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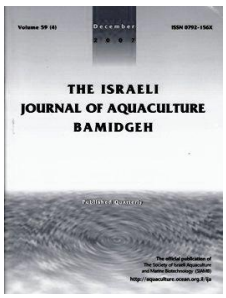

Zinc Supplementation and its Effect on Thermal Stress Resistance in *Carassius auratus* Fry

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Abstract

Goldfish (*Carassius auratus*) fry (80 mg) were fed purified diets based on casein as a protein source and containing different levels of supplementary zinc (0, 30, 60, 90, 120, 150 mg Zn/kg diet) for nine weeks. There were overt signs of zinc deficiency (suppressed growth, high mortality) in fish fed the zinc-deficient control diet, while the highest growth was obtained in fish fed the 60 mg Zn/kg diet. Alkaline phosphatase (ALP) activity in the muscle and super-oxide dismutase (SOD) activity in the liver were highest in fish fed the 60 mg Zn/kg diet and did not significantly improve beyond this level. Muscle and whole body zinc concentrations increased proportionately with the dietary zinc supplementation but iron and copper concentrations in fish tissue were not affected by the supplementary zinc. After the 9-week trial, twelve fish were subjected to thermal stress (28-32°C) for another four weeks to observe growth and the physiological response of the stress enzyme, SOD. After thermal stress, growth improved with the increase in zinc. Likewise, SOD activity in the fish liver increased, indicating that fish utilize more zinc in high temperatures to counteract stress.

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Introduction

Zinc is an essential trace element for living organisms because of its integral role in a number of metallo-enzymes, including dehydrogenases, aldolases, peptidases, phosphatases, and super-oxide dismutase (SOD). The amount of zinc in fresh (Spry et al., 1988) and saline (Wills and Sunda, 1984) water is insufficient to meet the requirements of growing fish. As an essential nutrient, zinc must be supplemented through the diet (NRC, 1981; Lall, 2000). Inadequate zinc supplementation can lead to common zinc deficiency symptoms such as growth retardation and high mortality in common carp (Ogino and Yang, 1979) and channel catfish (Gatlin and Wilson, 1983), while high incidences of eye cataracts and fin erosion were observed in rainbow trout (Ogino and Yang, 1978; Ketola, 1979). However, in fishes, high dietary zinc levels can negatively affect other elements such as iron (Ogino and Yang, 1978; Wekell et al., 1986; Spry et al., 1988) and copper (Knox et al*.,* 1984).

Dietary zinc requirements have been determined for commercially important *Lates calcarifer* and *Clarias batrachus* (Sapkale and Singh, 2011), *Eriocheir sinensis* (Sun et al., 2011), Nile tilapia (Eid and Ghonim, 1993; Do Carmo e Sa et al*.,* 2004), common carp (Ogino and Yang, 1979), and *Penaeus monodon* (Ali, 2000). Proper management of nutrition for ornamental fish can enhance growth, larvae survival, maturation, coloration, and breeding capacity. The zinc requirements of ornamental fish during the growth phase have been quantified for guppy (Saleas and Janssess, 2003). *Carassius auratus* of the Cyprinidae family is one of the most important ornamental fish in freshwater aquaria and has a physiology similar to other carps. Therefore, knowledge of the zinc requirements of *C. auratus* can serve as a reference for nutritional management of other carps. The aim of this study was to determine the optimum zinc requirement to support growth, survival, and the activity of metallo-enzymes in *C. auratus* fry. Optimization of the dietary zinc supplementation for this species is based not only on growth and survival but also on the level of deposition of zinc in the tissue and the interaction of zinc with copper and iron.

Materials and Methods

Diets. A basal diet (crude protein 30%) was formulated from purified ingredients (Lochmann and Phillips, 1994). Casein and gelatin were the protein sources (Table 1). The diet contained 6% oil as the lipid source, 30% dextrin as the carbohydrate source, and zinc-free mineral and vitamin premixes. To create five test diets, the basal diet was supplemented with five levels of zinc sulfate ($ZnSO_4 \bullet 7H_2O$), i.e., 30, 60, 90, 120, and 150 mg Zn/kg, while a corresponding amount of cellulose was removed. Dry ingredients were blended in a container and mixed with distilled water and oil (6%) to make a dough. About 450 ml distilled water was added per kg diet to achieve a consistency that would result in the production of stable pellets. The dough was placed in a cooker for 30 min, and the vitamin/mineral premixes, 125 mg ethoxyquin/kg diet, and calculated level of $ZnSO_4 \bullet 7H_2O$ were added. The dough was passed through a hand pelletizer to produce pellets that were air dried for 2 h, placed in a hot air oven at 50°C for 2 h, and stored at room temperature. The proximate compositions of the diets were analyzed according to AOAC (1995; Table 2). Zinc concentrations were measured by an atomic absorption spectrophotometer. The zinc concentration of the basal diet was 6.9 mg Zn/kg.

Experimental set up. The completely randomized design had six treatments with three replicates. Thirty goldfish (*Carassius auratus*) fry (77-80 mg) were randomly distributed into 18 glass aquaria (50 l), each, and conditioned on the basal diet for 7 days. The fish were then fed the experimental diets for 9 weeks. The diets were provided at 5% of the body weight per day, divided into two equal feedings. Fish were sampled every 15 days and the amount of feed was adjusted according to biomass. After sampling, fish were treated with methylene blue to prevent stress from handling and bacterial infection. The zinc concentration of the water was 11 µg/l prior to commencement of the experiment. Optimum water quality was maintained by continuous aeration and a 40% daily water exchange.

Table 1. Composition of the basal diet for goldfish (*Carassius auratus*) fry.

Ingredient	*g/kg diet*
Dextrin[1]	397.2
Casein[1]	280
Cellulose[1]	130
Gelatin[1]	64
Mineral premix[2]	44.52
Cod liver oil[3]	30
Sunflower oil[3]	30
Carboxy methyl cellulose	20
Vitamin premix[4]	10
DL-methionine[1]	1
L-tryptophan[1]	1
Ethoxyquin[1]	0.125

[1] Hi-media Laboratories Private Ltd.
[2] g/kg diet: $FeSO_4$ 0.2133, $CuSO_4$ 0.0167, $CaCO_3$ 10.667, KIO_3 0.0036, NaMoO 0.0049, $NaHCO_3$ 4, Na_2SeO_3 0.0008, $MgSO_4$ 4.6667, KH_2PO_4 10, $CoCl_2$ 0.0002, $MnSO_4.H_20$ 0.3533, $CaHP0_4$ 14.667
[3] From local market
[4] mg/kg diet: vitamin A 1.8, vitamin D 0.002, vitamin E 50, vitamin K 10, thiamin (vitamin B_1) 20, riboflavin (vitamin B_2) 20, D-calcium pantothenate (vitamin B_3) 50, niacin (vitamin B_5) 100, pyridoxine (vitamin B_6) 20, cyanocobalamin (vitamin B_{12}) 0.05, folacin (vitamin M) 5, choline chloride 550, ascorbic acid (vitamin C) 50, inositol 1

Enzyme activity analysis. At the end of the experiment, liver and muscle tissues were collected for enzyme activity and mineral assay. The samples were acid digested (5 ml HCl and 1 ml HNO_3) and the concentration of different elements in the muscle and whole body were measured with an atomic absorption spectrophotometer (Analyst 800, Perkin Elmer, USA). SOD activity in the liver and alkaline phosphatase (ALP) activity in the muscle were determined by the methods of Garen and Levinthal (1960) and Misra and Fridovich (1972), respectively.

After the 9-week feeding trial, 12 fish from the same experimental tanks were subjected to thermal stress by increasing the water temperature from 28°C to 32°C. The temperature of the experimental tanks was increased by 1°C every 5 days and the tanks were maintained at 32°C for another 10 days to determine the physiological response of the stress enzyme, i.e., SOD activity.

Statistical analysis. The least square means for each treatment group and ANOVA were determined by the PROC GLM procedure of SAS 9.1. Mean values of treatments were compared using Duncan's multiple range test. Differences were considered significant when $p<0.05$.

Table 2. Proximate compositions of experimental diets for goldfish fry, dry weight basis (means±SE).

	Zn level (mg/kg diet)					
Component (%)	*0*	*30*	*60*	*90*	*120*	*150*
Moisture	8.62±0.02	7.23±0.01	6.54±0.01	8.32±0.03	7.21±0.02	7.58±0.02
Crude protein	30.1±0.08	30.8±0.02	30.45±0.10	29.40±0.07	29.05±0.05	28.38±0.05
Crude fat	6.23±0.07	6.5±0.02	7.2±0.05	6.82±0.03	7.60±0.01	7.3±0.01
Ash	11.69±0.01	11.48±0.01	13.82±0.02	12.00±0.05	13.11±0.04	13.72±0.04
Total carbohydrates	43.36±1.20	43.99±1.15	41.99±1.23	43.46±0.90	43.05±1.25	43.02±1.25

Results

The weight gain of fry fed the basal diet was significantly lower than that of those fed the diets supplemented with 60 mg Zn or more (Table 3). The FCR was significantly higher in the control group than in treatments with 60 mg Zn or more. Growth increased with the dietary zinc supplementation up to 60 mg Zn/kg diet, then dropped. Deficiency symptoms, i.e., suppressed growth and reduced survival, were noticed in fish fed the basal diet. Survival was highest in fish fed the 60 mg Zn/kg diet. There was a gradual increase in zinc deposition in the fish body as the dietary zinc concentration increased but no significant differences in muscle or whole body copper concentrations. There were significant differences in muscle and whole body iron deposition, but no apparent trend.

Muscle ALP and liver SOD activity were determined relative to muscle protein concentrations to prevent differences caused by variations in the muscle protein concentration. The activity of both enzymes increased with zinc supplementation and was significantly higher in fish fed the 60 mg Zn/kg diet than in fish fed the control or 30 mg Zn diet but did not significantly differ from fish fed diets containing more than 60 mg Zn/kg diet. After thermal stress, the weight gain was highest in fish fed the 60 mg Zn/kg

diet while SOD activity in the liver increased with zinc supplementation and, while highest in fish fed the 150 mg Zn/kg diet, did not significantly differ from SOD activity in fish fed the 60 mg Zn diet.

Table 3. Growth, feed efficiency, tissue mineral concentrations, and enzyme activity in *Carassius auratus* fry fed diets containing different levels of zinc for 9 weeks (means±SE).

	Added dietary zinc (mg/kg diet)[1]					
	0	*30*	*60*	*90*	*120*	*150*
Mean wt gain (g)	0.51±0.04^{c}	0.55±0.01^{bc}	0.70±0.05^{a}	0.68±0.02^{a}	0.67±0.02^{a}	0.63±0.02^{ab}
SGR (%)[2]	3.12±0.10^{c}	3.18±0.01^{bc}	3.45±0.12^{a}	3.44±0.05^{a}	3.44±0.03^{a}	3.42±0.07^{ab}
FCR[3]	1.44±0.05^{a}	1.35±0.02^{ab}	1.10±0.05^{c}	1.14±0.02^{c}	1.18±0.02^{c}	1.24±0.03^{bc}
FCE[4]	69.45±2.44^{c}	73.93±1.30^{bc}	90.90±3.28^{a}	87.25±1.43^{a}	84.60±1.99^{a}	80.75±2.12^{ab}
Survival (%)	68.89±4.01^{b}	76.67±3.33^{ab}	85.11±2.22^{a}	82.22±2.22^{a}	81.11±4.00^{a}	81.11±2.94^{a}
Zinc (mg/kg body wt)						
Muscle	45.73±2.08^{f}	64.12±1.70^{e}	72.44±1.45^{d}	82.14±1.10^{c}	89.50±1.92^{b}	100.46±1.92^{a}
Whole body	54.22±1.07^{f}	70.2±0.60^{e}	81.12±0.04^{d}	89.41±0.55^{c}	95.99±0.50^{b}	101.84±1.33^{a}
Copper						
Muscle	8.29±0.17	8.62±0.11	8.13±0.08	8.67±0.29	9.10±0.20	8.93±0.13
Whole body	14.99±0.27	15.36±0.19	15.99±0.12	16.12±0.13	15.39±0.17	15.33±0.30
Iron						
Muscle	81.87±2.86^{b}	104.1±7.29^{a}	96.19±3.88^{a}	72.56±4.64^{b}	102.56±5.13^{a}	97.63±2.17^{a}
Whole body	87.12±0.79^{b}	78.22±1.29^{c}	88.50±1.50^{b}	97.57±1.08^{a}	100.43±2.33^{a}	91.48±1.49^{b}
Enzyme activity						
SOD in liver[5]	57.84±1.78^{c}	72.19±2.60^{b}	84.36±3.58^{a}	85.33±3.27^{a}	90.25±5.52^{a}	91.75±5.02^{a}
ALP in muscle[6]	6.77±0.35^{b}	6.80±0.480^{b}	9.26±0.60^{a}	9.83±0.48^{a}	9.8±0.58^{a}	10.27±0.88^{a}
After thermal stress (28-32°C) for one month						
Mean wt gain (g)	0.20±0.01^{d}	0.255±0.01^{c}	0.298±0.02^{a}	0.292±0.02^{ab}	0.290±0.02^{ab}	0.293±0.01^{ab}
SOD[5]	60.03±3.30^{d}	75.98±1.03^{c}	90.36±1.39^{ab}	92.37±4.36^{ab}	92.63^{a}±3.91^{ab}	96.63±6.26^{a}

Rows with different superscripts significantly differ (p<0.05)
[1] Basal diet contained 6.9 mg Zn/kg diet
[2] Specific growth rate
[3] Feed conversion ratio
[4] Feed conversion efficiency
[5] Super-oxide dismutase activity (unit enzyme activity/mg protein/min)
[6] Alkaline phosphatase (unit enzyme activity/mg protein)

Discussion

There was no significant weight gain above 60 mg Zn/kg diet indicating that this level was adequate for proper growth. Likewise, after a certain dietary level of zinc supplementation, growth did not improve in common carp (Jeng et al., 1981). Zinc deficiency induced retarded growth and high mortality in common carp (Ogino and Yang, 1978) while diets containing 50-200 mg Zn/kg produced high values for average body weight, growth factor, and feed efficiency in eel (Park and Shimizu, 1989) and for body weight in *Lates calcarifer* and *Clarias batrachus* (Sapkale and Singh, 2011), *Osteobrama belangeri* fry (Azad, 1997), and *Oreochromis niloticus* (Mahmoud, 2009). It might be that feed intake, and thereby weight gain, are influenced by the level of zinc through its role in enzymatic activities involved in metabolic and biochemical processes (Dabrowski et al., 1993; Guillaume et al., 2001).

Survival was significantly lower in the control group than in the other treatments. The high mortality in the control group can be attributed to zinc deficiency since optimum water quality was maintained in all tanks. High mortality has been associated with zinc deficiency in rainbow trout, common carp, and channel catfish (Ogino and Yang, 1978; Gatlin and Willson, 1983). The whole body zinc concentration in our study was significantly affected by the dietary zinc content, as reported by Hardy and Shearer (1985). The decreased amount of whole body zinc in the zinc deficient group indicates

that the growth retardation and significant mortality were caused by a zinc deficiency, as shown by Gatlin and Willson (1983) and Spry et al. (1988). In contrast, there was no significant improvement in the survival rate of juvenile Chinese mitten crab fed a dietary zinc content up to 100 mg Zn/kg (Sun et al., 2011).

The whole body and muscle iron concentrations did not show any trend in relation to the level of dietary zinc, indicating that there is no zinc-iron antagonistic activity in goldfish nutrition and that whole body and muscle iron concentration are unaffected by dietary zinc supplementation. Similarly, there was no correlation between iron and dietary zinc levels in Atlantic salmon (Maage and Julshamn, 1993) although, in some fish, iron levels are elevated in severe zinc deficiency (Ogino and Yang, 1978; Wekell et al., 1986; Spry et al., 1988). For example, there was a strong inverse relationship between whole body iron and whole body zinc in Nile tilapia (Chen and Pet, 2001).

Whole body and muscle copper concentrations were not significantly affected by the dietary zinc supplementation, indicating that there was no zinc-copper antagonistic activity. Similarly, there was no distinct copper-zinc antagonistic activity in channel catfish (Gatlin et al., 1989) and no adverse effect of high levels of dietary zinc on copper metabolism in rainbow trout (Ketola, 1979). On the other hand, there was evidence of copper-zinc antagonistic activity in rainbow trout (Knox et al., 1984).

SOD activity increased up to 60 mg Zn/kg diet, apparently the adequate level of zinc required to enhance SOD activity. SOD activity likewise increased in channel catfish with an increase in dietary zinc intake (Gatlin et al., 1989) and decreased with zinc deficiency in earlier studies (Dreosti and Record, 1979; Knox et al., 1984).

ALP is the most active phosphatase and catalyses the liberation of inorganic phosphate from esters such as glycerolphosphate and phenylphosphate. Zinc acts as a constituent of ALP (Hambidge et al., 1986) and influences its activity. In this study, the control diet produced poor ALP activity due to zinc deficiency. ALP activity increased with the increase in zinc supplementation and was highest in fish fed the 150 mg Zn/kg diet, although it did not significantly differ from ALP activity in fish fed the 60 mg diet. Likewise, in salmon, ALP activity increased with increased dietary zinc (Maage and Julshamn, 1993).

During metabolism, aerobic organisms continuously produce endogenous super-oxide radicals (ROS) that affect macromolecules such as carbohydrates, nucleic acid, and lipids during oxidative stress (Di Guilio et al., 1989; Finkel and Holbrook, 2000; Fang and Zheng, 2002). At high temperatures, production of ROS is enhanced in fishes under stress. To neutralize ROS, the activity of enzymes such as SOD increases. In the present study, there was an increase in SOD activity in all treatments after exposure to high temperatures, as found earlier by Parke (1978). Thus, at high temperatures, fish utilize more zinc to enhance the activity of the stress enzyme, SOD, to counteract stress. After thermal stress, the highest weight gain was observed in fish fed 60 mg Zn/kg diet; the lowest was in the control group. Again, beyond 60 mg Zn/kg diet, growth did not significantly improve indicating that, even in high temperatures, 60 mg Zn/kg diet is the optimum level of dietary zinc for proper growth.

In conclusion, dietary zinc significantly impacts growth, survival, and thermal stress resistance in *C. auratus* fry. Inclusion of 60 mg Zn/kg diet is optimum for growth and survival of *C. auratus* fry. At high temperatures, fish utilize more zinc to enhance the activity of the stress enzyme, SOD, to counteract stress.

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