

## Dietary zinc addition influenced zinc and lipid deposition in the fore- and mid-intestine of juvenile yellow catfish *Pelteobagrus fulvidraco*

Guang-Hui Chen<sup>1</sup>, Christer Hogstrand<sup>2</sup>, Zhi Luo<sup>1,3\*</sup>, Dian-Guang Zhang<sup>1</sup>, Shi-Cheng Ling<sup>1</sup> and Kun Wu<sup>1</sup>

<sup>1</sup>Key Laboratory of Freshwater Animal Breeding, Ministry of Agriculture of P.R.C., Fishery College, Huazhong Agricultural University, Wuhan 430070, People's Republic of China

<sup>2</sup>School of Medicine, King's College London, Diabetes and Nutritional Sciences Division, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH, UK

<sup>3</sup>Collaborative Innovation Center for Efficient and Health Production of Fisheries in Hunan Province, Changde 415000, People's Republic of China

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### Abstract

The present study explored the mechanisms of dietary Zn influencing Zn and lipid deposition in the fore- and mid-intestine in yellow catfish *Pelteobagrus fulvidraco*, and investigated whether the mechanism was intestinal-region dependent. For this purpose, yellow catfish were fed three diets containing Zn levels of 8.83, 19.20 and 146.65 mg Zn/kg, respectively. Growth performance, intestinal TAG and Zn contents as well as activities and mRNA expression of enzymes and genes involved in Zn transport and lipid metabolism in the fore- and mid-intestine were analysed. Dietary Zn increased Zn accumulation as well as activities of Cu-, Zn-superoxide dismutase and ATPase in the fore- and mid-intestine. In the fore-intestine, dietary Zn up-regulated mRNA levels of ZnT1, ZnT5, ZnT7, metallothionein (MT) and metal response element-binding transcription factor-1 (MTF-1), but down-regulated mRNA levels of ZIP4 and ZIP5. In the mid-intestine, dietary Zn up-regulated mRNA levels of ZnT1, ZnT5, ZnT7, MT and MTF-1, but down-regulated mRNA levels of ZIP4 and ZIP5. Dietary Zn reduced TAG content, down-regulated activities of 6-phosphogluconate dehydrogenase (6PGD), glucose-6-phosphate dehydrogenase (G6PD), malic enzyme (ME) and fatty acid synthase (FAS) activities, and reduced mRNA levels of 6PGD, G6PD, FAS, PPAR $\gamma$  and sterol-regulator element-binding protein (SREBP-1), but up-regulated mRNA levels of carnitine palmitoyltransferase IA, hormone-sensitive lipase (HSL $\alpha$ ), adipose TAG lipase (ATGL) and PPAR $\alpha$  in the fore-intestine. In the mid-intestine, dietary Zn reduced TAG content, activities of G6PD, ME, isocitrate dehydrogenase and FAS, down-regulated mRNA levels of 6PGD, G6PD, FAS, acetyl-CoA carboxylase a, PPAR $\gamma$  and SREBP-1, but up-regulated mRNA expression of HSL $\alpha$ , ATGL and PPAR $\alpha$ . The reduction in TAG content following Zn addition was attributable to reduced lipogenesis and increased lipolysis, and similar regulatory mechanisms were observed between the fore- and mid-intestine.

**Key words:** *Pelteobagrus fulvidraco*: Zinc: Intestinal regions: Zinc transport: Lipid metabolism

Zn is an essential micronutrient for all animals, including fish. It serves important functions in various metabolic pathways, such as transcriptional regulation, protein synthesis and cellular signal recognition<sup>(1,2)</sup>. Dietary Zn deficiency is associated with anorexia, poor appetite, weight loss and growth retardation<sup>(1,3,4)</sup>. However, Zn can be toxic at high concentrations<sup>(4–6)</sup>. Thus, as it is an essential but potentially toxic ion, a well-maintained Zn homeostasis is crucial for all organisms. Compared to terrestrial animals, fish can absorb Zn from the diet and water but, at least in freshwater fish, the diet is the main pathway for Zn absorption<sup>(7)</sup>. Thus, in fish as well as in mammals, regulation of intestinal Zn absorption is crucial for

health and survival<sup>(7)</sup>. Therefore, understanding the mechanisms of intestinal Zn uptake in fish is of considerable interest from both nutritional and toxicological perspectives. Studies suggested a morpho-functional specialisation of intestinal regions in fish with regard to ion transport<sup>(8–10)</sup>, and fish showed anterior–middle regionalisation of ion transport<sup>(11)</sup>. However, the mechanisms of absorption of mineral elements along the intestinal tract are far less understood<sup>(9,12)</sup>. Highlighting the relative importance of different sections of the intestine in ion transport processes, it is worth exploring the responsiveness of intestinal regionalisation to dietary mineral element addition.

**Abbreviations:** 6PGD, 6-phosphogluconate dehydrogenase; ACC, acetyl-CoA carboxylase; ATGL, adipose TAG lipase; CPT, carnitine palmitoyltransferase; FAS, fatty acid synthase; G6PD, glucose-6-phosphate dehydrogenase; HSL, hormone-sensitive lipase; ICDH, isocitrate dehydrogenase; ME, malic enzyme; MT, metallothionein; MTF-1, metal response element-binding transcription factor-1; SOD, superoxide dismutase; SREBP, sterol-regulator element-binding protein.

\* **Corresponding author:** Professor Z. Luo, fax +86 27 8728 2114, email luozhi99@mail.hzau.edu.cn; luozhi99@aliyun.com.

Zn metabolism in higher eukaryotes is complicated, being regulated by a complex interplay of uptake and efflux transporter proteins, coupled with metal-dependent transcriptional control of selected transport and storage proteins<sup>(13)</sup>. Recently, there have been advances in the understanding of genes and proteins involved in these processes and their regulation. ATPases are the membrane-bound enzymes responsible for the transport of ions through a biological membrane<sup>(14)</sup>. Cu-, Zn-superoxide dismutase (SOD) is a Zn-requiring enzyme that represents more than 90% of the total SOD in cells, and plays important roles in intervening in the first transformation by dismutation of the superoxide free radicals ( $O_2^{\cdot-}$ ) into  $H_2O_2$ <sup>(15)</sup>. The transport of Zn ions is controlled by two families of ion transporters, the ZnT (solute-linked carrier 30 family, SLC30A/ZnT) and Zips (solute-linked carrier 39 family, SLC39A/ZIP), which function in opposite directions of maintaining cellular Zn homeostasis<sup>(16,17)</sup>. The ZnT proteins, including ZnT1, ZnT5 and ZnT7, play critical roles in maintaining the cytoplasmic Zn balance by either transporting Zn out of cells or sequestering Zn into intracellular compartments<sup>(16–18)</sup>. By contrast, SLC39 family proteins, including ZIP4 and ZIP5, function to increase the cytosolic Zn concentration by promoting Zn import from the extracellular space or Zn release from organelles<sup>(19,20)</sup>. Metal response element-binding transcription factor-1 (MTF-1) functions as a cellular Zn sensor that coordinates the expression of genes involved in Zn homeostasis. Metallothioneins (MT) are small, cysteine-rich, metal-binding proteins that play an important role in Zn homeostasis and in detoxification of toxic metals<sup>(21)</sup>. At present, studies on teleost Zn transporters have been mainly limited to model species because of the limitation of genomic resource in non-model fish species. Recently, Jiang *et al.*<sup>(22)</sup> identified a set of thirty-seven Zn transporters in the common carp genome, including seventeen from the SLC30 family (ZnT) and twenty from SLC39 family (ZIP). However, the underlying molecular mechanisms involved in the regulation of intestinal Zn transporters in response to dietary Zn in non-model species remain largely unknown.

In fish, lipids are known to be used as energy reserves, and carry out a vast array of functions. Accordingly, as in mammals, lipid absorption in fish occurs predominantly in the proximal part of the intestine<sup>(23)</sup>. In mammals, studies indicated that Zn influences lipid metabolism<sup>(24,25)</sup>, indicating a close link between Zn and lipid metabolism. In general, lipid metabolism results from the balance between synthesis of fatty acids (lipogenesis) and fat catabolism via  $\beta$ -oxidation (lipolysis), and many key enzymes and transcriptional factors are involved in these metabolic processes. These enzymes include lipogenic enzymes (such as glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS)) and lipolytic enzymes (such as carnitine palmitoyl-transferase I (CPT I), hormone-sensitive lipase (HSL), adipose TAG lipase (ATGL))<sup>(26)</sup>. In addition, several transcription factors, such as PPAR and sterol-regulator element-binding protein (SREBP-1), play an intermediary role in lipid homeostasis, by orchestrating the gene transcription of enzymes involved in these pathways<sup>(27)</sup>.

Yellow catfish (*Pelteobagrus fulvidraco*), an omnivorous freshwater fish, is considered to be a good candidate for freshwater culture in China and other Asian countries because of its delicious meat and high market value. However, excessive lipid deposition in yellow catfish, which may affect the quality of harvest, is a problem. In our laboratory, Zheng *et al.*<sup>(4)</sup> found that dietary deficiency and excess of Zn exerted a profound effect on lipid deposition and metabolism in the liver and muscle of yellow catfish *P. fulvidraco*. In the present study, we hypothesise that there would be regional differences in Zn transport and lipid metabolism, due to a differential distribution of these specific carriers. Hence, the objectives of the present study were to explore the potential mechanisms of dietary Zn regulating Zn metabolism and lipid metabolism in the fore- and mid-intestine in yellow catfish, which provided new insights into Zn nutrition in fish.

## Methods

The experiment performed on animals followed the ethical guidelines of Huazhong Agricultural University for the care and use of laboratory animals, and the manuscript conformed to the Animal Research Reporting *In Vivo* Experiments (ARRIVE) Guidelines for Reporting Animal Research.

## Diet preparation

A total of three experimental diets were formulated with  $ZnSO_4 \cdot 7H_2O$  supplemented at levels of 0, 0.04 and 0.6 g/kg diet at the expense of cellulose (Table 1). Different Zn contents were added to the diets, based on our previous study<sup>(3)</sup>, in order to produce three different dietary Zn groups (Zn deficiency, adequate Zn and Zn excess, respectively). The formulation of the experimental diets was according to Luo *et al.*<sup>(3)</sup>. The formulated diets were dried at 80°C in an oven until the moisture was reduced to <10%. The dry pellets were

**Table 1.** Feed formulation and proximate analysis of experimental diets\*

Ingredients (g/kg)	Zn deficiency	Adequate Zn	Zn excess
Casein	360	360	360
Wheat starch	250	250	250
Fish oil	25	25	25
Soyabean oil	50	50	50
Ascorbyl-2-polyphosphate	10	10	10
NaCl	10	10	10
Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub> ·H <sub>2</sub> O	10	10	10
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0	0.04	0.6
Vitamin premix	5	5	5
Mineral premix (Zn-free)	5	5	5
Betaine	10	10	10
Gelatin	80	80	80
Cellulose	185	184.96	184.4
Proximate analysis (percentage of DM basis)			
Moisture content (%)	9.24	9.48	9.49
Crude protein (%)	41.28	41.34	40.92
Ash (%)	2.47	2.36	2.40
Lipid (%)	11.18	11.36	11.18
Zn (mg/kg)	8.83	19.20	146.65

\* Vitamin premix according to Luo *et al.*<sup>(3)</sup>; mineral premix according to Luo *et al.*<sup>(3)</sup> without Zn addition. ZnSO<sub>4</sub>·7H<sub>2</sub>O (≥99.0% in purity): Sinopharm Chemical Reagent Co. Ltd.

placed in plastic bags and stored at  $-20^{\circ}\text{C}$  until feeding. The final Zn concentrations in the experimental diets were analysed in triplicate using inductively coupled plasma (ICP) atomic emission spectrometry, and the contents were 8.83 (Zn deficiency), 19.20 (adequate Zn) and 146.65 (Zn excess) mg Zn/kg diet, respectively.

### Experimental procedures

The experiment was conducted in Panjin Guanghe Crab Co. Ltd, Panjin, China. Yellow catfish were obtained from a local fish pond (Panjin, China). They were transferred to indoor cylindrical fibreglass tanks (90 cm height, 80 cm diameter) for 2 weeks of acclimatisation. Afterwards, 216 uniform-sized fish (initial mean weight:  $0.81$  (SEM  $0.01$ ) g) were randomly assigned to nine fibreglass tanks with twenty-four fish per tank. The experiment was conducted in a semi-static aquarium system and continuously aerated to maintain dissolved  $\text{O}_2$  near saturation. The fish were fed to apparent satiation twice daily with two equal meals (09.00 and 16.00 hours) during the experiment. The amount of feed consumed by the fish in each tank was recorded daily and the dead fish was weighed, and feed intake (FI) and feed conversion rate (FCR) were calculated. Zn concentrations in water samples collected 10 min before and after feeding remained very low and negligible throughout the experiment. The experiment was continued for 8 weeks.

The experiment was conducted at ambient temperature and under a natural photoperiod (approximately 12 h light–12 h dark). Water quality parameters were monitored twice a week in the morning, and the ranges of the parameters were as follows: water temperature,  $23.1$ – $25.0^{\circ}\text{C}$ ; pH,  $8.1$ – $8.5$ ; dissolved  $\text{O}_2 \geq 6.00$  mg/l;  $\text{NH}_4\text{-N} \leq 0.06$  mg/l.

### Sampling

At the end of the 8-week period, 24 h after the last feeding, all fish were euthanised (MS-222 at 100 mg/l), counted and weighed to determine survival, weight gain (WG) and specific growth rate (SGR). Then, fish were dissected and the contents of the intestine were gently scraped off. The fore-intestine (from the end of the stomach to the first loop of the intestine) and mid-intestine (from the first loop to the last loop) were used for the following analysis. A total of four fish per tank were randomly collected and dissected on ice to obtain the fore- and mid-intestine samples for TAG analysis. For enzyme activity and mRNA expression assays, the fore- and mid-intestine samples of twelve fish from each tank (six fish for enzymatic activities and six fish for mRNA expression) were removed immediately using sterile forceps, frozen in liquid  $\text{N}_2$  and stored at  $-80^{\circ}\text{C}$  (not longer than 2 weeks) for further processing. Remaining samples were stored at  $-80^{\circ}\text{C}$  for determining the Zn content.

### Samples analysis

**Enzymatic activity assays.** For intestinal lipogenic enzyme analysis, the anterior- and mid-intestine samples were homogenised in three volumes of ice-cold buffer ( $0.02\text{ M}$  TRIS-HCl,  $0.25\text{ M}$  sucrose,  $2\text{ mM}$  EDTA,  $0.1\text{ M}$  sodium fluoride,  $0.5\text{ mM}$

phenylmethyl sulphonyl fluoride and  $0.01\text{ M}$ -mercapto-ethanol; pH 7.4), and centrifuged at  $20\,000\text{ g}$  at  $4^{\circ}\text{C}$  for 30 min. The supernatant was collected separately, and the activities of five lipogenic enzymes were immediately assayed spectrophotometrically. The reaction was started by addition of the tissue extract. The changes in absorbance at 340 nm were monitored at intervals of 15 s for 3 min. 6PGD and G6PD activities were determined by the method of Barroso *et al.*<sup>(28)</sup>, malic enzyme (ME) activity following Wise & Ball<sup>(29)</sup>, isocitrate dehydrogenase (ICDH) activity according to Bernt & Bergmeyer<sup>(30)</sup>, and FAS activity according to the method of Chang *et al.*<sup>(31)</sup> as modified by Chakrabarty & Leveille<sup>(32)</sup>. TAG content and activities of total ATPase and Cu-, Zn-SOD were determined using a commercial TAG assay Kit (A110-0), ATPase assay kit (A070-1) and a superoxide dismutase typed assay kit (A101-2) (Nanjing Jiancheng Bioengineering Institute), respectively. One unit of enzyme activity (U), defined as the amount of enzyme that converted  $1\text{ }\mu\text{mol}$  of substrate to product per min at  $30^{\circ}\text{C}$ , was expressed as units/mg of soluble protein. Soluble protein concentration of homogenates was determined using the method of Bradford<sup>(33)</sup> with bovine serum albumin as the standard. These analyses were conducted in triplicates.

**mRNA expression analysis (real-time fluorescence quantitative PCR).** Analyses at the gene-transcript levels were conducted using the real-time quantitative fluorescence PCR (qPCR) method. Frozen tissues were powdered in a liquid  $\text{N}_2$ -chilled mortar and pestle. Total RNA was extracted from tissues using TRIzol Reagent (Invitrogen) based on the acid guanidinium thiocyanate–phenol–chloroform extraction method. A quantity of  $2\text{ }\mu\text{g}$  of total RNA was used for reverse transcription with RevertAid™ Reverse Transcriptase (Fermentas) and an oligo-dT primer. qPCR assays were carried out in a quantitative thermal cycler (MyiQ™ 2 Two-Color Real-Time PCR Detection System; Bio-Rad) with a  $20\text{ }\mu\text{l}$  reaction volume containing  $10\text{ }\mu\text{l}$  of  $2 \times \text{SYBR}^{\text{®}}$  Premix Ex Taq™ (TaKaRa),  $0.4\text{ }\mu\text{l}$  of  $10\text{ mM}$  each of forward and reverse primers,  $1\text{ }\mu\text{l}$  diluted complementary DNA (cDNA) template (10-fold), and  $8.2\text{ }\mu\text{l}$  double-distilled  $\text{H}_2\text{O}$ . Primers used for qPCR analysis of genes are given in Table 2. The qPCR parameters consisted of initial denaturation at  $95^{\circ}\text{C}$  for 30 s, followed by forty cycles at  $95^{\circ}\text{C}$  for 5 s,  $57^{\circ}\text{C}$  for 30 s and  $72^{\circ}\text{C}$  for 30 s. All reactions were performed in duplicates and each reaction was verified to contain a single product of the correct size using agarose gel electrophoresis. A non-template control and dissociation curve were performed to ensure that only one PCR product was amplified and that stock solutions were not contaminated. Standard curves were constructed for each gene using serial dilutions of stock cDNA. The relative expression levels were calculated using the  $2^{-\Delta\Delta C_T}$  method<sup>(34)</sup> when normalising to the geometric mean of the best combination of two genes as suggested by geNorm<sup>(35)</sup>. Before the analysis, we performed an experiment to check the stability of housekeeping genes ( $\beta$ -actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein L7 (RPL7), beta-2-microglobulin (B2M), hypoxanthine-guanine phosphoribosyltransferase (HPRT), TATA-box-binding protein (TBP) and tubulin alpha chain (TUBA)), from which  $\beta$ -actin and B2M showed the most stable level of expression under the experimental conditions.



**Table 2.** Primers used for real-time quantitative fluorescence PCR analysis

Genes	Forward primer (5'→3')	Reverse primer (5'→3')	Size (bp)	Accession no.
<i>6PGD</i>	GCTCTGATGTGGCGAGGTGG	CGTAGAAGGACAGTGCAGTGG	216	JX992745
<i>G6PD</i>	CAGGAATGAACGCTGGGATG	TCTGCTACGGTAGGTCAGGTCC	249	JX992744
<i>FAS</i>	AACTAAAGGCTGCTGGTTGCTA	CACCTTCCCCTCACAAACCTC	141	JN579124
<i>ACCa</i>	GGGGTTTTACGCTGCTTC	GGTCTGATTGGGTGCTCCTG	165	JX992746
<i>CPT1A</i>	ATTTGAAGAAGCACCCAGAGTATGT	CCCTTTTATGGACGGAGACAGA	254	JQ074177
<i>HSLa</i>	GAAGGACAGGACAATGAGAAGC	TGTACCACCAGCCAAGGAGA	110	KJ588764
<i>ATGL</i>	TTGCGGAAATGTGATTGAGGT	CACGGAAGGCAGGAGGGA	291	KF614123
<i>PPAR<math>\alpha</math></i>	CGAGGATGGGATGCTGGTG	CGTCTGGGTGGTTCGTCTGC	323	JX992740
<i>PPAR<math>\gamma</math></i>	ACGCCCGTTTCGTTATCC	TGAGCAGATCACCTGGTCATTG	260	JX992741
<i>SREBP-1</i>	CTGGGTCATCGCTTCTTTGTG	TCCTTCGTTGGAGCTTTTGTCT	188	JX992742
<i>ZnT1</i>	CACAAATGCGGATAGTGGGA	GGTCACTTGGAGCAACTGAAAC	110	KY652749
<i>ZnT5</i>	AAGAAAGGACAGAAGGGGACG	ACCAAAGCGGAGCAGTCAA	281	KY652750
<i>ZnT7</i>	GAACTCCAGCTGCTTTGACC	CCGCCACATCTATCTGAACG	237	KY652751
<i>ZIP4</i>	CATTATAACTTCGCAGACGG	CCAGAAAGCAACCCAGATT	132	KY652752
<i>ZIP5</i>	CAGGACAGGAGATGGTTCAC	GGAAGGACGGCAGACTGATTAC	327	KY652753
<i>MT</i>	ATCCTTGCGAGTGCTCCA	GCAGGAATCGCCCTTACAC	158	EU124661.1
<i>MTF-1</i>	CGAGTTGATGTTGCAGAGCC	GCAGTATGAGGAAAGAAGGGA	294	KY652741
$\beta$ -Actin	GCACAGTAAAGGCGTTGTGA	ACATCTGCTGGAAGGTGGAC	136	EU161066
<i>18S-rRNA</i>	AGCTCGTAGTTGGATCTCGG	CGGGTATTTCAGGCGAGTTTG	196	KP938527
<i>RPL7</i>	GGCAAATGTACAGGAGCGAG	GCCTTGTGAGCTTGACGAA	199	KP938522
<i>B2M</i>	GCTGATCTGCCATGTGAGTG	TGCTGACACTGCAGCTGTA	186	KP938520
<i>HPRT</i>	ATGCTTCTGACCTGGAACGT	TTGCGGTTCACTGCTTTGAT	181	KP938523
<i>UBCE</i>	TCAAGAAGAGCCAGTGGAGG	TAGGGGTAGTCGATGGGGAA	150	KP938524
<i>TUBA</i>	TCAAAGCTGGAGTTCTCGGT	AATGGCCTCGTTATCCACCA	135	KP938526

*6PGD*, 6-phosphogluconate dehydrogenase; *G6PD*, glucose-6-phosphate dehydrogenase; *FAS*, fatty acid synthase; *ACCa*, acetyl-CoA carboxylase; *CPT1*, carnitine palmitoyltransferase I; *HSL*, hormone-sensitive lipase; *ATGL*, adipose TAG lipase; *SREBP-1*, sterol-regulator element-binding protein; *MT*, metallothionein; *MTF-1*, metal response element-binding transcription factor-1; *RPL7*, ribosomal protein L7; *B2M*, beta-2-microglobulin; *HPRT*, hypoxanthine-guanine phosphoribosyltransferase; *UBCE*, ubiquitin-conjugating enzyme; *TUBA*, tubulin alpha chain.

**Zinc content.** For the determination of Zn content, the fore- and mid-intestinal samples were digested in 3 ml concentrated nitric acid at 110°C for 72 h, and diluted to appropriate concentrations for Zn content using ICP-MS<sup>(36)</sup>. Quality assurance/quality control procedures included analysis of three method blanks (purified water), two certified biological reference tissues (DORM-2 and DORM-4; National Research Council of Canada) and two randomly selected duplicate samples per twenty samples. Recovery of Zn from certified biological reference tissues mentioned above ranged from 95 to 102%.

**Calculations**

$$SR \text{ (survival rate)} = (100 \times (\text{final fish number}) / (\text{initial fish number}))$$

$$WG \text{ (weight gain)} = ((\text{FBW} - \text{IBW}) / \text{IBW}) \times 100 \%$$

$$SGR \text{ (specific growth rate, \% / d)} = (100 \times (\ln(\text{FBW}) - \ln(\text{IBW})) / d)$$

$$FCR \text{ (\%)} = \text{feed intake} / ((\text{FBW} - \text{IBW} + \text{dead fish weight}), \text{ g}).$$

**Statistical analysis**

The results were presented as means with their standard errors. Before statistical analysis, all data were tested for normality of distribution using the Kolmogorov–Smirnov test. Then, data from each treatment were subjected to one-way ANOVA. When overall differences were significant ( $P < 0.05$ ), Duncan’s multiple range test was used to compare significant differences between the treatments. Statistical analysis was performed using SPSS 19.0 for Windows.

**Table 3.** Effect of dietary zinc levels on growth performance of yellow catfish after 8 weeks (Mean values with their standard errors;  $n$  3 replicate tanks, five fish were sampled for each tank)

	Diets					
	Zn deficiency		Adequate Zn		Zn excess	
	Mean	SEM	Mean	SEM	Mean	SEM
IBW	0.80	0.02	0.80	0.03	0.83	0.01
FBW	1.90	0.03	2.05	0.08	1.94	0.03
WG	138.4 <sup>a</sup>	6.1	155.2 <sup>b</sup>	3.0	143.8 <sup>a,b</sup>	4.1
SGR	1.73 <sup>a</sup>	0.05	1.87 <sup>b</sup>	0.02	1.71 <sup>a,b</sup>	0.02
FI	2.05	0.04	2.17	0.06	2.16	0.06
FCR	1.71	0.05	1.67	0.09	1.74	0.09
SR	92.22	1.11	94.45	2.22	91.11	2.22

IBW, initial mean body weight; FBW, final mean body weight; WG, weight gain; SGR, specific growth rate; FI, feed intake; FCR, feed conversion rate; SR, survival rate. <sup>a,b</sup> Mean values with unlike superscript letters were significantly different between the different dietary Zn groups ( $P < 0.05$ ).

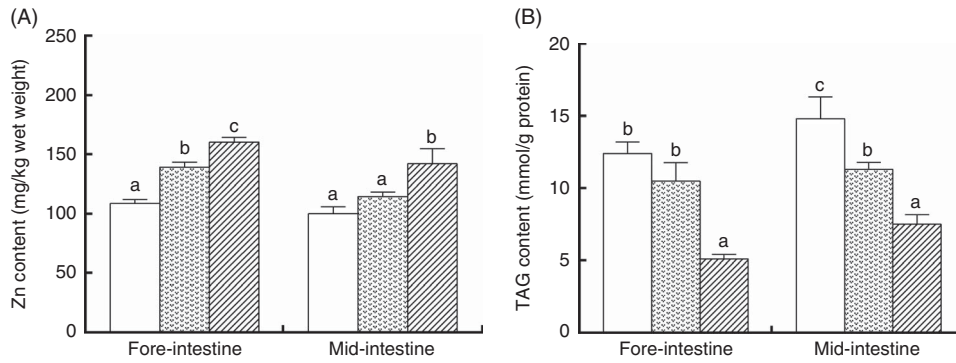
**Results**

**Growth performance**

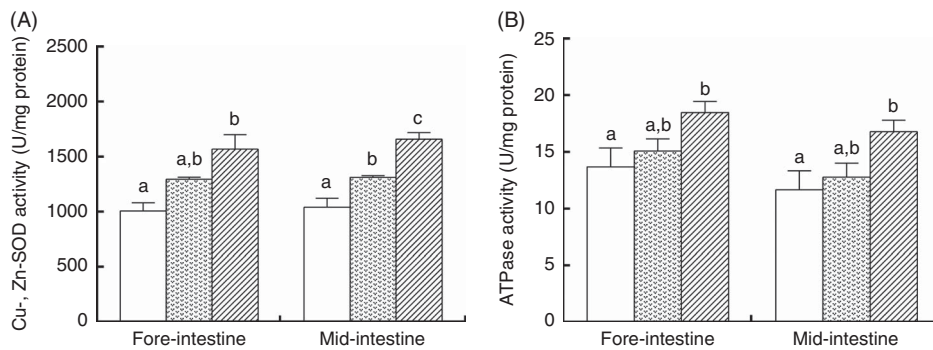
WG and SGR were numerically the highest in the adequate-Zn group but showed no significant differences between the other two groups (Table 3). FI, FCR and survival showed no significant differences between the three treatments.

**Zinc and TAG contents in the intestine**

In the fore-intestine and mid-intestine, Zn contents increased with increasing dietary Zn levels (Fig. 1(A)). In contrast, TAG



**Fig. 1.** Effect of dietary zinc levels on zinc (A) and TAG (B) contents in the intestine of yellow catfish. Values are means (*n* 3 replicate tanks), with standard errors represented by vertical bars. For zinc content analysis, four to six fish sampled for each tank; for TAG content, four fish were sampled for each tank. □, Zinc deficiency; ▨, adequate zinc; ▩, zinc excess. <sup>a,b,c</sup> Mean values with unlike letters were significantly different among three treatments (*P* < 0.05).



**Fig. 2.** Effect of dietary zinc levels on Cu-, Zn-superoxide dismutase (SOD) (A) and ATPase (B) activities in the intestine of yellow catfish. Values are means (*n* 3 replicate tanks, 6 fish were sampled for each tank), with standard errors represented by vertical bars. □, Zinc deficiency; ▨, adequate zinc; ▩, zinc excess. <sup>a,b,c</sup> Mean values with unlike letters were significantly different between the three treatments (*P* < 0.05).

contents in the fore-intestine and mid-intestine decreased with increasing dietary Zn levels (Fig. 1(B)).

#### Activities of intestinal enzymes involved in zinc transport

In the fore-intestine and mid-intestine, Cu-, Zn-SOD and total ATPase activities increased with increasing dietary Zn levels (Fig. 2).

#### mRNA expression levels of genes involved in zinc metabolism

In the fore-intestine, mRNA levels of ZnT1, ZnT5, ZnT7, MT and MTF-1 tended to increase with increasing dietary Zn levels (Fig. 3(A)). mRNA levels of ZnT1, ZnT5, ZnT7, MT and MTF-1 in the Zn-excess group were the highest and were significantly higher than those in the other two groups. By contrast, ZIP5 mRNA levels declined with increasing dietary Zn levels. ZIP4 and ZIP5 mRNA levels were the lowest for fish fed the Zn-excess diet and showed no significant difference between the other two groups.

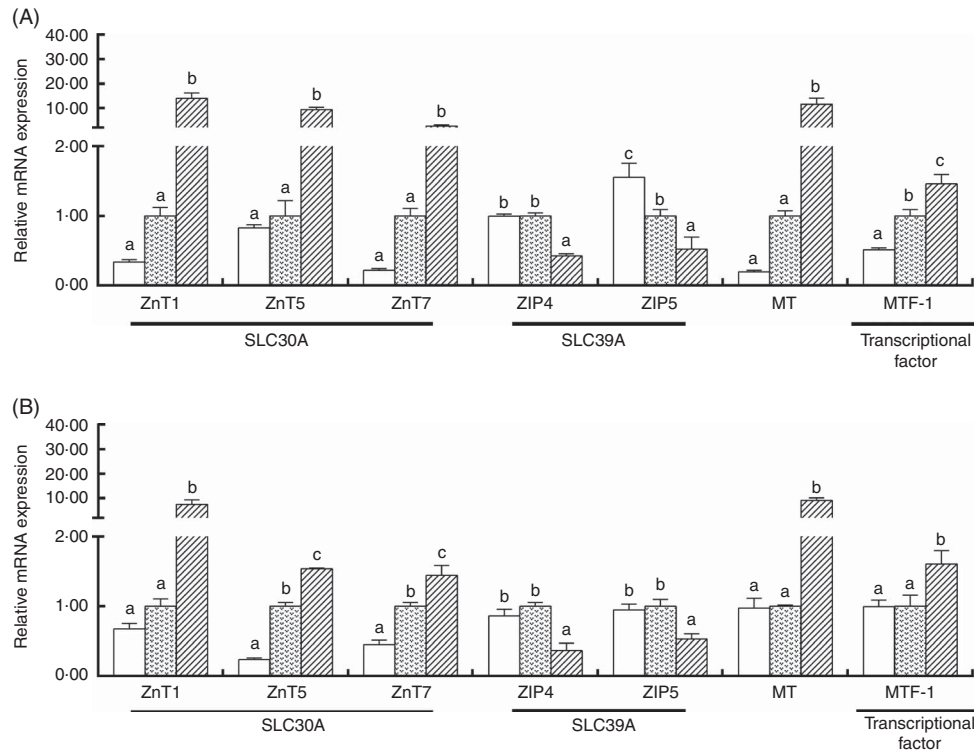
In the mid-intestine, dietary Zn addition up-regulated mRNA levels of ZnT1, ZnT5 and ZnT7 (Fig. 3(B)). mRNA levels of ZnT5 and ZnT7 in the Zn-excess group were the highest and were significantly higher than those in the other two groups.

mRNA levels of ZIP4 and ZIP5 were the lowest for fish fed the Zn-excess diet and showed no significant differences between the other two groups. ZnT1, MT and MTF-1 mRNA levels were the highest for fish fed the Zn-excess diet and showed no significant differences between the other two groups.

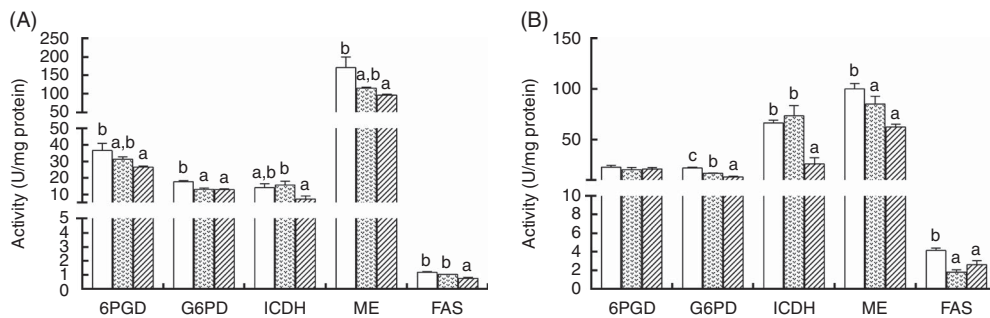
#### Intestinal lipogenic enzyme activities

In the fore-intestine, 6PGD, ME and FAS activities tended to decrease with increasing dietary Zn levels (Fig. 4(A)). G6PD activity in the Zn-deficiency group was significantly higher than that of the adequate- and excess-Zn groups. ICDH activity was the highest for fish fed the adequate-Zn diet and showed no significant differences between the other two groups. FAS activity in the Zn-excess group was the lowest and was significantly lower than that in the other two groups.

In the mid-intestine, G6PD and ME activities tended to decrease with increasing dietary Zn levels (Fig. 4(B)). G6PD and ME activities in the Zn-deficiency group was the highest and was significantly higher than that of the adequate- and excess-Zn groups. ICDH activity in the Zn-excess group was the lowest and it showed no significant difference between the adequate-Zn and Zn-deficiency groups. FAS activities were the highest in the Zn-deficiency group and they showed no significant differences between the other two groups. 6PGD showed no significant differences between the three treatments.



**Fig. 3.** Effect of dietary zinc levels on the mRNA levels of genes involved in zinc metabolism in the fore-intestine (A) and mid-intestine (B) of yellow catfish. Values are means ( $n$  3 replicate tanks, 6 fish were sampled for each tank), with standard errors represented by vertical bars. □, Zinc deficiency; ▨, adequate zinc; ▩, zinc excess; MT, metallothioneins; MTF-1, metal response element-binding transcription factor-1. mRNA expression values were normalised to  $\beta$ -actin and beta-2-microglobulin (*B2M*) expressed as a ratio of the control (control = 1). <sup>a,b,c</sup> Mean values with unlike letters were significantly different between the three treatments ( $P < 0.05$ ).



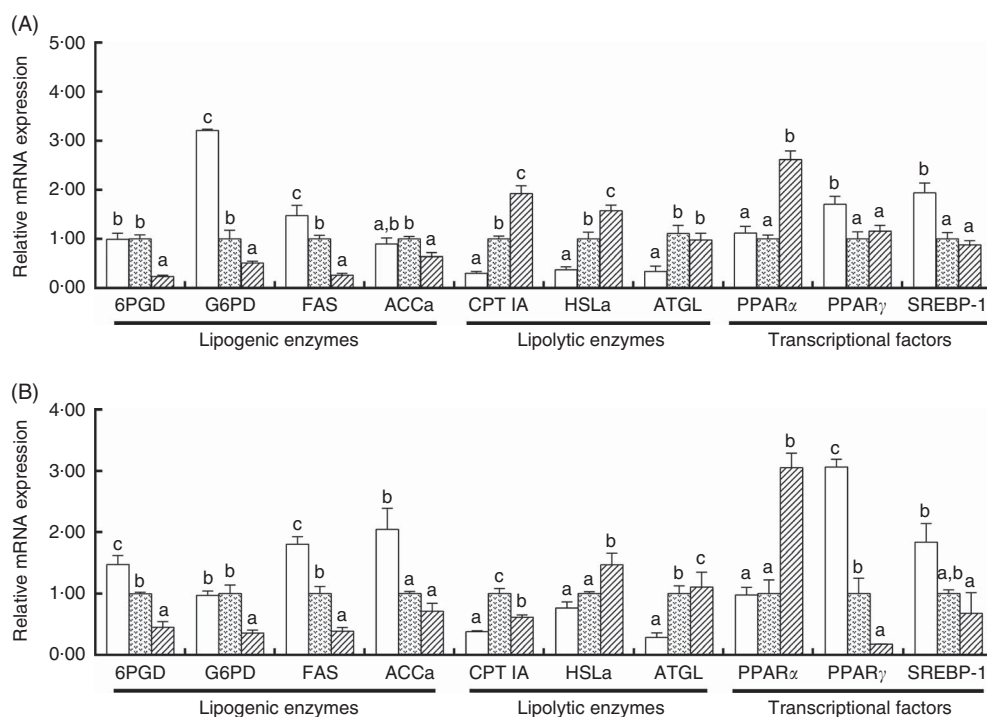
**Fig. 4.** Effect of dietary zinc levels on enzyme activities involved in lipid metabolism in the fore-intestine (A) and mid-intestine (B) of yellow catfish. Values are means ( $n$  3 replicate tanks, 6 fish were sampled for each tank), with standard errors represented by vertical bars. □, Zinc deficiency; ▨, adequate zinc; ▩, zinc excess; 6PGD, 6-phosphogluconate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; ME, malic enzyme; ICDH, isocitrate dehydrogenase; FAS, fatty acid synthase. <sup>a,b,c</sup> Mean values with unlike letters were significantly different between the three treatments ( $P < 0.05$ ).

### *mRNA expression levels of genes involved in lipid metabolism*

In the fore-intestine, mRNA levels of G6PD and FAS tended to decline, but mRNA levels of CPT IA and HSLA tended to increase with increasing dietary Zn levels (Fig. 5(A)). mRNA levels of G6PD, 6PGD, FAS and ACCa in the Zn-excess group were significantly lower than those of the adequate-Zn and Zn-deficiency groups. CPT IA, HSLA and ATGL mRNA levels for fish fed the Zn-deficient diet were significantly lower than those of the adequate- and excess-Zn groups. PPAR $\alpha$  mRNA levels were the highest for fish fed the Zn-excess diet, and showed no significant differences between the other two groups. mRNA

levels of PPAR $\gamma$  and SREBP-1 were the highest for fish fed the Zn-deficient diet and showed no significant differences between the other two groups.

In the mid-intestine, dietary Zn addition tended to down-regulate mRNA levels of 6PGD, FAS, ACCa, PPAR $\gamma$  and SREBP-1, but to up-regulate the mRNA expression of HSLA and ATGL (Fig. 5(B)). mRNA levels of G6PD, 6PGD and FAS in the Zn-excess group were significantly lower than those of the adequate-Zn and Zn-deficiency groups. ACCa mRNA level in the Zn-deficiency group was significantly higher than that in the other two groups. The highest PPAR $\alpha$  expression was observed for fish fed the Zn-excess diet and showed no significant differences between other two groups. CPT IA mRNA levels



**Fig. 5.** Effect of dietary zinc levels on the mRNA levels of genes involved in lipid metabolism in the fore-intestine (A) and mid-intestine (B) of yellow catfish. Values are means ( $n$  3 replicate tanks, 6 fish were sampled for each tank), with standard errors represented by vertical bars.  $\square$ , Zinc deficiency;  $\text{▨}$ , adequate zinc;  $\blacksquare$ , zinc excess; 6PGD, 6-phosphogluconate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; FAS, fatty acid synthase; ACCa, ACC, acetyl-CoA carboxylase; CPT I, carnitine palmitoyltransferase I; HSL, hormone-sensitive lipase; ATGL, adipose TAG lipase; SREBP-1, sterol-regulator element-binding protein. mRNA expression values were normalised to  $\beta$ -actin and beta-2-microglobulin (*B2M*) expressed as a ratio of the control (control = 1). <sup>a,b,c</sup> Mean values with unlike letters were significantly different between the three treatments ( $P < 0.05$ ).

were the highest for fish fed the adequate-Zn diet and lowest in the Zn-deficient group. PPAR $\gamma$  mRNA level for fish fed the Zn-deficient diet was significantly higher than that of the adequate-Zn and Zn-deficiency groups. SREBP-1 mRNA level was the highest for fish fed the Zn-deficient diet, but showed no significant differences between the other two groups.

## Discussion

Previous studies in yellow catfish have focused on the changes in lipid deposition and metabolism in the liver, muscle and adipose tissue<sup>(5,37,38)</sup>, and lipid metabolism in the intestine has been neglected. To our knowledge, this is the first study aimed at deducing the basic mechanisms of intestinal lipid metabolism in combination with Zn transport in a fish species.

In the present study, dietary Zn addition induced intestinal Zn accumulation, in agreement with other reports<sup>(39,40)</sup>. To explore the mechanism of Zn absorption and accumulation, we analysed the activities of enzymes and/or the mRNA expression of genes involved in Zn metabolism. Our results indicated that dietary Zn addition tended to increase total ATPase and Cu-, Zn-SOD activities. The increases in ATPase activity could possibly occur because of the maintenance of the ion flux<sup>(41)</sup>. Kavitha & Rao<sup>(42)</sup> suggested that the induction in antioxidant enzymatic activities could be an adaptive response to toxicant stress and to neutralise the impact of ROS generated. The report demonstrated that dietary Zn excess up-regulated mRNA levels of

ZnT1, ZnT5 and ZnT7, but down-regulated mRNA levels of ZIP4 and ZIP5 in the fore-intestine and mid-intestine. ZnT proteins are characterised by their ability to decrease the cytosolic Zn concentration by transporting Zn out of cells or into intracellular compartments<sup>(43)</sup>. In mammals and in zebrafish, dietary Zn regulates Zn-transport activities and Zn-transporter gene expression in the intestine<sup>(2,44)</sup>. Studies reported the up-regulation of mRNA levels of ZnT1<sup>(44,45)</sup>, which, in turn, reduced cytosolic Zn availability<sup>(19)</sup>. ZnT5 and ZnT7 are localised on the membrane of the Golgi apparatus as well as the cytoplasmic vesicles, and these transporters have been shown to transport Zn into the secretory pathway for Zn sequestration and/or Zn supply to the proteins that require Zn for structural formation or activities<sup>(17,43)</sup>. Thus, increased ZnT5 and ZnT7 mRNA expression will increase the transport of Zn from the cytosol into the secretory pathway for Zn sequestration and, accordingly, reduce Zn toxicity. These regulatory patterns would be consistent with functions of the corresponding proteins in Zn efflux from the cytosol, and reflect the need for greater Zn-export capacity from the cytosol, which helps delay intracellular Zn toxicity in the intestine. The present study indicated that dietary Zn addition down-regulated mRNA levels of ZIP4 and ZIP5 in the fore-intestine and mid-intestine. Increased ZIP4 mRNA expression results in increased dietary Zn absorption in response to Zn restriction, as suggested by several studies<sup>(16,46)</sup>. Kambe *et al.*<sup>(6)</sup> pointed out that ZIP5 was functional as a Zn importer and ZIP5 expression decreased in Zn-depleted environments. Taken together, these changes

suggested that the fractional Zn absorbed from the diet decreased and the export of Zn from the intestinal epithelium increased as dietary Zn load increased, indicating the presence of a mechanism for regulating the metabolism of dietary Zn. Our result showed that MT mRNA levels in the fore-intestine and mid-intestine were up-regulated when fish were fed with excessive dietary Zn, in agreement with many other studies<sup>(2,45,47)</sup>. In the present study, dietary Zn excess induced the up-regulation of MTF-1 mRNA level in the fore-intestine and mid-intestine, together with the up-regulation of ZnT1 and MT gene expression. MTF-1 is important for the induction of ZnT1 and MT expression by Zn, via binding to multiple metal-responsive elements in the 5' regulatory regions of their respective genes' promoter<sup>(47,48)</sup>. The activation of MTF-1 in response to Zn parallels increases in the relative rate of transcription of MT-1 and ZnT1<sup>(45,48,49)</sup>. Thus, these results reported, generally, similar rates in fore- and mid-intestinal segments, which was not surprising in view of the fact that activities and gene expression related to Zn uptake and transport were similar in the two segments, although exceptions had been reported.

The present study indicated that, in the fore-intestine, dietary Zn addition reduces TAG content, activities of 6PGD, G6PD, ME and FAS as well as down-regulated mRNA levels of 6PGD, G6PD and FAS. G6PD, 6PGD, ICDH, ME and FAS are the key regulatory enzymes and genes involved in lipogenesis<sup>(26)</sup>. The reduction in activities of lipogenic enzymes (6PGD, G6PD, ME and FAS) and gene expression (6PGD, G6PD and FAS) in response to dietary Zn addition would contribute to the reduced TAG content. Similarly, Eder & Kirchgessner<sup>(50)</sup> reported that dietary Zn-deficiency increased the activities of lipogenic enzymes (6PGD and G6PD) in rats. Our study also found that the changes in activities of FAS, 6PGD and G6PD were in parallel with the changes of their mRNA expression, suggesting that these enzymes were regulated by Zn at the transcriptional level. CPT I, HSL $\alpha$  and ATGL are three key genes involved in lipolysis<sup>(51,52)</sup>. The present study indicated that dietary Zn addition up-regulated mRNA levels of CPT I $\alpha$ , HSL $\alpha$  and ATGL in the fore-intestine. The up-regulation of these lipolytic enzymatic genes following dietary Zn addition would increase lipolysis, which, in turn, would reduce TAG content, as observed in the present study. Similarly, Zheng *et al.*<sup>(4)</sup> found that hepatic lipid contents declined with increasing dietary Zn levels, in parallel with increasing mRNA levels of CPT I $\alpha$ , G6PD and 6PGD, and reduced mRNA levels of ACC $\alpha$  and FAS, in yellow catfish. Our study indicated that, in the mid-intestine, dietary Zn addition reduced TAG contents, activities of G6PD, ME, FAS and ICDH and mRNA levels of 6PGD, G6PD, FAS and ACC $\alpha$ , but up-regulated mRNA levels of HSL $\alpha$ , ATGL and CPT I $\alpha$ . Thus, our study clearly suggested that dietary Zn supplementation decreased TAG content by up-regulating lipolysis and down-regulating lipogenesis in the mid-intestine of yellow catfish. We also found that changes in 6PGD activity in the mid-intestine were not attributable to the change of its mRNA expression. Similarly, enzymatic activities were not always accompanied by parallel changes in mRNA levels<sup>(53)</sup>. The mismatch between gene expression and enzyme-protein level may be involved in the time-lag effect between transcription

and translation and/or RNA stability<sup>(54)</sup>. On the other hand, our observations reveal the similar mRNA expression of genes involved in lipid metabolism between the fore- intestine and mid-intestine after diet-borne Zn exposure, indicating a similar regulatory mechanism in Zn-induced changes of lipid metabolism between the fore- and mid-intestine of yellow catfish.

It is well documented that expression patterns of lipid metabolic pathway genes are, primarily, governed by SREBP-1 and PPAR $\alpha$ <sup>(27,55)</sup>. The present study indicated that dietary Zn addition up-regulated PPAR $\alpha$  mRNA levels, but down-regulated mRNA levels of PPAR $\gamma$  and SREBP-1 in the fore-intestine. PPAR $\alpha$  plays key roles in the catabolism of fatty acids by increasing the expression of key lipolytic enzymes<sup>(56)</sup>. In the fore-intestine, the up-regulation of PPAR $\alpha$ , together with the increase in the transcriptional levels of lipolytic genes CPT I $\alpha$ , HSL $\alpha$  and ATGL, might contribute to the reduction of TAG content by Zn-added diets, which resulted in reduced TAG content in the fore-intestine. Similarly, Zheng *et al.*<sup>(4)</sup> indicated that the mRNA expression of PPAR $\alpha$  was significantly reduced during Zn deficiency and that this effect was reversible by Zn supplementation. SREBP-1 and PPAR $\gamma$  activate genes involved in lipogenesis<sup>(57,58)</sup>. SREBP-1 and PPAR $\gamma$  mediated TAG synthesis and accumulation by the regulation of genes involved in lipogenesis at the transcriptional level<sup>(59)</sup>. Thus, the reduction in TAG deposition in the fore-intestine by Zn addition might be explained by the suppression of both SREBP-1 and PPAR $\gamma$ , together with the concomitant reduction in the activities of lipogenic enzymes 6PGD, G6PD, ME and FAS, and mRNA levels of 6PGD, G6PD and FAS. Shen *et al.*<sup>(60)</sup> pointed out that the expression of PPAR $\gamma$  was significantly reduced during Zn deficiency. Amemiya-Kudo *et al.*<sup>(57)</sup> suggested that SREBP-1 positively regulated ACC through binding to regulatory sequences in the promoter, and subsequently enhanced the transcription level. By contrast, Zheng *et al.*<sup>(4)</sup> found that mRNA levels of PPAR $\alpha$  and SREBP-1 increased, but PPAR $\gamma$  mRNA levels reduced in the liver with increasing dietary Zn levels. In the mid-intestine, dietary Zn addition tended to down-regulate mRNA levels of PPAR and SREBP-1, in parallel with the reduced activities of G6PD, ME, FAS and ICDH, and mRNA levels of 6PGD, G6PD, FAS, ACC $\alpha$ . Thus, the present study indicated that PPAR $\alpha$ , PPAR $\gamma$  and SREBP-1 mediated the regulation of lipid deposition and metabolism in the fore- and mid-intestine in yellow catfish. Similarly, several studies reported that these signalling pathways widely mediated mineral element-induced changes in hepatic lipid deposition in fish<sup>(36,38)</sup>.

In conclusion, dietary Zn supplementation increased Zn accumulation but reduced TAG content in both the fore- and mid-intestine of yellow catfish. Increased Zn accumulation was attributable to the changes in enzymatic activities and mRNA expression of genes involved in Zn absorption and transportation. The reduced TAG content in both the fore- and mid-intestine of yellow catfish were attributable to the up-regulated lipolysis and down-regulated lipogenesis. Our observations also revealed the similar mRNA expression of genes involved in Zn and lipid metabolism between the fore- and mid-intestine after dietary Zn addition, indicating presence of the same or similar regulatory mechanisms of Zn and lipid metabolism in the fore- and mid-intestine of yellow catfish.



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Contributions of authors are as follows: Z. L. and G.-H. C. designed the experiment and analysed the data; G.-H. C. conducted the feeding trial, sample analysis and drafted the article; D.-G. Z. and S.-C. L. assisted with conducting the feeding trial and sample analysis; K. W. helped with analysis of gene expression and enzymatic activities; Z. L. and C. H. revised the manuscript; all authors read and approved the final paper.

The authors declare that there are no conflicts of interest.

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