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RESEARCH

Effects of Dietary Zinc on Growth, Haematological Indices, Digestive Enzyme Activity, Tissue Mineralization, Antioxidant and Immune Status of Fingerling *Heteropneustes fossilis*

Noorin Zafar[1,2] · Mukhtar A. Khan[1,2]

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Abstract
A 12 week feeding trial was conducted to evaluate the effects of dietary zinc levels on *Heteropneustes fossilis*. Triplicate groups of fish were fed isoproteic (CP; 400 g/kg) and isocaloric (GE; 17.89 kJ/g) diets increasing levels of zinc (0, 5, 10, 15, 20, 25, 30 mg/kg) achieved by supplementing zinc sulphate heptahydrate to basal diet. Analysed concentrations of zinc in diets were 10.68, 15.83, 21.34, 26.74, 30.61, 34.91 and 41.34 mg/kg. Growth indices increased linearly ($P<0.05$) up to 26.74 mg/kg Zn. The protein and ash content of whole body also improved significantly up to 26.74 mg/kg Zn. Whole body fat content showed inverse pattern. Haematological parameters also showed an improving trend with the increase in dietary zinc up to 26.74 mg/kg and then levelled off. Activities of antioxidant enzymes were improved with the increase in dietary zinc level up to 26.74 mg/kg followed by no significant change ($P>0.05$). Serum lysozyme activity also exhibited the similar pattern. Immune response in terms of the activities of lysozyme, alkaline phosphatase and myeloperoxidase was also improved with the increase in dietary zinc levels up to 26.74 mg/kg. Dietary zinc levels affected significantly the whole body as well as vertebrae mineralization. Broken-line regression analysis of weight gain, vertebrae zinc activity, serum superoxide dismutase and protease activity against increasing amounts of dietary zinc revealed that the inclusion of zinc in diet in the range of 26.82–29.84 mg/kg is optimum for growth, haematological indices, antioxidant status, immune response and tissue mineralization in fingerling *H. fossilis*. The information obtained from present study would be helpful in formulating the zinc-balanced commercial feeds to improve the growth and health status of this important fish, thus contributing to aquaculture production and strengthening the food security.

Keywords Zinc · Growth · Food security · Antioxidant status · Immune response · *Heteropneustes fossilis*

Introduction

There is a dire need of increasing the production of quality protein to feed the continuously increasing global population. Fish is considered as an ideal source of quality protein, lipid and micronutrients eliminating nutrient deficiency diseases. Aquaculture industry is contributing substantially in achieving food and nutritional security. Due to increased demand and stagnation in the supply from capture fishery, global aquaculture production needs to be boosted. In order to enhance the aquaculture production, development of species specific nutritionally-balanced commercial feeds is an important perquisite. For developing such feeds, data on nutrient needs of fish species to be cultured is essential. Most of the research carried in the past focused on species-specific macronutrient requirements such as protein, amino acid and lipid but information on micronutrients like mineral and vitamin which are also important for fish survival, growth, health and reproduction [1, 2] are scanty. Among minerals, zinc (Zn) is a vital micronutrient involved in a several metabolic pathways such as immunity, growth, energy metabolism and protein synthesis [3–10]. It is also a cofactor for various enzymes such as alcohol dehydrogenase, alkaline phosphatase, carbonic anhydrase and DNA polymerase [8, 11, 12]. Its function in antioxidant mechanism and haemoglobin synthesis has also been reported [7, 13–16]. Reduced growth, high mortality, cataract and dwarfism are the

✉ Mukhtar A. Khan
khanmukhtar@yahoo.com

[1] Fish Nutrition Research Laboratory, Aligarh Muslim University, Aligarh, India

[2] Department of Zoology, Aligarh Muslim University, Aligarh 202 002, India

main zinc deficiency signs [4, 6, 8, 11, 12, 15]. Additionally, zinc deficiency has been linked to low IGF-I, growth hormone binding protein mRNA and growth hormone receptor [17–19]. Zn also regulates the cellular activities by binding with specific membrane receptors, carriers and channels [20]. Zn is also involved in regulating cell proliferation, differentiation, apoptosis and the gene expression of metallothionein [21]. Furthermore, zinc status is also related to oxidative stress and interacts with various hormones [22, 23]. Excessive zinc levels not only increase the cost of the diet but also load it to the water body [24, 25] and may be toxic for fish [4, 12, 26]. Thus, inclusion of optimum amount of zinc in diet is essential to avoid zinc deficiency and toxicity in fish as well as in environment due to excess.

Aquaculture sector is facing several crucial challenges such as eutrophication and other water quality issue and diseases resulting to significant economic loss [27]. Hence, there is need to develop some strategies that can help to combat with these issues, so that there could be an efficient aquaculture production for the continuously growing population and the demand for quality protein, safe and healthy products for consumption. Minerals, organic substances, are one of the important nutrients. They participate is several crucial life processes such as skeleton formation, transmission of nerve impulse, maintenance of osmotic balance and muscle contraction etc. Among all the minerals, zinc is an essential trace element for all vertebrates including teleost. Zinc has several structural as well as functional roles regulate multiple metalloenzymes as a specific cofactor and catalyst. Deficiency of zinc can lead to several pathological conditions. Thus dietary supplementation of zinc is essential to meet the requirement and avoid zinc deficiency and can participate to combat with the effects of cellular stressors and other pathological factors.

Heteropneustes fossilis is freshwater, air breathing, omnivorous fish, commonly known as singhi. It has high demand in Southeast Asia and India [28–32]. It is hardy and can be cultured at high stocking rate [33, 34]. Consumers prefer it because of its taste and nutritional superiority due to high protein, low fat and high iron content [35]. Though, information on some of its nutrient requirement such as protein [36], vitamins [37], amino acids [38, 39] and minerals [29–31, 35] are available but information on its zinc requirement is not available. Hence, in present study effects of dietary zinc on growth, antioxidant status and immune response and optimum requirement of *H. fossilis* were evaluated.

Materials and Methods

Experimental Diets

Casein-gelatin-based isonitrogenous (400 g/kg CP) and isocaloric (17.89 kJ/g GE) diets containing graded concentrations of zinc (0, 5, 10, 15, 20, 25 and 30 mg/kg) were prepared by supplementing varying levels of zinc sulphate heptahydrate (Loba Chemie, pvt. Ltd.) at the cost of dietary cellulose (0, 21.9, 43.9, 65.9, 87.9, 109.9 and 131.9 mg/kg) to basal diet (Table 1). Diets were made in accordance with the previously established procedure by [35]. The diets were prepared and designated as Zn1, Zn2, Zn3, Zn4, Zn5, Zn6 and Zn7. Analysed values of zinc in these trial diets were 10.68, 15.83, 21.34, 26.74, 30.61, 34.91 and 41.34 mg/kg, respectively.

Table 1 Composition of the basal diet

Ingredients	g/kg dry diet
Casein[a]	400
Gelatin[b]	100
Dextrin	250.3
Corn oil	50
Cod liver oil	20
Mineral mix (zinc-free)[c,e]	40
Vitamin mix[d,e]	30
Carboxymethyl cellulose	50
α-Cellulose	59.7
$ZnSO_4.7H_2O$[f]	0
Analysed crude protein	400.43
Analysed lipid	69.95
Calculated gross energy (kJ/g)[g]	17.89
Estimated gross energy (kJ/g)[h]	17.97
Digestible energy (kJ/g)[i]	14.53

[a]Crude protein (760 g/kg); [a]crude protein (960 g/kg); [c,e]mineral mixture (g/kg of mineral premix): calcium biphosphate 135.7; calcium lactate 326.9; ferric citrate 29.7; potassium phosphate (dibasic) 239.8; sodium biphosphate 87.2; magnesium sulphate 132.0; sodium chloride 43.5; aluminium chloride.$6H_2O$ 0.15; potassium iodide 0.15; cuprous chloride 0.10; manganese sulphate.H_2O 0.80; cobalt chloride. $6H_2O$ 1.0; zinc sulphate.$7H_2O$ 0; α-cellulose 3; Loba Chemie, Mumbai, India; [e]Halver (2002); [d,e]vitamin mixture (30 g/kg of diet; 10 g vitamin mix + 20 g α-cellulose) choline chloride 5.0; meso-inositol 2.0; L-ascorbyl-2-polyphosphate 1.0; nicotinic acid 0.75; calcium D-pantothenate 0.5; riboflavin 0.2; menadione 0.04; pyridoxine hydrochloride 0.05; thiamine hydrochloride 0.05; folic acid 0.015; biotin 0.005; alpha-tocopheryl acetate 0.40; vitamin B_{12} (cyanocobalamin) 0.0001; Loba Chemie, India. [f]Zinc sulphate heptahydrate was added to trial diets at the levels of 0, 21.9, 43.9, 65.9, 87.9, 109.9 and 131.9 mg/kg, respectively, by replacing α-cellulose. [g]Calculated on the basis of fuel values 23.07, 20.18, 16.00 and 37.62 kJ/g for casein, gelatin, dextrin, and fat, respectively, as estimated on Gallenkamp ballistic bomb calorimeter; [h]estimated value of the basal diet on Gallenkamp ballistic bomb calorimeter; [i]digestible energy was calculated on the basis of physiological fuel values 18.83, 14.64 and 35.56 kJ/g for protein, carbohydrate and fat, respectively, as per Jauncey (1982) [130]

Experimental Design

Heteropneustes fossilis fingerling was shifted from fish hatchery to the feeding trial facility, disinfected with $KMnO_4$ solution (1:3000), and distributes in indoor tanks. During the acclimation period of 2 weeks, fish were offered H-440 [40] diet (casein-gelatin-based, 40 % crude protein). Acclimatized fish (7.54 ± 0.05 g, 10.5 ± 0.1 cm) were randomly stocked at the rate of 30 fish in triplicate groups in tanks (water volume 55 L) with a water flow of 1–1.5 L/min. During the experiment, water quality parameters were analysed as per APHA [41] and recorded range for water temperature and pH were 26.6–28.5°C and 7.1–7.3, respectively. The values of other parameters such as ammonia nitrogen, oxygen, alkalinity and carbon dioxide were found to range between 0.25–0.30, 5.6–6.4, 66.3–75.4 and 6.3–10.4 mg/L, respectively. The level of zinc in water ranged between 1.81 and 2.43 µg/L.

Feeding Trial and Growth Measurements

Fish were fed semi-moist diets at 8:00 and 16:00 h until they seemed satiated. It was ensured that the feed given was eaten fully. Before each feeding, faeces were removed. Fish were not fed on the day of recording mass weight. All groups of fishes were measured anesthetized in 100 mg/L tricaine methanesulfonate and their mass weights recorded. Proximate composition was done using standard methods [42].

Sample Collection

Fish were killed with a fatal dosage (200 mg/L) of MS-222 at the start and end of the trial. Six sub-samples from a pooled sample of 20 fish were taken and assessed for initial and final body composition. Twelve fish per replicate were collected randomly, killed and kept in a freezer at −20°C for estimating the mineral and proximate composition of whole body. Length and weight of 8 fish per replicate were noted to calculate the condition factor (CF). Blood samples were obtained from above 8 fish of each replicate in heparinized syringe by puncturing caudal vein and pooled. Three sub-samples from each pooled sample were subjected to analysis. Haematological analysis including red blood corpuscles (RBCs), haemoglobin (Hb) and haematocrit (Hct) was done in accordance with the methods used earlier [30]. Upon collection of blood samples, the viscera and liver were dissected out to determine the viscerosomatic index (VSI) and hepatosomatic index (HSI). The intestine and liver from same fish were used for mineral and digestive enzyme analysis. To extract the vertebrae sample from surrounding flesh, the same fish was microwaved for around 5 min to separate the surrounding flesh. Vertebrae were taken out by gently removing the spines, washed, dried at 105°C and defatted using a Soxhlet apparatus and then ground for mineral analysis. Mineral analysis of diets, whole body, serum, liver, and vertebrae samples was done using inductively coupled plasma atomic emission spectroscopy (ICP-AES) technique (SPECTRO Analytical Instruments GmbH, Germany). Superoxide dismutase (SOD) and catalase (CAT) activities in serum were assessed by using the method of Misra and Fridovich [43] and Aebi [44], respectively. Serum glutathione peroxidase (GPx) activity was measured using the method of Rotruck et al. [45]. Malondialdehyde (MDA) content was established as per the method of Utley et al. [46] as adopted by Fatima et al. [47]. The protease activity was assessed by following the method described by Moore and Stein [48]. The lipase activity was determined as per the method adopted by Seligman and Nachlas [49]. Amylase activity was determined by utilizing soluble starch as a substrate [50]. The activity of serum alkaline phosphatase (ALP) was estimated colorimetrically by adopting the method of Apines et al. [51]. Serum myeloperoxidase activity was assessed following the method of Quade and Roth [52], and turbidimetric method of Hultmark et al. [53] as adopted by Wang et al. [54] was used to assess the lysozyme activity.

Growth Assessment

Growth assessment was done by standard formulae used previously [30] which are given below:

Weight gain (WG; %) = Final body weight − Initial body weight/Initial body weight × 100

Feed conversion ratio (FCR) = Dry feed intake (g) / Wet weight gain (g)

Specific growth rate (SGR; %/day) = ln (Final body weight) − ln (Initial body weight)/No. of days ×100

Protein retention efficiency (PRE; %) = Protein gain/Protein fed × 100.

Protein gain (PG; g/fish) = Final body protein (%) × Final body weight (g) − Initial body protein (%) × Initial body weight (g)

Apparent zinc retention (%) = Final body zinc content – Initial body zinc content/Total zinc fed × 100

Hepatosomatic index (HSI; %) = Liver weight (g)/Body weight (g) × 100

Viscerosomatic index (VSI; %) = Viscera weight (g)/Body weight (g) × 100

Condition factor (CF; g/cm^3) = Body weight (g)/Body length (cm)^3× 100.

Mean corpuscular haemoglobin concentration (MCHC; g/dL) = Hb (g/dL) ×100/ Hct (%)

Mean corpuscular haemoglobin (MCH; pg) =Hb (g/dL) × 10/RBC (×10^6/mm^3)

Mean corpuscular volume (MCV; fL) = Hct (%) × 10/RBC (×10^6/mm^3)

Statistical Analysis

Data were evaluated for normality (Shapiro-Wilk test) and variance homogeneity (Levene's test) for equality before being subjected to a one-way analysis of variance. Tukey's test ($P<0.05$) was performed in comparing the differences among treatment means. Data acquired on several parameters in relation to increasing amounts of dietary zinc were submitted to various statistical models. Due to the highest R^2 value obtained in the Broken-line regression model [55] compared to other models, this model was used in determining the optimum dietary zinc requirements of *H. fossilis*. Origin (version 6.0; Origin Software, San Clemente, CA) was used to statistically analyse the growth data.

Results

Growth Performance

Results pertaining to growth parameters and conversion efficiencies of fingerling *H. fossilis* fed diets with different levels of zinc are listed in Table 2. Increase in dietary zinc significantly ($P<0.05$) affected the growth and conversion efficiencies of *H. fossilis*. Live weight gain (LWG, %), specific growth rate (SGR, %/day), protein retention efficiency (PRE, %), feed conversion ratio (FCR) and protein gain (PG, g/fish) improved with the increasing dietary zinc up to 26.74 mg/kg (Zn4) and remained relatively constant beyond that level. Broken-line regression analysis of LWG and PG against the increasing dietary zinc levels indicated the optimum zinc requirement for fingerling *H. fossilis* at 26.82 (Fig. 1) and 27.22 (Fig. 2) mg/kg of dry diet, respectively. Fish fed basal diet (Zn1) reflected maximum apparent zinc retention. Significant difference in zinc retention was evident in fish fed diet Zn1 and Zn2 followed by insignificant change ($P>0.05$) up to diet Zn4 and then significantly declined ($P<0.05$) to a minimum in fish fed diet containing 41.34 mg/kg zinc (Zn7).

Whole Body Composition and Biometric Indices

Effects different levels of zinc on body composition and biometric indices of *H. fossilis* are summarized in Table 3. Increase in the zinc significantly affected ($P<0.05$) whole body protein, fat and ash contents. However, moisture content did not differ significantly ($P>0.05$). A significant

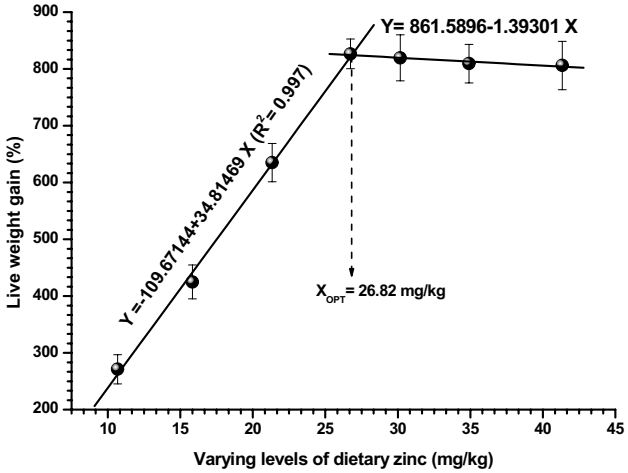

Fig. 1 Broken-line relationship of live weight gain to dietary zinc levels. Each point represents the mean of three replicates per treatment

Table 2 Growth performance and conversion efficiencies of fingerling *Heteropneustes fossilis* fed diets containing varying levels of zinc*,**

Varying levels of dietary zinc (mg/kg)							
	Zn1 (10.68)	Zn2 (15.83)	Zn3 (21.34)	Zn4 (26.74)	Zn5 (30.61)	Zn6 (34.91)	Zn7 (41.34)
Average initial weight (g/fish)	7.53±0.01	7.51±0.04	7.58±0.06	7.59±0.03	7.53±0.02	7.56±0.04	7.52±0.05
Average final weight (g/fish)	27.94±1.34^{d}	39.41±1.46^{c}	55.72±1.63^{b}	70.32±2.04^{a}	69.24±1.83^{a}	68.74±1.67^{a}	68.13±1.79^{a}
Live weight gain (%)	271.05±26.62^{d}	424.77±30.61^{c}	635.09±33.73^{b}	826.48±26.95^{a}	819.52±40.72^{a}	809.26±36.64^{a}	805.98±43.92^{a}
Specific growth rate (%/day)	1.56±0.07^{d}	1.97±0.08^{c}	2.37±0.09^{b}	2.65±0.07^{a}	2.64±0.10^{a}	2.63±0.09^{a}	2.62±0.07^{a}
Feed conversion ratio	3.66±0.16^{a}	2.93±0.19^{b}	2.31±0.11^{c}	1.77±0.09^{d}	1.78±0.11^{d}	1.79±0.13^{d}	1.81±0.12^{d}
Protein retention efficiency (%)	8.39±0.62^{d}	12.03±0.75^{c}	16.96±0.81^{b}	24.51±0.91^{a}	24.28±1.31^{a}	23.79±1.38^{a}	23.61±1.42^{a}
Protein gain (g/fish)	2.51±0.49^{d}	4.50±0.61^{c}	7.54±0.48^{b}	10.88±0.58^{a}	10.67±0.68^{a}	10.42±0.45^{a}	10.36±0.72^{a}
Apparent zinc retention (%)	67.73±1.63^{a}	59.03±1.37^{b}	57.90±0.93^{b}	60.89±0.91^{b}	52.51±0.95^{c}	46.17±0.68^{d}	38.42±0.73^{e}
Survival (%)	91.10±2.22^{b}	96.66±1.92^{a,b}	97.77±2.22^{a,b}	97.77±1.11^{a,b}	94.44±1.11^{a,b}	98.88±1.11^{a}	97.77±1.11^{a,b}

*Mean values of 3 replicates±SEM; **mean values sharing the different superscripts in the same row are significantly different ($P<0.05$)

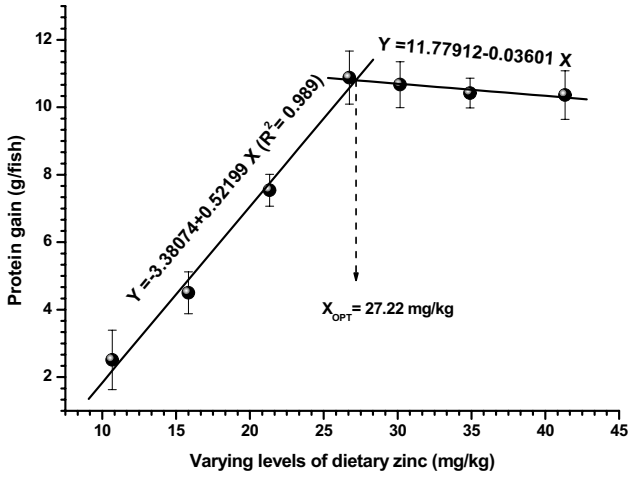

Fig. 2 Broken-line relationship of protein gain to dietary zinc levels. Each point represents the mean of three replicates per treatment

increase in whole body protein and ash was noted in fish fed diets with 10.68 mg/kg (Zn1) to 26.74 mg/kg zinc (Zn4) and then remained unchanged ($P>0.05$). Fat content was found to decrease ($P<0.05$) with the increase in dietary zinc concentration up to 26.74 mg/kg (Zn4) and then levelled off. Biometric indices were also significantly affected ($P<0.05$) by increasing dietary zinc levels. Hepatosomatic index (HSI) and viscerosomatic index (VSI) reflected a decreasing tendency ($P<0.05$) with incremental dietary zinc levels up to 26.74 mg/kg diet (Zn4) and then stabilized ($P>0.05$). However, condition factor (CF) improved ($P<0.05$) with the increase in dietary zinc levels up to 26.74 mg/kg (Zn4) and then steadied.

Haematological Indices

Dietary zinc levels affected the haematological status significantly ($P<0.05$) in terms of haemoglobin (Hb), red blood cell (RBCs) and haematocrit (Hct) as shown in Table 4. These parameters showed improvement ($P<0.05$) with the increasing concentration of zinc in the diet from 10.68 mg/kg (Zn1) to 26.74 mg/kg (Zn4) and further inclusion of zinc in the diet (Zn5-Zn7) did not change ($P<0.05$) the above parameter. MCH and MCV also showed the similar pattern. However, no change ($P>0.05$) in MCHC was noted.

Digestive Enzyme Activities

Intestinal protease, lipase and amylase activities were found to increase significantly ($P<0.05$) with the increasing dietary zinc levels up to 26.74 mg/kg (Table 4) indicating the improved intestinal health status. However, higher amounts of zinc did not reflect any significant change ($P>0.05$) in the activities of these enzymes. Broken-line regression of protease activity against increasing dietary zinc reflected the Zn requirement of fingerling *H. fossilis* to be 27.33 mg/kg (Fig. 3).

Whole Body and Tissue Mineralization

Significant difference in whole body, vertebrae and liver mineralization ($P<0.05$) were found in fish receiving diets with increasing levels of zinc (Table 5). The whole body zinc concentration increased ($P<0.05$) with the increase in dietary zinc up to 26.74 mg/kg diet (Zn4) and then stabilized. However, that in vertebrae increased up to 30.61 mg/kg (Zn5) beyond which it did not change ($P>0.05$). On the other hand, iron (Fe), copper (Cu) and calcium (Ca) in whole body and vertebrae reduced significantly ($P<0.05$) in fish fed above diets up to diet containing 30.61 mg/kg zinc compared to diets containing 10.68–26.74 mg/kg zinc. Liver Zn, Cu and Ca concentrations were not affected ($P>0.05$) by the increment in dietary zinc whereas Fe concentration tend to decline beyond the dietary zinc level of 26.74 mg/kg (Zn4). Regression analysis of vertebral zinc concentration against

Table 3 Whole body composition (wet basis) and biometric indices of fingerling *Heteropneustes fossilis* fed diets containing varying levels of zinc*,**

Varying levels of dietary zinc (mg/kg)								
	Initial	Zn1 (10.68)	Zn2 (15.83)	Zn3 (21.34)	Zn4 (26.74)	Zn5 (30.61)	Zn6 (34.91)	Zn7 (41.34)
Moisture (%)	73.85±4.42	72.54±2.21	73.12±1.23	73.19±2.78	72.61±3.42	73.21±3.58	72.68±2.95	72.65±3.62
Crude protein (%)	12.43±0.49	12.32±0.47^{d}	13.78±0.59^{c}	15.23±0.64^{b}	16.82±0.58^{a}	16.76±0.43^{a}	16.53±0.56^{a}	16.58±0.61^{a}
Crude fat (%)	5.85± 0.48	6.79±0.52^{a}	5.33± 0.49^{b}	4.12± 0.43^{c}	3.45± 0.39^{d}	3.22±0.28^{d}	3.38±0.23^{d}	3.19±0.27^{d}
Ash (%)	4.19± 0.25	3.47±0.47^{d}	4.36±0.32^{c}	5.12± 0.31^{b}	6.43±0.38^{a}	5.98±0.29^{a}	6.24±0.47^{a}	6.32±0.52^{a}
HSI (%)		2.47±0.17^{a}	2.18±0.12^{b}	1.87±0.11^{c}	1.46±0.09^{d}	1.43±0.1^{d}	1.51±0.12^{d}	1.49±0.08^{d}
VSI (%)		3.45±0.42^{a}	2.88±0.21^{b}	2.64±0.18^{c}	1.83±0.14^{d}	1.88±0.11^{d}	1.91±0.17^{d}	1.85±0.16^{d}
CF (g/cm^3)		1.19±0.07^{d}	1.27±0.03^{c}	1.43±0.05^{b}	1.58±0.04^{a}	1.57±0.07^{a}	1.61±0.06^{a}	1.55±0.05^{a}

*Mean values of 3 replicates±SEM; **mean values sharing the different superscripts in the same row are significantly different ($P<0.05$). *HSI*, hepatosomatic index; *VSI*, viscerosomatic index; *CF*, condition factor

Table 4 Haematological indices and digestive enzyme activities of fingerling *Heteropneustes fossilis* fed diets containing varying levels of zinc*,**

Varying levels of dietary zinc (mg/kg)							
	Zn1 (10.68)	Zn2 (15.83)	Zn3 (21.34)	Zn4 (26.74)	Zn5 (30.61)	Zn6 (34.91)	Zn7 (41.34)
Haemoglobin (g/dL)	6.05±0.12^{d}	7.01±0.11^{c}	8.72±0.12^{b}	10.84±0.34^{a}	10.73±0.27^{a}	10.80±0.36^{a}	10.39±0.28^{a}
RBCs (10^6/mm^3)	2.31±0.09^{c,d}	2.48±0.15^{c}	2.71±0.08^{b}	2.92±0.11^{a}	2.94±0.16^{a}	2.89±0.14^{a}	2.86±0.12^{a}
Haematocrit (%)	18.67±0.81^{d}	21.81±0.76^{c}	27.34±0.97^{b}	33.78±1.21^{a}	33.69±1.28^{a}	33.71±1.39^{a}	32.98±1.24^{a}
MCHC (g/dL)	32.40±1.35^{a}	32.14±1.28^{a}	31.89±1.32^{a}	32.08±1.57^{a}	31.84±2.05^{a}	32.03±2.11^{a}	31.50±1.97^{a}
MCH (pg)	26.19±0.56^{d}	28.26±0.52^{c}	32.17±0.81^{b}	37.12±1.3^{a}	36.49±1.41^{a}	37.37±1.73^{a}	36.32±1.23^{a}
MCV (fL)	80.82±2.45^{d}	87.94±2.87^{c}	100.88±3.23^{b}	115.68±3.78^{a}	114.59±3.95^{a}	116.64±4.68^{a}	115.31±4.53^{a}
Protease activity (U/mg protein)	19.45±0.75^{d}	23.73±0.83^{c}	27.03±1.09^{b}	32.89±1.22^{a}	32.48±1.39^{a}	33.17±1.24^{a}	32.57±1.31^{a}
Lipase, activity (U/mg protein)	0.39±0.02^{d}	0.66±0.03^{c}	0.81±0.05^{b}	1.17±0.05^{a}	1.22±0.06^{a}	1.15±0.13^{a}	1.19±0.17^{a}
Amylase activity (U/mg protein)	1.93±0.15^{d}	1.44±0.23^{c}	1.65±0.21^{b}	1.98±0.42^{a}	1.92±0.38^{a}	2.03±0.37^{a}	1.98±0.39^{a}

*Mean values of 3 replicates±SEM; **mean values sharing the different superscripts in the same row are significantly different (P<0.05). *MCHC*, mean corpuscular haemoglobin concentration; *MCH*, mean corpuscular haemoglobin; *MCV*, mean corpuscular volume

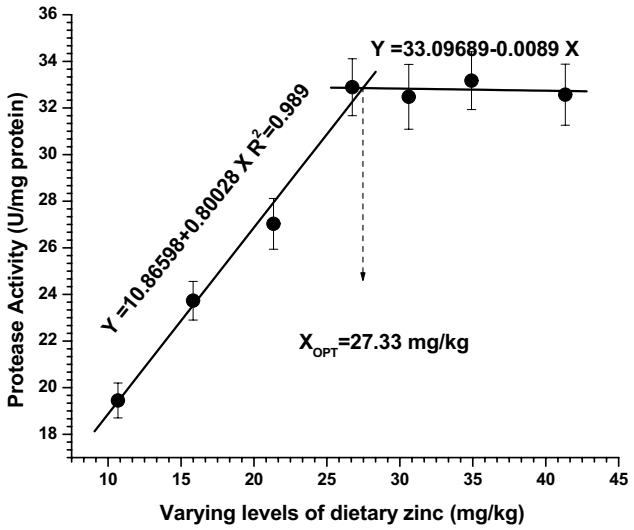

Fig. 3 Broken-line relationship of protease activity to dietary zinc levels. Each point represents the mean of three replicates per treatment

increasing dietary zinc levels revealed a need of 29.84 mg/kg (Fig. 4).

Antioxidant Enzyme Activities

Results are presented in Table 6. Increased amount of dietary zinc resulted into significant (P<0.05) increase in serum superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities up to 26.74 mg/kg, and further inclusion did not show any significant improvement in the activities of these enzymes. MDA content was found to be influenced by the dietary zinc levels. A significant (P<0.05) and continuous decrease in the MDA level was noted in fish fed diets with graded levels of dietary zinc. Dietary supplementation of zinc at the level of 26.74 mg/kg resulted to minimum MDA content, and no change (P>0.05) in the serum MDA levels was observed on further inclusion of dietary zinc. Broken-line regression analysis of serum SOD activity against increasing dietary zinc levels indicated the requirement at 26.66 mg/kg (Fig. 5).

Immune Parameters

Results pertaining to immune response of *H. fossilis* after feeding diets supplemented with zinc are presented in Table 6. Lysozyme activity increased (P<0.05) with the increase in dietary zinc concentration from 10.68 to 26.74 mg/kg and then remained unchanged. Myeloperoxidase (MPO) activity was also found to improve with increasing dietary zinc levels. Significantly higher (P<0.05) MPO activity was noted in fish fed diets with 26.74 mg/kg zinc compared to the group fed basal diet containing 10.68 mg/kg zinc. However, no significant change in MPO activity in fish fed higher concentration of zinc compared to those fed 26.74 mg/kg zinc was noted. Dietary zinc significantly affected the activity of alkaline phosphatase (ALP) enzyme. A significant increase (P<0.05) in the activity of enzyme was recorded with increasing dietary zinc concentration from 10.68 to 30.61 mg/kg. However, further inclusion of zinc in the diets at higher levels (34.91 and 41.34 mg/kg) did not show any significant change in the activity of ALP enzyme.

Discussion

The effects of rising concentration of zinc in the diets on growth, conversion efficiencies, haematological parameters, digestive enzymes activity, antioxidant status, mineralization and immune response vary among species due to several

Table 5 Effect of varying levels of dietary zinc on whole body, vertebrae, and liver mineralization of fingerling *Heteropneustes fossilis*[*,**]

Varying levels of dietary zinc (mg/kg dry diet)							
Whole body mineralization (wet basis)	**Zn1 (10.68)**	**Zn2 (15.83)**	**Zn3 (21.34)**	**Zn4 (26.74)**	**Zn5 (30.61)**	**Zn6 (34.91)**	**Zn7 (41.34)**
Zinc (mg/kg)	25.84±0.41^{d}	26.76±0.45^{c}	27.34±0.52^{b}	28.31±0.49^{a}	28.12±0.54^{a}	28.33±0.61^{a}	28.24±-0.63^{a}
Iron (mg/kg)	42.38±0.62^{a}	42.56±0.67^{a}	42.11±0.59^{a}	42.47±0.72^{a}	38.52±0.68^{b}	37.38±0.59^{b,c}	38.12±0.52^{b}
Copper (mg/kg)	6.85±0.11^{a}	6.44±0.21^{a}	6.49±0.19^{a}	6.78±0.22^{a}	5.12±0.27^{b}	4.97±0.18^{b}	4.83±0.24^{b}
Calcium (mg/kg)	10.23±0.29^{a}	10.14±0.31^{a}	10.38±0.22^{a}	10.21±0.19^{a}	9.87±0.17^{a,b}	8.74±0.09^{b}	8.79±0.11^{b}
Vertebrae mineralization (fat free, dry basis)	**Zn1 (10.68)**	**Zn2 (15.83)**	**Zn3 (21.34)**	**Zn4 (26.74)**	**Zn5 (30.61)**	**Zn6 (34.91)**	**Zn7 (41.34)**
Zinc (mg/kg)	161.72±2.55^{e}	173.34±2.75^{d}	188.47±3.09^{c}	197.29±3.37^{b}	205.83±3.29^{a}	203.47±3.12^{a}	203.65±3.14^{a}
Iron (mg/kg)	53.12±0.34^{a}	51.09±0.37^{b}	51.94±0.39^{b}	52.46±0.28^{a,b}	49.34±0.24^{c}	48.93±0.31^{c}	47.74±0.29^{c,d}
Copper (mg/kg)	12.43±0.09^{a}	12.12±0.11^{a}	11.85±0.08^{a}	12.19±0.13^{a}	10.76±0.16^{b}	10.83±0.10^{b}	9.83±0.13^{b,c}
Calcium (mg/kg)	204.43±2.34^{a}	201.45±2.51^{a}	197.38±2.29^{a}	200.67±1.93^{a}	191.43±1.89^{b}	189.13±2.04^{b}	190.33±1.78^{b}
Liver mineralization (wet basis)	**Zn1 (10.68)**	**Zn2 (15.83)**	**Zn3 (21.34)**	**Zn4 (26.74)**	**Zn5 (30.61)**	**Zn6 (34.91)**	**Zn7 (41.34)**
Zinc (mg/kg)	28.36±0.13	28.45±0.19	29.31±0.21	29.29±0.30	28.78±0.28	28.83±0.34	29.14±0.39
Iron (mg/kg)	139.52±1.34^{a}	137.33±1.54^{a}	138.43±1.19^{a}	138.64±1.12^{a}	119.48±1.41^{b}	118.38±1.38^{b}	109.34±0.04^{b,c}
Copper (mg/kg)	38.34±0.34	39.24±0.32	38.66±0.29	38.42±0.41	37.29±0.33	36.92±0.37	37.13±0.32
Calcium (mg/kg)	442.43±3.38	438.92±3.25	434.67±2.89	429.65±2.92	438.44±2.84	431.74±3.67	441.32±3.24

*Mean values of 3 replicates±SEM; **mean values sharing the different superscripts in the same column are significantly different (P<0.05)

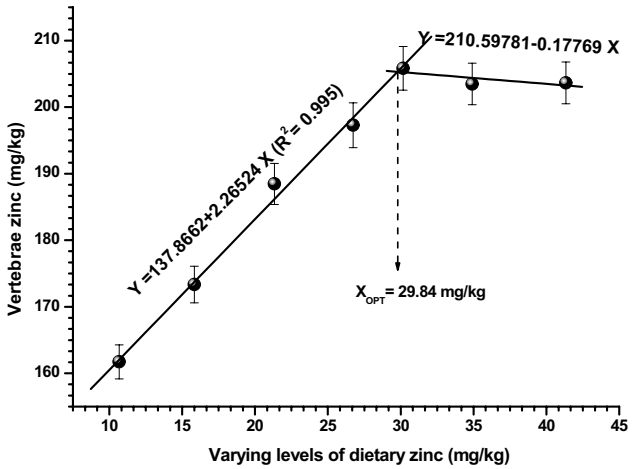

Fig. 4 Broken-line relationship of vertebrae zinc concentration to dietary zinc levels. Each point represents the mean of three replicates per treatment

factors. During the present study, zinc concentration of rearing water ranging 1.81–2.43 µg/L and zinc-deficient diets containing suboptimal levels of diets was noted to be insufficient to meet the metabolic need of *H. fossilis* resulting in zinc deficiency signs such as poor growth, conversion efficiencies, antioxidant status, immune response, haematological parameters and reduced zinc concentration in the whole body as well as other tissues. In the present study, zinc supplementation in *H. fossilis* diet enhanced the growth and improved the above parameters. Role of zinc in controlling the insulin-like growth factor I (IGF-I) [15, 19, 56] and modulating the expression of intestinal genes [57] for the incorporation of minerals might be the reason for its stimulative effects on growth. On subjecting the protein gain, live weight gain, intestinal protease action and vertebral zinc data against incremental concentrations of dietary zinc to broken line regression analysis, the requirement was found to be at 27.22, 26.82, 27.33 and 29.84 mg/kg diet, respectively.

The dietary zinc requirement for *H. fossilis* (26.82–29.84 mg/kg) obtained in this study is comparable to that stated for other species such as *Oreochromis niloticus* (30 mg/kg), *Clarias batrachus* (30 mg/kg), *Epinephelus malabaricus* (28.9–33.7 mg/kg) and *Acipenser baerii* (29.24–34.7 mg/kg) [8, 12, 58] but higher than that reported for *Pelteobagrus fulvidraco* (17–21 mg/kg), *Sciaenops ocellatus* (20 mg/kg), *Oreochromis aureus* (20 mg/kg), *Ictalurus punctatus* (20 mg/kg), *Cyprinus carpio* (15 mg/kg) and *Oncorhynchus mykiss* (15 mg/kg) [26, 59–62] and lower than that of *Rachycentron canadum* (42.9 mg/kg), *Lateolabrax japonicas* (103 mg/kg), *Cyprinus carpio* var. *jian* (42–49) and *Ctenopharyngodon idella* (55) [3, 63–65]. Similar results of increasing weight gain up to optimum were also stated in a study conducted on common carp [60], grouper [8], Nile tilapia [58, 66], yellow catfish [26], Jian carp [65], channel catfish [61], grass carp [3] and Siberian sturgeon [7]. However, no significant improvement of dietary zinc supplementation on the growth of blue tilapia [62], Atlantic salmon [67] and European sea bass [68] was recorded. In the above fishes, sufficient availability of zinc from basal diet and/or rearing water meeting the requirement may be the reason for no improvement in growth of the fish fed zinc-supplemented diets. However,

Table 6 Antioxidant status and immune response of fingerling *Heteropneustes fossilis* fed diets containing varying levels of zinc[*,**]

Varying levels of dietary zinc (mg/kg)							
	Zn1 (10.68)	Zn2 (15.83)	Zn3 (21.34)	Zn4 (26.74)	Zn5 (30.61)	Zn6 (34.91)	Zn7 (41.34)
SOD (U/mL)	55.34±1.57^{d}	62.54±1.49^{c}	74.39±1.67^{b}	83.68±1.81^{a}	82.56±1.78^{a}	83.49±1.82^{a}	83.87±1.84^{a}
CAT (U/ mL)	11.72±0.21^{d}	12.46±0.29^{c}	14.91±0.42^{b}	16.33±0.59^{a}	16.48±0.55^{a}	15.69±0.49^{a,b}	16.42±0.49^{a}
GPx (U/mL)	144.73±2.03 ^{d}	163.26±2.81^{c}	187.37±3.15^{b}	198.61±3.89^{a}	199.35±3.49^{a}	198.42±3.62^{a}	201.03±3.72^{a}
MDA (nmole/mL)	16.25±0.43^{a}	13.89±0.57^{b}	12.09±0.71^{b,c}	10.94±0.89^{d}	11.04±0.83^{d}	11.27±0.79^{d}	11.82±0.86^{c,d}
Lysozyme (U/mL)	138±2.09^{d}	179±2.83^{c}	216±3.24^{b}	241±3.57^{a}	239±3.51^{a}	238±2.97^{a}	239±2.95^{a}
ALP (U/L)	132.43±2.18^{e}	144.62±2.51^{d}	158.36±3.13^{c}	165.83±3.45^{b}	178.39±3.39^{a}	177.56±3.14^{a}	179.42±3.16^{a}
MPO (OD at 450 nm)	0.14±0.01^{c}	0.22±0.02^{b}	0.28±0.02^{b}	0.34±0.03^{a}	0.33±0.03^{a}	0.35±0.02^{a}	0.35±0.02^{a}

*Mean values of 3 replicates±SEM; **mean values sharing the different superscripts in the same row are significantly different ($P<0.05$). *ALP*, alkaline phosphatase; *SOD*, superoxide dismutase; *CAT*, catalase; *GPx*, glutathione peroxidase; *MDA*, malondialdehyde; *MPO*, myeloperoxidase

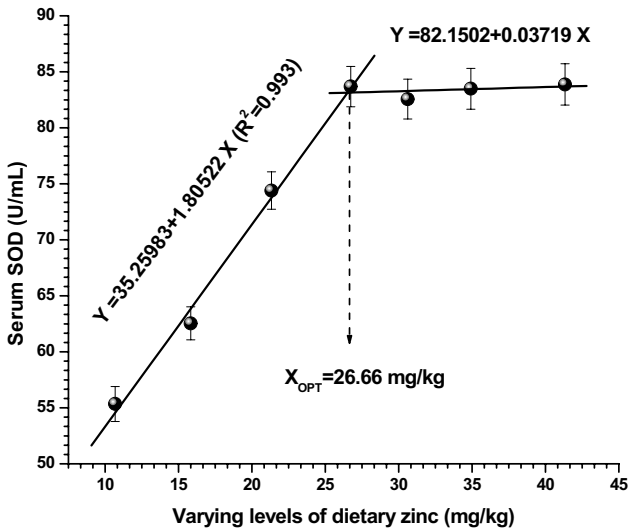

Fig. 5 Broken-line relationship of serum SOD activity to dietary zinc levels. Each point represents the mean of three replicates per treatment

feeding extremely high levels of zinc (176.7 and 334 mg/kg) resulted in reduced growth in Nile tilapia [15]. This reduction in growth may be because of zinc toxicity.

Many proteases, such as carboxypeptidases A and B, require zinc as a cofactor to function properly [66, 69]. Improvement in PRE with increasing zinc levels in diets may be due to an increase in the activity of carboxypeptidase resulting in an improvement in protein digestion as zinc acts as an essential cofactor for several metalloenzymes which take part in protein metabolism [8, 70]. Several zinc deficiency symptoms reported in fish species include cataract, anorexia, poor development, dermatitis, short trunk, low tissue zinc content and high mortality [12–15, 71–75]. In the current study, no overt deficient symptoms were identified except for anorexia, poor development, impaired antioxidant capacity, immune response, lower tissue mineralization and low Hb.

The highest apparent zinc retention was recorded in fish fed Zn1diet (basal diet) followed by a significant reduction in the efficiency of retention which was noted in Zn2 diet. However, no significant differences were noted among fish fed Zn2, Zn3 and Zn4 diet suggesting that the zinc retention efficiency in fish fed these diets was almost the same. This may be because of reduced zinc utilization efficiency with the increase in dietary zinc. In grass carp, a similar trend of zinc retention in reaction to increased dietary zinc levels was observed [3]. Increased intestinal absorption was also seen in broilers fed low levels of manganese in diet [76]. Lin et al. [74], Zhang et al. [77] and Zafar and Khan [29] also reported similar patterns of retention in hybrid tilapia, large yellow croaker and stinging catfish fed insufficient levels of manganese, respectively. No significant differences in growth and whole body zinc concentration of *H. fossilis* fed diets containing higher than optimum level (26.74 mg/kg) were evident indicating that an extra amount of zinc was excreted and not toxic.

Whole body composition is known to reflect the quality of fish as a product and that is why it is regarded as a crucial parameter in nutritional studies. A significant rise in whole body protein with the increase in dietary zinc concentrations up to optimum (26.74 mg/kg) was evident, indicating maximum protein retention efficiency at this dietary level of zinc (Table 2). This could explain the importance of zinc as an essential component of many proteins and its role in stabilization as well as adjustment of protein structure and functioning [78–80]. Increasing zinc levels, on the other hand, lowered total body fat. The greater body fat content observed in this study on *H. fossilis* fed Zn-deficient diets was also noted in yellow catfish [81]. Carnitine palmitoyltransferase (CPT I) is a regulatory enzyme mainly involved in the oxidation of long-chain fatty acids [82]. Decreased hepatic CPT I action was noted in fish fed zinc-deficient diets [81]. Thus, inhibited CPT I activity in fish fed zinc-deficient diets may be due to less lipid utilization and more lipid deposition, while increasing activity of CPT I with increasing dietary

zinc levels may result into increased lipolysis and decreased lipid deposition. Increased whole body fat in fish fed diets deficient in zinc was also recorded in the current study. The increase in dietary zinc levels did not affect the whole body moisture in *H. fossilis*. In hybrid tilapia, a similar tendency for total body hydration was observed [6]. Body ash in *H. fossilis* fed diets with increasing zinc increases up to Zn4 and then remained static. Several researchers such as Luo et al. [65] in yellow catfish and Moazenzadeh et al. [12] in Siberian sturgeon also noted comparable changes in whole body moisture, protein and fat of fish fed diets containing optimum level of zinc, whereas increasing dietary zinc levels did not exert any significant impact on whole body composition in studies conducted by Tan and Mai [83] on abalone and by Tan et al. [65] on Jian carp.

Biometric indices are generally used to demonstrate the health status of fish. HSI and VSI of *H. fossilis* decreased with the increase in dietary zinc up to 26.74 mg/kg diet. This decrease up to 26.74 mg/kg (Zn4) may be due to increased lipolysis and reduced deposition. However, no changes in HSI and VSI were reported with the increase of zinc in diet of yellow catfish [26], grass carp [3], Nile tilapia [15] and Siberian sturgeon [7]. Condition factor (CF) is an indicator of the robustness of fish which in H. *fossilis* increased up to optimum level and stabilized thereafter demonstrating that the fish were in good nutritional and physiological conditions at above dietary zinc level. Contrary to this, reduced CF was recorded in Siberian sturgeon fed higher levels of zinc in diets [7], whereas no major changes in the CF of yellow catfish [26] and grass carp [3] were reported with the increase in dietary zinc.

Tissue mineral concentration, enzyme activities and haematological indices are routinely used parameters in growth and nutritional studies [12–15, 60–68]. In the present study, digestive enzymes, haematological parameters, serum antioxidant enzymes activities, whole body, vertebrae and liver mineralization against increasing levels of dietary zinc were also used as response variables to assess the nutritional and physiological health status of *H. fossilis*. Digestive enzymes play a crucial role in the digestion of dietary components. Increased activity of intestinal enzymes is associated with enhanced digestive capability and growth performance. The activity level of fish digestive enzymes is regarded as a valuable comparative indication of food usage, digestive capability and growth performance [84, 85]. An increase in digestive enzyme activity, in the current study, with the increasing zinc concentrations in the diets up to optimum (Zn4) resulted in improved digestion and utilization of food and better intestinal health of fish. Several factors influence the activity of digestive enzymes, including life stage, diet, feeding management, and sampling time after feeding [86].

Haematological parameters are used in fish nutrition studies for describing or measuring the effects of diets on growth and physiological processes [6, 7, 15, 66]. In the current study, haemoglobin, RBCs, haematocrit, mean corpuscular haemoglobin and mean corpuscular volume increased with the rise in concentrations of dietary zinc up to 26.74 mg/kg (Zn4). The maximum values of the above parameters achieved at this level demonstrate that the inclusion of zinc at 26.74 mg/kg diet was adequate to optimize haematological parameters and prevent anaemic conditions. Haemoglobin production was reported to increase with increasing dietary zinc levels in this and other studies [7, 66, 87, 88]. Zinc plays a vital role in haemoglobin production through activation of enzyme D-aminolevulinic acid dehydrogenase, crucial for the production of porphobilinogen from two molecules of D-aminolevulinic acid [7]. Moreover, it is one of the important elements which affect the synthesis of superoxide dismutase and carbonic anhydrase in red blood cells [7, 15, 89]. Being an integral component of the antioxidant enzyme superoxide dismutase, zinc is crucial for erythrocyte membrane maintenance by protecting sulfhydryl groups from the harmful effects of superoxide free radicals [90].

Total body, spine and liver mineral levels have often been used to evaluate utilizable mineral intake in several studies [12, 15, 26, 51, 91]. Dietary zinc significantly affected mineralization in liver, vertebrae and whole body of *H. fossilis*. Whole body zinc concentration considerably enhanced with the increment in zinc up to 26.74 mg/kg diet (Zn4), and subsequently, at higher levels, no significant change was observed. Similar result was reported in *P. fulvidraco* [26, 81], *C. idella* [3] and *Oryzias melastigma* [5]. However, zinc concentration in vertebrae improved up to 30.61 mg/kg (Zn5) followed by no change. In Nile tilapia, a parallel increasing tendency was noted up to a specific threshold, followed by no significant difference in vertebral zinc concentration with increasing dietary zinc levels [15, 66]. Zinc stabilization in the whole body or vertebrae of fish receiving diets containing greater than optimal may be because of strong regulation of mineral homeostasis. The level of zinc in the liver of fish consumed diets containing increasing doses of zinc did not alter significantly. A similar pattern of liver zinc in reaction to rising dietary zinc levels was observed in *O. niloticus* [66], *C. idella* [3], *E. malabaricus* [8] and *E. coioides* [92]. A small quantity of zinc is retained in the liver and stomach, where it is connected to molecules known as metallothioneins. They promptly deliver zinc to the organism when it is required [92–95]. Hepatic metallothionein concentration due to its physiological relevance largely remains stable and does not vary with the source and zinc levels [96]. Thus, in the present study, constant liver zinc concentration with respect to an increase in dietary zinc points to the fact that the liver has strong regulation of zinc homeostasis.

Inclusion of dietary zinc beyond the requirement level (Zn4) decreased Fe, Ca and Cu concentrations in whole body and vertebrae which is in accordance with other

studies [15, 97–99]. Clearwater et al. [100] reported that the inclusion of higher amounts of zinc than optimum hampered the assimilation of other elements such as Fe, Ca and Mg. In liver, the concentration of Fe was found to reduce when *H. fossilis* were fed more than 26.74 mg/kg zinc. In tilapia, there was a negative connection between zinc in the diet and liver Fe (59). Cousins and Mc Mahon [101] also reported that Zn and Fe compete for binding sites on the divalent cations transporter I. This may be the reason for the lower Fe concentrations in the total body, vertebrae and liver of *H. fossilis* fed diets with greater than optimal Zn levels. Concentrations of other minerals such as Zn, Cu and Ca in the liver were not significantly affected by increasing dietary zinc levels. No significant change in muscle Ca concentration of Nile tilapia [66] fed diets with rising levels of zinc was noted.

Reactive oxygen species (ROS) are produced as by-products of metabolism. Normally, there is an equilibrium between production and elimination of these oxygen free radicals, but when production is higher than elimination, then it causes oxidative stress. These intermediate species attack cell membranes resulting in lipid peroxidation. Excess ROS generation in the metabolic system causes oxidative stress, which allows infections to infiltrate and infect the host [102]. Such excess ROS generation results into rise in the action of several antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase [103, 104] preventing the damaging effects of ROS and reducing lipid peroxidation [12]. Transmutation of superoxide radical into hydrogen peroxide and molecular oxygen is catalysed by superoxide dismutase. Furthermore, the catalase enzyme decomposes intracellular hydrogen peroxide (H_2O_2) into water and oxygen without creating free radicals. Antioxidant properties and involvement in the defence system are one of the crucial functions of zinc in fish [7, 105], and zinc is known to improve the antioxidant capability of fish by increasing the activities of catalase, glutathione peroxidase, and superoxide dismutase in the serum, liver, intestine, and muscle tissue of fish [12, 15, 106–108]. In the present study, increase superoxide dismutase and catalase activities in *H. fossilis* fed increasing dietary zinc up to optimum (Zn4) is in accordance with the results obtained in rainbow trout [109], yellow catfish [26], hybrid tilapia [6] and juvenile grouper [110]. However, contrary to our results, a decrease in CAT activity with the increase in dietary zinc concentrations was observed in Nile tilapia [15]. These authors speculated that the decrease in serum hydrogen peroxide due to the rise in superoxide dismutase and glutathione peroxidase activities may be the reason for reduced catalase activity with increased zinc concentration in diets.

Lipid peroxidation (LPO) is a damaging process caused by reactive oxygen species. The degree of LPO is measured by assessing the malondialdehyde (MDA) content which is the end product of LPO. The MDA content provides a well-situated index of lipid peroxidation [111] indicating the antioxidant capacity of fish. Serum MDA content was reduced with the increasing zinc levels up to the optimum level (Zn4). Fish receiving the optimum level of zinc in diet reflected lowest serum MDA content. There are several reports indicating the reduced MDA content in fish fed diets with adequate zinc. In juvenile Jian carp, suppressed lipid peroxidation and protein oxidation were observed by Feng et al. [106] when fed the optimum level of zinc. They concluded that optimum zinc improved the ability of Jian carp to scavenge hydroxyl ($OH^{\cdot}$) and superoxide (O_2^{-}) free radicals which are the most toxic oxygen species involved in oxidative damage. Onderci et al. [112] in laying hens and Kucukbay et al. [113] in rainbow trout also reported lower liver MDA levels when fed optimum dietary zinc. Luo et al. [114] and Jiang et al. [107] also observed a parallel phenomenon of reduction in MDA level in yellow catfish and blunt snout bream, respectively, when fed diet with an optimum level of zinc. A similar phenomenon was observed by Wu et al. [75] in Nile tilapia receiving optimum dietary zinc, however, they also reported a slight increase in MDA content when higher levels (76.37 mg/kg and 89.2 mg/kg) of dietary zinc were fed.

Alkaline phosphatase (ALP) activity showed improvement up to 30.61 mg/kg zinc with increasing dietary zinc levels and then stabilized. ALP activity is a sensitive tool for assessing zinc status [107, 115]. ALP is a homodimeric metalloenzyme having two Zn ions and one Mg ion at each active site. Zinc is a cofactor of ALP which stimulates the osteoblasts for bone formation [116] and enzyme aminoacyl-tRNA synthetase for protein synthesis [117]. Increased vertebrae zinc concentration and serum ALP activity in the present study also point to the role of zinc in bone formation. It has been noted that decreased plasma ALP activity is related to zinc deficit in fish [3, 66]. The reduced ALP activity in *H. fossilis* fed on basal diet indicates that the amount of zinc was inadequate which was increased with the rise in dietary zinc concentration up to optimum level (Zn4) and then stabilized. A similar pattern of improved ALP activity with increasing dietary zinc levels was also observed in channel catfish [61], Atlantic salmon [25], cobia [63], hybrid tilapia [74], grass carp [3], Nile tilapia [75], blunt snout bream [107] and Siberian sturgeon [12].

The immune system is a critical and essential defence mechanism. The environment has a large number of microorganisms and has a significant impact on the health status of fish. Nonetheless, fishes defend themselves and maintain basic health conditions due to intrinsic and unique defence mechanisms. In fish, innate immunity is faster than adaptive immunity, which takes much longer in mammals and is regarded as a critical defensive mechanism against infectious infections [118].

Potent antibacterial proteins present in secretions and tissues such as the kidney, alimentary tract, spleen, mucus, gills and serum include myeloperoxidase (MPO) and lysozyme [119]. Lysozyme catalyses the breakdown of a glycosidic link in the bacterial cell wall between N-acetylmuramic acid and N-acetylglucosamine. Lysozyme, a common invertebrate immunological enzyme, may kill bacteria by destroying the peptidoglycan layer of bacterial cell walls [120]. Zinc is essential for maintaining a healthy immune system, including both specific and non-specific immunity [121]. Several studies have reported that lysozyme activity can be used as a tool to evaluate the immune status of organisms [122, 123]. In the current study, dietary zinc supplementation boosted lysozyme activity up to optimum, but higher levels of zinc in the diet did not affect lysozyme action in fish. Parallel improvement in the activity of lysozyme activity was noted in some other previously conducted studies on other fish species [124]. Paripatananont and Lovell [125] reported that the amount of Zn in the diet improved the channel catfish resistance to harmful microorganisms by activating immunological responses. Myeloperoxidase (MPO) is a key enzyme secreted by polymorphic nuclear neutrophils that produce deadly oxidants that disrupt nitric oxide-dependent signalling pathways in the vasculature [126]. MPO is a crucial enzyme for many fish species because it boosts macrophages and neutrophil activity in the blood [126–128]. During a respiratory burst, MPO uses hydrogen peroxide to make hypochlorous acid [129]. The highest MPO activity detected in *H. fossilis* fed Zn4 diet signifies its well-developed immune status.

Conclusion

Results of the present study demonstrate the necessity of supplementing zinc in diet. Based on the findings of this study, inclusion of zinc in the range of 26.82–29.84 mg/kg diet is optimum for growth, conversion efficiencies, haematological parameters, antioxidant status, intestinal health, mineralization and immune response of *H. fossilis*.

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Author Contributions Noorin Zafar: Feeding trial; analyses; writing, original draft; funding acquisition

Mukhtar A Khan: Conceptualization, supervision; writing — editing

Data Availability All data utilized for this trial and to support the findings are available within the article.

Declarations

Conflict of Interest The authors declare no competing interests.

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