Effects of dietary zinc level on growth performance, lipolysis and expression of genes involved in the calcium/calmodulin-dependent protein kinase kinase-β/AMP-activated protein kinase pathway in juvenile Pacific white shrimp

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Abstract

The present study evaluated the effects of dietary Zn level on growth performance, serum and hepatopancreas metabolites, expression of genes involved in lipid and energy metabolism, and the signalling pathway of dietary Zn-induced lipolysis. Five isonitrogenous and isolipidic diets were formulated to contain different Zn levels: 46·4 (basal diet), 77·2, 87·0, 117·1 and 136·8 mg/kg, respectively. The results indicated that shrimp fed the diet containing Zn at 117·1 mg/kg 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 Zn level. Dietary Zn prevented the accumulation of free radicals and improved antioxidant activities by increasing Cu/Zn superoxide dismutase and reducing malondialdehyde in hepatopancreas. Dietary Zn supplementation enhanced lipase activity and adiponectin, which could promote TAG breakdown and fatty acid oxidation and lead to reduced lipid in hepatopancreas. The mRNA expressions of *ob-rb*, *adipor*, *camkkβ*, *ampk*, *cd36*, *mcd* and *cpt1* involved in Zn-induced lipid catabolism were up-regulated, and the expressions of *srebp*, *acc*, *fas* and *scd1* were down-regulated. The mRNA levels of SLC39 family genes (*zip3*, *zip9*, *zip11* and *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 Zn, lipid metabolites and expression of genes involved in lipogenesis and lipolysis in *Litopenaeus vannamei*.

Key words: *Litopenaeus vannamei*: Zinc: Lipolysis: Regulatory pathways: Zinc transporters

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[1–4]. Thus, Zn 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].

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 Zn nutrition in various crustaceans have mainly focused on requirement levels and/or immunity[1]. *L. vannamei* required 33 mg Zn/kg diet to maintain normal tissue mineralisation and growth[16]. A similar requirement value of 32–34 mg Zn/kg based on weight gain (WG) and whole-body Zn retention was reported for grass shrimp *Penaeus 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 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. Fishmeal, soyabean meal, poultry meal, soya protein concentrate, krill meal and peanut meal were used as protein sources; fish oil, soyabean oil and soya lecithin were used as lipid sources; and wheat flour was used as the carbohydrate source. Five experimental diets were formulated with $ZnSO_4.H_2O$ (Zn content = 35·5 %; Sinopharm Chemical Reagent Co. Ltd) as Zn source, with the analysed values of Zn being 46·4 (basal diet), 77·2, 87·0, 117·1 and 136·8 mg/kg 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 %) were 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) with pellet strands were cut into uniform sizes (1·5 and 2·5 mm diameter) (G-250, Machine Factory of South China University of Technology). Pellets were heated for 30 min at 90°C, then air-dried to approximately 10 % moisture, sealed in vacuum-packed bags and stored at −20°C until used for the feeding trial.

Shrimp rearing and experimental conditions

Juvenile *L. vannamei* were obtained from Chia-Tai Ningbo Company. Prior to the 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.) for 2 weeks to acclimatise to the experimental conditions. Juveniles (initial weight 1·33 (SE 0·01) g) were randomly distributed into 300-litre cylindrical

Table 1. Formulation and proximate compositions of the experimental diets

	Dietary Zn level (mg/kg)				
Ingredients (g/kg)	46·4	77·2	87·0	117·1	136·8
Fishmeal	200·00	200·00	200·00	200·00	200·00
Soyabean protein concentrate	60·00	60·00	60·00	60·00	60·00
Soyabean 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
Soyabean oil	15·00	15·00	15·00	15·00	15·00
Soya 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
$ZnSO_4.H_2O$	0·00	0·06	0·11	0·17	0·23
Proximate composition (DM %)					
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
Analysed Zn (mg/kg)	46·40	77·15	87·00	117·10	136·80

* Mineral premix (g/kg diet): NaCl, 0·74; K_2SO_4, 2·24; $MgSO_4.7H_2O$, 3·58; $FeC_6H_5O_7$, 0·29; $C_6H_{10}CaO_6.5H_2O$, 0·51; $MnSO_4.H_2O$, 0·12; $CuSO_4.5H_2O$, 0·16; KIO_3 (1 %), 0·02; Na_2SeO_3 (1 %), 0·07; $CoSO_4.7H_2O$, 0·02; zeolite, 2·25. The mineral premix does not supply Zn.
† Vitamin premix (mg/kg diet): D-calcium pantothenate, 120; inositol, 200; menadione, 60; nicotinic acid, 100; pyridoxine hydrochloride, 60; riboflavin, 50; thiamin nitrate, 60; all-rac-α-tocopherol, 100; cyanocobalamin, 0·1; biotin, 6·0; folic acid, 10; retinyl acetate, 5000 IU; cholecalciferol, 2000 IU.

fibre-glass tanks filled with 250 litres of seawater at a stocking density of thirty shrimp per tank, and each experimental diet was randomly assigned to five replicate tanks. Shrimp were fed three times/d (daily ration of 6–8 % of biomass) at 08.00, 12.00 and 17.00 hours, with the rations in the morning and evening being 70 % of the total given. Shrimp in each tank were weighed every 2 weeks, and the daily ration was 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 O_2 level was not <6·0 mg/l. During the experiment, photoperiod was maintained on a natural cycle, the temperature was 26–30°C, salinity was 23–27 g/l, pH was 7·6–7·8 and ammonia nitrogen concentration was lower than 0·05 mg/l. Salinity, pH, dissolved O_2 and ammonia nitrogen were measured by YSI Proplus (YSI, Yellow Springs). The duration of the feeding trial was 8 weeks.

Sample collection

A total of 120 juvenile *L. vannamei* were randomly sampled at the beginning of the feeding trial and frozen at −20°C for analysis of Zn 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, WG, specific growth rate, feed conversion ratio and feed intake. Furthermore, body length, whole-body and hepato-pancreas weight from four shrimp in each tank were taken to calculate condition factor and hepatosomatic index. Five shrimp from each tank were used to analyse the Zn concentration in tissues (whole body, hepatopancreas and shell). Haemolymph 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 5810 R). The supernatant was collected and stored at −80°C until analysis of haematological 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 analysed 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). Crude lipid was determined by the ether extraction method using Soxtec (Soxtec System HT6, Tecator). 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.

Zn concentrations in tissues (whole body, hepatopancreas and shell), experimental diets and water were measured using Inductively Coupled Plasma Optical Emission Spectrometer (PE 2100DV, Perkin Elmer) in Ningbo Institute of Materials Technology and Engineering, Chinese Academy of Sciences.

The concentration of Zn in seawater ranged from 1·8 to 2·0 μg/l among the five groups fed diets containing different Zn levels.

Haematological and hepatopancreas characteristic analysis

TAG, total cholesterol (CHOL), LDL-cholesterol and HDL-cholesterol contents in haemolymph were determined using an automatic chemistry analyzer (Hitachi 7600–110).

Hepatopancreas samples were homogenised on ice in nine volumes (w/v) of ice-cold physiological saline 8·9 g/ml and then centrifuged at 850 *g* for 10 min at 4°C. The resultant supernatant was collected, and aliquots were stored at −80°C until analysis. TAG, CHOL, LDL-cholesterol, HDL-cholesterol, lipase (LPS), NEFA, adiponectin (ADP), malondialdehyde (MDA), catalase and Cu/Zn superoxide dismutase (Cu/Zn SOD) were determined using the relevant diagnostic reagent kits (Nanjing Jiancheng Co.) according to the manufacturer's instructions.

Total RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from 10 to 20 mg hepatopancreas with Trizol reagent (TaKaRa) following the manufacturer's protocol. RNA was quantified by a NanoDrop spectrophotometer (Thermo Scientific NanoDrop 2000) 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 the Molecular Imager® Gel Doc™ XR System (Bio-Rad) to check integrity. Complementary DNA (cDNA) was generated from 1000 ng of DNAase-treated RNA and synthesised using a Prime Script™ RT Reagent Kit with gDNA Eraser (perfect real-time) (TaKaRa) according to the manufacturer's protocol, using Mastercycler nexus GSX1 PCR (Eppendorf). cDNA was diluted four times using RNA-free water.

The core fragments of all genes were obtained from the National Center for Biotechnology Information 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 normalise the template amount. The gene-specific primers used for mRNA quantification by real-time PCR were designed by Primer Premier 5.0 and are shown in Table 2. All primers were synthesised by BGI (The Beijing Genomics Institute). The quantitative PCR was carried out in a quantitative thermal cycler system (Roche, Light cyclern96) using SYBR Green I (Roche). The amplification was performed in a ninety-six-well plate in a 20 μl reaction volume containing 10 μl of 2 × SYBR Green I Master Mix (Roche), 1 μl (each) gene-specific forward and reverse primers (10 μm), 6 μl diethyl pyrocarbonate (DEPC) water and 2 μl of diluted cDNA. The real-time PCR programme was 95°C for 2 min, followed by forty-five 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 analysed according to the following equation $E = 10^{(-1/slope)} - 1$. During analysis, each sample was

Table 2. Real-time quantitative PCR primers for genes related to lipid and energy metabolism and *β-actin* of *Litopenaeus vannamei*

Gene	Primers (5′-3′)	Size (bp)	Tm (°C)	Accession no.
β-actin	F: CGAGGTATCCTCACCCTGAA	176	58·22	AF300705.2
	R: GTCATCTTCTCGCGGTTAGC		58·80	
srebp	F: ACCATTGCCACTCCCCTA	150	57·40	MG770374.1
	R: GTTGCGTTTCTCGCCTTT		56·67	
fas	F: CGTGACACCCCTTCCTCAC	196	60·00	HM595630.1
	R: GAGAGTGTGAGGAACATAGACCA		59·24	
ampk	F: CTTTGCTGATGCTAATGCCT	187	56·46	KP272116.1
	R: TCCTTTGGGTAGTCCACGAT		58·05	
ob-rb	F: CAACAGACATGGCAGGCATC	117	59·55	XM_027361398.1
	R: AGAACGGCCACCAGTTATTGT		59·93	
adipor	F: TCGCTGTTTGATCGACACC	241	58·18	KT372143.1
	R: TGAGTCGATGAGGGACTCGT		60·04	
cpt1	F: ACTCCCGATAAGCACACC	139	56·32	XM_027373671.1
	R: TTCATACATCCACCCCCT		54·22	
camkkβ	F: GAAACTTCCATTCCACGAT	182	52·94	XM_027368225.1
	R: TCACCCAGGGATGTTGCTT		58·84	
acc1	F: TGCATAGAAACGGCATTGCG	134	59·90	XM_027360190.1
	R: TTTGACACCTGAGCCAGACC		59·89	
scd1	F: TGTCTTACACCTTATCAATGGC	154	56·15	XM_027374708.1
	R: CGTTCGTATGTTCCTCTTCGTC		59·16	
mcd	F: AAGACCACAGGAAGGGACCA	114	60·40	XM_027376735.1
	R: GACACTTGAGATGCCACCCA		59·96	
cd36	F: AACCAAGGTCCTGACCATCAC	210	59·93	XM_027374896.1
	R: AGGTGAGAGTCGACGAGGAA		59·96	
zip3	F: GCAGAATGGGAAGAAGACCCA	125	60·00	XM_027367035.1
	R: ACCGGGTCTTCGAGGATACA		60·03	
zip9	F: TGACTATGTCCGAGGACCGT	201	60·03	XM_027354650.1
	R: GTCCGATGAGGTTGTGTGGT		59·97	
zip11	F: GGTCTGCTGTGGTGTTCCT	172	59·55	XM_027361505.1
	R: ACAAAAGCCCATTCCCCT		56·25	
zip14	F: TGCCAGAGGTGCTGTACTTG	108	59·96	XM_027375334.1
	R: GCAGACCATAGCCCCAAACT		60·03	

Tm, melting temperature; 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*, Ca/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.

run in triplicate and the E-values ranged from 95·1 to 103·6 %. The relative quantification method was used to analyse data with expression levels of target genes calculated using the $2^{-\Delta\Delta Ct}$ method as described by Livak & Schmittgen[19].

Calculations

Parameters were calculated as follows:

WG (%) = 100 × (final body weight (g) − initial body weight (g))/initial body weight (g).

Specific growth rate (%/d) = 100 × (Ln (final body weight (g)) − Ln (initial body weight (g)))/days.

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

Feed conversion ratio = feed consumption (g, dry weight)/(final body weight (g) − initial body weight (g)).

Feed intake (%/body weight day) = 100 × feed consumption (g, dry weight)/((initial body weight (g) + final body weight (g))/2)/days.

Hepatosomatic index (%) = 100 × (hepatopancreas wet weight (g))/(body wet weight (g)).

Condition factor (g/cm³) = 100 × body weight (g)/body length³ (cm³).

Deposition rate of Zn (%) = 100 × (final body weight (g) × final whole shrimp of Zn (mg/kg) − initial body weight (g) × initial whole shrimp of Zn (mg/kg))/(feed consumption (g) × feed Zn content (mg/kg)).

Statistical analysis

Results are presented as mean values with their standard errors. Data were checked for normality and homogeneity of variances and were normalised when appropriate. Proportional data were arcsine square root transformed before statistical analyses. Mean values were compared through one-way 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.) 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 Zn had higher WG and specific growth rate than those fed the other diets ($P < 0.05$). The lowest feed intake and feed conversion ratio were also observed in shrimp fed the diet supplemented with 117·1 mg/kg Zn. Moreover, shrimp fed the diet containing 46·4 mg/kg Zn had lower condition factor

Table 3. Effects of different dietary zinc levels on growth performance, feed utilisation and morphologic index of juvenile *Litopenaeus vannamei* (Mean values with their standard errors for five determinations)

	Dietary Zn level (mg/kg)										
	46·4		77·2		87·0		117·1		136·8		
Items	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	ANOVA *P*
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[b,c]	10·59	618·62[c]	5·23	584·62[b,c]	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 (%/d)	3·36[a]	0·03	3·51[b]	0·02	3·56[b,c]	0·04	3·65[c]	0·04	3·58[b,c]	0·05	0·002
FI (%/body weight day)	3·96[c]	0·08	3·76[b,c]	0·09	3·64[a,b]	0·05	3·50[a]	0·03	3·65[a,b]	0·09	0·040
FCR	1·54[c]	0·05	1·43[b]	0·04	1·37[a,b]	0·02	1·30[a]	0·01	1·37[a,b]	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/cm³)	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

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.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

Table 4. Effects of different dietary zinc levels on zinc concentration in tissues (mg/kg, wet weight) and zinc deposition rate of juvenile *Litopenaeus vannamei* (Mean values with their standard errors for five determinations)

	Dietary Zn level (mg/kg)										
	46·4		77·2		87·0		117·1		136·8		
Items	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	ANOVA *P*
Whole-body Zn (mg/kg)	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)	27·46[a]	0·94	29·38[a,b]	1·63	28·56[a,b]	0·68	31·84[b]	0·78	31·73[b]	1·38	0·052
Shell Zn (mg/kg)	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

[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

than those fed the other diets ($P < 0.05$). The hepatosomatic index was not significantly influenced by dietary Zn level.

Zinc concentration in tissues and zinc 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 and 136·8 mg/kg 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 Zn). The deposition rate of Zn significantly decreased as dietary Zn level increased from 46·4 to 136·8 mg/kg.

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 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 to 136·8 mg/kg, whereas moisture, protein and ash contents in hepatopancreas were not significantly affected by dietary Zn level.

Haematological metabolites and enzyme activity

Haematological metabolites related to lipid metabolism and health indicators are shown in Table 6. Haemolymph CHOL and HDL-cholesterol concentrations were significantly influenced by dietary Zn level ($P < 0.05$). Shrimp fed the diet containing 87·0 mg/kg Zn had the lowest CHOL and the highest HDL-cholesterol in haemolymph among all treatments. However, LDL-cholesterol and TAG concentrations and LPS activity in haemolymph were not significantly affected by dietary Zn level. In addition, shrimp fed the basal diet (46·4 mg/kg Zn) had the lowest Cu/Zn SOD and ceruloplasmin in haemolymph.

Hepatopancreas biochemical parameters

TAG, CHOL, HDL-cholesterol, MDA, NEFA, 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 Zn) had the highest CHOL, TAG, HDL-cholesterol and NEFA concentrations in hepatopancreas among all treatments. In addition, shrimp fed the diet containing 136·8 mg/kg Zn had lower HDL-cholesterol and NEFA concentrations in hepatopancreas than those fed the basal diet ($P < 0.05$). Shrimp fed the diet supplemented with 117·1 mg/kg Zn had the highest ADP concentration in hepatopancreas. LPS activity in hepatopancreas was significantly higher in Zn inclusion groups compared with the basal group

Table 5. Effects of different dietary zinc levels on proximate composition of whole body, hepatopancreas and muscle (%, wet weight) in juvenile *Litopenaeus vannamei*
(Mean values with their standard errors for five determinations)

| Items | Dietary Zn level (mg/kg) | | | | | | | | | | ANOVA *P* |
| | 46·4 | | 77·2 | | 87·0 | | 117·1 | | 136·8 | | |
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
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[b,c]	0·07	1·40[b]	0·07	1·11[a]	0·09	1·27[a,b]	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

[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

Table 6. Effects of different dietary zinc levels on haematological metabolites related to lipid metabolism and health indicators of juvenile *Litopenaeus vannamei*
(Mean values with their standard errors for five determinations)

| Items | Dietary Zn level (mg/kg) | | | | | | | | | | ANOVA *P* |
| | 46·4 | | 77·2 | | 87·0 | | 117·1 | | 136·8 | | |
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
Lipid metabolism											
CHOL (mmol/l)	0·56[b]	0·09	0·49[a,b]	0·11	0·37[a]	0·05	0·47[a,b]	0·05	0·46[a,b]	0·04	0·051
LDL-cholesterol (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-cholesterol (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[a,b]	0·81	15·96[a,b]	1·18	17·46[b]	1·52	16·31[a,b]	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

CHOL, total cholesterol; LPS, lipase; CP, ceruloplasmin; Cu/Zn SOD, Cu/Zn superoxide dismutase.
[a,b] Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

Table 7. Effects of different dietary zinc levels on hepatopancreas parameters related to lipid metabolism and health indicators of juvenile *Litopenaeus vannamei*
(Mean values with their standard errors for five determinations)

| Items | Dietary Zn level (mg/kg) | | | | | | | | | | ANOVA *P* |
| | 46·4 | | 77·2 | | 87·0 | | 117·1 | | 136·8 | | |
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
Lipid metabolism											
CHOL (mmol/g protein)	0·26[b]	0·05	0·18[a,b]	0·03	0·19[a,b]	0·01	0·16[a]	0·02	0·18[a,b]	0·02	0·047
LDL-cholesterol (mmol/g protein)	82·69	10·63	107·03	10·86	93·38	10·88	69·96	16·08	75·06	23·36	0·599
HDL-cholesterol (mmol/g protein)	0·15[b]	0·05	0·11[a,b]	0·04	0·10[a,b]	0·02	0·07[a,b]	0·02	0·01[a]	0·01	0·054
TAG (mmol/g protein)	0·45[b]	0·04	0·30[a,b]	0·11	0·23[a]	0·02	0·24[a]	0·05	0·29[a,b]	0·08	0·048
NEFA (μmol/g protein)	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 (μg/g protein)	4·18[a]	0·55	5·24[a]	0·87	6·06[a,b]	0·60	8·02[b]	1·09	4·14[a]	0·49	0·009
LPS (U/g protein)	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/mg protein)	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/mg protein)	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/mg protein)	19·08[a]	1·53	23·00[a]	2·18	22·28[a]	2·25	24·04[a,b]	2·64	30·04[b]	1·41	0·019

ADP, adiponectin; MDA, malondialdehyde; CAT, catalase.
[a,b] Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).
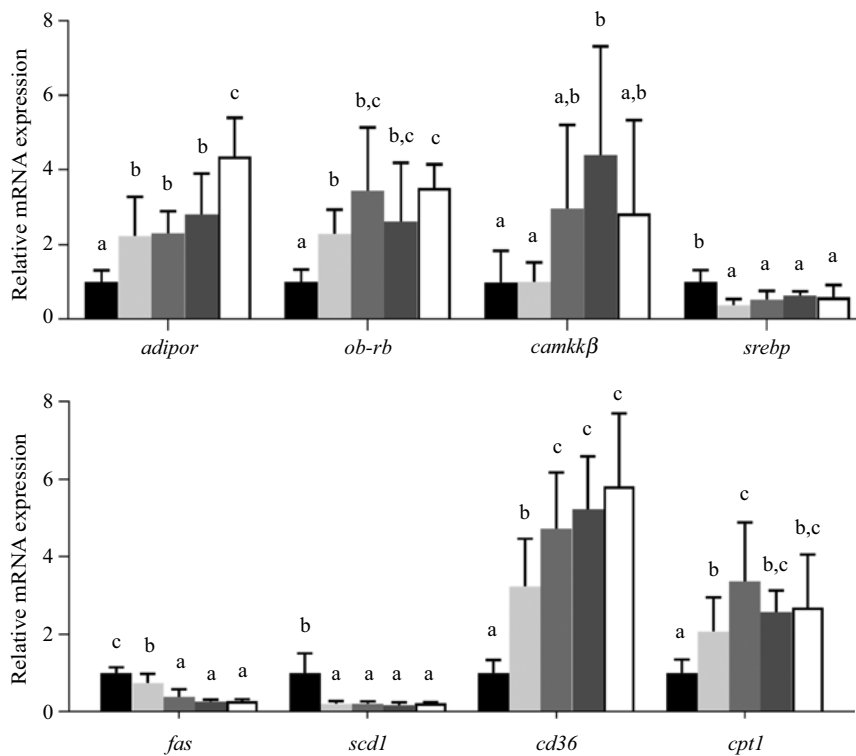
Fig. 1. mRNA levels of genes involved in lipid metabolism in the hepatopancreas of juvenile *Litopenaeus vannamei* fed the experimental diets. ■, 46·4 mg/kg zinc diet; ■, 77·2 mg/kg zinc diet; ■, 87·0 mg/kg zinc diet; ■, 117·1 mg/kg zinc diet; □, 136·8 mg/kg zinc diet. Expression values are normalised by *β-actin*-expressed transcripts. Relative fold difference among treatments are presented as means (*n* 8), with their standard errors. a,b,c Mean values with unlike letters were significantly different (*P* < 0·05; one-way ANOVA).

(*P* < 0·05), but LDL-cholesterol 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 catalase activity in hepatopancreas was not affected by dietary Zn level.

Gene expression

Transcript level of genes involved in lipid and energy metabolism in hepatopancreas is shown in Figs. 1 and 2. Transcript levels of *adipor*, *ob-rb* and *camkkβ* were significantly up-regulated with increasing dietary Zn level, and shrimp fed the basal diet (46·4 mg/kg 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, 87·0, 117·1 and 136·8 mg/kg Zn (Fig. 1).

Hepatopancreas transcript level of key enzymes involved in lipogenesis (*fas*, *scd1*), β-oxidation (carnitine palmitoyl transferase 1, *cpt1*) and lipolysis (*cd36*) was 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 to 136·8 mg/kg, with no significant differences in the expression of *fas* and *scd1* were observed in shrimp fed the diets containing 87·0, 117·1 and 136·8 mg/kg Zn. However, the expression levels of *cd36* and *cpt1* were significantly up-regulated with
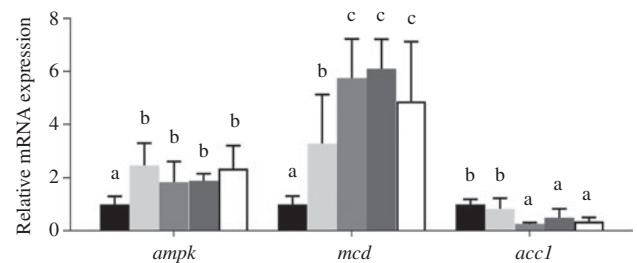
Fig. 2. mRNA levels of genes involved in energy metabolism in the hepatopancreas of juvenile *Litopenaeus vannamei* fed the experimental diets. ■, 46·4 mg/kg zinc diet; ■, 77·2 mg/kg zinc diet; ■, 87·0 mg/kg zinc diet; ■, 117·1 mg/kg zinc diet; □, 136·8 mg/kg zinc diet. Expression values are normalised by *β-actin*-expressed transcripts. Relative fold difference among treatments are presented as means (*n* 8), with their standard errors. a,b,c Mean values with unlike letters were significantly different (*P* < 0·05; one-way ANOVA).

increasing dietary Zn level, and shrimp fed the basal diet had lower expression of *cd36* and *cpt1* in hepatopancreas.

Dietary Zn level also affected the 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 to 77·2 mg/kg, whereas there were no significant differences in the expression of *ampk* and *mcd* when dietary Zn level exceeded 87·0 mg/kg. Shrimp fed the diets containing 46·4 and 77·2 mg/kg Zn had higher expression of *acc1* than those fed the other diets, while
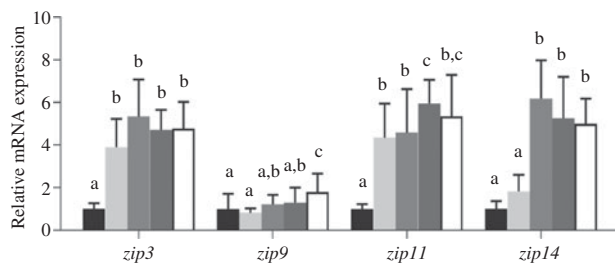
Fig. 3. mRNA levels of genes involved in SLC39 family genes (*zip3, zip9, zip11, zip14*) in the hepatopancreas of juvenile *Litopenaeus vannamei* fed the experimental diets. ■, 46·4 mg/kg zinc diet; ▨, 77·2 mg/kg zinc diet; ▥, 87·0 mg/kg zinc diet; ▦, 117·1 mg/kg zinc diet; □, 136·8 mg/kg zinc diet. Expression values are normalised by *β-actin*-expressed transcripts. Relative fold difference among treatments are presented as means (*n* 8), with their standard errors. a,b,c Mean values with unlike letters were significantly different (*P* < 0·05; one-way ANOVA).

no significant difference in the expression of *acc1* was observed in shrimp fed the diets with Zn above 87·0 mg/kg.

The mRNA expression of genes involved in the SLC39 family is 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*.

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 Zn improved growth performance and feed utilisation, 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 & Lawrence[16] reported that WG of Pacific white shrimp *Penaeus vannamei* was significantly affected by dietary Zn levels from 18 to 60 mg/kg 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 Zn, lower than the 117·1 mg/kg 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 Zn 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 & Sun[28] demonstrated that Zn firstly accumulated in the digestive tract, followed by skeletal tissue, and then skin and muscle in common carp *cyprinus carpio*, indicating that Zn in these tissues was useful indices for evaluating Zn 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 *P. monodon*[17] and juvenile grouper *Epinephelus malabaricus*[29]. Shiau & Jiang[17] reported that Zn concentration in hepatopancreas ranged from 0·6 to 21·8 μg/g when dietary Zn level increased from 7 to 127 mg/kg 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 in bone metabolism by stimulating collagen synthesis to increase Ca content in bone[30], invertebrates mainly deposit minerals in exoskeleton[29]. Therefore, the higher the ash content in whole body of shrimp fed the diet supplemented with Zn might be due to the increased Ca and Zn in shell.

Zn plays an important role in enhancing antioxidant status and regulating lipid metabolism[5]. In the present study, clinical haematological parameters demonstrated that dietary Zn could improve lipolysis and antioxidant responses by decreasing haematological CHOL and increasing ceruloplasmin 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 TAG content in juvenile goby *S. hasta*, which was considered to be due to the down-regulation of lipogenesis and increased lipolysis[31]. Similarly, dietary Zn of about 156 mg/kg reduced lipid content in liver and muscle and increased activities of CPT1 and lipoprotein LPS of liver in yellow catfish *P. fulvidraco*[11]. Furthermore, Wei *et al.*[12] reported that Zn reduced hepatic lipid deposition and activated lipophagy via Zn^{2+}/MTF-1/PPAα and Ca^{2+}/CaMKKβ/AMPK signalling pathways. In contrast, 8-week chronic Zn exposure induced lipid accumulation in yellow catfish *P. fulvidraco*[32]. Zheng *et al.*[33] also reported that dietary Zn at 155 mg/kg induced Zn accumulation in liver, muscle and whole body of yellow catfish *P. fulvidraco*. Thus, contradictory results on the influence of dietary Zn 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. TAG are major constituents of body lipid in humans and other animals, and TAG 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]. Lipid can be broken down to produce energy by LPS which catalyses the hydrolysis of TAG into glycerol and NEFA[35,36], then ADP breaks down NEFA[37]. A pharmacological study showed that administration of ADP to mice resulted in lower body weight associated with a reduction in NEFA and TAG[38]. The results of the present study showed that dietary Zn supplementation reduced hepatopancreas CHOL, HDL-cholesterol, TAG and NEFA and increased ADP content and LPS activity, suggesting that Zn supplementation could accelerate lipid breakdown by hydrolysing
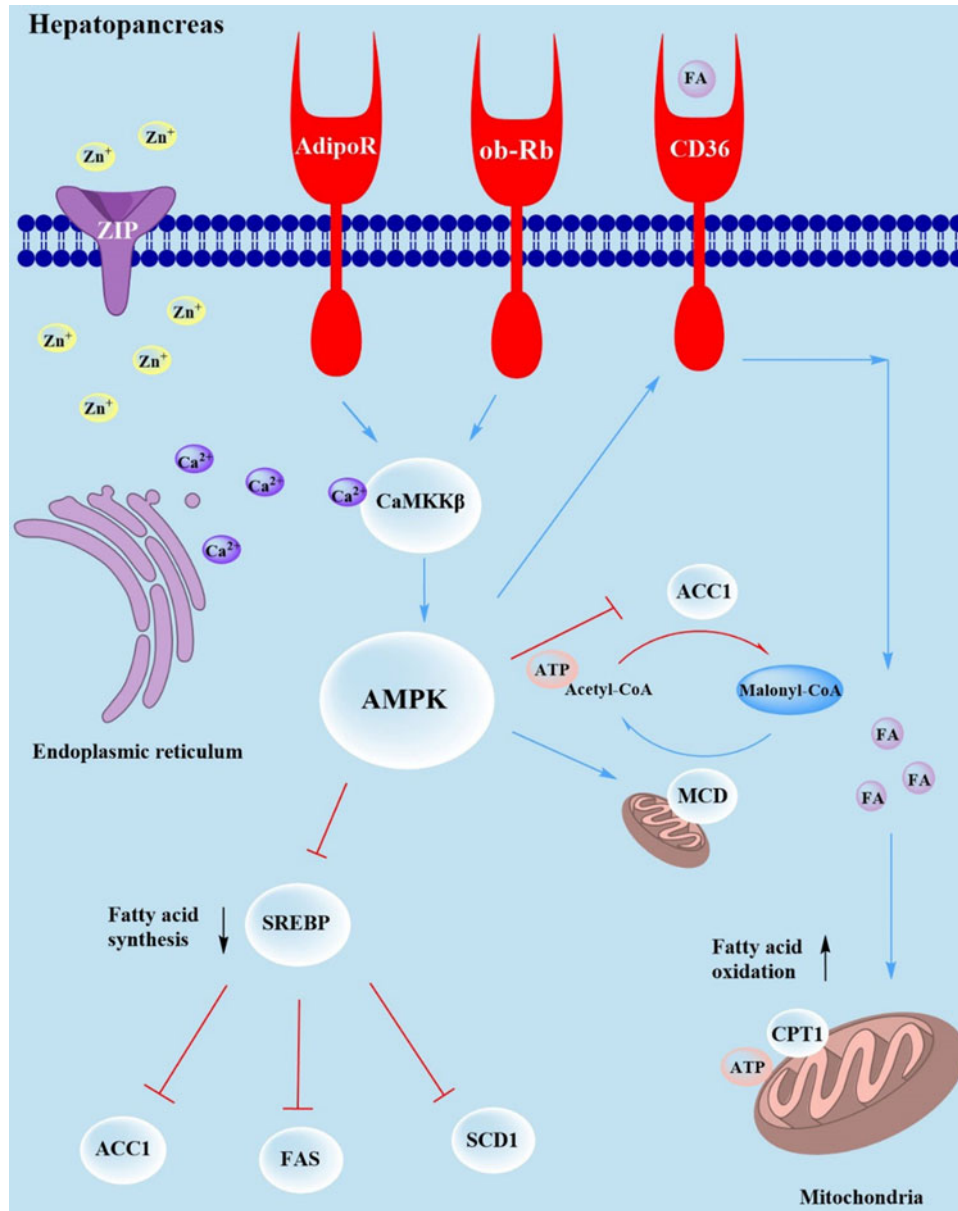
Fig. 4. Working model of how Zn^{2+} regulates lipophagy via the $Ca^{2+}/CaMKK\beta/AMPK$ axes. The blue lines indicate promotion, and the red lines indicate suppression. Extracellular Zn^{2+} activates SLC39 family genes (*zip3, zip9, zip11, zip14*) which increases intracellular Zn^{2+}, which promotes 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.

TAG through LPS activity to release NEFA, simultaneously increasing the content of ADP and promoting fatty acid oxidation, with the final manifestation being reduced lipid content of the hepatopancreas.

Many studies have demonstrated that the antioxidant defence system is associated with the physical health status of animals by inhibiting oxidation and removing excess reactive oxygen species [39–41]. Two of the essential antioxidant enzymes are catalase and ceruloplasmin. Catalase catalyses 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]. Reactive oxygen species can degrade polyunsaturated lipids forming MDA, a

marker for lipid oxidative stress[44]. In addition, Zn is an active central ion for Cu/Zn SOD, 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-recognised modulators of the guardians in lipid homoeostasis[46,47]. It can be activated by CaMKK[48,49] in response to increased intracellular Ca^{2+}. Activated $Ca^{2+}/CaMKK\beta$ 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+} homoeostasis, which is mediated by triggering sensitive Ca^{2+} pumps in the endoplasmic reticulum[12,52]. While Zn^{2+} absorption and homoeostasis 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 ADP[56]. Yamauchi *et al.*[57] reported that the ADP receptor (AdipoR) serves as the receptor for full-length ADP and activates AMPK. Iwabu *et al.*[58] showed that ADP induces extracellular Ca^{2+} influx by ADP 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 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 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 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 signalling 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 utilisation. Furthermore, incremental dietary Zn levels reduced total lipid, NEFA, CHOL and TAG levels in hepatopancreas. Importantly, dietary Zn-induced lipolysis was dependent on the activation of Zn 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.

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