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Effect of dietary zinc on the growth and metabolic enzyme activities of golden mahseer (*Tor putitora*) fry

Kaustubh Bhagawati[1*], N. K. Chadha[1], Debajit Sarma[3], M. S. Akhtar[3], Paramita B. Sawant[1] and Simanku Borah[2]

[1]Division of Aquaculture, Central Institute of Fisheries Education (ICAR), Versova, Mumbai – 400061 (Maharastra), INDIA
[2]Division of Fisheries Resource Management, Central Institute of Fisheries Education (ICAR), Versova, Mumbai – 400061 (Maharastra), INDIA
[3]Environmental Fish Biology and Nutrient Profiling Laboratory, ICAR-Directorate of Coldwater Fisheries Research Bhimtal, Nainital- 263136 (Uttarakhand), INDIA
*Corresponding author. E-mail: kaustabh.bhagawati@gmail.com

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Abstract: The golden mahseer (*Tor putitora*) is an important and high valued sport and food fish of national and international importance. Therefore for promotion of golden mahseer, proper mineral supplementation in early developmental stages is very important. The present study evaluated the effects of dietary zinc on growth, survival and physiological response of *Tor putitora* fry. One thousand eight hundred fry (avg.wt 54.35±3.09 mg) were randomly distributed into 6 treatment groups with triplicates each. Six iso-nitrogenous (40±0.02% to 41.44±0.01% crude protein) semi-purified diets were prepared with graded levels of dietary zinc. Zinc sulphate was added as the dietary zinc source to the basal diet. The results indicated that specific growth rate (SGR) was of value 2.52 ±0.23 gm of *T. putitora* fry which increased with dietary zinc levels up to a concentration of 40 mg Zn Kg^{-1} in diet. The zinc dependent metabolic enzymes and antioxidant enzymes was also observed to be highest in groups supplemented with 40 mg zinc/kg feed. The overall results of the present study suggest the efficacy of dietary zinc on growth, survival and physiological response of golden mahseer fry in order to garner the possibility of establishing the species to commercial aquaculture.

Keywords: Isonitrogenous, Metabolic enzymes, Specific growth rate, *Tor putitora*

INTRODUCTION

Tor putitora (golden mahseer) is a well-acknowledged, highly valued coldwater fish of India. Golden mahseer as a sport fish provides unparallel recreation to anglers from all over the world. As a food fish, it is highly esteemed and fetches the highest market price in north and north east of India. Despite its earlier abundance, *T. putitora* population has been declining in number and size in natural waters because of degradation of the aquatic environment and biological changes in the ecosystem. Therefore, the present concern is to conserve this species through artificial breeding, seed rearing and ranching into its natural habitats. But, the major bottleneck in successful seed propagation of this species is unavailability of suitable diet for larval rearing (Akhtar *et al*., 2013). Hence, a better understanding is required to study the nutritional role of trace minerals such as zinc where major research focus in needed, which will be essential for preparation of suitable larval diets.
To address the research problem a necessary understanding is required to formulate an artificial diet enriched with dietary zinc to know about the nutritional role of zinc in fish. Zinc is known to be an essential trace element for normal physiology, growth and development in terrestrial animals and fish (Liang *et al*., 2012). It serves as an integral part of nucleoproteins and regulates many metabolic processes of carbohydrate, lipid and protein metabolism (Lall, 2002). Zinc serves as a cofactor of many enzymes including carbonic anhydrase and is required for the activity of the antioxidant enzyme superoxide dismutase (Yousef *et al*., 2002). Zinc deficiency has been shown to produce impaired growth, increased mortality, eye cataract and poor feed utilization (Hasnat *et al*., 2012). Freshwater fish take up zinc from both water and food. However dietary zinc is more efficiently utilized to satisfy the nutritional requirement for the growing fish. Therefore in the present experiment, an attempt has been made to determine the optimum dietary zinc concentration on growth and physiological indices of the early developmental stages of golden mahseer (*T. putitora*) fry.

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MATERIALS AND METHODS

Experimental procedure: Fry of *T. putitora* (54.85±3.09 mg, average weight ± SE) were procured from mahseer hatchery of Directorate of Coldwater Fisheries Research (DCFR), Bhimtal, Uttarakhand, India, and were acclimatized for ten days in rectangular FRP tanks. The fish were fed with macerated goat liver and control diet during acclimatization period. Before start of the experiment, the tanks were cleaned and disinfected using potassium permanganate ($KMnO_4$) solution of 5 ppm concentration. All the experimental tanks were filled with water and kept for two days for conditioning before stocking the fishes. After 10 days of acclimation, fish were randomly distributed into six treatment groups in triplicates consisting of 18 uniform size (500 L capacity) plastic tanks (100 fish / tank). Each diet was assigned to triplicate tanks. The feeding trial lasted for 8 weeks. Feeding was done at *ad-libitum* twice in a day at 09:00 hours and at 17:00 hours so as to feed the fish near satiation. The fish were weighed every 2 weeks during the trial period. The water quality parameters were analyzed fortnightly and maintained at an optimum level for the proper growth and survival of the fishes. Water quality parameters were as follows: temperature, 18.45-24.35 ^{0}C; dissolved oxygen, 7.15-8.76 mg L^{-1}; pH, 7-9; total alkalinity, 116.20-144.40 mg L^{-1}; total hardness, 129.5 -155.5 mgL^{-1}; total ammonia-nitrogen, 0.006-0.01 mgL^{-1}. Zinc concentration of the rearing water was 0.01-0.03 mgL^{-1} during the trial period.

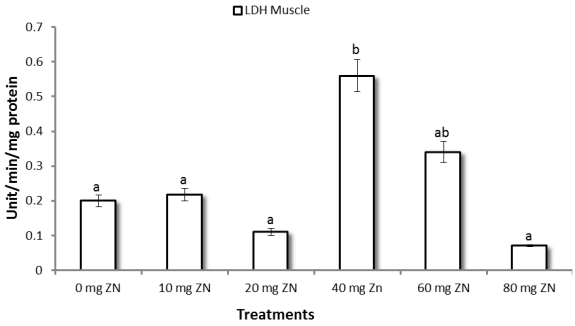

Fig.1. *Effect of dietary zinc on LDH (Lactate dehydrogenase) activity in muscle of T. putitora reared for a period of 8 weeks (60 days). Values in the same series with different superscript (a and b) signify statistical differences (p <0.05), Data expressed as mean ±S.E, n=6 (each replicate containing pooled samples of 15 fish).*

Experimental diet and diet preparation: Six isonitrogenous (40.00 ± 0.02 % to 41.44± 0.01 % crude protein) semi-purified experimental diets were formulated to contain graded levels of zinc by supplementing the basal diet with 0, 10, 20, 40, 60 and 80 mg kg^{-1} Zn

Table 1. Diet composition and proximate analysis of the experimental diets (% dry matter (DM) basis) fed to *T. putitora* fry during the experimental period.

Ingredients	Diets					
	1 (0 mg ZN)	**2** (10 mg ZN)	**3** (20 mg ZN)	**4** (40 mg ZN)	**5** (60 mg ZN)	**6** (80 mg ZN)
Casein (vitamin free)[a]	24	24	24	24	24	24
Dextrin[a]	14	14	14	14	14	14
Gelatin[a]	6	6	6	6	6	6
Egg albumin powder[a]	8	8	8	8	8	8
Fish meal	16	16	16	16	16	16
Cellulose[a]	6.80	6.79	6.78	6.76	6.74	6.72
Starch soluble[a]	10	10	10	10	10	10
Vitamin mix[b]	2	2	2	2	2	2
Mineral mix[c]	2	2	2	2	2	2
Soy lecithin[d]	2	2	2	2	2	2
Fish oil	7	7	7	7	7	7
Sod.alginate[d]	2	2	2	2	2	2
BetaineHCL[a]	0.1	0.1	0.1	0.1	0.1	0.1
ButylatedHydroxy Toluene (BHT)[a]	0.1	0.1	0.1	0.1	0.1	0.1
$ZnSO_4.7H_2O$ (mg/Kg)[e]	0	10	20	40	60	80
Total	100	100	100	100	100	100
Proximate composition of diets						
Moisture	8.13±0.01	8.20±0.01	8.02±0.02	8.14±0.01	8.16±0.01	8.06±0.03
Crude protein (CP)	40.06±0.02	41.44±0.01	41.15±0.34	40.43±0.34	40.67±0.03	41.15±0.23
Ether extract (EE)	10.17±0.010	10.16±0.008	11.13±0.003	10.14±0.017	10.16±0.020	11.13±0.014
Ash	3.49±0.010	2.82±0.023	3.13±0.014	3.13±0.003	2.09±0.008	3.11±0.012
Total carbohydrate	46.27±0.040	45.56±0.202	44.57±0.348	46.29±0.326	47.07±0.049	44.60±0.242
Zinc (mg/kg diet)	**16.7±0.06**	**28.0±0.09**	**42.1±0.08**	**59.3±0.08**	**72.1±0.07**	**92.5±0.04**

Table 2. The enzyme assays were analysed as per standard method.

S. N.	Enzymes assayed	References
1.	Lactate dehydrogenase (LDH)	Wroblewski and Ladue (1955).
2.	Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST)	Wotton (1964).
3.	Superoxide dismutase (SOD)	Misra and Fridovich (1972)
4.	Catalase (CAT)	Takahara *et al* (1960).
5.	ATPase	Fiske and Subbarow (1925).
6.	Acetylcholine Esterase (AchE)	Augustinsson (1957).
7.	Glycogen level	Hassid and Abraham (1957)
8.	Protein estimation	Lowry (1951)

Table 3. Growth parameters and % survival of *T. putitora* fry fed with zinc supplemented diets.

Treatments	% Body weight gain	SGR[1]	FCR[2]	PER[3]	% survival
T_1	273.40^{b}±7.99	2.20^{b}±0.18	3.36^{e}±0.25	0.74^{b}±0.07	98.00^{ab}±0.58
T_2	306.90^{c}±16.90	2.34^{c}±0.22	3.05^{d}±0.28	0.79^{b}±0.05	98.33^{b}±0.33
T_3	315.48^{c}±15.00	2.37^{cd}±0.16	2.80^{c}±0.22	0.86^{c}±0.06	98.67^{b}±0.89
T_4	354.28^{d}±18.85	2.52^{e}±0.23	2.31^{a}±0.17	1.06^{e}±0.07	99.33^{b}±0.67
T_5	345.89^{d}±21.45	2.49^{de}±0.19	2.62^{b}±0.21	0.94^{d}±0.07	99.33^{b}±0.33
T_6	237.90^{a}±14.82	2.02^{a}±0.14	3.80^{f}±0.13	0.64^{a}±0.05	96.41^{a}±0.34

Values in the same column with different superscripts (a, b, c, d, e and f) differ significantly ($p < 0.05$). Data expressed as Mean ± SE, n=3 (each replicate had 100 fish); [1]Specific growth rate; [2]Feed conversion ratio; [3]Protein efficiency ratio; T_1= 0 mg ZN; T_2= 10 mg ZN; T_3= 20 mg ZN; T_4= 40 mg ZN; T_5= 60 mg ZN; T_6= 80 mg ZN.

in the form of Zn sulphate ($ZnSO_4.7H_2O$). Ingredients such as casein, gelatin, cellulose, starch, dextrin, egg albumin powder, fish meal, fish oil, betaine chloride, soy lecithin, vitamin and mineral mixture (Zn free-manually prepared and components procured from Himedia Laboratories Ltd., Mumbai, India) were used for diet formulation. The diet composition and proximate analysis are presented in Table 1. All the ingredients were formulated using the square method of Hardy (1980). The moisture content was determined by drying at 105°C to a constant weight. Nitrogen content of the sample was estimated quantitatively by Kjeltec semi-automatic system (Pelican Equipment, Chennai, India) and crude protein was estimated by multiplying nitrogen percentage by a constant factor 6.25. Ether extract (EE) was measured by solvent extraction method (Socplus SCS-2, Pelican Equipment, Chennai, India) using diethyl ether (boiling point, 40–60 °C) as a solvent and ash content was determined by incinerating the samples in a muffle furnace (Macro Scientific Works, Delhi, India) at 600 °C for 6 hr. All the ingredients were analyzed for proximate composition prior to formulation of the test diets employing standard methods (AOAC, 2005).

Growth and survival study: Fish were weighed at the start and every 15 days interval till the termination of the experiment on the 60th day. The growth performances of fry were evaluated in terms of weight gain

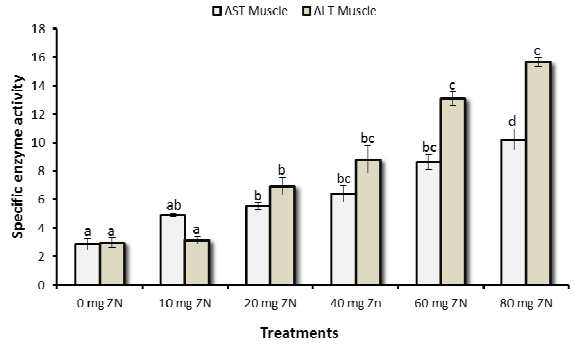

Fig. 2. *AST (Aspartate transaminase) and ALT (Alanine transaminase) activity of T. putitora fry treated with different concentrations of dietary zinc (values with different superscript differ significantly ($p < 0.05$) and express as mean ±SE) (n=6). Specific activities of enzymes expressed as AST as nano moles of oxaloacetate released/min/mg protein; ALT as nano moles of sodium pyruvate formed / mg protein/min.*

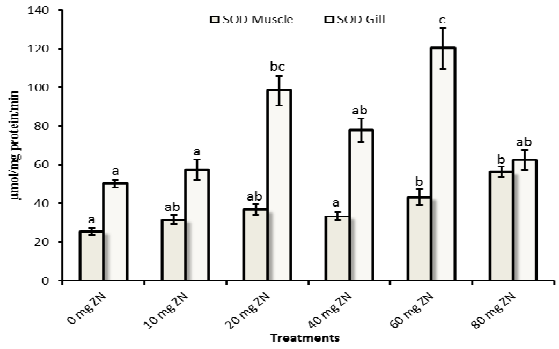

Fig. 3. *Superoxide dismutase (SOD) activity in muscle and gill of T. putitora fry treated with different concentrations of dietary zinc (values with different superscript differ significantly ($p < 0.05$) and express as mean ± SE) (n=6).Specific activities of enzymes are expressed as follows: SOD as unit activity (amount of protein required to give 50 % inhibition of epinephrine auto oxidation).*

Table 4. Effect of dietary zinc on ATPase activities in gill and AchE activities in brain of *T. putitora* fry reared for a period of 8 weeks.

Treatments	[4]ATPase	[5]AchE
	Gill	Brain
T_1	1.88^{ab}± 0.173	0.13^{a}±0.01
T_2	2.70^{b}±0.110	0.12^{a}±0.02
T_3	1.72^{ab}±0.051	0.24^{ab}±0.01
T_4	1.05^{a}±0.027	1.30^{b}±0.09
T_5	2.11^{ab}±0.176	3.01^{c}±0.24
T_6	1.37^{ab}±0.102	3.43^{d}±0.31
P value	0.001	0.001

Values in the same column with different superscript (a, b, c and d) differ significantly (p< 0.05). Data expressed as mean ± SE, n=3 (each replicate containing pooled samples of 15 fish) T_1= 0 mg ZN; T_2= 10 mg ZN; T_3= 20 mg ZN; T_4= 40 mg ZN; T_5= 60 mg ZN; T_6= 80 mg ZN; [4]Adenosine triphosphatase; [5]Acetylcholine esterase.

(%), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and survival (%). The growth parameters were analyzed employing standard methods of NRC, 2012.

Tissue homogenate preparation: At the end of feeding trial, 15 fish per treatment were sampled. The muscle, intestine, gill and brain of the fishes were dissected out carefully and weighed. It was homogenized with chilled sucrose solution (0.25M) in a glass tube using tissue homogenizer (Power Gen 500, Fisher Scientific, Germany). A 5% homogenate was prepared for all the tissues. The tubes were continuously kept in ice bath while homogenizing. The homogenate was centrifuged at 8000 rpm for 15 minutes in a centrifuge (Remi, Mumbai, India). The supernatant was kept frozen at -20^0C till further analysis.

Enzyme assays : The enzyme assays were analysed as per standard method as mentioned in Table 2.

Statistical analysis: Data were analyzed by one-way analysis of variance (ANOVA) and the significant difference between the treatments was determined by Duncan's Multiple Range Test (DMRT) using SPSS (Version 16). The level of significance employed was 0.05.

RESULTS AND DISCUSSION

Growth and survival study: The growth parameters and % survival of the experimental animals of different groups at the end of feeding trials are shown in the (Table 3). Supplementation of dietary zinc significantly (p < 0.05) affected the weight gain% and specific growth rate (SGR) of the experimental groups. The highest (p< 0.05) weight gain % and SGR were found in the group fed diet with 40 mg Zn kg^{-1} diet (T_4). The lowest (p< 0.05) value was observed in T_5 group fed diet with 80 mg Zn kg^{-1} diet. In the present experiment, percentage weight gain and specific growth rate of *T. putitora* fry increased with dietary zinc levels up to a concentration of 40 mg kg^{-1} dry diet and decreased thereafter. The results indicated that supplemental zinc could improve growth performance of *T. putitora* fry up to a certain extent which is in consistence with the results obtained on *Cyprinus carpio* (Tan *et al.*, 2011) and *Carracius auratus* (Hasnat *et al*., 2012). FCR was recorded a decreasing trend with increase in dietary zinc supplementation up to 40 mg Zn kg^{-1} diet and increased thereafter which signify statistical (p< 0.05) differences among the zinc treated groups. PER were significantly higher for fish fed the diet supplemented with 40 mg Zn kg^{-1} diet (T_4) than those fed the zinc-un supplemented diet (p< 0.05). Lowest % survival was recorded in the T_6 group and highest % survival was observed in the T_4 and T_5 groups which did not vary significantly (p >0.05) among other zinc fed groups. The FCR and PER enhanced with increasing dietary zinc levels upto a certain limit. A similar trend was

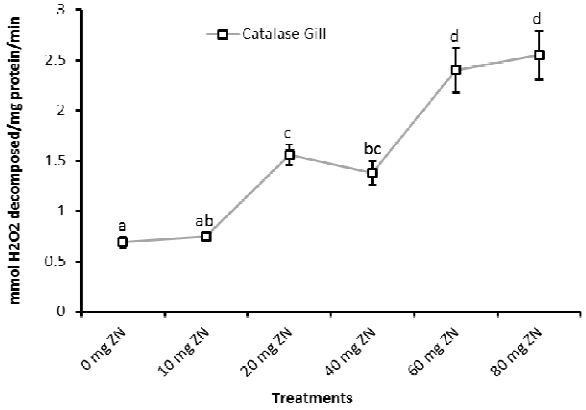

Fig. 4. *Effect of dietary zinc on catalase activity in gill of T. putitora reared for a period of 8 weeks. Values in the same series with different superscript (a, b, c and d) signify statistical differences (p <0.05), Data expressed as mean ± S.E, n=6 (each replicate containing pooled samples of 15 fish).*

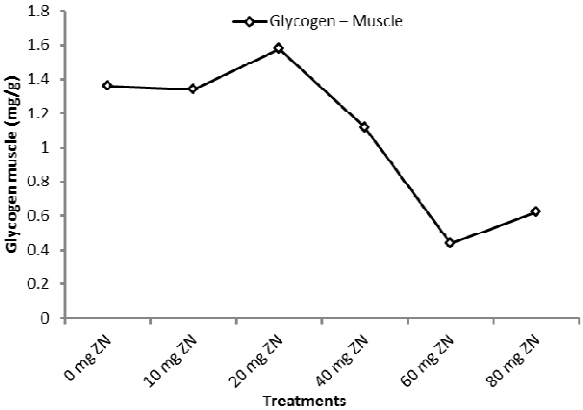

Fig. 5. *Effect of dietary zinc on glycogen level in muscle of T. putitora reared for a period of 8 weeks. Data expressed as mean ± S.E, n=6 (each replicate containing pooled samples of 15 fish).*

found in *Cyprinus carpio* (Tan *et al*., 2011) and *Ctenopharyngodon idella* (Liang *et al.,* 2012). These studies suggested that feed intake and weight gain are influenced by levels of dietary zinc.

Enzyme assays: Dietary zinc supplementation significantly ($p < 0.05$) affected lactate dehydrogenase (LDH) activity in muscle of *T. putitora* fry (Fig. 1) in different experimental groups. LDH activity was recorded highest ($p < 0.05$) in 40 mg ZN group. Lowest value was observed in 80 mg ZN among all the treatment groups. LDH plays a key role in energy metabolism and acts a pivotal enzyme between glycolytic pathway and Kreb's cycle (Murray *et al*., 2003). The LDH activity in muscle was observed to be highest in groups supplemented with 40mg zinc/kg feed. The inhibition of LDH activity was observed in groups deficient in dietary zinc levels. Present results are in accordance with previous findings of Daniela *et al*. (2012) who affirmed that inhibition of LDH activity may be due to ion imbalance or intracellular action of metal subsequent to initial tissue damage. Effects of dietary zinc on the muscle AST and ALT activity are presented in Fig. 2. Highest ($p < 0.05$) AST activity was observed in 80 mg ZN group exposed to higher concentration of dietary zinc and lower was observed in control (0 mg ZN) group. An increasing trend was found in AST activity with higher inclusion of dietary zinc which varies significantly among the treatment groups. Similar trend was also noticed for ALT activity in muscle of dietary zinc fed *T. putitora* fry which activity increased concomitantly in highest zinc supplemented group. This increase in activity signifies greater mobilization of amino acids for glucose production via gluconeogenesis to cope up with stress (Chatterjee *et al*., 2004). Knox and Greengard (1965) also reported that elevated level of transaminase activity would lead to increased feeding of keto acids into TCA cycle thereby affecting oxidative metabolism. However no parallel report is available on the dietary zinc on AST and ALT enzyme activity to substantiate our findings.

Dietary zinc supplementation has significant ($p < 0.05$) influence on SOD activity in gills of treated groups (Fig 3). Highest gill SOD activity was observed in 60 mg Zn group fed with 60 mg Zn kg^{-1} diet that varied significantly from all other groups. Lowest value was observed in the control group (0 mg ZN) fed without zinc. In the muscle, SOD activity exhibited significant ($p < 0.05$) variation among the treatments. In the muscle the highest and lowest SOD activity was observed in 80 mg ZN and 40 mg ZN groups, respectively. The present results showed that the activities of SOD were affected during a 60 days exposure period to dietary zinc. The SOD activity was observed to be low in groups fed with 40 mg Zn kg^{-1} diet which showed significant difference in response to control and groups fed with higher concentration of zinc. The results in the present study are in agreement with the findings of (Hasnat *et al.,* 2012), who reported increasing trend of SOD activity in channel catfish and goldfish with increase in dietary zinc intake. These changes indicated a possible mechanism of oxidative stress generated by dietary zinc on *T. putitora* fry. The gill catalase activity was significantly ($p < 0.05$) affected by dietary zinc supplementation (Fig 4). Gill catalase activity was recorded highest in 80 mg ZN fed groups that varied significantly among all other groups except 60 mg ZN. Many environmental pollutants, including pesticides and heavy metals are capable of inducing oxidative stress in fish (Pandey *et al.*, 2003; Monteiro *et al.*, 2006). The present study reports that the gill catalase activity was significantly higher in group exposed to higher concentration of dietary zinc. However, our findings are not in agreement with the results obtained by Kong *et al*. (2012) who reported that CAT activity was inhibited in the developing embryos of *Carassius auratus* under mercury exposure. Therefore the complicated action involved in CAT activity makes it difficult to completely elucidate, which requires further studies on the response mechanism of CAT in the early developmental stage of fish under heavy metal exposure in order to provide additional evidence.

Tissue glycogen: The muscle glycogen level of the experimental groups is presented in Fig. 5. Dietary zinc supplementation has significant ($p < 0.05$) effect on glycogen level in muscle. Highest muscle glycogen level was observed in 20 mg ZN followed by lowest observed in 60 mg ZN group. Tissue glycogen is commonly measured parameter of stress response (Manush *et al*., 2005). Muscle is the chief component on which the nutritive value of fish may be assessed and glycogen is the main components of this tissue. Increase in glucose and concomitant decrease in glycogen on exposure to various environmental toxicants like heavy metals Zn, Ni, Cr and pesticides like sulphoxides, phosphamidon carbonate has been reported by (Sarma *et al.,* 2013) and in muscle tissues of *Labeo rohita* exposed to pesticides stress (Akhtar *et al*., 2012). .

In the present study, reduction of gill ATPase activity may be due to alterations in the structure and functions gill plasma membrane or may be due to direct inhibition of zinc on the enzymes. Further research is required to validate these conclusions. Acetylcholine esterase (AchE) is one of the most widely used enzyme biomarker for environmental pollution (Vani *et al*., 2011). Reduction in AchE enzyme activity indicates an accumulation of acetylcholine in the brain tissue, interfering with energy metabolism of the nervous system, preventing transmission of nervous impulses, and thereby causing behavioral alteration (Sarma *et al*., 2010). In agreement with the above studies, the present results also evidenced that AchE activity decreased significantly with the increase in dietary zinc concentrations indicating an inhibitory effect of higher dietary

zinc exposure on AchE activity.

Conclusion

For propagation of golden mahseer (*Tor putitora*), proper zinc supplementation in diet for feeding the fishes is very important. To our knowledge this is the first report on studying the effect of optimum dietary zinc supplementation on the early developmental stages of golden mahseer. The present study concluded that dietary supplementations of zinc at the level 40 mg Zn kg^{-1} in diet increased the growth efficiency, enzymatic responses and survival rate of fry of golden mahseer. Enzymatic responses in nutritional studies are useful indices of defining the functional capacities of tissues. The use of enzymatic responses in the present study signifies the physiological responses of *T. putitora* fry, which favours dietary supplementations of zinc at the level 40 mg Zn kg-1. The results of the present study will enable the scientific community to formulate a suitable diet for larval stages of golden mahseer which will be a boon for propagation and conservation of golden mahseer (*T. putitora*) so as to garner the possibility of establishing the species to commercial aquaculture.

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