Accepted manuscript

Effects of dietary Zn level on growth performance, lipolysis and expression of genes involved in the Ca^{2+} / CaMKKβ / AMPK pathway in juvenile Pacific white shrimp

Bo Shi[1], Min Jin[1*], Lefei Jiao[1], Mónica B. Betancor[2], Douglas R. Tocher[2], Qicun Zhou[1*]

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

E-mail address:

jinmin@nbu.edu.cn (Min Jin)

zhouqicun@nbu.edu.cn (Qicun Zhou)

Abbreviations: *adipor*, adiponectin receptor; *ob-rb*, leptin receptor; *camkkβ*, calcium/calmodulin dependent protein kinase kinase; *srebp*, sterol regulatory element-binding protein; *fas*, fatty acid synthase; *scd1*, stearoyl-CoA desaturase; *cd36*, cluster of differentiation 36; *cpt1*, carnitine palmitoyl transferase 1; *ampk*, 5'-AMP-activated protein kinase; *mcd*, malonyl-CoA decarboxylase; *acc*1, acetyl-CoA carboxylase 1; *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.

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/S0007114520001725
The British Journal of Nutrition is published by Cambridge University Press on behalf of The Nutrition Society

Abstract

The present study evaluated the effects of dietary zinc level on growth performance, serum and hepatopancreas metabolites, and expression of genes involved in lipid and energy metabolism, and the signal pathway of dietary Zn-induced lipolysis. Five isonitrogenous and isolipidic diets were formulated to contain different zinc levels: 46.4 (basal diet), 77.2, 87.0, 117.1, and 136.8 mg kg^{-1}, respectively. The results indicated that shrimp fed the diet containing zinc at 117.1 mg kg^{-1} had higher weight gain and specific growth rate, and the lowest feed intake and feed conversion rate, than shrimp fed the other diets. The deposition rate of Zn in whole body significantly decreased with increasing dietary zinc level. Dietary Zn prevented the accumulation of free radicals and improved antioxidant activities by increasing Cu/Zn superoxide dismutase and reducing malonaldehyde in hepatopancreas. Dietary Zn supplementation enhanced lipase activity and adiponectin, which could promote triglyceride breakdown and fatty acid oxidation and lead to reduced lipid in hepatopancreas. The mRNA expressions of *ob-rb*, *adipor*, *camkkβ*, *ampk*, *cd36*, *mcd*, *cpt1* involved in Zn-induced lipid catabolism were up-regulated, and expressions of *srebp*, *acc*, *fas* and *scd1* were down-regulated. The mRNA levels of SLC39 family genes (*zip3*, *zip9*, *zip11*, *zip14*) in hepatopancreas were up-regulated with increasing dietary Zn level. The results demonstrated that dietary Zn level could significantly affect growth performance, tissue deposition of zinc, lipid metabolites and expression of genes involved in lipogenesis and lipolysis in *Litopenaeus vannamei*.

Keywords: *Litopenaeus vannamei*; Zinc; Lipolysis; Regulatory pathways; Zn transports

Introduction

Zinc (Zn) is required for normal growth and development, being an essential nutrient for nearly all organisms, and is most notably involved as an important component of over 300 enzymes and 1000 transcription factors [1, 2-4]. Thus, zinc serves as a catalytic or structural cofactor in several enzyme systems as a component of metalloenzymes that are involved in many physiological and metabolic pathways including carbohydrate and protein metabolism [4, 5], and especially lipid metabolism [6-9]. As Zn has been demonstrated to be involved in lipid metabolism of mammals, the effect of dietary Zn supplementation on lipid metabolism has been recently studied in aquatic animals, such as goby *Synechogobius hasta* [10] and yellow catfish *Pelteobagrus fulvidraco* [11, 12].

The AMPK-activated protein kinase (AMPK) is a crucial cellular energy sensor and is composed of a catalytic α-subunit and two regulatory β- and γ-subunits [13]. Upon energy deficiency, AMPK is phosphorylated at Thr^{172} in the catalytic α-subunit by calmodulin-dependent protein kinase kinase-β (CaMKKβ) [13]. Activated AMPK leads to a concomitant activation of ATP-producing catabolic pathways such as fatty acid oxidation and glycolysis, and inhibition of energy-consuming biosynthetic pathways like protein, glycogen and fatty acid synthesis [14]. Thus, AMPK not only restrains lipid biosynthesis pathways, but also activates fatty acid oxidation-related genes to maintain energy *in vivo* [15]. A previous study demonstrated that dietary Zn had a potent effect in lowering levels of hepatic lipids by up-regulating the activity of AMPK [12]. *In vivo* and *in vitro* studies have demonstrated that Zn plays an essential protective role in the regulation of lipid accumulation and metabolism in fish [12]. However, in contrast there are no studies on the relationship between Zn and lipid metabolism in shrimp.

Litopenaeus vannamei is an important cultured shrimp worldwide, accounting for 80 % of total penaeid shrimp production due to its great economic value and rapid growth rate [1]. Nutrition and feeding of *L. vannamei* under semi-intensive or intensive conditions have received a great deal of attention. Studies on zinc nutrition in various crustaceans have mainly focused on requirement levels and/or immunity [1]. *L. vannamei* required 33 mg Zn kg^{-1} diet to maintain normal tissue mineralization and growth [16]. A similar requirement value of 32-34 mg Zn kg^{-1} based on weight gain and whole-body zinc retention was reported for grass shrimp *P. monodon* [17]. However, until now, the regulation of lipid metabolism by dietary Zn has not been explored in *L. vannamei*. Therefore, the aim of the present study was to determine the relationship between Zn and lipid

metabolism, including the Zn-induced Ca^{2+} / CaMKKβ / AMPK pathway, to reveal the mechanism of Zn-induced lipolysis in *L. vannamei.*

Methods

Ethics statement

The study was performed in strict accordance with the Standard Operation 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.

Experimental diets

The formulation and proximate composition of the diets are presented in Table 1. Fish meal, soybean meal, poultry meal, soy protein concentrate, krill meal and peanut meal were used as protein sources; fish oil, soybean oil and soy lecithin were used as lipid sources, and wheat flour was used as the carbohydrate source. Five experimental diets were formulated with $ZnSO_4 \cdot H_2O$ (Zn content = 35.5 %; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) as Zn source, with the analyzed values of Zn being 46.4 (basal diet), 77.2, 87.0, 117.1 and 136.8 mg kg^{-1} diet, respectively. All dry ingredients were ground through 80-mesh and weighed according to the formulation. The mineral and vitamin premixes were mixed thoroughly by the progressive enlargement method, and then lipid and distilled water (35%) added. The ingredients were mixed in a Hobart type mixer and cold-extruded pellets produced (F-26, Machine Factory of South China University of Technology, Guangzhou, China) with pellet strands cut into uniform sizes (1.5 mm and 2.5 mm diameter) (G-250, Machine Factory of South China University of Technology, Guangzhou, China). Pellets were heated for 30 min at 90 °C, and then air-dried to approximately 10 % moisture, sealed in vacuum-packed bags and stored at -20 °C until used for the feeding trial.

Table 1 insert here

Shrimp rearing and experimental conditions

Juvenile *L. vannamei* were obtained from Chia-Tai Ningbo Company (Ningbo, China). Prior to start of the feeding trial, the shrimp were reared in cement pools and fed a commercial feed (40 % protein, 8 % lipid; Yue-Hai Aquafeed Corp., Jiaxiang, China) for two weeks to acclimatize to the experimental conditions. 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, and each experimental diet randomly assigned to five replicate tanks. Shrimp were fed three times a day (daily ration of 6-8 % of biomass) at 8:00, 12:00 and 17:00, with the rations in the morning and evening being 70 % of the total given. Shrimp in each tank were weighed every two weeks and the daily ration adjusted accordingly. Dead shrimp were immediately removed, weighed and recorded. All tanks were cleaned daily by siphoning out the waste material and exuviae, and over 70 % of the tank seawater was exchanged daily prior to the morning feed. The seawater in the tanks was provided with continuous aeration through air stones and dissolved oxygen level was not less than 6.0 mg L^{-1}. During the experiment, photoperiod was maintained on a natural cycle, the temperature was 26 to 30 °C, salinity was 23 to 27 g L^{-1}, pH was 7.6 to 7.8, and ammonia nitrogen concentration was lower than 0.05 mg L^{-1}. Salinity, pH, dissolved oxygen and ammonia nitrogen were measured by YSI Proplus (YSI, Yellow Springs, Ohio, USA). The duration of the feeding trial was 8 weeks.

Sample collection

One hundred and twenty juvenile *L. vannamei* were randomly sampled at the beginning of the feeding trial and frozen at -20 °C for analysis of zinc in whole body. At the termination of the experiment, shrimp were fasted for 24 h before sampling. All shrimp from each tank were counted and weighed to determine survival, weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR) and feed intake (FI). Furthermore, body length, whole body and hepatopancreas weight from four shrimp in each tank were taken to calculate condition factor (CF) and hepatosomatic index (HSI). Five shrimp from each tank were used to analyze the Zn concentration in tissues (whole body, hepatopancreas and shell). 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). The supernatant was collected and stored at -80 °C until analysis of hematological characteristics. Hepatopancreas samples were also collected and stored at -80 °C until analysis of lipid metabolism-related parameters and gene expressions.

Proximate composition and mineral concentration analysis

Crude protein, crude lipid, ash and moisture contents of the diets and shrimp tissues (whole body, hepatopancreas and muscle) were analyzed by standard methods of the AOAC [18]. Crude protein (N × 6.25) was determined using the Dumas combustion method with an auto-protein analyzer (FP-528,

Leco, USA). Crude lipid was determined by the ether extraction method using Soxtec (Soxtec System HT6, Tecator, Hoganas, Sweden). Moisture content was determined by drying the samples to a constant weight at 105 °C, and ash content was determined in a muffle furnace at 550 °C for 8 h.

Zinc concentrations in tissues (whole body, hepatopancreas and shell), experimental diets and water were measured using ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometer, PE 2100DV, Perkin Elmer, USA) in Ningbo Institute of Materials Technology and Engineering, Chinese Academy of Sciences (Ningbo, China). The concentration of Zn in seawater ranged from 1.8 μg L^{-1} to 2.0 μg L^{-1} among the five groups fed diets containing different Zn levels.

Hematological and hepatopancreas characteristic analysis

Triacylglycerol (TAG), total cholesterol (CHOL), low-density lipoprotein-cholesterol (LDL-C) and high-density lipoprotein-cholesterol (HDL-C) contents in hemolymph were determined using an automatic chemistry analyzer (Hitachi 7600-110, Tokyo, Japan).

Hepatopancreas samples were homogenized on ice in 9 volumes (w:v) of ice-cold physiological saline 8.9 g mL^{-1} and then centrifuged at 850×g for 10 min at 4 °C. The resultant supernatant was collected and aliquots stored at -80 °C until analysis. TAG, CHOL, LDL-C, HDL-C, lipase (LPS), non-esterified fatty acids (NEFA), adiponectin (ADP), malonaldehyde (MDA), catalase (CAT) and Cu/Zn superoxide dismutase (Cu/Zn SOD) were determined using the relevant diagnostic reagent kits (Nanjing Jiancheng Co., Nanjing, China) according to the manufacturer's instructions.

Total RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from 10-20 mg hepatopancreas with Trizol reagent (TaKaRa, Japan) following the manufacturer’s protocol. RNA was quantified by a NanoDrop spectrophotometer (Thermo Scientific NanoDrop 2000, USA) at 260 and 280 nm, and RNA ratio (A260:A280) between 1.9 and 2.0 was used for further experiments. The isolated RNA quality was electrophoresed on a 1.2 % denatured agarose gel through Molecular Imager® Gel Doc™ XR System (Bio-Rad, USA) to check integrity. cDNA was generated from 1000 ng of DNAase-treated RNA and synthesized using Prime Script™ RT Reagent Kit with gDNA Eraser (perfect real-time) (TaKaRa, Japan) according to the manufacturer's protocol, using Mastercycler nexus GSX1 PCR (Eppendorf, Germany). cDNA was diluted four times using RNA-free water.

The core fragments of all genes were obtained from the National Center for Biotechnology

Information (NCBI) database. β-actin (GenBank accession no. AF300705.2), a housekeeping gene whose expression was found to be unaffected by dietary treatment in the present experiment, was used as an endogenous reference to normalize the template amount. The gene-specific primers used for mRNA quantification by RT-PCR were designed by Primer Premier 5.0 and are shown in Table 2. All primers were synthesized by BGI (The Beijing Genomics Institute, Shenzhen, China). The qPCR was carried out in a quantitative thermal cycler system (Roche, Light cyclern96, Switzerland) using SYBR Green I (Roche, Switzerland). The amplification was performed in a 96-well plate in a 20 μl reaction volume containing 10 μl of 2 × SYBR Green I Master Mix (Roche, Switzerland), 1 μl (each) gene-specific forward and reverse primers (10 μm), 6 μl DEPC-water and 2 μl of 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 (2-, 4-, 8-, 16- 32- and 64-fold dilutions in triplicate) of the 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 95.1 % to 103.6 %. The relative quantification method was used to analyze data with expression levels of target genes calculated using the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen [19].

Table 2 insert here

Calculations

Parameters were calculated as follows:

WG (%) = 100 × [final body weight (g) - initial body weight (g)] / initial body weight (g).

SGR (%/day) = 100 × [Ln (final body weight (g)) - Ln (initial body weight (g))] /days.

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

FCR = feed consumption (g, dry weight) / [final body weight (g) - initial body weight (g)]

FI (%/body weight day) =100 × feed consumption (g, dry weight) / [(initial body weight (g) + final body weight (g)) / 2] / days

HSI (%) = 100 × [hepatopancreas wet weight (g)] / [body wet weight (g)].

Condition factor (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})).

Statistical analysis

Results are presented as mean values with standard error (S.E.M.). Data were checked for normality and homogeneity of variances, and were normalized when appropriate. Proportional data were arcsine square root transformed before statistical analyses. Mean values were compared through one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test. The level of significance was set at $P < 0.05$. All statistical analyses were conducted using the SPSS 20.0 software package (IBM Crop., USA) for Windows.

Results

Growth performance and morphometric index

Survival ranged from 92 % to 94.7 %, and there were no significant differences among treatments (Table 3). Shrimp fed the diet containing 117.1 mg kg^{-1} Zn had higher WG and SGR than those fed the other diets ($P < 0.05$). The lowest FI and FCR were also observed in shrimp fed the diet supplemented with 117.1 mg kg^{-1} Zn. Moreover, shrimp fed the diet containing 46.4 mg kg^{-1} Zn had lower condition factor (CF) than those fed the other diets ($P < 0.05$). The hepatosomatic index was not significantly influenced by dietary Zn level.

Table 3 insert here

Zn concentration in tissues and Zn deposition rate

Zn concentration in tissues and Zn deposition rate of *L. vannamei* are presented in Table 4. Zn concentration in whole body was not significantly affected by dietary Zn level. Shrimp fed the diets containing 117.1 mg kg^{-1} and 136.8 mg kg^{-1} Zn had the highest Zn concentrations in hepatopancreas and shell among the treatments, and the lowest Zn concentrations in hepatopancreas and shell occurred in shrimp fed the basal diet (46.4 mg kg^{-1} Zn). The deposition rate of Zn significantly decreased as dietary Zn level increased from 46.4 mg kg^{-1} to 136.8 mg kg^{-1}.

Table 4 insert here

Proximate compositions of tissues

Moisture, protein, lipid and ash contents of muscle were not significantly influenced by dietary Zn level (Table 5). Shrimp fed the basal diet (46.4 mg kg^{-1} Zn) had lower ash content in whole body than those fed the other diets ($P < 0.05$), however, there were no significant differences of moisture,

protein and lipid contents in whole body among all treatments. Lipid content in hepatopancreas significantly decreased as dietary Zn level increased from 46.4 mg kg^{-1} to 136.8 mg kg^{-1}, whereas, moisture, protein and ash contents in hepatopancreas were not significantly affected by dietary Zn level.

Table 5 insert here

Hematological metabolites and enzyme activity

Hematological metabolites related to lipid metabolism and health indicators are shown in Table 6. Hemolymph CHOL and HDL-C concentrations were significantly influenced by dietary Zn level ($P < 0.05$). Shrimp fed the diet containing 87.0 mg kg^{-1} Zn had the lowest CHOL and the highest HDL-C in hemolymph among all treatments. However, LDL-C and TAG concentrations and lipase (LPS) activity in hemolymph were not significantly affected by dietary Zn level. In addition, shrimp fed the basal diet (46.4 mg kg^{-1} Zn) had the lowest Cu/Zn SOD and ceruloplasmin (CP) in hemolymph.

Table 6 insert here

Hepatopancreas biochemical parameters

TAG, CHOL, HDL-C, MDA, non-esterified free fatty acids (NEFA), adiponectin (ADP) concentrations and LPS, Cu/Zn SOD activities in hepatopancreas were significantly influenced by dietary Zn level (Table 7). Shrimp fed the basal diet (46.4 mg kg^{-1} Zn) had the highest CHOL, TAG, HDL-C and NEFA concentrations in hepatopancreas among all treatments. In addition, shrimp fed the diet containing 136.8 mg kg^{-1} Zn had lower HDL-C and NEFA concentrations in hepatopancreas than those fed the basal diet ($P < 0.05$). Shrimp fed the diet supplemented with 117.1 mg kg^{-1} Zn had the highest ADP concentration in hepatopancreas. LPS activity in hepatopancreas significantly higher in Zn inclusion groups compared with the basal group ($P < 0.05$), but LDL-C concentration in hepatopancreas was not affected by dietary Zn level. Shrimp fed the basal diet had the highest MDA concentration and the lowest Cu/Zn SOD activity in hepatopancreas, but CAT activity in hepatopancreas was not affected by dietary Zn level.

Table 7 insert here

Gene expression

Transcript level of genes involved in lipid and energy metabolism in hepatopancreas are shown in Figs 1 and 2. Transcript levels of *adipor*, *ob-rb* and *camkkβ* were significantly up-regulated with

increasing dietary zinc level, and shrimp fed the basal diet (46.4 mg kg^{-1} Zn) had the lowest expression levels of *adipor*, *or-rb* and *camkkβ* in hepatopancreas. In contrast, the opposite was the case for hepatopancreas sterol regulatory element-binding protein (*srebp*) mRNA level, with expression level of *srebp* being significantly down-regulated in shrimp fed the diet containing 77.2 mg kg^{-1}, 87.0 mg kg^{-1}, 117.1 mg kg^{-1} and 136.8 mg kg^{-1} Zn (Fig. 1).

Hepatopancreas transcript level of key enzymes involved in lipogenesis (*fas*, *scd1*), β-oxidation (carnitine palmitoyl transferase 1, *cpt1*) and lipolysis (*cd36*) were significantly affected by dietary Zn level (Fig. 1). The expression levels of *fas* and *scd1* were significantly down-regulated as dietary Zn level increased from 46.4 mg kg^{-1} to 136.8 mg kg^{-1}, with no significant differences in expression of *fas* and *scd1* were observed in shrimp fed the diets containing 87.0 mg kg^{-1}, 117.1 mg kg^{-1} and 136.8 mg kg^{-1} Zn. However, the expression levels of *cd36* and *cpt1* were significantly up-regulated with increasing dietary Zn level, and shrimp fed the basal diet had lower expression of *cd36* and *cpt1* in hepatopancreas.

Dietary Zn level also affected expression of genes involved in energy metabolism (Fig. 2). The expression levels of *ampk* and *mcd* in hepatopancreas were significantly up-regulated as dietary Zn level increased from 46.4 mg kg^{-1} to 77.2 mg kg^{-1}, whereas there were no significant differences in expression of *ampk* and *mcd* when dietary zinc level exceeded 87.0 mg kg^{-1}. Shrimp fed the diets containing 46.4 mg kg^{-1} and 77.2 mg kg^{-1} Zn had higher expression of *acc1* than those fed the other diets, while no significant difference in expression of *acc1* was observed in shrimp fed the diets with Zn above 87.0 mg kg^{-1}.

The mRNA expression of genes involved in the SLC39 family was shown in Fig. 3. The mRNA expression levels of *zip3*, *zip9*, *zip11* and *zip14* in hepatopancreas were significantly up-regulated as dietary Zn level increased. Shrimp fed the basal diet had lowest expression levels of *zip3*, *zip9*, *zip11* and *zip14*.

Fig 1-3 insert here

Discussion

The dietary requirement for Zn has been quantified for a variety of marine shrimp fed semi-purified or commercial diets. The requirement of Zn was demonstrated to be substantially higher when fed with practical diets compared with purified diets [20-22]. In the present study, 117.1 mg kg^{-1} Zn improved growth performance and feed utilization, and higher or lower dietary Zn reduced growth

and feed efficiency of juvenile *L. vannamei*, similar to results reported previously for this shrimp species [16]. Davis and Lawrence [16] reported that weight gain of Pacific white shrimp *Penaeus vannamei* was significantly affected by dietary zinc levels from 18 mg kg^{-1} to 60 mg kg^{-1} in shrimp fed a casein/gelatin-based semi-purified diet. In that study, best growth was obtained in *P. vannamei* fed a diet supplemented with 33 mg kg^{-1} Zn, lower than the 117.1 mg kg^{-1} Zn found in the present study. This may be due to different feed ingredients. The fishmeal and plant protein sources used in diets in the present study may contain anti-nutritional factors (e.g. tri-calcium phosphate from hard tissues and phytic acid from plant meals), which would form complexes with Zn and inhibit zinc bioavailability [23-25]. Thus, higher levels of supplemented Zn were required to overcome the depressed bioavailability caused by the presence of anti-nutritional factors in the commercial diet [16, 26].

In addition to growth indicators, tissue Zn deposition is also used to evaluate the Zn nutritional status of animals [27]. Jeng and Sun [28] demonstrated that zinc firstly accumulated in the digestive tract, followed by skeletal tissue, and then skin and muscle in common carp *cyprinus carpio*, indicating that zinc in these tissues were useful indices for evaluating zinc status. In the present study, incremental dietary Zn significantly increased Zn concentrations in hepatopancreas and shell, and did not reach a plateau implying that increasing dietary Zn level promoted Zn deposition in these tissues. These results were in accordance with previous studies on grass shrimp *Penaeus monod*on [17] and juvenile grouper *Epinephelus malabaricus* [29]. Shiau and Jiang [17] reported that Zn concentration in hepatopancreas ranged from 0.6 μg g^{-1} to 21.8 μg g^{-1} when dietary Zn level increased from 7 mg kg^{-1} to 127 mg kg^{-1} in juvenile grass shrimp. Chen *et al* [29] reported that Zn concentrations in vertebra and scale significantly increased with increasing dietary Zn level in juvenile grouper. However, no differences were found in whole body Zn concentrations in this study, suggesting that this was not a sensitive indicator for evaluating Zn status in *L. vannamei*. While Zn involved bone metabolism by stimulating collagen synthesis to increase calcium content in bone [30], invertebrates mainly deposit minerals in exoskeleton [29]. Therefore, the higher ash content in whole body of shrimp fed the diet supplemented with Zn might be due to the increased calcium and Zn in shell.

Zinc plays an important role in enhancing antioxidant status and regulating lipid metabolism [5]. In the present study, clinical hematological parameters demonstrated that dietary Zn could improve lipolysis and antioxidant responses by decreasing hematological CHOL and increasing CP and

Cu/Zn SOD. In addition, the lipid content of hepatopancreas was significantly reduced by increased dietary Zn level. Similar results were reported in previous studies, in which waterborne Zn exposure decreased anterior intestine TG content in juvenile goby *synechogobius hasta*, which was considered to be due to down-regulation of lipogenesis and increased lipolysis [31]. Similarly, dietary Zn of around 156 mg kg^{-1} reduced lipid content in liver and muscle, and increased activities of CPT1 and lipoprotein lipase of liver in yellow catfish *Pelteobagrus fulvidraco* [11]. Furthermore, Wei *et al* [12] reported that zinc reduced hepatic lipid deposition and activated lipophagy via Zn^{2+}/MTF-1/PPAα and Ca^{2+}/CaMKKβ/AMPK signaling pathways. In contrast, 8-week chronic Zn exposure induced lipid accumulation in yellow catfish *Pelteobagrus fulvidraco* [32]. Zheng *et al* [33] also reported that dietary Zn at 155 mg kg^{-1} induced Zn accumulation in liver, muscle and whole body of yellow catfish *Pelteobagrus fulvidraco*. Thus, contradictory results on the influence of dietary zinc on lipid metabolism have been reported in different fish species, which may reflect differences due to species, trial duration or other experimental conditions. In the present study, some hepatic lipid metabolism-related parameters were determined to confirm the relationship between Zn and lipid metabolism. Triglycerides are major constituents of body lipid in humans and other animals, and triglyceride content can reflect the state of lipid metabolism [31]. Cholesterol is an essential structural component of animal cell membranes, and excessive accumulation of cholesterol in cells results in disorders of lipid metabolism [34]. Fatty acids can be broken down to produce energy by lipase catalyzes the hydrolysis of TG into glycerol and free fatty acids (FFA) [35, 36], then adiponectin breakdown free fatty acids [37]. A pharmacological study showed that administration of adiponectin to mice resulted in lower body weight associated with a reduction in free fatty acids and triglycerides [38]. The results of the present study showed that dietary Zn supplementation reduced hepatopancreas CHOL, HDL-C, TAG and NEFA, and increased ADP content and LPS activity, suggesting that Zn supplementation could accelerate lipid breakdown by hydrolyzing triglycerides through lipase activity to release free fatty acids, simultaneously increasing the content of adiponectin and promoting fatty acids oxidation, with the final manifestation being reduced lipid content of the hepatopancreas.

Many studies have demonstrated that the antioxidant defense system is associated with the physical health status of animals by inhibiting oxidation and removing excess reactive oxygen species (ROS) [39-41]. Two of the essential antioxidant enzymes are catalase and ceruloplasmin. Catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen, protecting the cell from oxidative

damage [42], and ceruloplasmin can serve as a scavenger of superoxide radicals [43]. ROS can degrade polyunsaturated lipids forming malondialdehyde, a marker for lipid oxidative stress [44]. In addition, zinc is an active central ion for Cu/Zn superoxide dismutase, which converts highly reactive superoxide anion radical (O_2^-) to less reactive hydrogen peroxide (H_2O_2) [45]. In the present study, shrimp fed the basal diet had the lowest Cu/Zn SOD activity and the highest MDA content in hepatopancreas, indicating that dietary Zn supplementation could prevent the accumulation of free radicals and improve antioxidant activities in hepatopancreas of juvenile *L. vannamei*.

AMPK is one of the most well-recognized modulators of the guardians in lipid homeostasis [46, 47]. It can be activated by calmodulin-dependent protein kinase (CaMKK) [48, 49] in response to increased intracellular Ca^{2+}. Activated Ca^{2+}/CaMKKβ phosphorylates the subunit of AMPK and forms a multimeric protein complex comprising (Ca^{2+}/CaMKKβ)/AMPK [50, 51]. In this process, Zn^{2+} is a significant factor in regulating cytosolic Ca^{2+} homeostasis, which is mediated by triggering sensitive Ca^{2+} pumps in the endoplasmic reticulum [12, 52]. While, Zn^{2+} absorption and homeostasis depend on ZIP transporters (known as SLC39 family), which promote uptake of extracellular Zn^{2+} and release of vesicular Zn^{2+} into the cytosol, eventually leading to increased free Zn^{2+} in the cytoplasm [53, 54]. At the whole-body level, AMPK is regulated by a diverse range of hormones, including leptin [55] and adiponectin [56]. Yamauchi *et al* [57] reported that the adiponectin receptor (AdipoR) serves as the receptor for full-length adiponectin and activates AMPK. Iwabu *et al* [58] showed that adiponectin induces extracellular Ca^{2+} influx by adiponectin receptor, which was necessary for subsequent activation of Ca^{2+}/ CaMKKβ and AMPK.

Under the condition of low energy, AMPK phosphorylates specific enzymes to increase ATP generation and decrease ATP consumption which by accelerating the decomposition of fatty acids and inhibiting the synthesis of fatty acids [59]. AMPK inhibits fatty acid synthesis by inducing the inhibitory phosphorylation of acetyl-CoA carboxylase (ACC) and sterol regulatory element-binding protein (SREBP) [46]. SREBP is a family of transcription factors that regulate the expression of enzymes required for endogenous fatty acid synthesis, affecting multiple genes such as ACC1, FAS and SCD1[60-63]. AMPK is also associated with activates lipid catabolism. *In vivo*, AMPK activated CD36 (also known as fatty acid translocase), which increases cellular fatty acid uptake [64]. Once inside cells, fatty acids are transported into the mitochondria interior by carnitine palmitoyl transferase-1(CPT1) for β-oxidation and accompanied with the production of large amounts of ATP [46, 65].

AMPK also directly involved in energy metabolism by increasing intracellular acetyl-CoA content. Bergeron *et al* [66] reported that decreased malonyl-CoA occurs as a result of the action of malonyl-CoA decarboxylase (MCD), which prevents acetyl-CoA conversion to malonyl-CoA, which may be regulated by AMPK. In the present study, mRNA expression levels of the SLC39 family such as *zip3*, *zip9*, *zip11* and *zip14* were significantly higher in shrimp fed dietary Zn supplementation compared with the basal group, indicating that more Zn^{2+} was transferred into the cell. However, intracellular Zn^{2+} promotes the release of Ca^{2+} from the endoplasmic reticulum, followed by Ca^{2+} / CaMKKβ / AMPK singling pathway induced lipophagy, which was confirmed by increased mRNA levels of *camkkβ* and *ampk*. Then, activated *ampk* inhibited the transcription factor *srebp* binding with *fas*, *acc1* and *scd1*, which ultimately led to decreased expression of these genes. In the meantime, activated *ampk* promoted the expression of *cd36* and *cpt1*, enhancing fatty acid oxidation to produce ATP. Finally, *ampk* inhibited the expression of *acc1* and promoted the expression of *mcd*, increasing the content of intracellular acetyl-CoA that, in turn, might affect energy metabolism (Fig.4.).

Conclusion

In the present study, dietary Zn level affected growth performance, Zn deposition in tissues (hepatopancreas and shell), and lipid metabolites, and we identified a novel mechanism of Zn-induced lipolysis. Dietary deficient or excessive Zn retarded growth and reduced feed utilization. Furthermore, incremental dietary Zn levels reduced total lipid, non-esterified free fatty acids, total cholesterol and triglyceride levels in hepatopancreas. Importantly, dietary Zn-induced lipolysis was dependent on the activation of zinc transporters (SLC39 family) and involved up-regulation of the Ca^{2+} / CaMKKβ / AMPK pathway. Further studies on the relationship between dietary Zn and energy products, cytokines, adipokines, and receptors are required to fully explain the role of Zn in energy metabolism. Modulation of Zn status may become a new target for the prevention and treatment of metabolic disorders. Deeper knowledge and understanding of the physiological functions of Zn transporters and the ability to control their activity may be an important factor in the mechanism of coordinated lipid metabolism in hepatopancreas or liver mediated by Zn-induced lipophagy.

Acknowledgments

This study was supported by National Key R & D Program of China (2018YFD0900400), China Agriculture Research System (CARS-48), Nature Science Foundation of Zhejiang Province (LY17C190002), Key Research Program of Zhejiang Province of China (2018C02037), Zhejiang Aquaculture Nutrition & Feed Technology Service Team (ZJANFTST2017-2). This research was also sponsored by the K. C. Wong Magna Fund in Ningbo University. The authors are grateful to Lihui Zhu for helping with chemical analysis.

Conflict of Interest

The authors declared that there were no conflicts of interest.

Authorship

B.S. formulated the research question, designed the study, carried out the study, analyzed the data and wrote the article. M.J. designed the study, assisted in the correction and developed the questions. M.B.B. developed the questions, and revised the manuscript. D.R.T. assisted in developing the research questions and revising the manuscript. Q.Z. formulated the research question, designed the study, and revised the manuscript. All the authors read and approved the final version of the manuscript.

References

1. National Research Council (2011) *Nutrient requirements of Fish and Shrimp*. Washington DC: The National Academies Press. pp. 176-179.
2. Cherasse Y & Urade Y (2017) Dietary zinc acts as a sleep modulator. *Int J Mol Sci* **18**, 2334.
3. King JC, Brown KH, Gibson RS *et al*. (2016) Biomarkers of nutrition for development (bond)-zinc review. *J Nutr* **146**, 858-885.
4. Kambe T, Tsuji T, Hashimoto A, *et al*. (2015) The physiological, biochemical, and molecular roles of zinc transporters in zinc homeostasis and metabolism. *Physiol Rev* **95**, 749-784.
5. Olechnowicz J, Tinkov A, Skalny A, *et al*. (2018) Zinc status is associated with inflammation, oxidative stress, lipid, and glucose metabolism. *J Physiol Sci* **68**, 19-31.
6. Dieck HT, Frank Döring, Roth HP, *et al*. (2003) Changes in rat hepatic gene expression in response to zinc deficiency as assessed by DNA arrays. *J Nutr* **133**, 1004-1010.
7. Li X, Guan Y, Shi X, *et al*. (2013) Effects of high zinc levels on the lipid synthesis in rat hepatocytes. *Biol Trace Elem Res* **154**, 97-102.
8. Ranasinghe P, Wathurapatha WS, Ishara MH, *et al*. (2015) Effects of zinc supplementation on serum lipids: a systematic review and meta-analysis. *Nutr Metab* **12**, 1-16.
9. Tayeb W, Nakbi A, Cheraief I, *et al*. (2013) Alteration of lipid status and lipid metabolism, induction of oxidative stress and lipid peroxidation by 2, 4-dichlorophenoxyacetic herbicide in rat liver. *Toxicol Mech Methods* **23**, 449-458.
10. Zheng JL, Luo Z, Chen QL, *et al*. (2011) Effect of waterborne zinc exposure on metal accumulation, enzymatic activities and histology of *Synechogobius Hasta*. *Ecotoxicol Environ Saf* **74**, 1864-1873.
11. Zheng JL, Luo Z, Hu W, *et al*. (2015) Different effects of dietary Zn deficiency and excess on lipid metabolism in yellow catfish *Pelteobagrus Fulvidraco*. *Aquaculture* **435**, 10-17.
12. Wei CC, Luo Z, Hogstrand C, *et al*. (2018) Zinc reduces hepatic lipid deposition and activates lipophagy via Zn^{2+}/MTF-1/PPARα and Ca^{2+}/CAMKKβ/AMPK pathways. *FASEB J* **32**, fj.201800463.
13. Carling D (2004) The AMP-activated protein kinase cascade-a unifying system for energy control. *Trends Biochem Sci* **29**, 18-24.
14. Hardie DG, Ross FA & Hawley SA (2012) AMPK: a nutrient and energy sensor that maintains

energy homeostasis. *Nat Rev Mol Cell Biol* **13**, 251-262.

15. Angin Y, Beauloye C, Horman S, *et al*. (2016) Regulation of carbohydrate metabolism, lipid metabolism, and protein metabolism by AMPK. *Springer International Publishing Switzerland* **10**7, 23-43.
16. Davis DA, Lawrence AL & Gatlin D (1993) Evaluation of the dietary zinc requirement of *Penaeus vannamei* and effects of phytic acid on zinc and phosphorus bioavailability. *J World Aquac Soc* **24**, 40-47.
17. Shiau SY & Jiang LC (2006) Dietary zinc requirements of grass shrimp, *Penaeus monodon*, and effects on immune responses. *Aquaculture* **254**, 470-482.
18. Association of Official Analytical Chemists (2000) *Official Methods of analysis*. Washington, DC: AOAC.
19. Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402-408.
20. Delbert M, Gatlin III & Wilson RP (1984). Zinc supplementation of practical channel catfish diets. *Aquaculture* **41**, 31-36.
21. Hardy RW & Shearer KD (1985) Effect of dietary calcium phosphate and zinc supplementation on whole body zinc concentration of rainbow trout (*Salmo gairdneri*). *Can J Fish Aquat Sci* **42**, 181-184.
22. Satoh S, Tabata K, Izume K, *et al*. (1987) Effect of dietary tri-calcium phosphate on availability of zinc to rainbow trout (*Salmo gairdnerii*). *Nippon Suisan Gakkaishi* **53**, 1199-2005.
23. Apines MJ, Satoh S, Kiron V, *et al*. (2003) Availability of supplemental amino acid-chelated trace elements in diets containing tricalcium phosphate and phytate to rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* **225**, 431-444.
24. Richardson NL, Higgs DA, Beames RM, *et al*. (1985) Influence of dietary calcium, phosphorus, zinc and sodium phytate level on cataract incidence, growth and histopathology in juvenile chinook salmon (*Oncorhynchus tshawytscha*). *J Nutr* **115**, 553-567.
25. Satoh S, Tabata K, Izume K, *et al*. (1987) Effect of dietary tricalcium phosphate on availability of zinc to rainbow trout (*Salmo gairdneri*). *Bull Jpn Soc Sci Fish* **53**, 1199-1205.
26. Gatlin DM & Phillips HF (1989) Dietary calcium, phytate and zinc interactions in channel catfish. *Aquaculture* **79**, 259-266.
27. Forbes RM, Parker HM & Erdman JW (1984) Effects of dietary phytate, calcium and

magnesium levels on zinc bioavailability to rats. *J Nutr* **114**, 1421-1425.

28. Jeng SS & Sun LT (1981) Effects of dietary zinc levels on zinc concentrations in tissues of common carp. *J Nutr* **111**, 134-140.
29. Chen HY, Cheng YC, Hu LC *et al*. (2014) Dietary zinc requirements of juvenile grouper, *Epinephelus Malabaricus*, *Aquaculture* **432**, 360-364.
30. Oner G, Bhaumick B & Bala RM (1984) Effect of zinc deficiency on serum somatomedin levels and skeletal growth in young rats. *Endocrinology* **114**, 1860–1863.
31. Ling SC, Luo Z, Chen GH, *et al*. (2017) Waterborne Zn influenced Zn uptake and lipid metabolism in two intestinal regions of juvenile goby *synechogobius hasta*. *Ecotoxicol Environ Saf* **148**, 578-584.
32. Zheng JL, Luo Z, Liu CX, *et al*. (2013) Differential effects of acute and chronic zinc (Zn) exposure on hepatic lipid deposition and metabolism in yellow catfish *Pelteobagrus fulvidraco*. *Aquat Toxicol* **132-133**, 173-181.
33. Zheng JL, Luo Z, Wei HU, *et al*. (2015) Differential effects of dietary Zn deficiency and excess on carnitine status, kinetics and expression of CPT1 in yellow catfish *Pelteobagrus fulvidraco*. *Aquaculture* **188**, 24-30.
34. Maxfield FR & Tabas I (2005) Role of cholesterol and lipid organization in disease. *Nature* **438**, 612-621.
35. Svendsen A (2000) Lipase protein engineering. *Biochim Biophys Acta* **1543**, 223-228.
36. Zechner R, Strauss JG, Haemmerle G, *et al*. (2005) Lipolysis: pathway under construction. *Curr Opin Lipidol* **16**, 333-340.
37. Diez J & Iglesias P (2003) The role of the novel adipocyte-derived hormone adiponectin in human disease. *Eur J Endocrinol* **148**, 293-300.
38. Fruebis J, Tsao TS, Javorschi S, *et al*. (2001) Proteolitic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *PNAS* **98**, 2005-2010.
39. Li XM, Ma YL & Liu XJ (2007) Effect of the Lycium barbarum polysaccharides on agerelated oxidative stress in aged mice. *J Ethnopharmacol* **111**, 504-511.
40. Dawood MAO, Koshio S, Ishikawa M, *et al*. (2015) Effects of heat killed Lactobacillus plantarum (LP20) supplemental diets on growth performance, stress resistance and immune response of red sea bream, *Pagrus major*. *Aquaculture* **442**, 29-36.

41. Ding Z, Zhang Y, Ye J, *et al*. (2015) An evaluation of replacing fish meal with fermented soybean meal in the diet of Macrobrachium nipponense: growth, nonspecific immunity, and resistance to Aeromonas hydrophila. *Fish Shellfish Immunol* **44**, 295-301.
42. Chelikani P, Fita I & Loewen PC (2004) Diversity of structures and properties among catalases. *Cell Mol Life Sci* **61**,192-208.
43. Goldstein IM, Kaplan HB, Edelson HS, *et al*. (1979) Ceruloplasmin: A scavenger of superoxide anion radicals. *J Biol Chem* **254**, 4040-4045.
44. Lushchak VI (2011) Environmentally induced oxidative stress in aquatic animals. *Aquat Toxicol* **101**, 0-30.
45. Li HT, Jiao M, Chen J, *et al*. (2010) Roles of zinc and copper in modulating the oxidative refolding of bovine copper, zinc superoxide dismutase. *Acta Biochim Biophys Sin* **42**, 183-194.
46. Jeon & Sang-Min (2016) Regulation and function of AMPK in physiology and diseases. *Exp Mol Med* **48**, e245.
47. Carling & David (2017) AMPK signalling in health and disease. *Curr Opin Cell Biol* **45**, 31-37.
48. Hurley RL, Anderson KA, Franzone JM, *et al*. (2005) The Ca^{2+}/Calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases. *J Biol Chem* **280**, 29060-29066.
49. Woods A, Dickerson K, Heath R, *et al*. (2005) Ca^{2+}/calmodulin-dependent protein kinase kinase-β acts upstream of amp-activated protein kinase in mammalian cells. *Cell Metab* **2**, 21-33.
50. Green MF, Anderson KA & Means AR (2011) Characterization of the CAMKKβ-AMPK signaling complex. *Cell Signal* **23**, 2005-2012.
51. Zhao F, Huang W, Zhang Z, *et al*. (2016) The CAMKKβ-AMPK signaling pathway in prostate cancer cells. *Oncotarget* **7**, 5366-5382.
52. Sensi SL, Yin HZ, Carriedo SG, *et al*. (1999) Preferential Zn^{2+} influx through Ca^{2+}-permeable AMPK/kainate channels triggers prolonged mitochondrial superoxide production. *Proc Natl Acad Sci USA* **96**, 2414-2419.
53. Jeong J & Eide DJ (2013) The SLC39 family of zinc transporters. *Mol Aspects Med* **34**, 612-619.
54. Ntgen S & Monika (2014) The families of zinc (SLC30 and SLC39) and copper (SLC31) transporters. *Curr Top Membr* **73**, 321-355.
55. Minokoshi Y, Kim YB, Peroni OD, *et al*. (2002) Leptin stimulates fatty-acid oxidation by

activating AMP-activated protein kinase. *Nature* **415**, 339-343.

56. Yamauchi T, Kamon J, Minokoshi Y, *et al*. (2002) Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* **8**, 1288-1295.
57. Yamauchi T, Kamon J, Ito Y, *et al*. (2003) Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* **423**, 762-769
58. Iwabu M, Yamauchi T, Okadaiwabu M, *et al*. (2010) Adiponectin and AdiporR1 regulate PGC-1alpha and mitochondria by Ca $^{(2+)}$ and AMPK / SIRT1. *Nature* **464**, 1313-1319.
59. Herzig Sébastien & Shaw RJ (2017) Ampk: guardian of metabolism and mitochondrial homeostasis. *Nat Rev Mol Cell Biol* **19**, 121-135.
60. Zhang JJ, Hao JJ, Zhang YR, *et al*. (2017) Zinc mediates the SREBP-SCD axis to regulate lipid metabolism in Caenorhabditis elegans. *J Lipid Res* **58**, 1845-1854.
61. Zhao Y, Li H, Zhang Y, *et al*. (2016) Oncoprotein HBXIP modulates abnormal lipid metabolism and growth of breast cancer cells by activating the LXRs/SREBP-1c/FAS signaling cascade. *Cancer Res* **76**, 4696-4707.
62. Hao J, Zhu L, Zhao S, *et al*. (2011) PTEN ameliorates high glucose-induced lipid deposits through regulating SREBP-1/FASN/ACC pathway in renal proximal tubular cells. *Exp Cell Res* **317**, 1629-1639.
63. Xu H, Wang J, Chang Y, *et al*. (2014) Fucoidan from the sea cucumber *Acaudina molpadioides* exhibits anti-adipogenic activity by modulating the WNT/β-catenin pathway and down-regulating the SREBP-1c expression. *Food Funct* **5**, 1547-1555.
64. Habets DDJ, Coumans WA, Hasnaoui ME, *et al*. (2009) Crucial role for LKB1 to AMPKα2 axis in the regulation of CD36-mediated long-chain fatty acid uptake into cardiomyocytes. *Acta Biochim Biophys* **1791**, 212-219.
65. Townsend KL, An D, Lynes MD, *et al*. (2013) Increased mitochondrial activity in BMP7-treated brown adipocytes, due to increased CPT1- and CD36-mediated fatty acid uptake. *Antioxid Redox Signal* **19**, 243-257.
66. Bergeron R, Ren JM, Cadman KS *et al*. (2001) Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. *Am J Physiol Endocrinol Metab* **281**, 1340-1346.

Figure legends

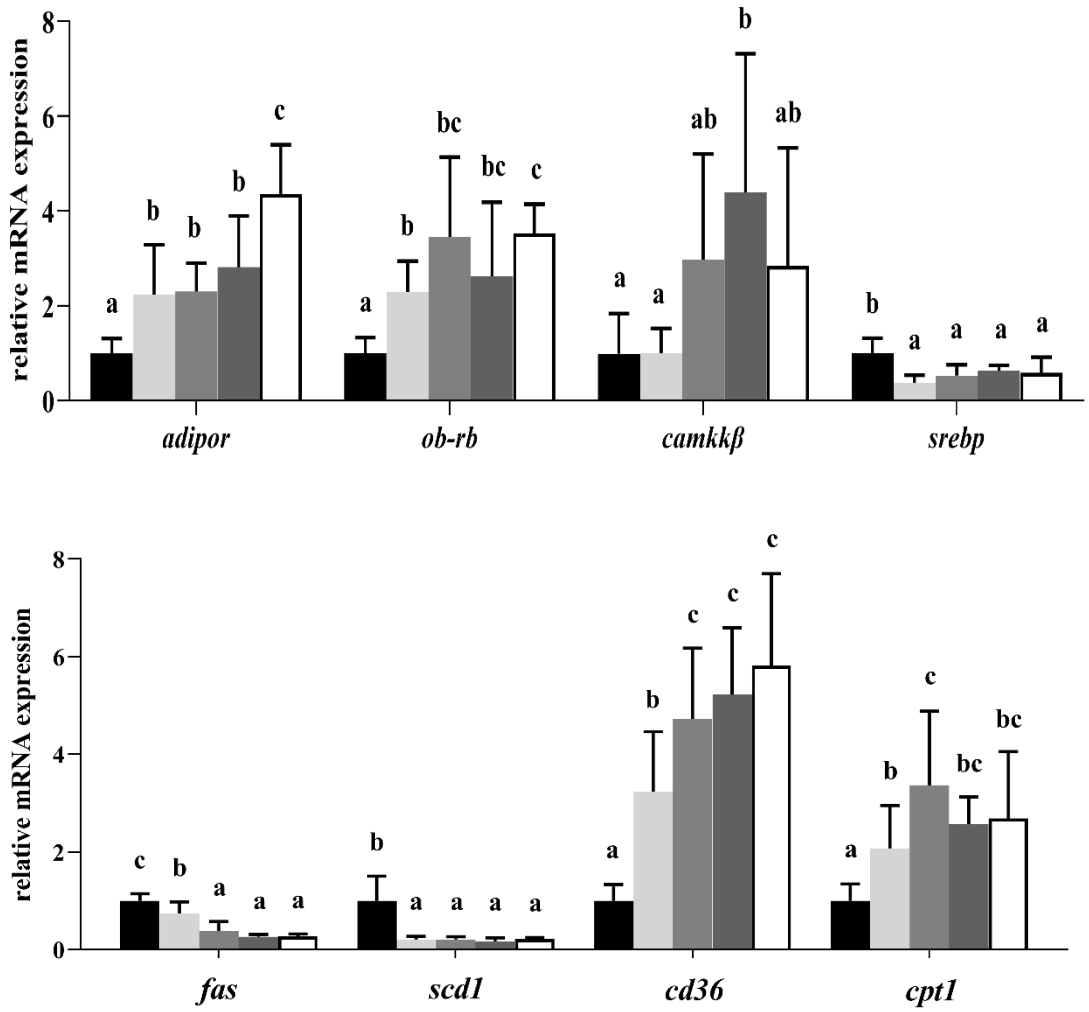

Fig. 1. mRNA levels of genes involved in lipid metabolism in the hepatopancreas of juvenile *L.vannamei* fed the experimental diets. ■ , 46.4mg kg^{-1} Zn diet; ■ , 77.2mg kg^{-1} Zn diet; ■ ,87.0 mg kg^{-1} Zn diet; ■, 117.1 mg kg^{-1} Zn diet; □, 136.8 mg kg^{-1} Zn diet. Expression values are normalised by *β-actin*-expressed transcripts. Relative fold difference among treatments are presented as means (n = 8), with their standard error. Significant differences at $P < 0.05$ (one-way ANOVA).

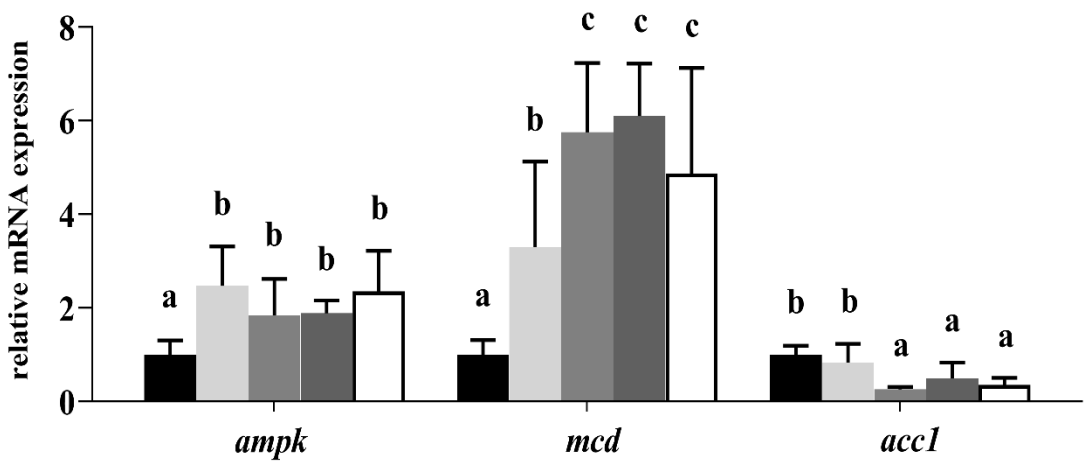

Fig. 2. mRNA levels of genes involved in energy metabolism in the hepatopancreas of juvenile *L. vannamei* fed the experimental diets , 46.4mg kg^{-1} Zn die , 77.2mg kg^{-1} Zn d ; ,87.0 mg kg^{-1} Zn et; , 117.1 mg kg^{-1} Zn diet; , 136.8 mg kg^{-1} Zn diet. Expression values are normalized by *β-actin*-expressed transcripts. Relative fold difference among treatments are presented as means (n = 8), with their standard error. Significant differences at $P < 0.05$ (one-way ANOVA).

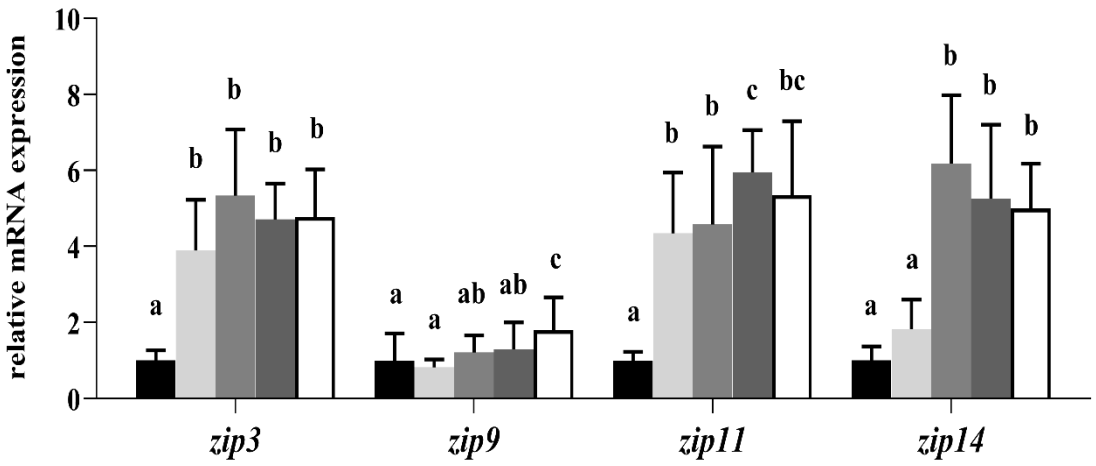

Fig. 3. mRNA levels of genes involved in SLC39 family genes (*zip3, zip9, zip11*, *zip14*) in the hepatopancreas of juvenile *L. vannamei* fed the experimental diets. ■, 46.4mg kg^{-1} Zn diet; ■, 77.2mg kg^{-1} Zn diet; ■ , 87.0 mg kg^{-1} Zn diet; ■ , 117.1 mg kg^{-1} Zn diet; ■ , 136.8 mg kg^{-1} Zn diet. Expression values are normalized by *β-actin*-expressed transcripts. Relative fold difference among treatments are presented as means (n = 8), with their standard error. Significant differences at $P < 0.05$ (one-way ANOVA).

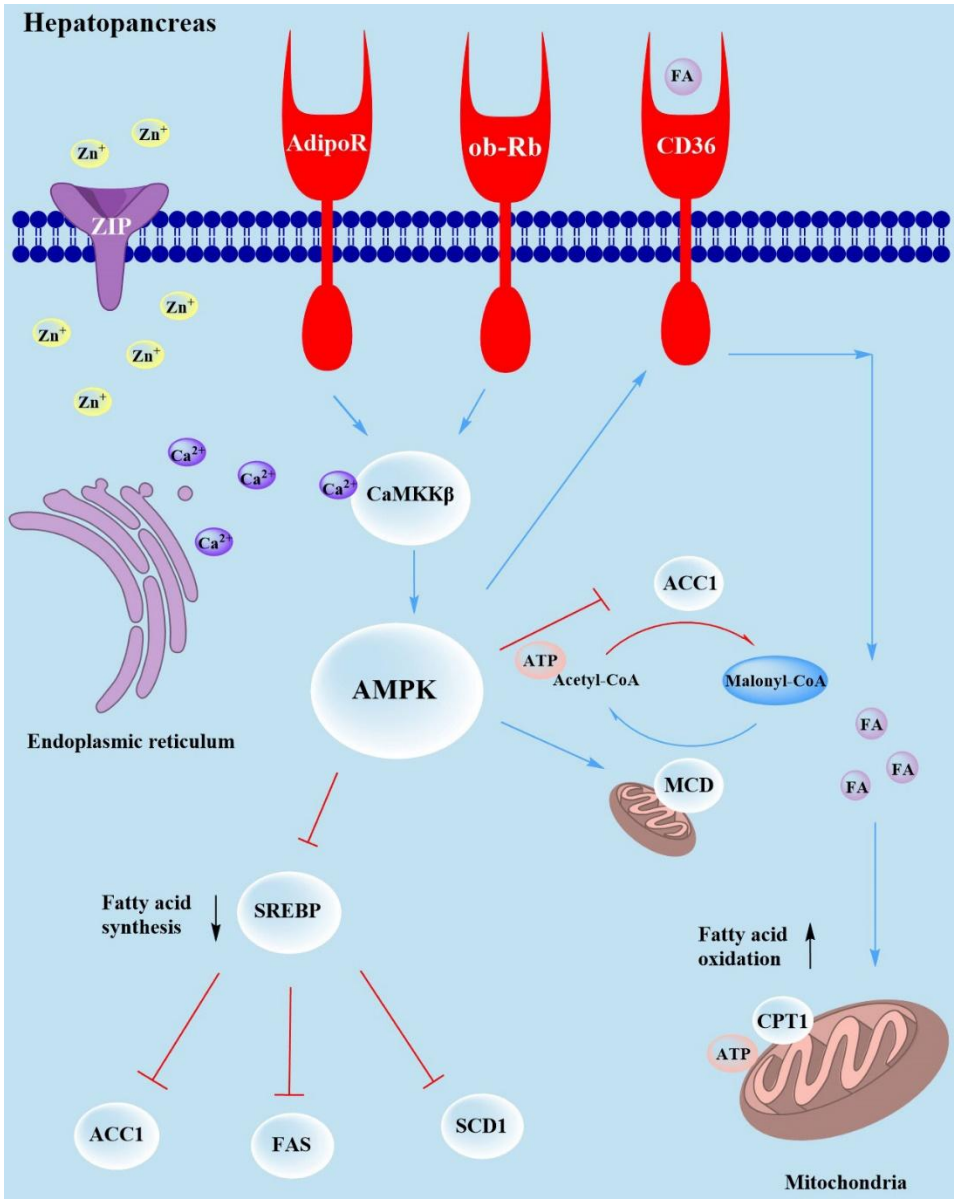

Fig. 4. A working model of how Zn^{2+} regulates lipophagy via the Ca^{2+} / CaMKKβ / AMPK axes. The blue lines indicate promotion and the red lines indicate suppression. Extracellular Zn^{2+} activates SLC39 family genes (*zip3*, *zip9*, *zip11*, *zip14*) which increases intracellular Zn^{2+}, which promotes the release of Ca^{2+} and activation *ampk* via *camkkβ*, reducing the mRNA expression of *fas*, *acc1* and *scd1*, resulting in inhibition of fatty acid synthesis. Meanwhile, *ampk* activates *cd36*, which increase the mRNA expression of *cpt1* and enhancement of fatty acid oxidation. Moreover, activated *ampk* promotes the mRNA expression of *mcd*, which might affect energy metabolism.

Table 1

Formulation and proximate compositions of the experimental diets.

Ingredients (g kg^{-1})	Dietary zinc level (mg kg^{-1})				
	46.4	77.2	87.0	117.1	136.8
Fish meal	200.00	200.00	200.00	200.00	200.00
Soybean protein concentrate	60.00	60.00	60.00	60.00	60.00
Soybean meal	230.00	230.00	230.00	230.00	230.00
Poultry meal	60.00	60.00	60.00	60.00	60.00
Krill meal	30.00	30.00	30.00	30.00	30.00
Peanut meal	50.00	50.00	50.00	50.00	50.00
Wheat flour	286.75	286.75	286.75	286.75	286.75
Fish oil	15.00	15.00	15.00	15.00	15.00
Soybean oil	15.00	15.00	15.00	15.00	15.00
Soy lecithin	20.00	20.00	20.00	20.00	20.00
Mineral premix*	10.00	10.00	10.00	10.00	10.00
Vitamin premix†	5.00	5.00	5.00	5.00	5.00
Ca $(H_2PO_4)_2$	15.00	15.00	15.00	15.00	15.00
Choline chloride	3.00	3.00	3.00	3.00	3.00
Astaxanthin	0.25	0.25	0.25	0.25	0.25
$ZnSO_4 \cdot H_2O$	0.00	0.06	0.11	0.17	0.23
Proximate composition (dry matter %)					
Crude protein	42.85	42.56	43.06	42.78	42.89
Crude lipid	8.22	7.85	8.01	8.92	8.07
Moisture	11.45	12.08	12.05	11.56	11.98
Ash	11.23	11.34	11.28	11.36	11.42
Analyzed zinc (mg kg^{-1})	46.40	77.15	87.00	117.10	136.80

* Mineral premix (g kg^{-1} diet): NaCl, 0.74; K_2SO_4, 2.24; $MgSO_4 \cdot 7H_2O$, 3.58; $FeC_6H_5O_7$, 0.29; $C_6H_{10}CaO_6 \cdot 5H_2O$, 0.51; $MnSO_4 \cdot H_2O$, 0.12; $CuSO_4 \cdot 5H_2O$, 0.16; KIO_3 (1%), 0.02; Na_2SeO_3 (1%), 0.07; $CoSO_4 \cdot 7H_2O$, 0.02; zeolite, 2.25. The mineral premix does not supply Zn.

† Vitamin premix (mg kg^{-1} diet): D-Ca pantothenate, 120; inositol, 200; menadione, 60; nicotinic acid, 100; pyridoxine hydrochloride, 60; riboflavin, 50; thiamin nitrate, 60; all-rac-a-tocopherol, 100; cyanocobalamin, 0.1; biotin, 6.0; folic acid, 10; retinyl acetate, 5000 IU; cholecalciferol, 2000 IU.

Table 2

Real-time quantitative PCR primers for genes related to lipid and energy metabolism and *β-actin* of *L.vannamei*.

Gene	Primers (5′-3′)	Size (bp)	TM (°C)	Accession no.
β-actin	F: CGAGGTATCCTCACCCTGAA R: GTCATCTTCTCGCGGTTAGC	176	58.22 58.80	AF300705.2
srebp	F: ACCATTGCCACTCCCCTA R: GTTGCGTTTCTCGCCTTT	150	57.40 56.67	MG770374.1
fas	F: CGTGACACCCCTTCCTCAC R: GAGAGTGTGAGGAACATAGACCA	196	60.00 59.24	HM595630.1
ampk	F: CTTTGCTGATGCTAATGCCT R: TCCTTTGGGTAGTCCACGAT	187	56.46 58.05	KP272116.1
ob-rb	F: CAACAGACATGGCAGGCATC R: AGAACGGCCACCAGTTATTGT	117	59.55 59.93	XM_027361398.1
adipor	F: TCGCTGTTTGATCGACACC R: TGAGTCGATGAGGGACTCGT	241	58.18 60.04	KT372143.1
cpt1	F: ACTCCCGATAAGCACACC R: TTCATACATCCACCCCCT	139	56.32 54.22	XM_027373671.1
camkkβ	F: GAAACTTCCATTCCACGAT R: TCACCCAGGGATGTTGCTT	182	52.94 58.84	XM_027368225.1
acc1	F: TGCATAGAAACGGCATTGCG R: TTTGACACCTGAGCCAGACC	134	59.90 59.89	XM_027360190.1
scd1	F: TGTCTTACACCTTATCAATGGC R: CGTTCGTATGTTCCTCTTCGTC	154	56.15 59.16	XM_027374708.1
mcd	F: AAGACCACAGGAAGGGACCA R: GACACTTGAGATGCCACCCA	114	60.40 59.96	XM_027376735.1
cd36	F: AACCAAGGTCCTGACCATCAC R: AGGTGAGAGTCGACGAGGAA	210	59.93 59.96	XM_027374896.1
zip3	F: GCAGAATGGGAAGAAGACCCA R: ACCGGGTCTTCGAGGATACA	125	60.00 60.03	XM_027367035.1
zip9	F: TGACTATGTCCGAGGACCGT R: GTCCGATGAGGTTGTGTGGT	201	60.03 59.97	XM_027354650.1
zip11	F: GGTCTGCTGTGGTGTTCCT R: ACAAAAGCCCATTCCCCT	172	59.55 56.25	XM_027361505.1
zip14	F: TGCCAGAGGTGCTGTACTTG R: GCAGACCATAGCCCCAAACT	108	59.96 60.03	XM_027375334.1

F, forward primer; R, reverse primer; *srebp*, sterol regulatory element-binding protein; *fas*, fatty acid synthase; *ampk*, 5'-AMP-activated protein kinase; *ob-rb*, leptin receptor; *adipor*, adiponectin receptor; *cpt1*, carnitine palmitoyl transferase 1; *camkkβ*, calcium/calmodulin dependent protein kinase kinase; *acc1*, acetyl-CoA carboxylase 1; *scd1*, stearoyl-CoA desaturase; *mcd*, malonyl-CoA decarboxylase; *cd36*, cluster of differentiation 36; *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.

Table 3

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

Items	Dietary zinc level (mg kg^{-1})										ANOVA *P*
	46.4		77.2		87.0		117.1		136.8		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
IBW(g)	1.34	0.00	1.34	0.01	1.33	0.00	1.34	0.01	1.33	0.01	0.942
WG (%)	522.40^{a}	15.21	565.87^{b}	15.20	586.20^{bc}	10.59	618.62^{c}	5.23	584.62^{bc}	15.28	0.010
Survival (%)	94.67	1.33	93.33	1.49	93.33	1.83	93.33	1.83	92.00	2.26	0.886
SGR (%/day^{-1})	3.36^{a}	0.03	3.51^{b}	0.02	3.56^{bc}	0.04	3.65^{c}	0.04	3.58^{bc}	0.05	0.002
FI (%/body weight day)	3.96^{c}	0.08	3.76^{bc}	0.09	3.64^{ab}	0.05	3.50^{a}	0.03	3.65^{ab}	0.09	0.040
FCR	1.54^{c}	0.05	1.43^{b}	0.04	1.37^{ab}	0.02	1.30^{a}	0.01	1.37^{ab}	0.04	0.002
HSI (%)	4.79	0.27	5.49	0.12	5.14	0.21	5.31	0.33	5.38	0.46	0.532
CF (g/cm^{3})	0.58^{a}	0.03	0.64^{b}	0.01	0.65^{b}	0.01	0.64^{b}	0.01	0.64^{b}	0.01	0.051

Mean values with their standard errors for five determinations. ^{a,b,c} Mean values within a row with different superscript letters were significantly different ($P < 0.05$).

IBW, initial mean body weight; WG, weight gain; SGR, specific growth rate; FI, feed intake; FCR, feed conversion ratio; HSI, hepatosomatic index; CF, condition factor.

Table 4

Effects of different dietary zinc levels on Zn concentration in tissues (mg kg^{-1}, wet weight) and Zn deposition rate of juvenile *L.vannamei*

Items	Dietary zinc level (mg kg^{-1})										ANOVA *P*
	46.4		77.2		87.0		117.1		136.8		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Whole-body Zn (mg kg^{-1})	12.14	0.25	11.76	0.15	12.31	0.47	12.13	0.18	12.63	0.23	0.319
Hepatopancreas Zn (mg kg^{-1})	27.46^{a}	0.94	29.38^{ab}	1.63	28.56^{ab}	0.68	31.84^{b}	0.78	31.73^{b}	1.38	0.052
Shell Zn (mg kg^{-1})	50.78^{a}	0.95	54.97^{a}	1.08	51.96^{a}	2.79	61.52^{b}	1.26	61.89^{b}	1.83	0.003
Deposition rate of Zn (%)	17.08^{c}	0.83	10.64^{b}	0.31	10.37^{b}	0.56	7.99^{a}	0.16	6.77^{a}	0.21	0.000

Mean values with their standard errors for five determinations. ^{a,b,c} Mean values within a row with different superscript letters were significantly different ($P < 0.05$).

Table 5

Effects of different dietary zinc levels on proximate composition of whole body, hepatopancreas and muscle (%, wet weight) in juvenile *L.vannamei*

Items	Dietary zinc level (mg kg^{-1})										ANOVA *P*
	46.4		77.2		87.0		117.1		136.8		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Whole body											
Moisture	77.70	0.23	77.40	0.33	77.51	0.28	77.93	0.19	76.71	0.12	0.292
Protein	16.36	0.21	15.83	0.73	15.93	0.22	15.36	0.16	15.78	0.11	0.057
Lipid	1.70	0.04	1.66	0.09	1.67	0.05	1.65	0.05	1.66	0.04	0.986
Ash	2.04^{a}	0.10	2.67^{b}	0.11	2.69^{b}	0.03	2.68^{b}	0.07	2.92^{b}	0.17	0.000
Hepatopancreas											
Moisture	76.58	0.54	76.46	0.65	76.80	0.12	76.82	0.87	76.64	0.48	0.943
Protein	18.02	1.22	17.86	0.53	17.10	0.43	17.87	0.72	17.78	0.25	0.747
Lipid	1.63^{c}	0.09	1.48^{bc}	0.07	1.40^{b}	0.07	1.11^{a}	0.09	1.27^{ab}	0.04	0.001
Ash	0.84	0.02	0.86	0.04	0.95	0.04	0.94	0.03	0.92	0.03	0.120
Muscle											
Moisture	77.69	0.34	77.03	0.53	77.64	0.48	77.48	0.58	77.09	1.88	0.927
Protein	17.65	0.21	17.76	0.09	17.58	0.17	17.84	0.34	17.99	0.66	0.923
Lipid	0.87	0.06	0.94	0.05	0.91	0.06	0.93	0.08	0.92	0.08	0.960
Ash	1.55	0.02	1.56	0.05	1.53	0.05	1.53	0.05	1.62	0.11	0.873

Mean values with their standard errors for five determinations. ^{a,b,c} Mean values within a row with different superscript letters were significantly different ($P < 0.05$).

Table 6

Effects of different dietary zinc levels on hematological metabolites related to lipid metabolism and health indicators of juvenile *L. vannamei*.

Items	Dietary zinc level (mg kg^{-1})										ANOVA *P*
	46.4		77.2		87.0		117.1		136.8		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Lipid metabolism											
CHOL (mmol/L)	0.56^{b}	0.09	0.49^{ab}	0.11	0.37^{a}	0.05	0.47^{ab}	0.05	0.46^{ab}	0.04	0.051
LDL-C (mmol/L)	0.13	0.04	0.15	0.03	0.12	0.03	0.14	0.01	0.12	0.02	0.881
HDL-C (mmol/L)	0.05^{a}	0.01	0.07^{a}	0.01	0.13^{b}	0.01	0.11^{b}	0.01	0.11^{b}	0.01	0.000
TAG (mmol/L)	0.46	0.06	0.44	0.04	0.34	0.04	0.42	0.06	0.39	0.05	0.521
LPS (U/L)	26.42	7.67	24.79	7.95	57.72	16.26	45.66	13.66	39.52	5.60	0.223
Health indicators											
CP (U/L)	13.81^{a}	0.69	15.88^{ab}	0.81	15.96^{ab}	1.18	17.46^{b}	1.52	16.31^{ab}	1.16	0.027
Cu/Zn SOD (U/ml)	38.56^{a}	0.65	45.10^{b}	0.86	46.44^{b}	1.09	46.15^{b}	1.06	46.27^{b}	0.91	0.034

Mean values with their standard errors for five determinations. ^{a,b} Mean values within a row with different superscript letters were significantly different ($P < 0.05$).

CHOL, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; TAG, triacylglycerol; LPS, lipase; CP, ceruloplasmin; Cu/Zn SOD, Cu/Zn superoxide dismutase.

Table 7

Effects of different dietary zinc levels on hepatopancreas parameters related to lipid metabolism and health indicators of juvenile *L.vannamei*.

Items	Dietary zinc level (mg kg^{-1})										ANOVA *P*
	46.4		77.2		87.0		117.1		136.8		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Lipid metabolism											
CHOL (mmol/gprot)	0.26^{b}	0.05	0.18^{ab}	0.03	0.19^{ab}	0.01	0.16^{a}	0.02	0.18^{ab}	0.02	0.047
LDL-C (mmol/gprot)	82.69	10.63	107.03	10.86	93.38	10.88	69.96	16.08	75.06	23.36	0.599
HDL-C (mmol/gprot)	0.15^{b}	0.05	0.11^{ab}	0.04	0.10^{ab}	0.02	0.07^{ab}	0.02	0.01^{a}	0.01	0.054
TAG (mmol/gprot)	0.45^{b}	0.04	0.30^{ab}	0.11	0.23^{a}	0.02	0.24^{a}	0.05	0.29^{ab}	0.08	0.048
NEFA (umol/gprot)	677.17^{b}	44.96	462.14^{a}	40.20	503.07^{a}	55.27	488.05^{a}	71.27	492.08^{a}	57.36	0.033
ADP (ug/gprot)	4.18^{a}	0.55	5.24^{a}	0.87	6.06^{ab}	0.60	8.02^{b}	1.09	4.14^{a}	0.49	0.009
LPS (U/gprot)	5.12^{a}	0.42	11.49^{b}	1.40	11.72^{b}	2.04	10.79^{b}	1.55	12.00^{b}	2.00	0.020
Health indicators											
MDA (nmol/mgprot)	3.74^{b}	0.07	2.61^{a}	0.07	2.49^{a}	0.10	2.49^{a}	0.12	2.42^{a}	0.06	0.000
CAT (U/mgprot)	1.55	0.06	1.45	0.02	1.49	0.05	1.48	0.03	1.38	0.09	0.375
Cu/Zn SOD (U/mgprot)	19.08^{a}	1.53	23.00^{a}	2.18	22.28^{a}	2.25	24.04^{ab}	2.64	30.04^{b}	1.41	0.019

Mean values with their standard errors for five determinations. ^{a,b} Mean values within a row with different superscript letters were significantly different ($P < 0.05$).

NEFA, non-esterified fatty acids; ADP, adiponectin; MDA, malonaldehyde; CAT, catalase.