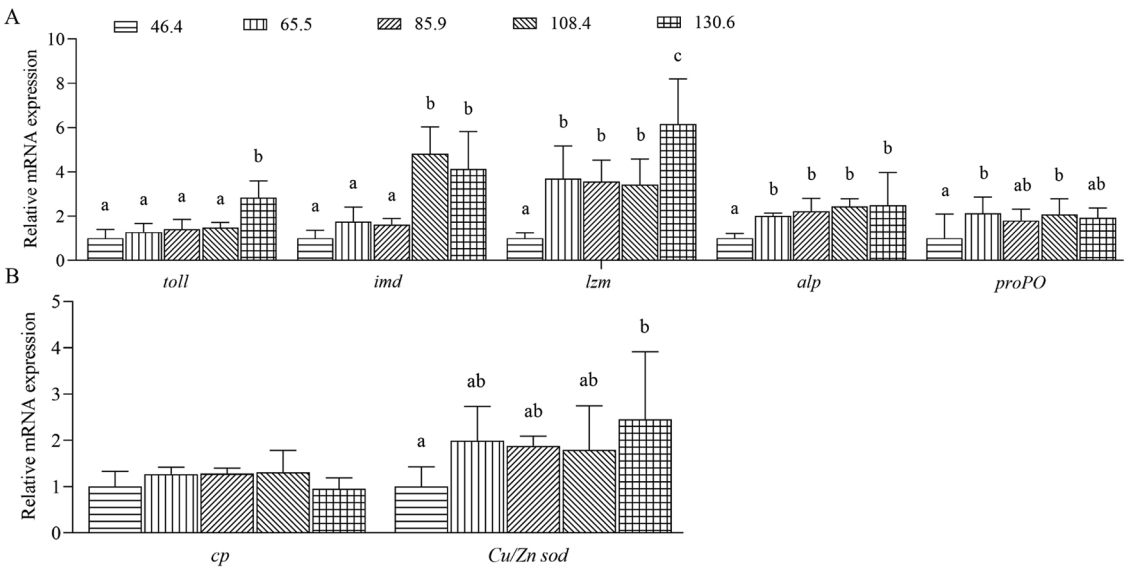


**Fig. 5.** Effects of dietary zinc level on relative mRNA expression levels of genes involved in innate immunity (**A**) and antioxidant ability (**B**) of hepatopancreas of juvenile *L. vannamei*. The gene expression of shrimp fed the basal diet (46.4 mg $kg^{-1}$ Zn) were set at 1. Expression values are normalized by $\beta$-actin-expressed transcripts. Values are means ± S.E.M. (n = 5), and bars bearing different letters are significantly different by ANOVA and Duncan's multiple-range test ($P < 0.05$).

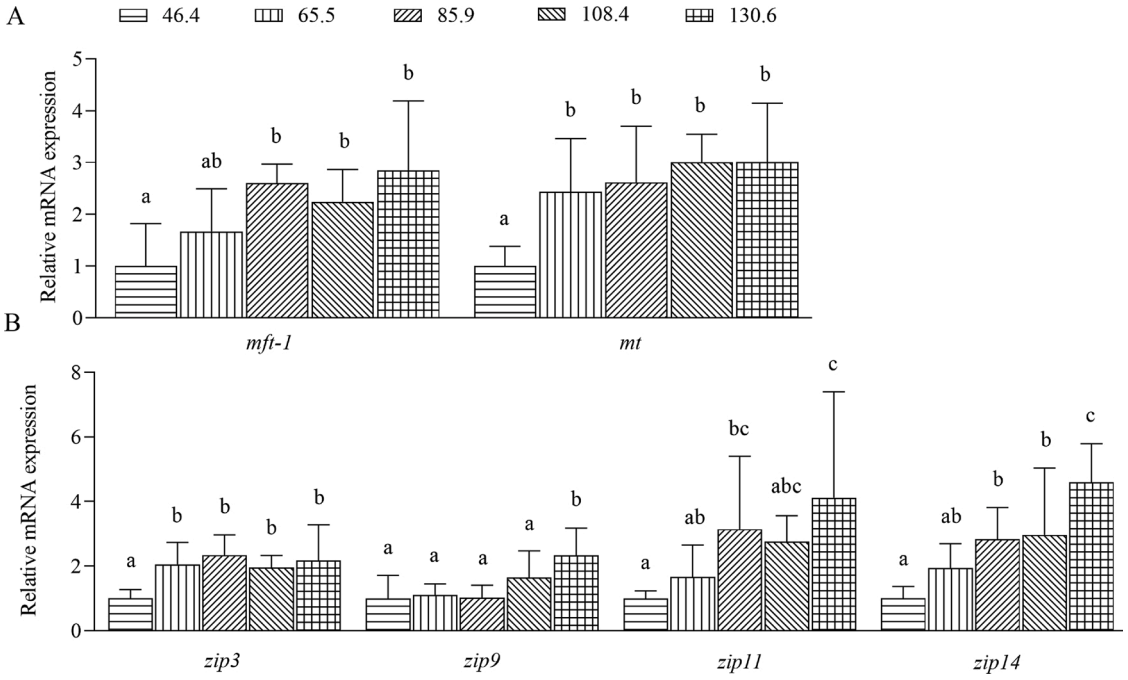


**Fig. 6.** Effects of dietary zinc level on relative mRNA expression levels of genes involved in zinc homeostasis (**A**) and SLC39 family genes (*zip3*, *zip9*, *zip11*, *zip14*) (**B**) of hepatopancreas of juvenile *L. vannamei*. The gene expression in shrimp fed the basal diet (46.4 mg $kg^{-1}$ Zn) were set at 1. Expression values are normalized by $\beta$-actin-expressed transcripts. Values are means ± S.E.M. (n = 5), and bars bearing different letters are significantly different by ANOVA and Duncan's multiple-range test ($P < 0.05$).

## 4. Discussion

### 4.1. Dietary zinc promoted growth and Zn deposition in tissues

Numerous studies have demonstrated that insufficient dietary zinc resulted in poor growth and high mortality in Pacific white shrimp (Davis and Lawrence, 1993), grass shrimp (Shiau and Jiang, 2006) and various fish species (Gatlin et al., 1991; Luo et al., 2001; Do Carmo e Sá et al., 2004; Liang et al., 2012). The present study showed a significant reduction in growth performance in shrimp fed the unsupplemented diet, suggesting dietary zinc deficiency retarded growth and reduced feed utilization in Pacific white shrimp. Davis and Lawrence (1993) reported that 33 mg $kg^{-1}$ Zn appeared to satisfy the zinc requirement of Pacific white shrimp fed a casein/gelatin-based semi-purified diet, which is markedly lower than the 104.8 mg $kg^{-1}$ Zn found in the present study. The higher requirement value established in the present study is likely due to the different feed ingredients. Plant protein sources were used in the practical feeds containing phytate which can form insoluble complexes with zinc, reducing bioavailability (Gatlin and Phillips, 1989; Ma et al., 2014; Chen et al., 2014). Consistent with this, Davis and

Lawrence (1993) reported that 200 mg $kg^{-1}$ zinc supplementation was required to overcome 1.5 % phytate in the diet. In the present study, Zn concentration in hepatopancreas and carapace significantly increased with increasing dietary Zn level, indicating they could be sensitive indicators for evaluating Zn status in Pacific white shrimp, consistent with previous studies in grass shrimp (Shiau and Jiang, 2006) and juvenile grouper *Epinephelus malabaricus* (Chen et al., 2014). Some studies reported that Zn concentration in hepatopancreas of grass shrimp was sensitive to different dietary Zn levels, moreover, Zn concentration in vertebrae and scales increased significantly with increasing dietary Zn level in juvenile grouper (Shiau and Jiang, 2006; Chen et al., 2014).

### 4.2. Zinc promoted immune response and antioxidant capacity by modulating zinc signaling

Invertebrates survive without adaptive immunity, mainly depending on a fixed non-specific immune mechanism (Gatlin and Phillips, 1989; Chen et al., 2014). The first line of innate defense is the melanization of pathogens, which is controlled by the prophenoloxidase (proPO) activation cascade generating polyphenol oxidase and monophenyl oxidase (Söderhäll and Cerenius, 1998; Cerenius et al., 2008; Amparyup et al., 2013). A study has confirmed that melanization mediated by the proPO system is an important composition in shrimp humoral and hepatopancreas immune responses (Amparyup et al., 2013). In this study, incremental dietary Zn significantly increased the expression of *proPO* in hepatopancreas and the activity of polyphenol oxidase in hemolymph and hepatopancreas, indicating zinc could improve innate immunity by activating the proPO system. In addition, the humoral immune response contains defense enzymes such as ALP, ACP and LZM (Qin et al., 2018). Alkaline phosphatase is a Zn (II) metalloenzyme with two Zn ions at the active center, and the activity of ALP is regulated by saturation of Zn (Millán, 2006; Anand and Srivastava, 2012). The present study showed shrimp fed the diets supplemented with zinc had higher activity and mRNA expression level of *alp* in hepatopancreas, which was consistent with previous studies (Apines et al., 2001; Do Carmo e Sá et al., 2004; Jiang et al., 2016; Moazenzadeh et al., 2018; Musharraf and Khan, 2019). Thus, the increased activity of ALP may be due to the enhanced saturation of zinc active centers (Anand and Srivastava, 2012). Lysozyme, a typical immune enzyme of invertebrates, can kill bacteria by attacking the peptidoglycan layer of bacterial cell walls (Bayarri et al., 2014). Studies have reported that functional immune status of shrimp could be evaluated by lysosome activity (Burge et al., 2007; Yao et al., 2008). In the present study, shrimp fed the diets supplemented with zinc had higher activity and mRNA expression of *lzm* in hepatopancreas, indicating dietary zinc supplementation enhanced immune function of Pacific white shrimp. The main regulatory pathways of innate immunity of shrimp are the Toll pathway and the IMD pathway, both of which are activated and work together upon immune challenge (Li and Xiang, 2013). However, these two pathways play different defense functions in invertebrates, the former mainly defends against fungi, gram-positive bacteria, and viruses, while the latter targets gram-negative bacterial (Rutschmann et al., 2002; Zambon et al., 2005; Avadhanula et al., 2009). In the present study, the expression levels of *toll* and *imd* in hepatopancreas were significantly up-regulated as dietary zinc level increased, and lowest expression levels were observed in shrimp fed the basal diet, suggesting that zinc may affect immune function of shrimp through the Toll and IMD pathways, although further in-depth studies are required.

Reactive oxygen species (ROS) including superoxide anion, hydroxyl radical and hydrogen peroxide are products of cellular oxygen metabolism (Weissman et al., 2007). Excessive production of ROS results in oxidative stress and is deleterious to cell structure (Valko et al., 2007). Malondialdehyde is a final product of ROS and a biomarker for lipid peroxidation (Janero, 1990). Furthermore, antioxidant defenses including enzymes SOD, CAT and CP, SOD converts highly reactive superoxide anion radicals to less reactive hydrogen peroxide, which generates water, molecular oxygen and H donors under the action of CAT (Matés et al., 1999). Cu/Zn SOD, also known as SOD1, contains a binuclear Cu/Zn site in each subunit, which is responsible for catalyzing the disproportionation of superoxide to hydrogen peroxide and oxygen (Sea et al., 2014; Estácio et al., 2015). In the present study, shrimp fed the diets supplemented with zinc had higher activity of Cu/Zn SOD and lower content of MDA in hepatopancreas, suggesting that zinc could protect Pacific white shrimp from oxidative stress by increasing Cu/Zn SOD, resulting in reduced MDA. These results were consistent with previous studies in Nile tilapia *Oreochromis niloticus* (Do Carmo e Sá et al., 2004), Jian carp *Cyprinus carpio* (Feng et al., 2011), yellow catfish *Pelteobagrus fulvidraco* (Luo et al., 2001), grass carp *Ctenopharyngodon idella* (Wu et al., 2014) and Indian major carp *Labeo rohita* (Musharraf and Khan, 2019).

Transport of $Zn^{2+}$ into the cell depends on the zinc importer (ZIP, SLC39), which control the input of Zn from the extracellular environment or regulate the release of Zn from the organelles (Rink and Gabriel, 2000). ZIPs are involved in many cellular responses, including the regulation of enzymes (Fukada et al., 2011). ZIP3, ZIP9, ZIP11 and ZIP14 are localized in cell membrane, and ZIP9 and ZIP11 are localized to the Golgi apparatus (Matsuura et al., 2009). Studies have suggested that the members of ZIP family display specific changes at the transcriptional level in response to Zn stimuli (Chen et al., 2018). Zn supplementation up-regulated mRNA expression level of *zip9* to increase Zn in intestinal epithelial cells of yellow catfish (Chen et al., 2019). Wang et al. (2004) reported that the mRNA expression level and activity of ZIP3 is regulated by dietary Zn. Furthermore, Zn addition increased ZIP11 and ZIP14 mRNA and protein expression, with a marked difference recorded in the liver (Jenkitkasemwong et al., 2012; Yu et al., 2013). In this study, the mRNA expression levels of *zip3*, *zip9*, *zip11* and *zip14* were significantly up-regulated as dietary Zn increased, indicating that dietary zinc supplementation up-regulated mRNA expression of SLC39 family genes (*zip3*, *zip9*, *zip11*, *zip14*) to transport Zn into the cytosols and release Zn from the Golgi apparatus.

The metal responsive transcription factor 1 (MTF-1) is a zinc dependent transcription factor heralded as the molecular switch that coordinated the cellular defense against excess metal ions (Laity and Andrews, 2007; Hogstrand et al., 2008). MTF-1 can be activated directly by Zn to drive MT, which is a superfamily of intracellular metal binding proteins involved in the homeostasis of essential metals and metal detoxification (Vallee, 1995; Nordberg and Nordberg, 2000; Sims et al., 2012). MT combines with Zn, the primary binding ion to reduce free metals in cells (Coyle et al., 2002). Previous studies demonstrated that zinc induced the transcriptional activation of *mt* through *mtf-1* to regulate the homeostasis of metals (Chen et al., 1999; Dong et al., 2014; Chen et al., 2020). Overall, results of the present study showed that mRNA expression levels of the SLC39 family genes such as *zip3*, *zip9*, *zip11* and *zip14* were significantly up-regulated as dietary Zn increased, indicating that more $Zn^{2+}$ could be transported into cells. Intracellular $Zn^{2+}$ promotes the formation of Cu/Zn SOD and ALP, which was confirmed by the increase of activities and mRNA expression levels. Excess $Zn^{2+}$ in the cell activates *mtf-1* to promote the expression of *mt*, and consequently stored $Zn^{2+}$ in metallothioneins (Fig. 7).

## 5. Conclusion

In conclusion, based on quadratic regression analysis of WG against dietary zinc level, the optimal zinc requirement was estimated to be 104.8 mg $kg^{-1}$ (~10.5 μg $g^{-1}$ body weight) for juvenile Pacific white shrimp. Feeding diets with 108.4–130.6 mg $kg^{-1}$ Zn could maintain normal immune responses and antioxidant ability, confirmed by the increased activities of PPO, LZM, CP, and reduced MDA in hepatopancreas. Importantly, dietary zinc could activate Cu/Zn SOD and ALP by mediating zinc signaling. A deeper knowledge of the relationship between dietary zinc and the Toll and IMD pathways will provide novel insights into the role of Zn as a signaling molecule in health and diseases

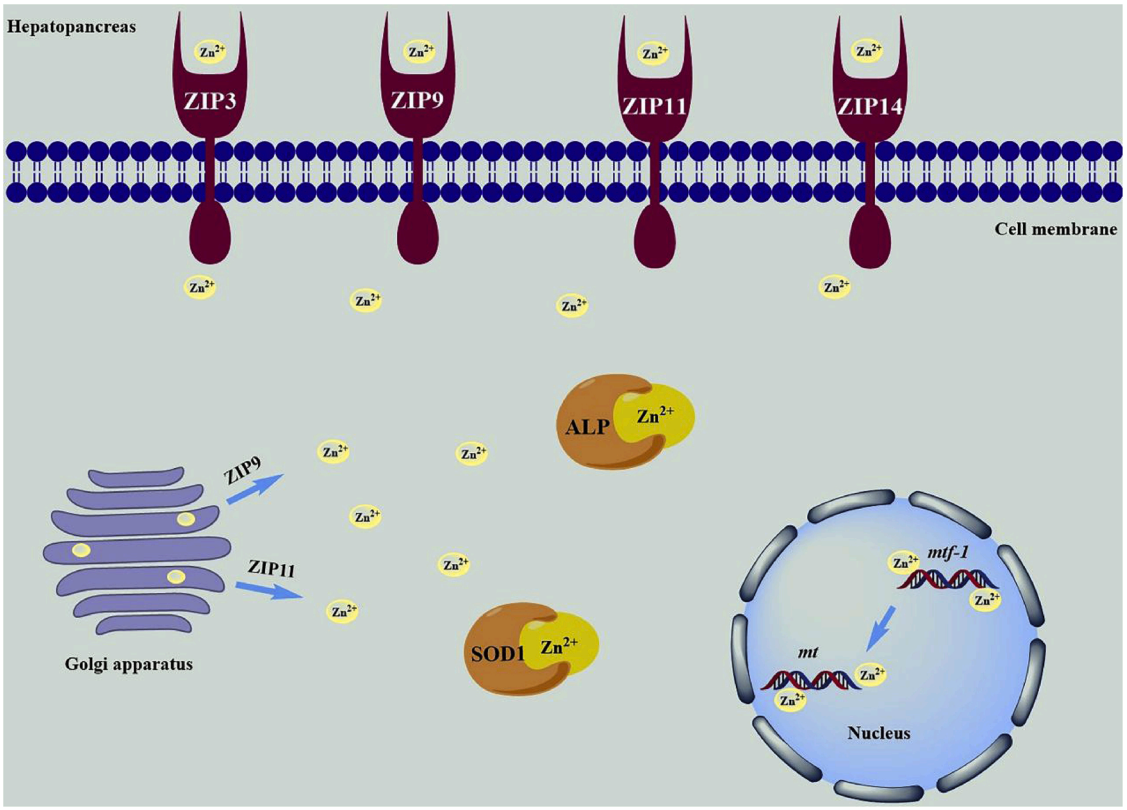


**Fig. 7.** A working model of $Zn^{2+}$ mobilization and storage. The blue lines indicate promotion. Extracellular $Zn^{2+}$ activates SLC39 family genes (*zip3*, *zip9*, *zip11*, *zip14*) to transfer $Zn^{2+}$ into cells and promote the release of $Zn^{2+}$ from the Golgi apparatus. Intracellular $Zn^{2+}$ binds to ALP and SOD1 (Cu/Zn SOD) to regulate the activities of immune and antioxidant enzymes. Excess $Zn^{2+}$ combines with *mtf-1* to promote the expression of *mt*, and consequently be stored in *mt* to maintain zinc homeostasis in cells.

in Pacific white shrimp.

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## Ethics statement

All experimental procedures complied with the Standard Operation Procedures (SOPs) of the Guide for Use of Experimental Animals of Ningbo University. The study was approved by the Scientific Ethics Committee for Experiments on Animals of Ningbo University.

## CRediT authorship contribution statement

**Bo Shi:** Conceptualization, Software, Validation, Writing - original draft. **Fangmin Xu:** Software, Writing - review & editing. **Qicun Zhou:** Validation, Resources, Writing - review & editing, Supervision, Funding acquisition. **Melanie K. Regan:** Software, Writing - review & editing. **Mónica B. Betancor:** Writing - review & editing, Supervision. **Douglas R. Tocher:** Writing - review & editing, Supervision. **Mihai Sun:** Validation. **Fanyi Meng:** Validation. **Lefei Jiao:** Supervision. **Min Jin:** Resources, Writing - review & editing, Supervision, Funding acquisition.

## Declaration of Competing Interest

The authors declared that there were no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aqrep.2021.100638.

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