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Diet supplementation of organic zinc positively affects growth, antioxidant capacity, immune response and lipid metabolism in juvenile largemouth bass, *Micropterus salmoides*

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Abstract

Zn is an important trace element involved in various biochemical processes in aquatic species. An 8-week rearing trial was thus conducted to investigate the effects of Zn on juvenile largemouth bass (*Micropterus salmoides*) by feeding seven diets, respectively, supplemented with no Zn (Con), 60 and 120 mg/kg inorganic Zn (Sul60 and Sul120), and 30, 60, 90 and 120 mg/kg organic Zn (Bio30, Bio60, Bio90 and Bio120). Sul120 and Bio120 groups showed significantly higher weight gain and specific growth rate than Con group, with Bio60 group obtaining the lowest viscerosomatic index and hepatosomatic index. 60 or 90 mg/kg organic Zn significantly facilitated whole body Zn retention. Up-regulation of hepatic superoxide dismutase, glutathione peroxidase and catalase activities and decline of malondialdehyde contents indicated augmented antioxidant capacities by organic Zn. Zn treatment also lowered plasma aminotransferase levels while promoting acid phosphatase activity and hepatic transcription levels of *alp1*, *acp1* and *lyz-c* than deprivation of Zn. The alterations in whole body and liver crude lipid and plasma TAG contents illustrated the regulatory effect of Zn on lipid metabolism, which could be possibly attributed to the changes in hepatic expressions of *acc1*, *pparγ*, *atgl* and *cpt1*. These findings demonstrated the capabilities of Zn in potentiating growth and morphological performance, antioxidant capacity, immunity as well as regulating lipid metabolism in *M. salmoides*. Organic Zn could perform comparable effects at same or lower supplementation levels than inorganic Zn, suggesting its higher efficiency. 60 mg/kg supplementation of organic Zn could effectively cover the requirements of *M. salmoides*.

Key words: **Organic zinc: Largemouth bass: Antioxidant capacity: Immune response: Lipid metabolism**

Zinc (Zn) is a trace element which participates in various fundamental biochemical processes in vertebrates. It is an indispensable component of certain metalloenzymes including alkaline phosphatase (ALP) and superoxide dismutase (SOD)[1,2], which makes it a pivotal nutrient to cope with oxidative stress and strengthen immunity. Several researches have elaborated that Zn deficiency in animals results in oxidative damage by oxygen-free radicals[3], impairs resistance to infection diseases[4] and also increases the risk of DNA damage which could ultimately develop into cancer[5]. Thus, it can be summarised that appropriate Zn absorption is essential for maintaining a normal immune system in animals. Nevertheless, it is not advisable to include excessive Zn in daily feed intake either, which may trigger physiological disorders including disrupted mitochondrial homoeostasis[6], elevated blood pressure level[7] and Cu deficiency[8]. Therefore, it is of great necessity to find out what level of Zn supplementation can fulfil the basic need of different species to optimise their growth performance and immune response. Several studies have already revealed the recommended Zn supplementation levels of some aquatic animals. Luo *et al.* reported that 17·12–20·86 mg/kg Zn supplementation was adequate for satisfying the need of *Pelteobagrus fulvidraco*[9], while Shi *et al.* stated that 104·8 mg/kg Zn addition, for *Litopenaeus vannamei*, was the optimal requirement level

Abbreviations: ACP, acid phosphatase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAT, catalase; GSH-PX, glutathione peroxidase; MDA, malondialdehyde; HSI, hepatosomatic index; SOD, superoxide dismutase; Cu-Zn SOD, Cu-Zn superoxide dismutase.

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based on growth performance[10]. Other studies on *Labeo rohita*, *Epinephelus malabaricus* and *Ctenopharyngodon idella* also reported optimal dietary Zn levels of 47·85–52·93 mg/kg, 28·9–33·7 mg/kg and 55·1 mg/kg, respectively[11–13].

Constrained by the limited production as well as the booming requirement of fishmeal in aquaculture industry, substituting fishmeal for plant-derived protein has been increasingly adopted as a feasible strategy to overcome the shortage of fishmeal. However, it should be noted that phytic acid in plant-derived protein can impede the absorption and utilisation of inorganic Zn by animals[14,15], which possibly urges higher levels of Zn supplementation in feed[16]. However, high dietary Zn levels have been proven to have environmentally damaging effects. Excessive Zn would pollute water environments, which could be deleterious or even lethal for aquatic animals, since they are exposed to Zn beyond proper levels continuously. Researchers have illustrated that high-level Zn exposure might lead to tissue accumulation of metal, impair antioxidant activities, negatively influence lipid metabolism and deposition, and also develop histopathological changes in gills and spleen[17–20]. Considering the relatively low efficiency of inorganic Zn to cover basic requirement of aquatic animals, organic Zn has started to be included in aquatic feed over the past few decades because of its greater bioavailability. This explains why less organic Zn was needed to achieve the same growth performance than inorganic Zn[21,22]. Currently, organic Zn is mainly used in the form of amino acid or peptide chelated Zn. It has been reported in aquaculture research that organic Zn facilitates better growth parameters and immune response when supplemented at same level in diets as inorganic Zn[23,24]. Hence, the appropriate supplementation levels of organic Zn remain to be investigated in certain aquatic animals, since the existing standards for inorganic Zn addition are presumed to mismatch the proper amount of organic Zn for optimised rearing performance.

Largemouth bass, an important carnivorous economic fish, is extensively reared in southern China with potential for developing a greater culture industry because of its high nutritional value and promising economic benefits. Nevertheless, its inefficiency at absorbing carbohydrates constrains the application of low fishmeal aquatic feed[25,26]. To be specific, it is very likely to trigger disorder in lipid and carbohydrate metabolism due to the high level of carbohydrates in diets, and meanwhile, inflammation in fish body can be caused by increasing the use of plant-derived dietary ingredients. Hence, in the present study, we explored the regulatory effects of Zn treatment in modulating growth, antioxidant capacity, immunity and lipid metabolism, as well as comparing the difference of such effects in fish fed with organic or inorganic Zn. The results of this study will benefit us in understanding how Zn form and level correlate with physiological changes in fish, as well as promoting rational utilisation of Zn in aquatic feeding.

Materials and methods

Experimental diets

Seven isonitrogenous and isolipidic diets were formulated according to Table 1. Zn sulphate was added to the diets of Sul60 and Sul120 groups, respectively, at the doses of 264 mg/kg and 528 mg/kg to guarantee the supplementation of inorganic Zn at 60 mg/kg and 120 mg/kg. Bioplex-Zn (including 15 % proteinate Zn; Alltech) were also provided to Bio30, Bio60, Bio90 and Bio120 diets at the doses of 200 mg/kg, 400 mg/kg, 600 mg/kg and 800 mg/kg to achieve the desired organic Zn levels. The determined Zn contents in seven diets were 57, 116, 176, 95, 114, 147 and 178 mg/kg, respectively. Pelleted feed was made and utilised in this experiment. Dry ingredients were first ground into fine powder and then thoroughly mixed with fish oil and soyabean lecithin for 10 min. For every kilogram of feed ingredient mixture, 0·4 l water was added to obtain a moist dough, which was then extruded through the pelletizer to form 2·0 mm pellets under 80°C. The pellets were dried at 16°C in well-ventilated environment until moisture content dropped below 100 g/kg and stored at −20°C for future use.

Fish and experimental conditions

Largemouth bass juveniles were purchased from Shunye Fishery Company and then reared in cement tanks for 20 d while feeding a commercial diet (Tongwei Agriculture Development Co Ltd) to acclimate them to the experimental conditions. A total of 630 juveniles (initial body weight: 8·92 ± 0·01 g) that were not wounded and of similar size were selected and randomly allocated to twenty-one cement tanks of 100 litres at a density of thirty fish per tank. Each dietary treatment was provided to three replicate tanks. Fish were fed to satiation twice a day at 09.00 and 16.00 for 8 weeks. Recirculating water system was utilised during the feeding trial, with water temperature maintained at 24–28°C, pH at 7·9–8·2, dissolved oxygen concentration at about 9·0 mg/l and ammonia nitrogen level lower than 0·2 mg/l. Water was refreshed once a week, and faeces were removed timely for preventing deterioration of water quality and potential adverse effects on fish.

Sample collection

At the termination of the feeding trial, fish were fasted for 24 h and the total weight and number of fish in each tank were recorded for statistical analysis of parameters of growth performance. Fish were then anesthetised using MS-222. Four fish from each tank were randomly taken for the determination of morphological parameters including condition factor, visceral somatic index, hepatosomatic index (HSI) and intraperitoneal fat ratio by measuring their body length, body weight, visceral mass weight, hepatic weight and mesenteric fat weight. Blood samples were collected before dissection from the caudal vein of the same four fish from each tank using sterile 1 ml syringes and then centrifuged at 6000 rpm for 10 min at 4°C. Plasma samples were obtained by aspirating the upper layer into a new centrifuge tube and storing it at −80°C until biochemical analysis. For each tank, the same four fish were further dissected to get liver samples for the analysis of crude lipid contents, antioxidant capacity, immunity and gene expression level, which were quickly frozen in liquid N_2 after collection. Another three fish from each tank were collected for whole body proximate composition and mineral concentration analysis.

Table 1. Ingredients and proximate composition of experiment diets (g 100 g^{-1} diet)

	Con	Sul60	Sul120	Bio30	Bio60	Bio90	Bio120
Wheat flour	12·3	12·2736	12·2472	12·2798	12·2596	12·2404	12·2202
White fishmeal	40	40	40	40	40	40	40
Krill meal	5	5	5	5	5	5	5
Beer yeast	5	5	5	5	5	5	5
Soyabean meal	16	16	16	16	16	16	16
Wheat gluten	10	10	10	10	10	10	10
Fish oil	6	6	6	6	6	6	6
Soyabean lecithin	1	1	1	1	1	1	1
Multivitamin*	1	1	1	1	1	1	1
Multimineral† (deprived of Zn)	1	1	1	1	1	1	1
Choline chloride (50%)	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Monocalcium phosphate	1	1	1	1	1	1	1
Vitamin C	0·2	0·2	0·2	0·2	0·2	0·2	0·2
Sodium alginate	1	1	1	1	1	1	1
Bioplex-Zn (15 %)‡	0	0	0	0·02	0·04	0·06	0·08
ZnSO4·7H2O	0	0·0264	0·0528	0	0	0	0
Sum	100·00	100·00	100·000	100·00	100·00	100·00	100·000
Proximate composition (g 100 g^{-1} diet)							
Moisture	8·58	8·35	9·57	10·51	9·62	9·21	9·04
Crude protein	50·94	50·93	51·46	51·30	53·23	51·52	52·17
Crude lipid	11·38	11·61	11·56	11·51	11·30	11·67	11·90
Zn (mg kg^{-1} diet)	57	116	176	95	114	147	178

* Multi-vitamin (kg^{-1} diet): vitamin B_1, 30 mg; vitamin B_2, 60 mg; vitamin B_6, 20 mg; nicotinic acid, 200 mg; calcium pantothenate, 100 mg; inositol, 100 mg; biotin, 2·5 mg; folic acid, 10 mg; vitamin B_{12}, 0·1 mg; vitamin K3, 40 mg; vitamin A, 10000 μg; vitamin D3, 2000 μg; vitamin E, 160 μg.
† Multimineral (kg^{-1} diet): $MgSO_4 \cdot 7H_2O$, 1090 mg; KH_2PO_4, 932 mg; $NaH_2PO_4 \cdot 2H_2O$, 432 mg; $FeC_6H_5O_7 \cdot 5H_2O$, 181 mg; $ZnCl_2$, 80 mg; $CuSO_4 \cdot 5H_2O$, 63 mg; $AlCl_3 \cdot 6H_2O$, 51 mg; $MnSO_4 \cdot H_2O$, 31 mg; KI, 28 mg; $CoCl_2 \cdot 6H_2O$, 6 mg; $Na_2SeO_3 \cdot H_2O$, 0·8 mg.
‡ Provided by Beijing Alltech Biological Products Co Ltd, China.

Proximate composition analysis

Proximate composition analysis was conducted according to standard methods of Association of Official Analytical Chemists (AOAC) for experimental diets, whole body and liver samples. Crude protein content was determined using Dumas combustion method (Dumas nitrogen analyzer, N pro (DT Ar/He Basic), Gerhardt GMBH & CO. KG). Crude lipid content was detected with Soxhlet extraction method (Soxtec System HT6, Tecator AB). Moisture content was calculated by drying samples at 105°C until constant weight was reached.

Mineral detection

Whole body and feed samples were fully dried and ground into powder, and then 0·1 g of every sample was weighed and completely nitrified in heating nitric acid for collecting colourless and transparent nitrification liquid product. The contents of Zn, Fe and Cu in whole body and Zn in feeds were then detected by utilising Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES, Optima8300, Perkin-Elmer).

Analysis of plasma and hepatic biochemical parameters

The enzyme activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), ALP and acid phosphatase (ACP) as well as the contents of TAG, total cholesterol, HDL-cholesterol, LDL-cholesterol and blood glucose (GLU) were all detected in plasma using diagnostic reagent kits (Nanjing Jiancheng Bioengineering Institute) following the manufacturer's instructions.

A block of liver sample of about 0·5 g was homogenised at 4°C with nine volumes (w:v) of precooled PBS (pH 7·4), and then centrifuged at 3000 rpm for 10 min at 4°C before collecting the supernatant and storing at −80°C as aliquots for future analysis. Hepatic enzyme activities of total superoxide dismutase (T-SOD), Cu-Zn superoxide dismutase (Cu-Zn SOD), glutathione peroxidase (GSH-PX), catalase (CAT) and the level of malondialdehyde (MDA) and total antioxidant capacity were also determined using corresponding reagent kits (Nanjing Jiancheng Bioengineering Institute).

Total RNA extraction, RT and quantitative real-time PCR

Total RNA was extracted from liver samples with RNAeasy™ Animal RNA isolation kit (Beyotime Biotechnology). 1·0 % agarose gel electrophoresis was performed for evaluating the quality of extracts, and a Nanodrop spectrophotometer (Nanodrop 2000, Thermo Scientific) was utilised to determine the concentration of total RNA product. Next, *Evo M-MLV* Reverse transcription reagent kit (Accurate Biology) was used to remove genomic DNA, and reverse transcription reactions were performed under the guidance of manufacturer's instruction to synthesise cDNA from total RNA product. Quantitative real-time PCR was performed on LightCycler 480 II quantitative real-time system (Roche Diagnostics). Each reaction system had a volume of 10 μl containing 5·0 μl 2 × SYBR Green *Pro Taq* HS Premix (SYBR Green *Pro Taq* HS Premix qPCR reagent kit, Accurate Biology), 0·2 μl forward primer, 0·2 μl reverse primer, 2·6 μl DEPC water and 2·0 μl cDNA which has been pre-diluted with nine volumes (v/v) of DEPC water. Reactions on every cDNA sample were all carried out in triplicate. The reaction procedure started with the step of pre-incubation at 95°C for 10 min, followed by the step of amplification, which were forty cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 30 s, and the whole

Table 2. Sequences of gene primers for quantitative real-time PCR

Genes	Forward primers (5' to 3')	Reverse primers (5' to 3')	Genbank No.
alp1	AGGGACTCCGAGACTGATCC	CTGGTCATGAGATCTGCCCG	XM_038696721·1
acp1	GTGAAAAACGCCAAGGCACAG	TCAAAGTCCTCGTCACTCCCA	XM_038726999·1
lyz-c	ATTGAAAAAGGTTTCGGGGCTC	CATTCAGCAAAGTACTCAGAGGC	XM_038713041·1
lyz-g	AGTCCAGGGCCGGAAATGTA	TGTCCACCTCCGTTTGGATT	XM_038713810·1
Cu-Zn sod	TGGCAAGAACAAGAACCACA	CCTCTGATTTCTCCTGTCACC	XM_038708943·1
gsh-px	GTATGTCCGTCCAGGGAATGG	TCCTACAGACGGGACTCCAAA	XM_038697220·1
cat	ATCCCTGTGGGCAAAATGGT	CGGTGACGATGTGTGTCTGG	XM_038704976·1
acc1	ATCCCTCTTTGCCACTGTTG	GAGGTGATGTTGCTCGCATA	XM_038709727·1
fas	CAGCCCTTGACTCATTCCG	CGCAGACTACGACCCGACAG	XM_038735140·1
atgl	CCATGATGCTCCCCTACACT	GGCAGATACACTTCGGGAAA	XM_038705351·1
cpt1	TTCCCCTTTATTGACTTTGGC	AGAACTTCCCTTTGTCCCTGTAA	XM_038705334·1
pparα	CCACCGCAATGGTCGATATG	TGCTGTTGATGGACTGGGAAA	XM_038705496·1
pparγ	CCTGTGAGGGCTGTAAGGGTTT	TTGTTGCGGGACTTCTTGTGA	XM_038695875·1
srebp-1	AGTCTGAGCTACAGCGACAAGG	TCATCACCAACAGGAGGTCACA	XM_038699585·1
ef-1α	GGCTGGTATCTCCAAGAACG	GTCTCCAGCATGTTGTCWCC	KT827794·1

procedure was terminated by running standard melting curves and analysing the amplification efficiency. The results were processed using $2^{-\Delta\Delta Ct}$ method for comparing the relative expression of target genes in different samples. The primers utilised in this study were designed on NCBI, and their sequence information is listed in Table 2.

Statistical analysis

All results are presented as means ± SEM of three replication tanks (n 3). Data were analysed using one-way ANOVA and further examined by Duncan's multiple tests by SPSS 23.0 (IBM) for discovering differences that were statistically significant ($P < 0·05$) between data from different groups.

Results

Growth performance and morphological parameters

The effects of different forms and doses of Zn on growth performance and morphological parameters of *M. salmoides* are shown in Tables 3 and 4. Fish in the Con group had the lowest final body weight, weight gain and specific growth rate among all seven groups, with fish fed diets of Sul120 and Bio120 being significantly higher than those fed the Con diet in these parameters ($P < 0·05$). No significant differences were found in feed conversion ratio between Con group and Zn supplementing groups ($P > 0·05$); however, the feed conversion ratio of fish in groups Bio90 and Bio120 were significantly lower than that of group Sul60 ($P < 0·05$). No statistical differences were recorded among all treatment groups in survival rate ($P > 0·05$), which ranged from 86·67 % to 98·89 %.

Visceral somatic index and HSI were both at their minimum in the Bio60 group, with visceral somatic index of Bio60 group being significantly lower than that of Con, Sul60, Sul120, Bio90 and Bio120 groups ($P < 0·05$), and HSI of Bio60 group being significantly lower than that of Bio120 group ($P < 0·05$). HSI of Bio60 group was also lower than that of Con group, albeit not significant ($P > 0·05$). In inorganic Zn-supplemented groups, higher visceral somatic index and HSI were both recorded in Sul120 group than in Sul60 group ($P > 0·05$), while within the organic Zn-supplemented groups, these two parameters were lower at low Zn levels and became higher as organic Zn leveled up in the diets, with the turning point existing in Bio60 group. No statistical differences were observed in condition factor or intraperitoneal fat ratio of fish in any diet ($P > 0·05$).

Whole body and liver proximate composition analysis

As shown in Table 5, whole body crude protein content was the lowest in Con group, and the level of crude protein of Bio60 group was significantly higher than that of Con group ($P < 0·05$). The crude lipid content in whole body was generally lower when supplemented with Zn, with the contents of Sul120 and Bio60 groups being significantly lower than that of Con group ($P < 0·05$). As for liver samples, Bio60 group had significantly lower crude lipid contents than Bio120 group ($P < 0·05$). Crude lipid content of Bio60 group was also lower than that of Con group, although no statistical differences were recorded ($P > 0·05$). No statistical differences were found in moisture contents of whole body and liver among the treatment groups ($P > 0·05$).

Whole body mineral concentration

As Table 6 presents, the concentrations of Zn, Fe and Cu in whole body are analysed. The concentration of Zn in whole body increased in all Zn-supplemented groups, with the Zn retention level of Bio60 and Bio90 being significantly higher than that of Con group ($P < 0·05$). Bio60 group had higher Zn retention level in whole body than Sul60 group, although no significant difference existed ($P > 0·05$). No significant differences existed between Con group and the other groups in Fe concentration level ($P > 0·05$), but a decreasing trend was both shown in groups supplemented with higher level of inorganic or organic Zn. The concentration of Fe of Bio120 group was significantly lower than that of Bio90 group ($P < 0·05$), while the concentration of Fe in Sul120 group decreased when compared with Sul60 group ($P > 0·05$). No remarkable differences were shown in whole body Cu concentration among all the treatments ($P > 0·05$).

Table 3. The effects of different sources and doses of Zn supplementation on growth performance of *Micropterus salmoides*

	IBW* (g)		FBW† (g)		WG‡ (%)		SGR§ (%/d)		FCR‖		SR¶ (%)	
Groups	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Con	8·93	0·03	35·49	0·74^{a}	297·73	8·80^{a}	2·60	0·04^{a}	1·25	0·08^{ab}	94·44	4·01
Sul60	8·93	0·01	40·61	0·19^{ab}	354·93	2·61^{ab}	2·86	0·01^{ab}	1·35	0·15^{b}	86·67	8·39
Sul120	8·92	0·02	41·82	1·14^{b}	367·85	12·70^{b}	2·91	0·05^{b}	1·10	0·08^{ab}	93·33	3·85
Bio30	8·93	0·02	40·52	0·28^{ab}	353·57	2·98^{ab}	2·85	0·01^{ab}	1·12	0·04^{ab}	98·89	1·11
Bio60	8·94	0·03	39·10	0·25^{ab}	338·01	2·04^{ab}	2·79	0·01^{ab}	1·09	0·04^{ab}	96·67	3·33
Bio90	8·90	0·02	40·03	2·82^{ab}	349·62	30·80^{ab}	2·83	0·13^{ab}	1·06	0·06^{a}	96·67	3·33
Bio120	8·89	0·02	42·19	3·67^{b}	374·82	41·51^{b}	2·92	0·17^{b}	1·06	0·05^{a}	92·22	1·11

IBW, initial body weight; FBW, final body weight; WG, weight gain; SGR, specific growth rate; FCR, feed conversion ratio; SR, survival rate.
Results are presented as 'mean ± SEM' (n 3), and mean values on the same row with different letters indicate significant differences ($P < 0·05$), while with same letters or no letters mean no significant differences ($P > 0·05$).
* IBW (g) = initial body weight/initial number of fish.
† FBW (g) = final body weight/final number of fish.
‡ WG rate (%) = 100 × (final body weight−initial body weight)/initial body weight.
§ SGR (%/d) = 100 × (Ln (final individual weight)−Ln (initial individual weight))/number of feeding days.
‖ FCR = feed consumed/weight gain.
¶ SR (%) = 100 × (final number of fish)/(initial number of fish).

Table 4. The effects of different sources and doses of Zn supplementation on morphological indices of *Micropterus salmoides*

	CF* (g cm^{-3})		VSI† (%)		HSI‡ (%)		IPF§ (%)	
Groups	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Con	1·96	0·05	7·85	0·40^{bc}	2·63	0·32^{ab}	0·74	0·18
Sul60	1·98	0·02	7·85	0·18^{bc}	2·58	0·17^{ab}	0·87	0·03
Sul120	2·06	0·03	8·14	0·26^{c}	3·06	0·27^{ab}	0·85	0·12
Bio30	1·92	0·05	7·37	0·27^{ab}	2·72	0·23^{ab}	0·79	0·08
Bio60	1·96	0·04	6·91	0·17^{a}	2·35	0·02^{a}	0·71	0·07
Bio90	1·96	0·07	7·73	0·08^{bc}	2·81	0·11^{ab}	0·79	0·08
Bio120	1·96	0·05	8·22	0·11^{c}	3·19	0·33^{b}	0·90	0·07

CF, condition factor; VSI, viscerosomatic index; HSI, hepatosomatic index; IPF, intraperitoneal fat ratio.
Results are presented as 'mean ± SEM' (n 3), and mean values on the same row with different letters indicate significant differences ($P < 0·05$), while with same letters or no letters mean no significant differences ($P > 0·05$).
* CF (g cm^{-3}) = 100 × (body weight, g)/(body length^{3}, cm^{3}).
† VSI (%) = 100 × (viscera weight, g)/(whole body weight, g).
‡ HSI (%) = 100 × (liver weight, g)/(whole body weight, g).
§ IPF (%) = 100 × (intraperitoneal fat weight, g/whole body weight, g).

Plasma biochemical parameters

The effects of different forms and doses of Zn on immunity response, lipid status and blood glucose in plasma are shown in Table 7. The activities of both ALT and AST were at their highest level in Con group, with the activity of ALT being significantly lower in all inorganic Zn-supplemented groups as well as Bio60 group ($P < 0·05$). The lowest activity of AST was recorded in the Bio30 group, and both Bio30 and Bio60 groups showed a significant difference with Con group ($P < 0·05$). As expected, the fluctuation of activities of ALP and ACP showed the opposite trend from ALT and AST. The activity of ACP in Bio60 group was significantly higher than that in Con group ($P < 0·05$). Although being not statistically different, the activities of ALP in Zn-supplemented groups were generally higher than that of Con group ($P > 0·05$).

Plasma lipid status also differed among the Zn-supplemented groups. The plasma TAG levels were significantly lower in fish fed Bio60 and Bio90 diets compared with those fed the Con diet ($P < 0·05$). Unlike TAG, plasma total cholesterol contents showed no significant differences among the seven treatment groups ($P > 0·05$). The plasma of fish in Bio90 group contained more HDL-cholesterol than that in Con group ($P < 0·05$). Although no significant difference was occurred, the plasma HDL-cholesterol level of fish in Bio60 and Bio120 groups was higher than that of fish fed inorganic Zn diets of the same supplemental doses, respectively ($P > 0·05$). As for LDL-cholesterol, its content in plasma showed no significant differences among all the treatments ($P > 0·05$). Moreover, the contents of GLU in plasma were generally lowered with Zn supplementation, with the GLU level of Bio60 group being significantly lower than that of Con group ($P < 0·05$).

Hepatic antioxidant capacity

The activities of hepatic antioxidant enzymes are presented in Table 8. Fish in Bio60 group had higher activity of T-SOD than those in all other groups except Sul60 ($P < 0·05$). The highest activity of Cu-Zn SOD was also recorded in liver of fish fed Bio60 diet, although there was no statistical difference in Cu-Zn SOD activity among the seven groups. The activity of GSH-PX in Con group was the lowest among the seven treatment groups, with fish in Sul60, Bio30 and Bio60 group all having significantly higher levels of GSH-PX activity than those in Con

Table 5. The effects of different sources and doses of Zn supplementation on whole body proximate composition of *Micropterus salmoides*

	Con		Sul60		Sul120		Bio30		Bio60		Bio90		Bio120	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Whole body														
Moisture (%)	72·18	0·55	72·24	0·65	72·55	0·41	72·20	0·32	72·79	0·53	72·44	0·47	72·81	0·55
Crude protein (% dry weight)	59·05	0·89a	59·74	0·52ab	61·06	0·26ab	60·89	0·49ab	61·99	0·40b	60·25	1·27ab	60·57	0·27ab
Crude lipid (% dry weight)	24·42	0·75c	24·12	0·80bc	21·61	0·66a	23·45	1·44abc	22·08	0·51ab	24·16	0·21bc	24·15	0·07bc
Liver														
Moisture (%)	68·92	1·28	69·41	1·53	68·64	1·60	69·35	0·54	68·61	0·38	68·55	0·98	68·98	1·25
Crude lipid (% dry weight)	5·42	0·61ab	4·18	0·81ab	5·15	0·62ab	4·51	0·29ab	3·98	0·35a	4·98	0·67ab	5·98	0·46b

Results are presented as 'mean ± SEM' (n 3), and mean values on the same line with different letters indicate significant differences ($P < 0·05$), while with same letters or no letters mean no significant differences ($P > 0·05$).

Table 6. The effects of different sources and doses of Zn supplementation on whole body mineral concentrations of *Micropterus salmoides*

	Con		Sul60		Sul120		Bio30		Bio60		Bio90		Bio120	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Zn (mg kg^{-1})	18·73	1·26a	19·70	3·18ab	25·20	0·49abc	23·23	1·15abc	28·65	1·60bc	29·10	5·10c	27·72	1·75abc
Fe (mg kg^{-1})	36·50	4·62abc	34·43	0·17abc	26·96	2·11a	25·51	2·51a	40·09	2·60bc	44·26	1·04c	29·64	6·20ab
Cu (mg kg^{-1})	15·00	4·05	13·29	4·11	13·20	3·71	11·37	0·43	12·59	1·54	13·06	1·91	10·47	1·53

Results are presented as 'mean ± SEM' (n 3), and mean values on the same line with different letters indicate significant differences ($P < 0·05$), while with same letters or no letters mean no significant differences ($P > 0·05$).

Table 7. The effects of different sources and doses of Zn supplementation on plasma biochemical parameters of *Micropterus salmoides*

	Con		Sul60		Sul120		Bio30		Bio60		Bio90		Bio120	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
ALT (U L^{-1})	21·45	0·84c	16·34	1·59a	16·90	1·52ab	17·58	0·72abc	16·07	1·31a	18·89	0·40abc	20·66	0·57bc
AST (U L^{-1})	8·51	1·23b	7·29	0·29ab	5·64	0·31ab	4·37	0·30a	4·72	0·03a	5·60	1·57ab	5·99	1·21ab
ALP (U L^{-1})	727·73	34·46	957·72	125·63	986·04	87·82	912·24	106·87	1006·92	55·09	998·06	147·59	778·65	67·33
ACP (U L^{-1})	952·81	104·37a	1046·23	29·56ab	1152·63	85·87ab	1219·65	99·47ab	1288·29	109·14b	1049·47	54·08ab	1020·23	72·36ab
TAG (mmol l^{-1})	3·59	0·04b	3·15	0·23ab	2·87	0·44ab	2·52	0·29ab	2·29	0·49a	2·35	0·42a	2·61	0·17ab
Total cholesterol (mmol l^{-1})	9·77	0·74	11·56	1·41	10·04	0·70	12·04	0·99	10·35	1·05	10·40	0·43	11·72	0·42
HDL-cholesterol (mmol l^{-1})	4·13	0·55a	5·00	0·42ab	5·34	0·83ab	6·71	0·77ab	6·08	0·39ab	7·18	1·31b	6·38	0·81ab
LDL-cholesterol (mmol l^{-1})	1·76	0·11	1·65	0·29	1·29	0·05	1·23	0·08	1·93	0·09	1·65	0·28	1·93	0·08
GLU (mmol l^{-1})	5·34	0·26b	4·37	0·38ab	4·16	0·17ab	5·05	0·57ab	3·68	0·28a	4·94	0·16ab	5·22	0·86ab

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; ACP, acid phosphatase.
Results are presented as 'mean ± SEM' (n 3), and mean values on the same line with different letters indicate significant differences ($P < 0·05$), while with same letters or no letters mean no significant differences ($P > 0·05$).

Table 8. The effects of different sources and doses of Zn supplementation on hepatic antioxidant capacities of *Micropterus salmoides*

	Con		Sul60		Sul120		Bio30		Bio60		Bio90		Bio120	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
T-SOD (U $mgprot^{-1}$)	443·16	35·71a	512·31	24·29ab	463·66	3·86a	473·66	3·96a	564·40	39·78b	460·94	14·44a	451·24	9·51a
Cu-Zn SOD (U $mgprot^{-1}$)	275·92	8·00	297·30	16·25	283·60	22·97	293·65	11·72	324·02	9·17	280·60	18·59	280·10	8·24
GSH-PX (U $mgprot^{-1}$)	18·87	1·24a	26·39	1·84bc	21·18	1·06ab	25·60	2·39bc	28·90	2·07c	24·54	2·84abc	23·83	1·03abc
CAT (U $mgprot^{-1}$)	6·87	0·45abc	6·36	0·76ab	6·26	0·80a	9·51	0·43d	9·12	1·26cd	8·81	0·06bcd	5·77	0·60a
MDA (nmol $mgprot^{-1}$)	0·80	0·10b	0·65	0·05ab	0·74	0·12ab	0·67	0·06ab	0·64	0·04ab	0·44	0·06a	0·67	0·21ab
T-AOC (mmol $gprot^{-1}$)	0·12	0·01a	0·15	0·01ab	0·14	0·01ab	0·16	0·01b	0·17	0·01b	0·14	0·00ab	0·14	0·02ab

T-SOD, total superoxide dismutase; Cu-Zn SOD, Cu-Zn superoxide dismutase; GSH-PX, glutathione peroxidase; CAT, catalase; MDA, malondialdehyde; T-AOC, total antioxidant capacity.
Results are presented as 'mean ± SEM' (n 3), and mean values on the same line with different letters indicate significant differences ($P < 0·05$), while with same letters or no letters mean no significant differences ($P > 0·05$).

group ($P < 0{\cdot}05$). The GSH-PX activity of Bio60 group was also significantly higher than that of Sul120 group ($P < 0{\cdot}05$). The inorganic Zn-supplemented groups presented no significant differences in hepatic CAT activity when compared with Con group ($P > 0{\cdot}05$), while CAT activity of Bio30 group was significantly higher than Con, Sul60, Sul120 and Bio120 groups ($P < 0{\cdot}05$), with Bio60 group also having higher CAT activity than Sul60, Sul120 and Bio120 groups ($P < 0{\cdot}05$). Interestingly, the CAT activity gradually lessened as the dose of organic Zn went up. Zn treatment also generally decreased hepatic MDA levels, while only fish in Bio90 group contained significantly lower MDA than those in Con group ($P < 0{\cdot}05$). Unsurprisingly, the levels of total antioxidant capacity were the highest in Bio30 and Bio60 groups, which were significantly greater than that in Con group ($P < 0{\cdot}05$), with all other treatment groups being intermediate.

Expression of immunity and antioxidant capacity-related genes

The hepatic transcription levels of immunity-related genes are shown in Fig. 1. Zn inclusion generally increased the expression of *alp1* and *acp1*. However, only Bio60 and Bio120 groups showed significant differences from Con group for *alp1* and Bio30 group for *acp1* ($P < 0{\cdot}05$). Supplementation of Zn affected the expression of *lyz-c* but not *lyz-g*. The expression of *lyz-c* in Bio60 group was the highest among all treatments and was also significantly higher than that in Con, Sul60 and Bio90 groups ($P < 0{\cdot}05$). No statistical difference was found among the seven groups for the expression of *lyz-g* ($P > 0{\cdot}05$).

As Fig. 2 shows, the transcription levels of antioxidant capacity-related genes were also affected by the Zn supplementation strategies. The expression of *Cu-Zn sod* was the highest in Bio60 group, with significantly greater expression ($P < 0{\cdot}05$) than Con, Sul120, Bio30 and Bio90 groups. The expression levels of *gsh-px* in Sul60, Bio60 and Bio120 groups were all significantly higher than that in Con group ($P < 0{\cdot}05$), which was the lowest among all the groups. Unlike what the enzyme activity has shown, the transcription level of *cat* presented no significant differences among all treatments ($P > 0{\cdot}05$).

Expression of lipid metabolism-related genes

The hepatic transcription levels of lipolysis and lipogenesis-related genes are presented in Fig. 3 and Fig. 4. For lipolysis genes, all Zn-supplemented groups had numerically higher expression levels of *atgl* in liver as compared with the control. However, only the level of Bio120 group was significantly higher than that of Con group ($P < 0{\cdot}05$). Expression level of *cpt1* was significantly higher in fish fed Bio90 diet compared with fish fed the Con, Sul60, Bio60 and Bio120 diets ($P < 0{\cdot}05$), while *pparα* expression exhibited no statistical differences in transcription level among the dietary treatments ($P > 0{\cdot}05$).

In regard to lipogenesis genes, all Zn-supplemented groups except Sul60 group had a lower expression level of *acc1* than Con group, while only the level of Bio120 group was significantly lower than that of Con group ($P < 0{\cdot}05$). Additionally, the level of *acc1* in Sul60 group was significantly higher than those of Sul120, Bio90 and Bio120 groups ($P < 0{\cdot}05$). Significantly lower expression of *fas* was found in Sul120 group compared with Bio30 and Bio90 groups ($P < 0{\cdot}05$), while no statistical difference existed between control and experimental groups ($P > 0{\cdot}05$). Fish fed Bio60 diet had significantly lower expression level of *pparγ* than those from Con, Sul60 and Bio30 groups ($P < 0{\cdot}05$), with the level of *pparγ* in Sul120 group being also significantly lower than that in Sul60 and Bio30 groups ($P < 0{\cdot}05$). Unlike *pparγ*, there were no significant differences in expression levels of *srebp1* between fish fed Con diet and Zn-supplementing diets ($P > 0{\cdot}05$).

Discussion

Zn is an important trace element required in several physiological and biochemical reactions which take place in various tissues in animals[27]. To date, several researches have verified the essential roles of Zn in aquatic animals like tilapia[28], rainbow trout[29] and snakehead[30], mainly focusing on its effects in enhancing growth performance, antioxidant capacity, innate immunity and metabolism. In the present study, we first compared the difference in growth parameters of *M. salmoides* fed Con diet or diets supplemented with Zn products, elucidating that Zn inclusion generally enhanced the growth rate, which was indicative of improved growth performance. Meanwhile, it has been revealed as well that the addition of organic Zn could achieve higher feed efficiency than utilising Zn sulphate in diets. 60 mg/kg of organic Zn supplementation also affected the morphological features of fish more remarkably than inorganic Zn in our research, highlighting the function of organic Zn in the development of fish body. Other researches have given substantial evidences of comparable results in multiple aquatic species including fish[12,31,32] and several crustaceans[10,24,33], which generally supports the functions of Zn in increasing specific growth rate and feed efficiency. Some researches also pointed out that Zn deficiency could lead to abnormality and retardation in physical development and immunity[34,35]. There was a large body of literature indicating that organic Zn had higher bioavailability than Zn sulphate[21,22,36], which likely explained the difference in feed conversion ratio after being fed with Zn of different doses and forms. Zn sulphate is more likely than organic Zn to be chelated with phytic acid in plant feed sources like soyabean meal, which negatively affects its absorption by intestinal tissues and thus leads to higher supplementing levels of inorganic Zn to compensate for the lower bioavailability[14,16,37].

Results from former studies have already determined the influence of Zn in regulating crude lipid contents in whole body and tissues[9,13,20,38]. However, Zn treatments can have promoting or decreasing effects in different kinds of aquatic animals, and the intrinsic regulating mechanism of Zn on lipid deposition have not yet been clarified. In our study, we demonstrated that lipid storage in whole body of *M. salmoides* would be lowered with supplementation of 60 mg/kg organic Zn or 120 mg/kg Zn sulphate, following the same pattern as what was discovered in *P. fulvidraco* and *L. rohita*[9,11], but contrasting with *Scophthalmus maximus* and *C. idella*[13,39]. The crude lipid contents in liver first decreased and then increased again with the increment of organic Zn, suggesting the optimal lipid-lowering

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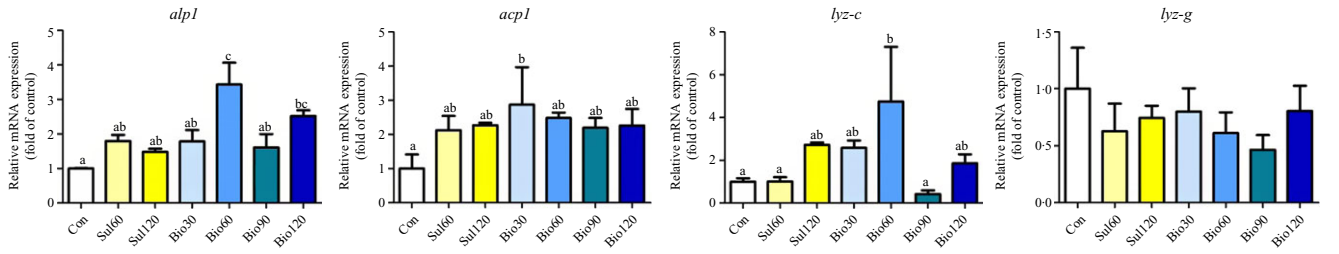

Fig. 1. The relative expression of hepatic immunity-related genes in *Micropterus salmoides* feeding different sources and doses of Zn. Results are presented as 'mean ± SEM' (n 3), and data bars topped with different letters indicate significant differences ($P < 0{\cdot}05$), while with same letters or no letters mean no significant differences ($P > 0{\cdot}05$).

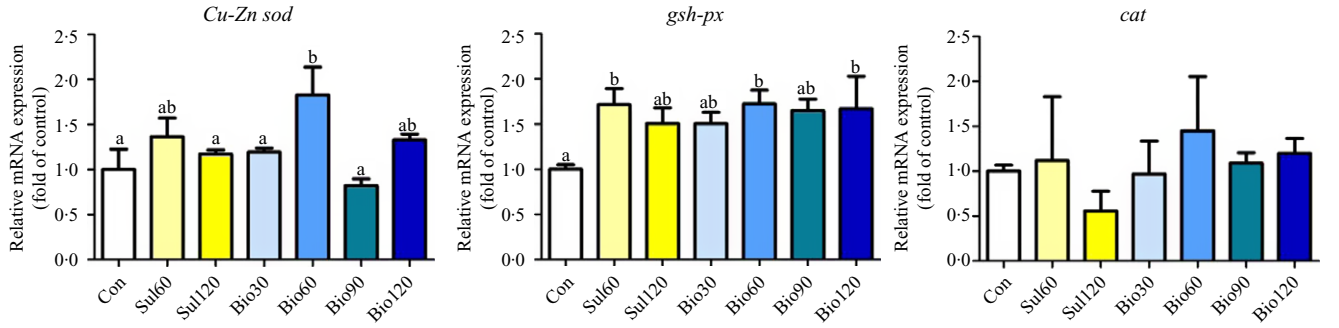

Fig. 2. The relative expression of hepatic antioxidant capacity-related genes in *Micropterus salmoides* feeding different sources and doses of Zn. Results are presented as 'mean ± SEM' (n 3), and data bars topped with different letters indicate significant differences ($P < 0{\cdot}05$), while with same letters or no letters mean no significant differences ($P > 0{\cdot}05$).

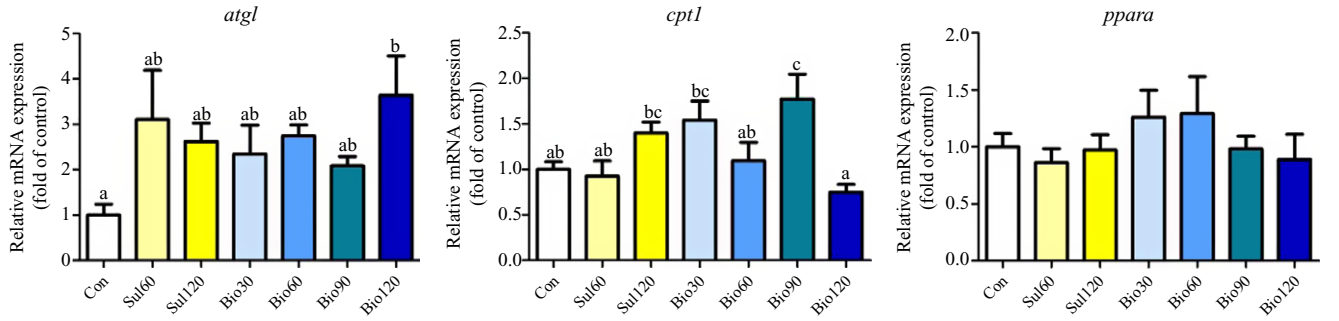

Fig. 3. The relative expression of hepatic lipolysis-related genes in *Micropterus salmoides* feeding different sources and doses of Zn. Results are presented as 'mean ± SEM' (n 3), and data bars topped with different letters indicate significant differences ($P < 0{\cdot}05$), while with same letters or no letters mean no significant differences ($P > 0{\cdot}05$).

effect by 60 mg/kg organic Zn in our experiment. Research on *P. fulvidraco* reported as well the reduction of hepatic lipid contents by dietary Zn addition[20], while study on *C. idella* pointed out that hepatic lipid contents were increased by supplementing higher level of Zn[13]. It could be postulated that such discrepancies originated from the differences in factors like trophic levels, experimental feed compositions and metabolism capability of certain nutrients of various kinds of fish.

Analysis of mineral concentration in whole body also shed light on Zn requirement of *M. salmoides* and variance in bioavailability of inorganic and organic Zn. In our experiment, higher level of inorganic Zn inclusion allowed more Zn accumulation in whole body, while the retention of Zn was first increased and then reached a plateau at 90 mg/kg with the increment of organic Zn, which indicated the saturation of Zn in fish body. Such results were consistent with what were discovered in whole body, liver, scales, vertebrae and serum of *L. rohita*[11], whole body, scales and vertebrae of *C. idella*[13], vertebrae and scales of *E. malabaricus*[12] and whole body of *Oryzias melastigma*[40], in which Zn concentrations all increased initially when started raising the supplement level of Zn and then remained stable after reaching the level of Zn which was sufficient for growth performance and tissue mineralisation. However, some researches revealed that Zn contents might also remain unaffected by the treatment of Zn. Hybrid snakehead showed no significant difference in Zn contents in whole body, vertebrae and liver when fed with different dietary level of Zn[30], and Zn concentration of *L. vannamei* was also reported to be not sensitive to the variation of dietary Zn in whole body[10], which showed inconsistency in response to different level of Zn exposure in multiple aquatic

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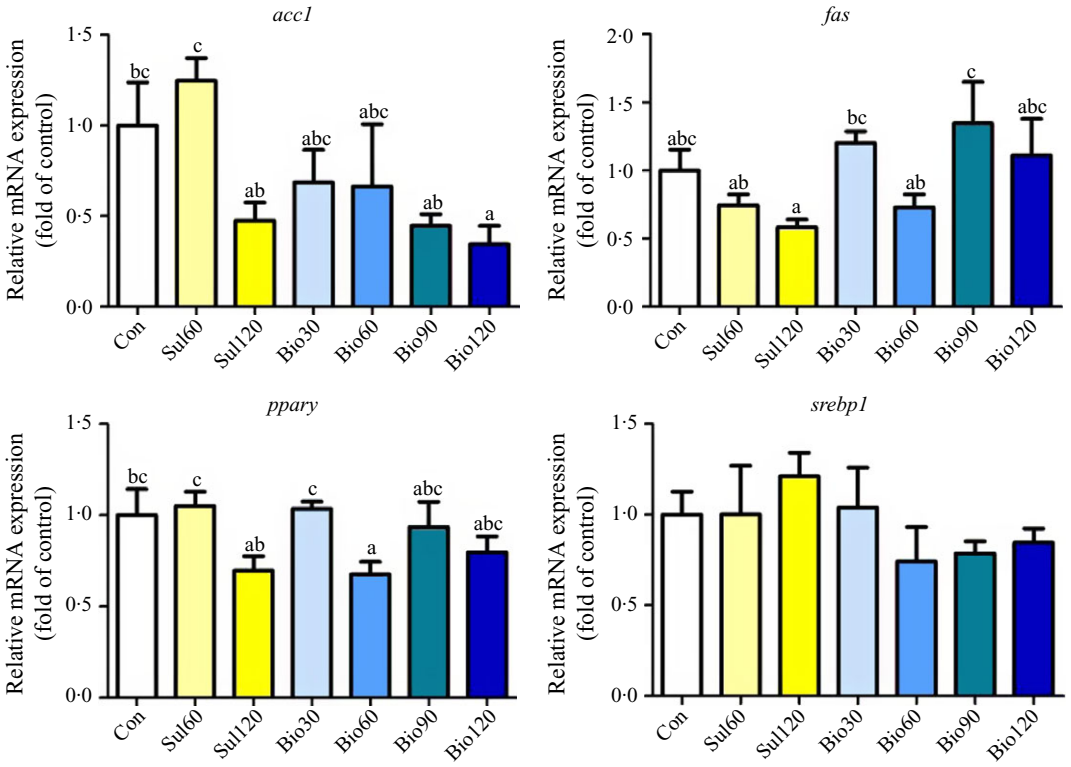

Fig. 4. The relative expression of hepatic lipogenesis-related genes in *Micropterus salmoides* feeding different sources and doses of Zn. Results are presented as 'mean ± SEM' (n 3), and data bars topped with different letters indicate significant differences ($P < 0{\cdot}05$), while with same letters or no letters mean no significant differences ($P > 0{\cdot}05$).

animals. It could be illustrated as well from our results that organic Zn exhibited higher bioavailability than inorganic Zn, as a supplement level of 90 mg/kg organic Zn in diets would be sufficient for the proper requirement of *M. salmoides*, while treatment of 120 mg/kg inorganic Zn retained less Zn than that of 60 and 90 mg/kg organic Zn, although no significant differences were recorded. Similar conclusion was obtained in the research on *Huso huso*, which was found to retain Zn methionine more efficiently than Zn sulphate in muscle[23]. Study on *Ictalurus punctatus* also elucidated that Zn methionine potently met the requirements of Zn with much lower supplement level than Zn sulphate[21].

Some previous researches have elucidated that a high level of dietary intake of Zn inhibited the absorption and deposition of Cu, another vital trace element for animals[9,30,40]. In our research, such trend appeared to be less remarkable, even though the Cu content slightly declined in Zn-supplemented groups when compared with Con group. We might speculate that Cu retention was less susceptible to the level of Zn supplementation in *M. salmoides* than in other aquatic species. The influence of Zn treatment on Fe retention also varied in different species. Fe contents in whole body of *L. rohita* and bone of *Oreochromis niloticus* decreased with the increment in Zn level[11,31], while Fe concentration remained constant in whole body of *O. melastigma* and soft body of abalone under Zn treatment of different levels[22,40]. In general, the supplementation of Zn had no significant effect on altering the content of Fe in our research. Although higher levels of organic Zn supplementation facilitated the retention of Fe in whole body when compared with the supplementing level of 30 mg/kg, such supplementation level should not exceed 90 mg/kg, which negatively affected the deposition of Fe, proven by a significant decline in Fe content of Bio120 group. Since Fe content in Sul120 group was also lower than that in Sul60 group, it could be preliminarily concluded that it was of no use to raise the supplementing level of Zn for optimising Fe retention in *M. salmoides*, while Zn overdosing would even damage the retention of Fe. The inverse relationship between Zn and Fe might be explained by the existence of competitive inhibition between certain cations because of their similarities in characteristics of structures[11]. Therefore, it was suggested that organic Zn supplementation would be sufficient at 60 mg/kg and should be maintained at no more than 90 mg/kg for preventing retarded effects in morphology, lipid deposition and mineralisation, although 120 mg/kg Zn barely show negative effects on growth performance.

In our study, we evaluated as well the effects of Zn on immunity of *M. salmoides* by detecting activities and gene expression of antioxidant enzymes and immunity-related enzymes. The maintenance of antioxidant capacity of organisms including aquatic animals is modulated by the well-organised antioxidant system consisting of SOD, GSH-PX, CAT and other factors[41]. SOD are pivotal enzymes that initially perceive the emergence of exorbitant oxygen-free radicals in animals. They defend against oxidative stress by scavenging superoxide anion radicals and converting them into hydrogen peroxide and oxygen, thus reducing the possibility of generating reactive oxygen species

and reactive nitrogen species[42]. SOD require the existence of metal ions including Cu^{2+} and Zn^{2+} or Mn^{2+} in the catalytic centre as cofactors, which divides them into different isoforms[2]. In our study, total SOD activity was enhanced in fish of Zn-supplemented groups, but its impact became less apparent with higher Zn doses. Cu-Zn SOD activity, specifically, exhibited a similar trend in response to higher doses of Zn supplementation. GSH-PX and CAT collaborate to facilitate the breakdown of H_2O_2 by producing H_2O[43]. GSH-PX generally completes this reaction by involving glutathione (GSH) as a key reductant, which forms GSSG with targeted oxidants being eliminated. This takes place ubiquitously in various cell compartments, and it is understood that GSH-PX also exhibits activities towards lipid hydroperoxides[44,45]. The activities of GSH-PX and CAT could be both elevated by Zn treatment. To be specific, either inorganic or organic Zn could elevate the activity of GSH-PX when supplemented at no more than 60 mg/kg, while the activity of CAT was significantly promoted only by supplementation of 30 mg/kg organic Zn. It was suggested that either form of Zn was capable of positively affecting the antioxidant system, while organic Zn seemed to stimulate higher activity of CAT than inorganic Zn. Lipid oxidation occurs when being exposed to oxidative stress, which usually generates MDA by peroxidation of PUFA, a typical product recognised as a reliable biomarker for evaluating the severity of oxidative stress[46], and higher MDA concentrations are always linked to higher risks of health problems and diseases[47]. The decline of MDA in our study thus suggested the alleviation of oxidative stress by Zn treatment, with organic Zn showing greater capability in reducing lipid peroxidation. Total antioxidant capacity is considered a credible index to evaluate the antioxidant status of certain animals and describe the overall ability of alleviating oxidative stress[48]. From our study, Zn treatment did beneficially affect the antioxidant capacity of *M. salmoides*, with the effect of organic Zn slightly surpassing that of inorganic Zn. Our analysis on relative quantitative expression of antioxidant genes further validated these results. The involvement of Zn promoted the mRNA expression of both *Cu-Zn sod* and *gsh-px*, but these effects did not increase with higher levels of Zn supplementation. A downward trend was recorded in the expression of *Cu-Zn sod* when the supplementing doses of organic Zn exceeded 60 mg/kg. We concluded from these results that certain amounts of Zn could fortify the antioxidant ability of *M. salmoides*, while excessive level of dietary Zn was not profitable for maintaining the antioxidant system in fish body. The importance of Zn in enhancing antioxidant capacity has been demonstrated in previous studies in *Oreochromis mossambicus*[28], *Oncorhynchus kisutch*[49], *Epinephelus coioides*[36], *Megalobrama amblycephala*[38] and *Paramisgurnus dabryanus*[50]. Some of these researches also reported the increase of antioxidant enzyme activity followed by a stable plateau or decline as the supplementation of Zn kept leveling up, with some other researches elaborating the decline of MDA contents by Zn in *L. rohita*[11], *L. vannamei*[10] and *M. amblycephala*[38]. Together with our study, these findings indicate that the addition of dietary Zn should be controlled in an appropriate range. Over-supplementation is unlikely to bring benefits and may actually be harmful to the oxidative stress defence system.

Phosphatases exert great influences in maintaining normal physiological properties in aquatic animals and can be divided into ALP and ACP by their differing optimal pH for catalytic activities[51]. They, together with lysozymes, are crucial molecules involved in humoral innate immunity. ALP, a type of metalloenzyme, requires Zn^{2+} and Mg^{2+} to coordinate with specific amino acid residues. It shows anti-inflammatory activity partly by mediating the detoxification of LPS through dephosphorylation[1,52]. ACP also targets the phosphate bonds of external entities for conducting hydrolysis and advance their degradation[51,53]. Results of our study showed that the activities of both ACP and ALP were promoted with the inclusion of both inorganic and organic Zn, yet a decline could be seen when supplemented with higher levels of organic Zn. It also seemed that organic Zn enhanced ACP activity better than inorganic Zn when supplemented at the same level (60 mg/kg). Similar results were obtained when evaluating the transcription level of genes encoding the two phosphatases, as the level of all Zn inclusion groups exceeded that of the control group, but incremental Zn supplementation did not result in higher levels of *alp1* and *acp1*, which suggested the development of potentially negative effects on immunity caused by too much Zn supplementation. Previous researches have shown that the activity of ALP could be positively regulated by Zn in several tissues including serum[11,13,31], liver or hepatopancreas[49,50] and intestine[32]. Meanwhile, research in *L. vannamei* also detected the elevation of ACP activity with Zn supplementation[37]. Some studies described ALP activity remaining unaltered or being lowered following Zn supplementation at higher levels[36,50], which resembled what we obtained in our experiment, reemphasising the importance of avoiding over-supplementation of Zn in aquatic feed. As an indispensable component of innate immune response in fish species, lysozymes mainly serve as a defender against the infection of microbial pathogens by hydrolysing the peptidoglycan in pathogen cell walls[54]. Lysozymes are classified into different types based on the discrepancies in structures, catalysis and immunization[55], and among them chicken type (Lyz-c) and goose type (Lyz-g) are expressed in vertebrates. In the present study, two types of lysozymes were affected by the supplementation of Zn in different ways. To be specific, the mRNA expression of *lyz-c* was up-regulated with organic Zn at first and then declined at higher Zn levels, while the expression level of *lyz-g* remained relatively stationary notwithstanding the Zn level. It could be postulated that organic Zn could enhance the immunity of *M. salmoides* partly through specifically stimulating the function of *lyz-c* at certain dietary levels. Several studies have recognised the promoting effect of organic Zn on lysozyme activity in *H. huso*[23], *Siganus rivulatus*[27] and *L. vannamei*[10] and expression levels of lysozyme genes in the hepatopancreas of *L. vannamei*[10,37]. However, research has yet to elucidate the types of lysozymes influenced by Zn treatment in different animals. ALT and AST are both sensitive biomarkers for indicating liver injuries[56]. Detection of aminotransferases in the plasma strongly reflects the damage of hepatocellular membrane which makes it possible for enzymes to enter serum by passing through the leaks on the cell surface[57]. A decline in the concentration of both ALT and AST was detected in all Zn-supplemented groups in the present study, from which we could infer that Zn likely

functioned in maintaining the integrity and proper function of cell barrier. The AST level indicated best maintenance of cellular membrane function at 30 mg/kg of organic Zn supplementation. A study in *H. huso* also presented the results that organic Zn treatment obtained lower serum AST and ALT levels than supplementing with Zn sulphate or no Zn supplementation[23]. Such results were consistent with the previously discussed immunological and antioxidant parameters in liver and plasma of *M. salmoides*. Together, these results holistically concluded that no more than 60 mg/kg of organic or inorganic Zn addition could help aquatic animals effectively mitigate endogenous oxidative stress or exogenous pathogen invasion, which lessens the possibility of suffering from worsening physiological conditions and positively regulates immunity.

HDL are lipid carriers that show both metabolic and immunity-regulating properties. They can guarantee proper lipoprotein metabolism through modifying cholesterol efflux and thus reject abnormal cellular cholesterol accumulation[58]. Moreover, they potently inhibit oxidation, inflammation and apoptosis with the help of multiple molecules including apolipoprotein[59]. Conversely, LDL can cause disorders in cholesterol transport and induce inflammation as well as apoptosis after being oxidised, which has long been associated with CVD and atherosclerosis[60,61]. Zn showed great ability in increasing the concentration of HDL-cholesterol in the present study, with HDL-cholesterol levels being generally higher with organic Zn than inorganic Zn, while no prominent difference was seen in LDL-cholesterol level with any Zn level or form compared with the control group. Since no remarkable difference was present in total cholesterol whether supplementing Zn or not, we assumed that Zn inclusion helped maintain normal cholesterol metabolism and transport by regulating the existing forms instead of the amount of cholesterol. Additionally, the concentrations of TAG and GLU in plasma were both lower with Zn supplementation, which might represent the role of Zn in boosting the metabolism as well as limiting the accumulation of lipid and carbohydrate in plasma. This postulation could be supported by the changes in whole body and liver crude lipid contents by Zn treatment. Researches in *M. amblycephala* and *L. vannamei* also found decreasing level of TAG[37,38], while research in *C. idella* contradicted our results in showing a converse trend in TAG content in plasma relative to Zn supplementation level[13]. We tend to attribute such inconsistency to the difference in feeding habits among species and their capabilities of tolerating relatively high carbohydrate levels in diets. In our present study, the level of carbohydrates in experimental diets was believed to exceed the optimal amount for *M. salmoides* according to the study of Amoah *et al.*[62] and Lin *et al.*[26], which confirmed the poor ability of *M. salmoides* to utilise carbohydrates. Some other researches have pointed out that *M. salmoides* might cope with excessive carbohydrates by affecting lipid metabolism, which was mainly manifested in altered expression of lipid metabolism-related genes[63,64]. Therefore, it would be necessary to determine the expression change in metabolism-related genes for further exploration of the effects of Zn in regulating nutrient metabolism, especially exploiting the potential functions of Zn in maintaining metabolism homoeostasis with high carbohydrate diets. Here, we focused mainly on the regulatory effects of Zn on lipid metabolism, as TAG in plasma and crude lipid in liver were both affected by Zn addition. *acc1* and *fas* are genes coding for key enzymes that regulate *de novo* lipogenesis, converting acetyl coenzyme A to fatty acids of longer carbon strands[65], while *atgl* and *cpt1* are genes coding for lipolysis-related enzymes, which respectively target and regulate the biological reaction processes of TAG hydrolysis to generate diglyceride and fatty acid transport through mitochondrial membrane for β-oxidation[66,67]. Zn treatment remarkably augmented lipolysis and attenuated lipogenesis according to our results, which was supported by the change in expression level of *atgl*, *cpt1* and *acc1*. Concomitantly, the decline of plasma TAG in Zn-supplemented groups also revealed the decreasing activity of lipid synthesis and storage in fish body, which was consistent with what we concluded above. Zn is a crucial trace element required for building up 'zinc finger' domains of multiple proteins including PPAR, suggesting its necessity in maintaining the normal biological function of PPAR. Deprivation of Zn may lead to dysfunction of PPARα[68]. PPARα regulates the expression of certain downstream genes in the liver and enhances fatty acid oxidation and ketogenesis under a fasting state, as well as controlling fatty acid transport and energy expenditure[69,70]. PPARγ mainly provokes adipogenesis in adipose tissues and induces *de novo* lipogenesis in hepatocytes, and it also promotes lipid deposition in the liver[68,71]. Our study elucidated that Zn showed higher preference for regulating the transcription of *pparγ*, especially with the supplementation of 60 mg/kg organic Zn, while the transcription of *pparα* was not notably affected by Zn treatment. Such results conveyed the message that Zn might preferentially restrict the activities of enzymes which stimulate lipogenesis and prompt lipid deposition, making PPARγ one of the key transcription factors to modulate lipid homoeostasis in liver tissues. Sterol regulatory element-binding protein 1 (SREBP1) is also a transcription factor which exhibits regulatory activities on fatty acid and TAG synthesis by targeting various lipogenic genes in liver and adipose tissue[72]. Our study was unable to record a difference in *srebp1* expression, which might be ascribed to the selective modulation of Zn on lipogenesis, requiring further experimental exploration for better understanding. In summary, we put a preliminary judgement that Zn is capable of regulating lipid metabolism by modulating lipogenesis and lipolysis, which might potentially provide new strategies for dealing with low availability of carbohydrates in *M. salmoides* and possibly other carbohydrate intolerant species.

Conclusion

Zn is a crucial trace element for aquatic animals. Fish deprived of Zn intake are more vulnerable to oxidative stress and immunological damage, which makes them susceptible to developing metabolic disorders. Our study demonstrated that Zn supplementation provided benefits to growth performance, Zn retention, antioxidant capacity, innate immunity and lipid metabolism in *Micropterus salmoides*. For several parameters, supplementation of same or lower levels of organic Zn achieved a more significant beneficial difference than Zn sulphate, which could be due to the higher bioavailability of organic Zn. It should also be noted that organic Zn supplementation levels are

recommended to be maintained at 60 mg/kg and should be limited within 90 mg/kg in diets of *M. salmoides*, as higher levels of Zn generally brought no incremental benefits, and for some variables, lessened positive changes were recorded, which potentially suggested the fully saturation of Zn levels. This research also provides insights into the role of Zn in modulating lipid metabolism, which requires further exploration.

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X. H., Y. Z., G. L. and J. N. designed the experiment. X. H. carried out the feeding trial. X. H., A. C., Z. L., Z. Z., Y. L., H. W., Z. W. and Y. W. collected the samples and performed the experiment. X. H. completed the data analysis and wrote this manuscript. All authors contributed to the article and approved the final version of the manuscript.

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