Fish and Shellfish Immunology 84 (2019) 38–47

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Effect of dietary selenium on immuno-biochemical plasticity and resistance against *Aeromonas veronii biovar sobria* in fish reared under multiple stressors

Neeraj Kumar*, Narendra Pratap Singh

ICAR-National Institute of Abiotic Stress Management (NIASM), Baramati, Pune, 413115, India

ARTICLE INFO

Keywords:
Selenium
Growth performance
Stress biomarkers
Immunity
Pb
Temperature

ABSTRACT

The present investigation aims to study role of dietary selenium (Se) on growth performance, oxidative stress markers (catalase, superoxide dismutase and glutathione-s-transferase), stress biomarkers [blood glucose, cortisol and heat shock protein (HSP 70) and immunological status, Nitro blue tetrazolium (NBT), total protein, albumin, globulin, A/G ratio, total immunoglobulin and vitamin C] and survival of fish after *Aeromonas veronii biovar sobria* challenged. *Pangasianodon hypophthalmus* was treated with lead (Pb, 4 ppm), and high temperature (34 °C) for 60 days. The growth performance was reduced with declined in feed intake, growth rate and feed efficiency in case of group exposed with Pb alone and concurrent exposure to Pb high temperature (34 °C). The Se has immunomodulatory properties however, supplementation of the dietary Se @ 1 and 2 mg/kg diet has been realistically improved growth performance up to 240%, elevated antioxidative status in different tissues, and immunological status were also improved significantly in the *P. hypophthalmus*. The bacterial challenged with *A. veronii biovar sobria* in the *P. hypophthalmus* resulting in less cumulative mortality (%) and high relative (%) survival has been observed with supplementation of dietary Se @ 1 and 2 mg/kg diet. The bioaccumulation of Pb in muscle tissue has been also drastically reduced with supplementation of dietary Se in feed. Hence, overall results indicated that, dietary Se @ 1 and 2 mg/kg have ability to enhanced overall performance and alleviated multiple stresses in *P hypophthalmus*.

1. Introduction

Selenium (Se) is the most important nutritional components for all living organism including human and animal [1]. It has three important biological functions such as (a) Se essential for normal growth and development with trace level (b) nutritional and supra-nutritional levels can be stored in tissue and helps in body homeostatic maintenance (c) higher level can creates toxicity and resultant in deleterious effects [2]. It is very sharp and thin line between safe and toxic level of Se hence it is important question to be addressed when consider the role of selenium in dietary supplements [3]. Therefore, it is an important that toxicological hazard, due to the very narrow range between its essentiality and toxicity [4]. Se have very important biological role such as functional component for selenoenzymes as glutathione peroxidase, thioredoxin reductase and also incorporate into protein as selenocysteine and prevents oxidative damage to body tissues [5]. It is also an important role in regulation of thyroid hormone metabolism, cell growth and antioxidant defence systems and prevents cells against oxidative stress damage [6]. Se is necessary for growth, fertility, and immune system in animals and humans [7]. The living organism needs limit oxygen requirement but in case of higher oxygen concentration generates reactive oxygen species (ROS) which can cause oxidative stress [8–10]. The oxidative stress is resultant of either ROS overproduction or less antioxidant defense system whereas, oxygen is necessary for oxidative metabolism. Generally, antioxidative defense mechanisms are divided into enzymatic and non-enzymatic systems. The enzymatic systems covered ROS detoxification through enzymatic cascades system leading to complete detoxification by reacting directly with ROS or acting as redox regulators such as catalase helps in the detoxification of hydrogen peroxide and also adaptation to endogenous oxidative stress and lipid peroxidation [11,12]. The other one non-enzymatic antioxidative systems which are not as specific as enzymatic, but nevertheless, they are in the first line of antioxidative defense and are therefore high importance in cellular response to oxidative stress. Such as to neutralize adverse effects of ROS, the living system uses antioxidative defense systems including various enzymes like catalase, superoxide dismutase, glutathione-S-transferase and peroxidase, etc. similarly, selenium is also an important antioxidant located at the catalytic site of thioredoxin reductase and glutathione peroxidase enzymes [13].

* Corresponding author. ICAR-National Institute of Abiotic Stress Management, Malegaon, Baramati, Pune, 413115, India.
E-mail address: neeraj_journal@live.in (N. Kumar).

https://doi.org/10.1016/j.fsi.2018.09.065
Received 6 August 2018; Received in revised form 20 September 2018; Accepted 23 September 2018
Available online 24 September 2018

The aquatic water bodies are totally unpredictable due to sudden change in temperature and contamination source, hence the aquatic animal especially fish are affected badly. Since, last two decade, the temperature has been exceptionally risen [14–16]. Moreover, it is one of the most important abiotic stress factor which known to modify the chemistry of a number of chemical pollutants resulting in significant alterations in their toxicities. Due to higher temperature, the increases in rate of uptake of inorganic and organic pollutants such as lead (Pb) through changes in ventilation rate in response to an increased metabolic rate and decrease in oxygen solubility [11,17]. The toxicity of heavy metal depends on the organisms tolerable limit and beyond which it becomes toxic. In the aquatic water bodies, rising temperature enhances the toxicity of chemical contaminant [11,18–20] to the fishes. The inorganic contamination such as lead (Pb) is belong to major heavy metal that lead to impart contamination of aquatic ecosystem owning to its occurrence in both physical and chemical forms, which adversely affects the health status of fish at concentration higher than normal [17]. Although Pb is very toxic to fishes at higher level, but lower level also creates muscular and neurological degeneration and destruction, growth inhibition, mortality, as well as reproductive problems [21].

The ability of fish to protect themselves through immunological system against several stresses such as abiotic and biotic stress (bacterial infection). During protection of the body the immune response itself produce countless oxidative compound in order to kill invasion agents [22], hence ROS compounds cannot distinguish self and non-self target molecules and immune defense may also act as a source of oxidative stress and resulting in mounting an immune response has energetic and physiological costs that can decrease growth performance and impact reproduction [23]. In this prospect fish may acquire antioxidant compounds but they cannot syntheses such as selenium, however, these nutrient provided through their food and environment [24,25]. To complete this gap, we investigated on dietary selenium to improve antioxidant and immunological system of pathogen challenged *Pangasianodon hypophthalmus* and to elucidate if selenium can counteract these responses elicited by exposure of *P. hypophthalmus* to abiotic and biotic stress.

2. Materials and methods

2.1. Ethics statement

The use of animals conforms to the existing laws in India. The care and treatment of animals used in this study were in accordance with the guidelines of the committee for the purpose of control and supervision of experiments on animals, [Ministry of Environment & Forests, Animal Welfare Division, Government of India]. The study protocol and experimental endpoints were approved by the institute research committee and the authorities of the ICAR-National Institute of Abiotic Stress Management, Baramati, Pune, India.

2.2. Fish and experimental design

Experimental fish (*Pangasianodon hypophthalmus*) was obtained from local market Nil Aquarium, Baramati, Pune, Maharashtra, India. Fish was transported in healthy condition at wet laboratory, ICAR-National Institute of Abiotic Stress Management, Baramati, Pune. The fishes were quarantine with salt solution and $KMnO_4$ and kept in FRP tank (Fibre Reinforced Plastic, Circular, 500 L) for a period of one month prior to the experiment. The fifteen fishes were randomly distributed into 21 rectangular plastic (125 L capacity) tanks in triplicate form followed by completely randomized design reared for 60 days. Aeration was provided with compressed air pump to all the experimental tank and manual water exchange (two third) was carried out at every second day. The experimental setup were designed as fed with control diet and treated with normal water (Ctr/L0), fed with control diet and treated with lead (Ctr/L), fed with control diet and concurrent exposed to lead and high temperature (34 °C) (Ctr/LT), fed with Se @ 1 mg/kg diet and reared under normal water (Se-1 mg/kg/L0), fed with Se @ 2 mg/kg diet and reared under normal water (Se-2 mg/kg/L0), concurrent exposed to Pb and temperature (34 °C) and fed with Se @ 1 mg/kg (LT-Se 1 mg/kg) and concurrent exposed to Pb and temperature (34 °C) and fed with Se @ 2 mg/kg (LT-Se 2 mg/kg) diet. The lead was treated to experimental water at the level of 1/21th of 96 h LC_{50} [17] using lead nitrate. The temperature was recorded in the range of 26.4–28.8 °C in normal and 34 ± 0.85 °C in temperature treated groups. The temperature was maintained with the help of thermostatic rod heater. Water quality parameters viz. dissolved oxygen and temperature, pH (digital pH meter), free carbon dioxide (titrimetric method), total hardness (carbonate hardness test kit, Merck, Germany), ammonia (at 635 nm by phenate method), nitrite and nitrate (543 nm wave length) were recorded as per APHA [26].

2.3. Feed preparation

The selenium supplemented diet was formulated with inclusion of 0, 1 and 2 mg/kg diet with iso-caloric and iso-nitrogenous form and sodium selenite was used as source of selenium in the diet. The feed ingredient such as pelleted diet, quality fish meal, soybean meal, groundnut meal, wheat flour, sunflower oil and cod liver oil were procured from local market. The selenium free vitamin mineral mixtures were prepared manually along with ascorbyl phosphate (SD Fine Ltd., Mumbai, India). The feed ingredients were mixed properly and made dough, pelleted, air dried and kept in hot air oven at 60 °C until dry (Table 1).

2.4. Tissue homogenate preparation and blood collection

At the end of the experiment, fish was anesthetized with clove oil at 50 μl L^{-1} and collected tissues such as gill, liver, brain and kidney under aseptic conditions. The tissues were homogenized (5% w/v) in chilled sucrose (0.25 M) and EDTA solution (1 mM) in tube using tissue homogenizer (Omni Tissue Master Homogenize, Kennesaw, GA). During homogenization, the tubes were kept on ice to avoid denaturation of the enzymes from the sample. The homogenates sample were centrifuge at 5000 rpm for 20 min at 4 °C in a cooling centrifuge (Eppendorf AG, 5430R, Hamburg, Germany). The enzymes supernatants were collected after centrifugation and stored at −20 °C until further analysis. The blood was collected from same fish which has been selected for tissue collection from each treatment in replicate and

Table 1
Ingredients composition of the different experimental diets fed to *Pangasianodon hypophthalmus* during the experimental period of 60 days.

	Control	Selenium-1 mg	Selenium-2 mg
Soybean meal[a]	35.5	35.5	35.5
Fish meal[a]	20	20	20
Groundnut meal[a]	10	10	10
Wheat flour[a]	26.470	26.469	26.468
Sunflower oil[a]	4.5	4.5	4.5
Cod liver oil[a]	1.5	1.5	1.5
Vitamin + mineral mix[b]*	2	2	2
Vitamin C[c]	0.03	0.03	0.03
Selenium	0	0.001	0.002
	100	100	100

Composition of vitamin mineral mix (quantity/250 g starch powder): vitamin A 55,00,00 IU; vitamin D3 11,00,00 IU; vitamin B1:20 mg, vitamin B2 2,00 mg; vitamin E 75 mg; vitamin K 1,00 mg; vitamin B12 0.6 mcg; calcium pantothenate 2,50 mg; nicotinamide 1000 mg; pyridoxine: 100 mg; Mn 2700 mg; I 1,00 mg; Fe 750 mg; Cu 200 mg; Co 45 mg; Ca 50 g; P 30 g.

[a] Procured from local market.

[b] Prepared manually and all components from Himedia Ltd.

[c] SD Fine Chemicals Ltd., India.

another two fish were used for serum collection. The Lowry method [27] was used for protein analysis from the tissues.

2.5. Sample preparation for Se and Pb analysis

The tissues samples (0.5 g) were collected during end of the experimental period for analysis of Se and Pb followed by acidic digestion in microwave digestion system (Microwave Digestion System, Model START-D, SN- 135177, Milestone, USA). The acidic composition was used in the form of HNO_3 and H_2O_2 in 5:1 for digestion. The completely digested samples were allowed to cool to room temperature, then, digested samples were filtered with Whatman paper (pore size-0.45 μm) and finally volume made up to 50 ml to proceed further for Se and Pb analysis through Inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7700 series, Agilent Technologies, USA) [9,17,28]. Selenium concentrations in feed and muscle tissue and Pb concentrations in water and muscle tissue were measured through ICP-MS. Multi-element Calibration Standard (Agilent Technologies, USA) solutions of 10 μg/ml was used to prepare calibration curve. The calibration curves with $R^2 > 0.999$ were accepted for concentration calculation.

2.6. Growth performance study

Fishes were weighed at the start and every 15 day interval thereafter till 60th day. At the end of the experiment, fish were weighed individually. The growth performance of fish was evaluated in terms of weight gain (%), feed conversion ratio (FCR), protein efficiency ratio (PER) and specific growth rate (SGR).

Weight gain (%) = Final body weight (FBW)-Initial body weight (IBW)/Initial body weight (IBW) × 100
FCR = Total dry feed intake (g)/wet weight gain (g)
SGR = 100 (ln FBW-ln IBW)/number of days
PER = Total wet weight gain (g)/crude protein intake (g)

2.7. Antioxidant enzyme activities

The enzyme activity of superoxide dismutase (SOD) (EC 1.15.1.1) was measured at 480 nm for inhibition of amount of enzymes needed to inhibit reaction by 50% [29]. The enzymatic activity of catalase (EC 1.15.1.1) was based on decomposition of H_2O_2 at 240 nm [30]. The activity of gluthathione-S-transferase (GST) (EC 2.5.1.18) was measured at 412 nm, following the initiate the reaction between glutathione (GSH) and 1-chloro-2,4-dinitrobenzene to form thioether glutathione dinitrobenzene [31].

2.8. Neurotransmitter enzyme activities

Acetyl cholinesterase (AChE; EC 3.1.1.7) was measured by the method of Hestrin et al. [32]. The activity was spectrophotometrically measured as the increase in absorbance of the sample at 540 nm. Acetylcholine chloride and dithiobisnitrobenzoic acid were used as substrate.

2.9. Cortisol and HSP-70

Cortisol was quantified in the serum of fish from the different experimental groups through ELISA. The quantification was done using commercially available Cortisol EIA kit (Catalog no. 500360), procured from Cayman Chemicals, USA. The assay was performed according to the protocol provided along with the kit. The absorbance was read in the ELISA plate reader (Biotek India Pvt. Ltd.). The expression of HSP-70 (EIA kit, catalog no. EKS-700B) in gill and liver was determined as per the manufacturer's instructions (Bioguenix/Enzo Life Science, Mumbai, India). The absorbance was read in the ELISA plate reader (Biotek India Pvt. Ltd.).

2.10. Respiratory burst activity, serum protein and A:G ratio

The respiratory burst activity assay was done by the method of Secombes [33] as modified by Stasiack and Baumann [34]. Total plasma protein was quantified colorimetrically by BCA method using Genei protein estimation kit (Cat.No. 105569). Albumin was quantified using bromocresol green binding method by Doumas et al., [35]. Globulin was quantified by subtracting albumin values from total plasma protein. Albumin/globulin ratio (A/G ratio) was determined through dividing albumin values by globulin values.

2.11. Myeloperoxidase content and total immunoglobulin level

Total myeloperoxidase was measured according to Quade and Roth [36] with some modifications [37] and total immunoglobulin level was determined as per modified method of Anderson and Siwicki [38].

2.12. Blood glucose

Blood glucose level was estimated by the method of Nelson [39] and Somoyogi [40]. Blood was de-proteinized with zinc sulphate and barium hydroxide, filtered and the supernatant is used for glucose estimation. The absorbance was recorded at 540 nm against the blank.

2.13. Challenge study with Aeromonas veronii biovar sobria

After 60 days of feeding trial 7 fishes per group were challenged with virulent *A. veronii biovar sobria* obtained from Aquatic Animal Health Management Division, Central Institute of Fisheries Education, Mumbai. Initially the pathogenic isolates of *A. veronii biovar sobria* were grown on a nutrient broth for 24 h at 37 °C in a BOD incubator and harvested by centrifuging the culture broth at 10,000 rpm for 10 min at 4 °C. The cells were then washed thrice in sterile PBS (pH 7.2) and final concentration was maintained at 10^8 CFU ml^1. The fish in each experimental group were intraperitoneally injected with 0.15 ml of bacterial suspension. Mortality was observed for a week. Tissues were taken from the dead fish for bacteriological culture to confirm *A. veronii biovar sobria* as the cause of death. The cumulative mortality (%) and relative percent survival (RPS) in different treatment groups were calculated by the following formula:

$$\text{Cumulative mortality (\%)} = \frac{\text{Total mortality in each treatment after challenge} \times 100}{\text{Total no. of fish challenged for the same treatments}}$$

$$\text{Relative \% survival} = \frac{\text{Mortality (\%) Control} - \text{Mortality (\%) Treatment} \times 100}{\text{Mortality (\%) Control}}$$

2.14. Statistics

The data were analyzed by Statistical Package for the Social Sciences (SPSS) version 16 (SPSS, Chicago, IL). The one way ANOVA (Analysis of variance) was used to see the treatment effect. Duncan's multiple range tests were used to see the significant difference between the means if any, and the comparisons were made at the 5% probability level.

3. Results

3.1. Growth performance

The growth performance (weight gain %, FCR, PER and SGR) of *P. hypophthalmus* concurrently exposed to lead (Pb) and high temperature (34 °C) and fed with dietary selenium for 60 days are presented in

Table 2
Effect of dietary Se on weight gain%, FCR PER and SGR of *Pangasianodon hypophthalmus* exposed to Pb and high temperature for 60 days.

Treatments	Weight gain (%)	FCR	PER	SGR
Ctr/Ctr	76.07b ± 4.18	2.60b ± 0.11	1.11b ± 0.06	0.94b ± 0.04
Ctr/Pb	41.14a ± 4.03	4.44c ± 0.42	0.66a ± 0.03	0.57a ± 0.05
Ctr/Pb-T	36.27a ± 2.40	4.92c ± 0.25	0.58a ± 0.02	0.52a ± 0.03
Se-1	229.26c ± 14.41	1.27a ± 0.07	2.26c ± 0.13	1.98c ± 0.12
Se-2	216.93c ± 6.98	1.34a ± 0.03	2.13c ± 0.09	1.92c ± 0.04
Se-1/Pb-T	217.53c ± 11.23	1.30a ± 0.06	2.23c ± 0.16	1.92c ± 0.12
Se-2/Pb-T	240.10c ± 14.54	1.25a ± 0.05	2.28c ± 0.08	2.04c ± 0.15
P-Value	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$

Values in the same column with different superscript (a, b, c, d) differ significantly ($p < 0.01$). Data expressed as Mean ± SE (n = 3).

Table 2. The weight gain %, PER and SGR were noticeably ($p < 0.01$) higher with Se @ 1 and 2 mg/kg diet group with or without exposed to Pb and high temperature (34 °C) in compared to control and other groups. The overall 215% better growth performance has been observed in Se fed group in compared to control group. Similarly, FCR was significantly ($p < 0.01$) lowered with Se supplementation in compared to control and exposure group. The growth performance of *P. hypophthalmus* drastically affected with concurrent exposure to Pb and high temperature (34 °C) fed with control diet. In case of Se @ 1 and 2 mg/kg diet were significantly ($p < 0.01$) similar growth performance observed in *P. hypophthalmus* after 60 days feeding trial.

3.2. Antioxidative status

The oxidative stress such as catalase, superoxide dismutase (SOD) and glutathione-S-transferase (GST) in the liver, gill and kidney of *P. hypophthalmus* exposed to lead (Pb) and high temperature (34 °C) and fed with Se for 60 days are presented in Tables 3 and 4. The catalase, SOD and GST in the liver, gill as well as kidney were remarkably ($p < 0.01$) higher in Pb and high temperature exposure group fed with control diet. The supplementation of Se @ 1 and 2 mg/kg diet with or without stressors were noticeably ($p < 0.01$) reduced the catalase, SOD and GST in the liver, gill as well as kidney of *P. hypophthalmus*. The overall results of oxidative stress indicate that the use of dietary Se reduced the oxidative stress in exposure to Pb and high temperature.

3.3. Neurotransmitter status

The neurotransmitter status in terms of acetylcholine esterase (AChE) in brain of *P. hypophthalmus* is presented in Fig. 1. The brain AChE activities were remarkably ($p < 0.01$) inhibited in the Pb exposed alone and concurrent exposed to Pb and high temperature and fed with control diet. The remarkably ($p < 0.01$) improved the brain AChE activities with supplementation of dietary Se @ 1 and 2 mg/kg diet without stressors group (Pb and high temperature) in compared to control and exposure group and Se @ 1 and 2 mg/kg diet with stressors group were significantly ($p < 0.01$) higher than all other groups.

3.4. Stress biomarkers

The stress biomarkers including blood glucose, cortisol, HSP 70 in gill and liver as well as vitamin C in muscle and brain of *P. hypophthalmus* exposed to lead (Pb) and high temperature (34 °C) and fed with Se diet for 60 days are presented in Table 4 and Fig. 2. The blood glucose and cortisol were remarkably ($p < 0.01$) higher in the exposure group of Pb and high temperature and fed with control diet. The level of blood glucose and cortisol were noticeably improved ($p < 0.01$) with supplementation of dietary Se @ 1 and 2 mg/kg diet with or without stressors group in compared to all others group. The HSP 70 in gill and liver were significantly elevated ($p < 0.01$) with exposure to Pb and high temperature and fed with the control diet, but the level of HSP 70 were significantly improved ($p < 0.01$) with supplementation of dietary Se @ 1 and 2 mg/kg diet with or without stressors group. Similarly, vitamin C in muscle and brain were remarkably ($p < 0.01$) improved with Se @ 1 and 2 mg/kg diet.

3.5. Immunological status

Immunological status such as nitroblue tetrazolium (NBT), serum total protein, albumin, globulin, A:G ratio, total immunoglobulin and myeloperoxidase activity of *P. hypophthalmus* exposed to lead (Pb) and high temperature (34 °C) and fed with Se for 60 days are presented in Table 5 and Fig. 3. The supplementation of dietary Se @ 1 and 2 mg/kg diet were remarkably ($p < 0.01$) higher NBT value, total protein, albumin, globulin, total immunoglobulin and myeloperoxidase of *P. hypophthalmus* exposed and or unexposed to Pb and high temperature in compared to all other groups. The level of all immunological status has been drastically ($p < 0.01$) affected with exposure to Pb and high temperature. Similarly, A:G ratio was noticeably ($p < 0.01$) reduced with supplementation of dietary Se @ 1 and 2 mg/kg diet with or without exposure to Pb and high temperature.

3.6. Selenium and Pb concentration in the fish muscle and experimental water

The selenium and Pb concentration in the fish muscle at the end of the experiment and experimental water at the interval of 20th, 40th and 60th days are presented in Figs. 4–6. The Se concentration in fish muscle was followed as 0.24, 0.24, 0.19, 0.32, 0.54, 0.49 and 0.64 mg/kg in group such as control, Pb exposed group, concurrent exposure to Pb and high temperature group, Se @1 mg/kg, and Se @2 mg/kg diet with or without exposure to Pb and high temperature respectively. Similarly bioaccumulation of Pb in fish muscle follows as control, Pb

Table 3
Effect of dietary Se on catalase and superoxide dismutase in liver, gill and kidney of *Pangasianodon hypophthalmus* exposed to lead and temperature (34 °C) toxicity for 60 days.

Treatments	Catalase (CAT)			Superoxide dismutase (SOD)		
	Liver	Gill	Kidney	Liver	Gill	Kidney
Ctr/Ctr	11.76b ± 1.14	14.69b ± 1.19	15.08c ± 0.69	58.15b ± 0.57	34.19b ± 0.53	49.38b ± 1.03
Ctr/Pb	22.46c ± 1.12	24.03c ± 0.58	28.48d ± 0.59	63.24c ± 1.11	39.27c ± 0.94	60.12c ± 1.06
Ctr/Pb-T	26.98d ± 1.26	32.39d ± 1.01	31.68e ± 0.77	65.89d ± 0.81	41.43c ± 1.45	59.65c ± 0.99
Se-1	8.49a ± 0.80	9.27a ± 0.37	7.04a ± 0.42	50.53a ± 0.76	28.42a ± 1.01	45.34a ± 1.29
Se-2	8.55a ± 0.37	9.95a ± 0.98	8.02 ab ± 0.35	49.24a ± 1.04	28.36a ± 1.28	43.17a ± 1.08
Se-1/Pb-T	6.96a ± 0.63	9.76a ± 0.51	8.83b ± 0.55	48.14a ± 0.58	27.14a ± 0.68	45.65a ± 0.75
Se-2/Pb-T	8.36a ± 0.48	9.49a ± 0.49	8.94b ± 0.50	49.36a ± 0.81	27.04a ± 0.56	49.15a ± 1.28
P-Value	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$

Values in the same column with different superscript (a, b, c, d) differ significantly ($p < 0.01$). Data expressed as Mean ± SE (n = 6). Catalase and SOD: Units/mg protein.

Table 4
Effect of dietary Se on Glutathione-S-transferase (GST) in liver, gill and kidney, blood glucose, cortisol and HSP 70 in gill and liver of *Pangasianodon hypophthalmus* exposed to lead and temperature (34 °C) toxicity for 60 days.

Treatments	Glutathione-S-transferase (GST)			Blood Glucose	Cortisol	HSP	
	Liver	Gill	Kidney			Gill	Liver
Ctr/Ctr	1.19b ± 0.12	1.28b ± 0.09	1.11b ± 0.04	69.72b ± 0.92	49.43b ± 0.68	21.07b ± 1.49	21.50b ± 1.47
Ctr/Pb	1.92c ± 0.07	2.15c ± 0.05	1.80c ± 0.05	88.75c ± 4.23	65.63c ± 2.02	30.70c ± 1.01	27.90c ± 0.87
Ctr/Pb-T	2.34d ± 0.06	3.04d ± 0.13	2.52d ± 0.04	98.53d ± 2.28	78.03d ± 1.14	37.43d ± 2.08	33.20d ± 0.60
Se-1	0.56a ± 0.04	0.59a ± 0.02	0.60a ± 0.03	56.06a ± 0.94	36.80a ± 0.15	14.10a ± 0.31	14.40a ± 0.42
Se-2	0.57a ± 0.05	0.51a ± 0.03	0.58a ± 0.03	56.24a ± 0.47	36.47a ± 0.28	13.73a ± 0.20	13.92a ± 0.26
Se-1/Pb-T	0.57a ± 0.02	0.60a ± 0.04	0.57a ± 0.02	57.30a ± 0.94	36.83a ± 0.41	13.93a ± 0.23	13.90a ± 0.21
Se-2/Pb-T	0.56a ± 0.03	0.57a ± 0.03	0.56a ± 0.01	57.83a ± 1.80	37.60a ± 0.55	13.77a ± 0.19	13.97a ± 0.15
P-value	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$

Values in the same column with different superscript (a, b, c, d) differ significantly ($p < 0.01$). Data expressed as Mean ± SE (n = 6) Glutathion-S-transferase (GST): Units/mg protein, Blood glucose: mg/dl, Cortisol and HSP (Heat shock protein): ng/ml.

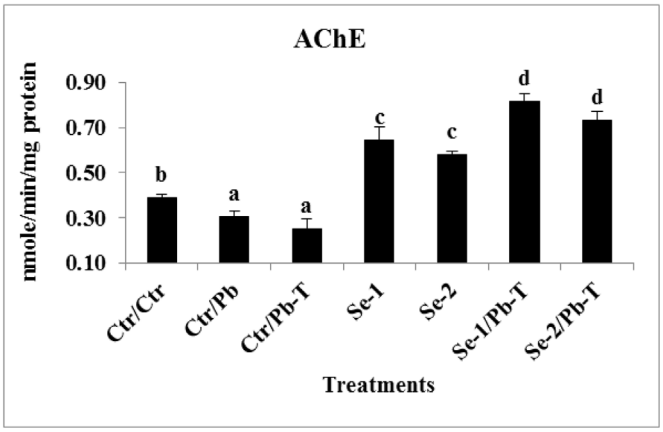

Fig. 1. Effect of dietary Se on brain AChE of *Pangasianodon hypophthalmus* during 60 days feeding trial.
Values in the figure with different superscript (a, b, c, d) differ significantly ($p < 0.01$). Data expressed as Mean ± SE (n = 6).

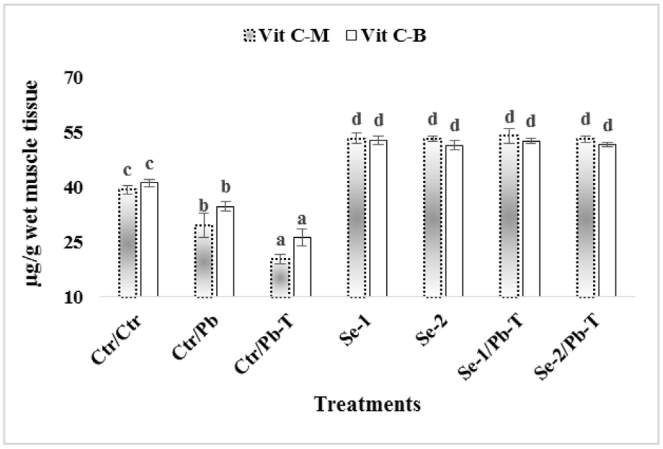

Fig. 2. Effect of dietary Se on vitamin C in muscle and brain of *Pangasianodon hypophthalmus* during 60 days feeding trial.
Values in the figure with different superscript (a, b, c, d) differ significantly ($p < 0.01$). Data expressed as Mean ± SE (n = 6).

exposed group, concurrent exposure to Pb and high temperature group, Se-1 mg/kg, and Se-2 mg/kg diet with or without exposure to Pb and high temperature as 0.61, 4.93, 7.56, 0.48, 0.55, 1.11 and 1.02 mg/kg diet group respectively.

Se concentration in experimental water during 20th, 40th and 60th days in experimental group such as control, Pb exposed group, concurrent exposure to Pb and high temperature group, Se-1 mg/kg, and Se-2 mg/kg diet with or without exposure to Pb and high temperature as 0.69, 0.72, 0.51, 0.68, 0.48, 0.84 and 0.48 µg/L, 0.57, 0.27, 0.35, 0.49, 0.96, 0.56 and 0.67 µg/L and 0.63, 0.34, 0.38, 1.86, 10.60, 0.84 and 1.07 µg/L respectively were found. Similarly, the Pb concentration in experimental water in treated group such as control, Pb exposed group, concurrent exposure to Pb and high temperature group, Se-1 mg/kg, and Se-2 mg/kg diet with or without exposure to Pb and high temperature as 7.58, 51, 75, 4.62, 2.68, 39.43 and 40.15 µg/L at 20 days, 3.10, 49.56, 69.14, 14.56, 18.88, 30.58 µg/L and 65.91 at 40 days and 1.44, 65.56, 87.56, 2.63, 2.03, 10.76 and 15.28 µg/L at 60 days respectively were observed.

3.7. Selenium concentration in feed

Selenium concentration in the experimental feed was found to be highest in Se @ 2 mg/kg diet (1.78 mg) followed by Se @ 1 mg/kg diet (1.23 mg) and the control diet (0.34 mg) (Fig. 4).

3.8. Bacterial infection in P. hypophthalmus

P. hypophthalmus was fed with Se @ 1 and 2 mg/kg diet with or without exposure to Pb and high temperature for 60 days and after that bacterial infection (*Aeromonas veronii biovar sobria*) were given and cumulative mortality (%) as well as relative survival (%) (RPS) were observed (Fig. 7). The cumulative mortality (%) was highest in Pb and high temperature exposure group and fed with control diet (79%) followed by Pb exposed alone (54%) and the minimum cumulative mortality (%) were observed in the Se @ 1 mg/kg diet (29%) followed by Se @ 2 mg/kg diet. Similarly the RPS was highest in Se @ 1 mg/kg (30%) followed by Se @ 2 mg/kg diet and least RPS were observed in the group exposed to Pb and high temperature and fed with control diet.

4. Discussion

The aquatic water bodies are dynamic in nature, including interference of abiotic and biotic factor such as temperature and chemical contaminant. These factors effect on overall growth and reproductive performance of the fish. The nutritional approach is the suitable option to minimize such kinds of abiotic and biotic factors. In this line, Se is the one of the best nutritional components for alleviating such kinds of stress. It is an essential nutrient for human and animal including fish for playing an important role in biological activities in trace levels for normal growth and development, required for homeostatic functions, but higher concentration can be resultant in deleterious effects [2]. In the present study, we have used two levels of dietary Se @ 1 and 2 mg/kg diet. There are very thin line of safe level and toxic level for Se but it is essential nutrient for animal feed. Dietary Se @ 1 and 2 mg/kg diet enhanced growth performance (weight gain %, FCR, PER and SGR) in

Table 5
Effect of dietary Se on Immunological status (NBT, total protein, albumin, globulin, A:G ratio and total immunoglobulin) of *P. hypophthalmus* exposed to lead and temperature (34 °C) toxicity for 60 days.

Treatments	NBT	Total Protein	Albumin	Globulin	A:G	Total Immunoglobulin
Ctr/Ctr	0.49b ± 0.04	0.77c ± 0.05	0.29b ± 0.01	0.48b ± 0.04	0.62b ± 0.03	0.52b ± 0.03
Ctr/Pb	0.33a ± 0.01	0.60b ± 0.03	0.22a ± 0.01	0.38 ab ± 0.03	0.60b ± 0.05	0.19a ± 0.02
Ctr/Pb-T	0.30a ± 0.01	0.45a ± 0.05	0.17a ± 0.02	0.28a ± 0.02	0.63b ± 0.01	0.10a ± 0.03
Se-1	0.75c ± 0.02	1.17d ± 0.04	0.40c ± 0.01	0.77c ± 0.05	0.52a ± 0.02	0.80c ± 0.04
Se-2	0.73c ± 0.03	1.24d ± 0.04	0.40c ± 0.02	0.84c ± 0.04	0.48a ± 0.04	0.95d ± 0.05
Se-1/Pb-T	0.74c ± 0.05	1.16d ± 0.03	0.40c ± 0.01	0.77c ± 0.05	0.52a ± 0.02	0.95d ± 0.06
Se-2/Pb-T	0.73c ± 0.03	1.28d ± 0.11	0.42c ± 0.02	0.86c ± 0.03	0.49a ± 0.04	1.06d ± 0.04
P-Value	p < 0.01	p < 0.01	p < 0.01	p < 0.01	p < 0.01	p < 0.01

Values in the same column with different superscript (a, b, c, d, e) differ significantly (p < 0.01). Data expressed as Mean ± SE (n = 6).

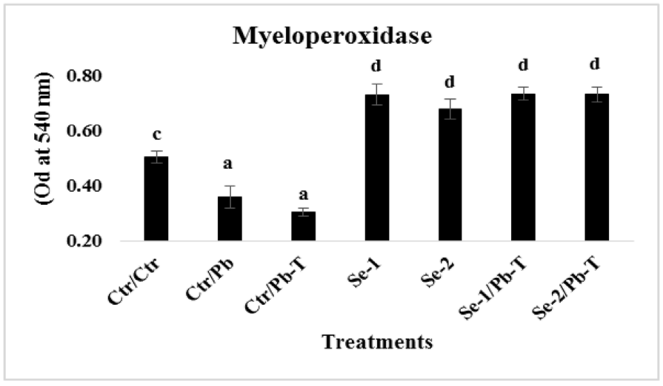

Fig. 3. Effect of dietary Se on myeloperoxidase of *Pangasianodon hypophthalmus* during 60 days feeding trial.
Values in the figure with different superscript (a, b, c, d) differ significantly (p < 0.01). Data expressed as Mean ± SE (n = 6).

Pb and high temperature exposure condition. There are several other research works which is supported our finding [41–43], that use of Se in diet responsible for enhancement of growth in fish but it is in a safe environment (no any kinds of abiotic and biotic stress). The role of Se in biological function such as enzymatic oxidation-reduction, nucleic acid metabolism and in promoting the activity of easily oxidized substances as carotenoids and vitamin A and also may increase protein and water in the cells might be reason for enhancing growth performance in the fish in Pb and high temperature (34 °C) exposure condition. Based on other research work through world wide, it is suggested that due to use of nutritional supplements enhanced growth performance in fish reared under abiotic stress condition [8,44–46].

The oxidative stress arises when the level of reactive oxygen species (ROS) elevated inside the body due to normal and or low amount of antioxidants present in the body system [9,11]. The ROS is the resultant of aerobic metabolism and resulting in elevated level of lipid peroxidation of the membrane and may damage proteins, enzymes, carbohydrates, and DNA. In addition, ROS is the important for energy

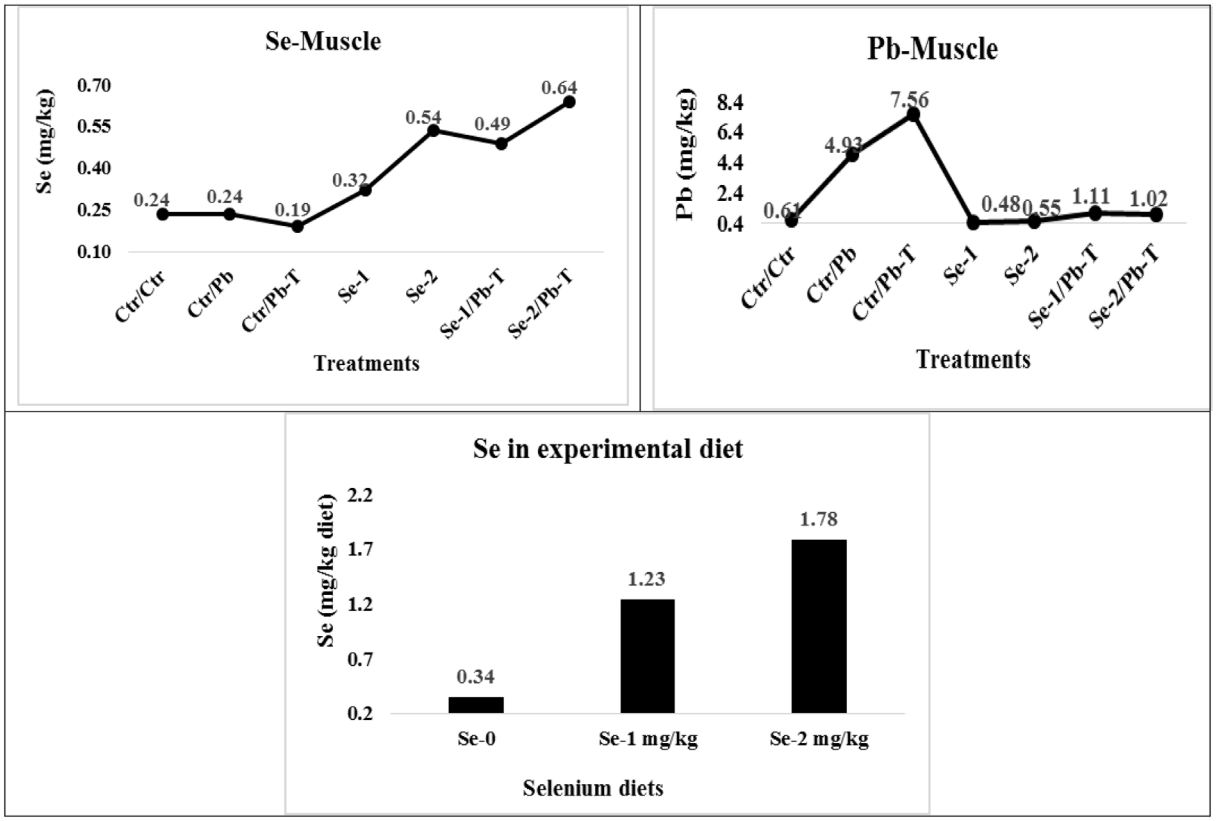

Fig. 4. Se concentration in diet and muscle as well as Pb in fish muscle on post 60 days experimental period.
Data expressed as Mean ± SE (n = 3).

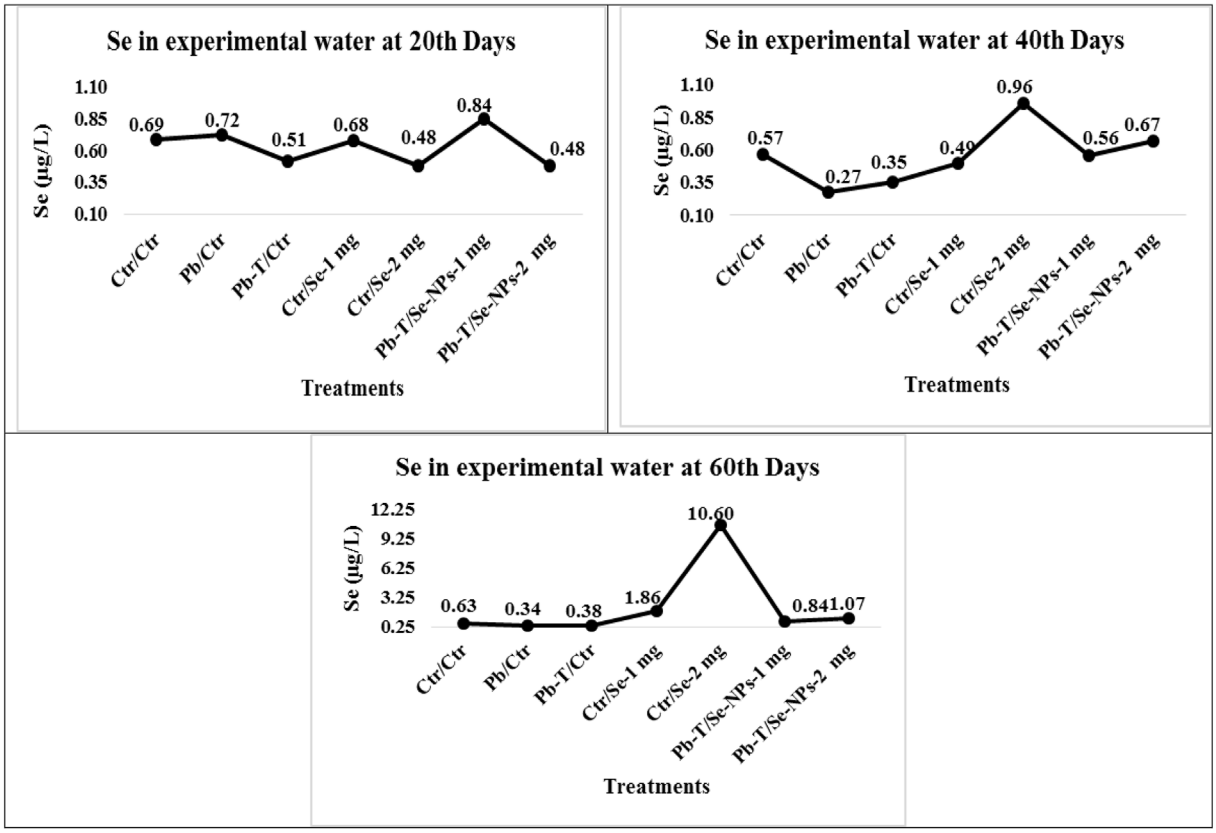

Fig. 5. Bioaccumulation of the Se in experimental water on 20th, 40th, and 60th days duration.
Data expressed as Mean ± SE (n = 3).

production, phagocytosis, cell growth regulation, intercellular signaling, and synthesis of important biological substances [47]. In the present study, Pb and high temperature treated group showed elevated levels of catalase, SOD and GST in the liver, gill and kidney, however, it may be due to the generation of uncontrolled free radicals in the fish tissue. Our previous finding also found similar results, when fishes exposed to Pb and high temperature/endosulfan [8,48,49]. Dietary supplementation of Se @ 1 and 2 mg/kg diet fed and exposed to Pb and high temperature to *P. hypophthalmus* was remarkable reduced the oxidative stress (catalase, SOD and GST) in the liver, gill and kidney tissue; might be due to utilization of free radical defense systems against oxidative stress [50]. It is also well understood that Se has antioxidant effect due to selenoproteins like selenocysteine. it is active components of glutathione peroxidase and supports in neutralization of adverse effects of reactive oxygen species with the help of catalase, superoxide dismutase and glutathione-S-transferase [13].

The Se is essential for function of central nervous systems (CNS), in which most of the selenoproteins shown to be expressed in the brain [51] in particularly, cortical and hippocampal neurons [52]. The Se have essential role in maintaining brain motor performance, coordination, memory, cognition and diverse functions of the CNS. Exposure to contaminant resulting in inhibited neurotransmitter enzymes such as acetylcholine esterase (AChE), similarly, the AChE activities has been inhibited in the present study due to Pb and high temperature exposure. It might be due to greater availability of synaptic AChE, which is responsible for the noted decrease the defecation cycle time. We confirmed that Se interfere with cholinergic system, in which neurotransmitter (acetylcholine esterase) has been found elevated in the Se @ 1 and 2 mg/kg diet with or without stressors group. In our earlier study use of nutritional component enhanced AChE activities in brain of fishes exposed to abiotic and biotic factors [11,53].

The stress biomarkers such as blood glucose, cortisol, HSP 70 in gill and liver as well as vitamin C in muscle and brain are very sensitive which was remarkable affected with exposure to Pb and high temperature [11,54,55]. It might be due to denatured proteins resulting from the accumulation of toxic ROS induce heat shock protein expression [56]. The cortisol and blood glucose are highly interdependent as cortisol increase the glucose production and also increase viz. gluconeogenesis and glycogenolysis [57]. In the present study, the elevated level of glucose and cortisol levels might be due to excess energy needed in metabolic defense during stress condition. Further, blood glucose levels are related with HSP 70 expression, as it protect from regulation of blood glucose [58]. Supplementation of dietary Se @ 1 and 2 mg/kg diet reduced the level of stress biomarkers, it might be due to role of Se in regulation and control of the body's antioxidant glutathione and glutathione peroxidase system, which plays a major role in the control of ROS [59]. It is also an important component of metalloenzyme cofactor and allosteric components that function in electron transfer. In the present study, the vitamin C has been remarkably depleted with exposure to Pb and high temperature but supplementation with dietary Se @ 1 and 2 mg/kg diet improved vitamin C in muscle and brain. It may be due to vitamin C and Se has complementary role in several enzymatic process such as thioredoxin reductase can regenerate L-ascorbic acid from dehydroascorbic acid [60] and the ascorbyl free radical [61]. The vitamin C is important stress biomarkers important for collagen synthesis that is useful for metabolism of steroids detoxification of xenobiotics and also plays a crucial role in protections of the cell against oxidative injury [46]. This indicates that Se supplementation helps to conserve ascorbate content of tissues during stress to some extent.

The immunological status (NBT, total protein, albumin, globulin, A:G ratio, total immunoglobulin and myeloperoxidase) of *P. hypophthalmus* has been drastically reduced with exposure to Pb and high temperature [11] but supplementation with dietary Se @ 1 and 2 mg/

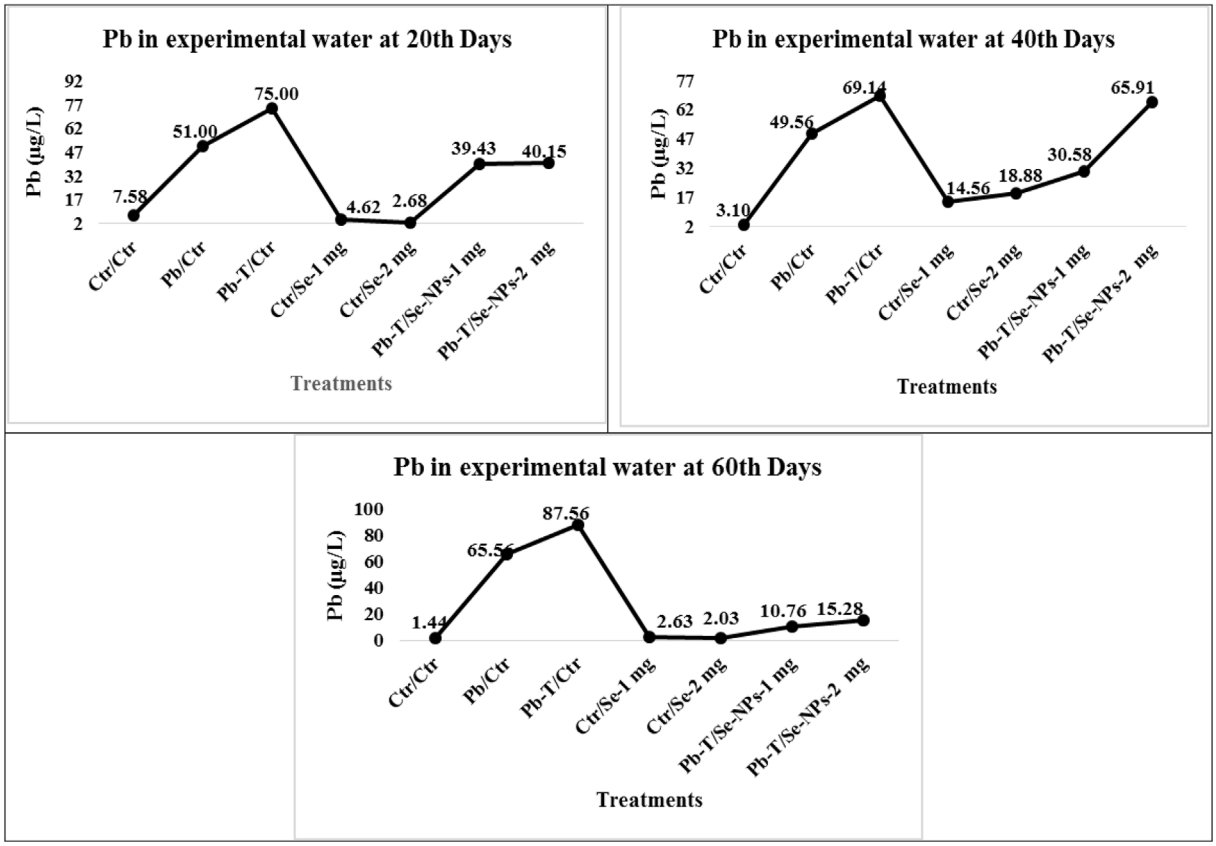

Fig. 6. Bioaccumulation of the Pb in experimental water on 20th, 40th, and 60th days duration.
Data expressed as Mean ± SE (n = 3).

kg diet enhanced immunological status [62]. It might be due to role of Se in the integral parts of selenomethionine, and methyl selenocysteine. It is very much needed for up-regulating selenoenzymes in fish with higher bioavailability and lower toxicity [63]. Several studies have been conducted on nutritional supplementation which led to improvement in immunological parameters (NBT, total protein, A: G ratio and blood glucose) such as yeast [64–66] and probiotics and vitamin C [67]. Generally, the fish innate immune system is the key factor for maintaining integrity based on phagocytosis and secretion of soluble antimicrobial molecules and is characterized by non-specific immunity. The NBT value measured in terms of respiratory burst activity of phagocytes produced by intracellular superoxide radicals. The total protein in blood component is indicators of good health status of the fish and higher serum protein, globulin and lowered albumin globulin ratio are strong indicators of better immunity [68]. Similarly, the natural antibodies (immunoglobulin) has wide function in defence-related activities such as limiting the dispersal of infectious agents, killing of microbes, and other potential pathogens, repair of tissue damage, and restoration of the healthy state. In the present study, the higher immunoglobulin was observed in the supplemented group of dietary Se @ 1 and 2 mg/kg diet. The myeloperoxidase is also immune parameter and indicators for strong immunity; it is haemoprotein, utilizes hydrogen peroxide during respiratory burst to produce hypochlorous acid [69] which are potent oxidants known to have several cytotoxic effects on mammalian and bacterial cells [70]. The above finding clearly indicates the anti-stress and immuno-boosting abilities of dietary Se supplementation leading to mitigate the multiple stressors.

After 60 days of feeding trial the *P. hypophthalmus* has been infected with bacteria *A. veronii biovar sobria*. It has been observed that Supplementation of dietary Se @ 1 and 2 mg/kg diet has been reported

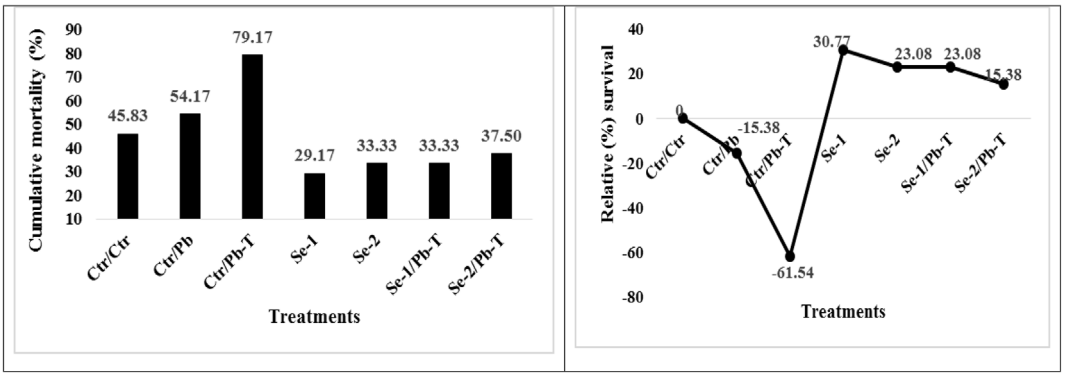

Fig. 7. Effect of dietary Se on Relative % Survival and Cumulative mortality of *Pangasianodon hypophthalmus* post 60 days feeding trial.
Data expressed as Mean ± SE (n = 3).

highest relative % survival and lowest cumulative mortality (%). The results showed that use of Se in the diet has enhanced immune system of the fish might be due to Se modulate immune function through regulation of redox-sensitive transcription factors [71]. In addition, Se acts as anti-oxidant and anti-inflammatory agent which has an important role in the reduction of hydrogen peroxide, lipid, phospholipid and hydroperoxides The pathogenic infection has been reduced with supplementation of dietary Se @ 1 and 2 mg/kg diet might be due to complement system (C3), which has the capacity to kill and reduced the bacteria by creating pores in their surface membranes. This property of C3 systems might be due to activation of C3 by microorganism and antibody-antigen (Ag-Ig) complexes formation [72,73].

In the present study, we have also determined the bioaccumulation of Se and Pb in experimental water at 20, 40 and 60th days. The higher concentration of Se was found in the Se fed treatments and Pb was highest in the concurrent exposure to Pb and high temperature group and fed with control diet. The highest muscle Se concentration was found in the Se @ 2 mg/kg diet and exposed to Pb and high temperature and highest Pb concentration was found in the exposure to Pb and high temperature and fed with control diet.

5. Conclusions

The heavy metal contamination with high temperature exposure in water bodies are really lethargic combination, in which its effect on fish growth performance, anti-oxidative status, innate immunity, other stress markers (Cortisol, HSP 70 and vitamin C) and pathogenic infection in fish. As per IPCC, the global temperature including aquatic ecosystems has rising up day by day and wide fluctuation in the temperature variation has been observed. In this condition, what approaches, we should plan for less impact of such abiotic and biotic stress on aquatic organism, fish. As a scientist, we planned nutritional strategies to mitigate such stress (abiotic and biotic stress). In this investigation, we used dietary selenium for enhancement of growth performance, biochemical attributes, anti-oxidative status, immunological status and reducing chances of pathogenic infection (*A. veronii biovar sobria*) to the *P. hypophthalmus*. The selenium has several crucial biological functions involved in the mitigation of such abiotic (Pb and high temperature) and biotic stress (pathogenic infection). Moreover, based on the present results concluded that dietary selenium @ 1 and 2 mg/kg diet is suitable for mitigation of abiotic and biotic stress. The same formulation can be used for feed formulation in the aquaculture industries for culture practices of *P. hypophthalmus*.

Conflicts of interest

None.

Acknowledgement

The authors express sincere gratitude to the Director, ICAR-National Institute of abiotic Stress Management, Baramati, Pune for providing all the facilities to conduct the present work. The financial assistance has been provided by Indian Council of Agricultural Research (ICAR), New Delhi, India as institutional project (#IXX09673) is highly acknowledged. I also place a record of thanks to, Mrs. Yogita, and Mr. Yuvraj Sanas for their technical assistance.

References

[1] P.F. Surai, Selenium in Nutrition and Health, Nottingham University Press, Nottingham, 2006, pp. 363–588.
[2] S.J. Hamilton, Review of selenium toxicity in the aquatic food chain, Sci. Total Environ. 326 (2004) (2004) 1–31.
[3] H. Li, J. Zhang, T. Wang, W. Luo, Q. Zhou, G. Jiang, Elemental selenium particles at nano-size (Nano-Se) are more toxic to Medaka (*Oryzias latipes*) as a consequence of hyper-accumulation of selenium: a comparison with sodium selenite, Aquat. Toxicol. 29 (89) (2008) 251–260 (4).
[4] A. Kouba, J. Velisek, A. Stara, J. Masojidek, P. Kozak, Supplementation with sodium selenite and selenium-enriched microalgae biomass show varying effects on blood enzymes activities, antioxidant response, and accumulationin common barbel (*Barbus barbus*), BioMed Res. Int. 2014 (2014) 408270.
[5] J.T. Rotruck, A.L. Pope, H.E. Ganther, Selenium: biochemical role as a component of glutathione peroxidase, Science 179 (1973) 585–590.
[6] S.G. Patching, P.H. Gardiner, Recent developments in selenium metabolism and chemical speciation: a review, J. Trace Elem. Med. Biol. 13 (4) (1999) 193–214.
[7] G.N. Schrauzer, P.F. Surai, Selenium in human and animal nutrition: resolved and unresolved issues. A partly historical treatise in commemoration of the fiftieth anniversary of the discovery of the biological essentiality of selenium, dedicated to the memory of Klaus Schwarz (1914-1978) on the occasion of the thirtieth anniversary of his death, Crit. Rev. Biotechnol. 29 (1) (2009) 2–9.
[8] N. Kumar, S. Gupta, N.K. Chandan, M. Aklakur, A.K. Pal, S.B. Jadhao, Lipotropes Protect against Pathogen-Aggravated Stress and Mortality in Low Dose Pesticide-Exposed Fish, PLoS One 9 (4) (2014) e93499.
[9] N. Kumar, K.K. Krishnani, K.K. Meena, S.K. Gupta, N.P. Singh, Oxidative and cellular metabolic stress of *Oreochromis mossambicus* as biomarkers indicators of trace element contaminants, Chemosphere 171 (2017) 265–274.
[10] S. Bhattacharjee, Reactive oxygen species and oxidative burst: roles in stress, senescence and signal transduction in plants, Curr. Sci. 89 (2005) 113–121.
[11] N. Kumar, K.K. Krishnani, P. Kumar, A.K. Jha, S.K. Gupta, N.P. Singh, Dietary zinc promotes immuno-biochemical plasticity and protects fish against multiple stresses, Fish Shellfish Immunol. 17 (62) (2017) 184–194.
[12] N. Kumar, K.K. Krishnani, S.K. Gupta, N.P. Singh, Cellular stress and histopathological tools used as biomarkers in *Oreochromis mossambicus* for assessing metal contamination, Environ. Toxicol. Pharmacol. 49 (2017) 137–147.
[13] M.A. Reeves, P.R. Hoffmann, The human selenoproteome: recent insights into functions and regulation, Cell. Mol. Life Sci. 66 (2009) 2457–2478.
[14] T.P. Barnett, D.W. Pierce, K.M. AchutaRao, P.L. Gleckler, B.D. Santer, J.M. Gregory, W.M. Washington, Penetration of human induced warming into the world's oceans, Science 309 (2005) 284–287.
[15] D. Schiedek, B. Sundelin, J.W. Readman, R.W. Macdonald, Interactions between climate change and contaminants, Mar. Pollut. Bull. 54 (12) (2007) 1845–1856.
[16] S.F.B. Tett, P.A. Stott, M.R. Allen, W.J. Ingram, J.F.B. Mitchell, Causes of twentieth-century temperature change near the Earth's surface, Nature 339 (1999) 569–572.
[17] N. Kumar, K.K. Krishnani, M.P. Brahmane, S.K. Gupta, P. Kumar, N.P. Singh, Temperature induces lead toxicity in Pangasinodon hypophthalmus: an acute test, antioxidative status and cellular metabolic stress, International J. Environ. Sci. Tech. (2017), https://doi.org/10.1007/s13762-017-1364-5.
[18] N. Kumar, K. Ambasankar, K.K. Krishnani, P. Kumar, M.S. Akhtar, S. Bhushan, P.S. Minhas, Dietary pyridoxine potentiates thermal tolerance, heat shock protein and protect against cellular stress of Milk fish (*Chanos chanos*) under endosulfan-induced stress, Fish Shellfish Immunol. 55 (2016) 407–414.
[19] N. Kumar, P.S. Minhas, K. Ambasankar, K.K. Krishnani, R.S. Rana, Dietary Lecithin Potentiates thermal tolerance and Cellular Stress protection of Milk fish (*Chanos chanos*) reared under low dose endosulfan induced stress, J. Therm. Biol. 46 (2014) 40–46.
[20] R.W. Patra, J.C. Chapman, R.P. Lim, P.C. Gehrke, The effects of three organic chemicals on the upper thermal Tolerances of four freshwater fishes, Environ. Toxicol. Chem. 26 (7) (2007) 1454–1459.
[21] I.S. Rabitto, J.R.A. Silva, H.C. de Assis, E.E. Pelletier, F.M. Akaishi, A. Anjos, M.A. Randi, C.A. Oliveira Ribeiro, Effects of dietary Pb(II) and tributyltin on neotropical fish, *Hoplias malabaricus*: histopathological and biochemical findings, Ecotoxicol. Environ. Saf. 60 (2005) 147–156.
[22] J.D. Biller-Takahashi, L.S. Takahashi, F.E. Mingatto, E.C. Urbinati, The immune system is limited by oxidative stress: dietary selenium promotes optimal anti-oxidative status and greatest immune defense in pacu *Piaractus mesopotamicus*, Fish Shellfish Immunol. 47 (1) (2015) 360–367.
[23] G. Sorci, B. Faivre, Inflammation and oxidative stress in vertebrate host parasite systems, Philos. Trans. R. Soc. 364 (2009) 71–83.
[24] M.E. Yonar, S.M. Yonar, S. Silici, Protective effect of propolis against oxidative stress and immunosuppression induced by oxytetracycline in rainbow trout (*Oncorhynchus mykiss*, W.), Fish Shellfish Immunol. 31 (2011) 318–325.
[25] J.Y. Ko, E.A. Kim, J.H. Lee, M.C. Kang, J.S. Lee, J.S. Kim, W.K. Jung, Y.J. Jeon, Protective effect of aquacultured flounder fish-derived peptide against oxidative stress in zebrafish, Fish Shellfish Immunol. 36 (2014) 320–323.
[26] APHA-AWWA-WEF, in: L.S. Clesceri, A.E. Greenberg, A.D. Eaton (Eds.), Standard Methods for the Estimation of Water and Waste Water, twentieth ed., American Public Health Association, American Water Works Association, Water Environment Federation, Washington DC, 1998.
[27] O.H. Lowry, N.J. Ronebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[28] N. Kumar, K.K. Krishnani, N.P. Singh, Oxidative and cellular stress as bioindicators for metals contamination in freshwater mollusk *Lamellidens marginalis*, Environ. Sci. Pollut. Res. (2017), https://doi.org/10.1007/s11356-017-9266-0.
[29] H.P. Misra, I. Fridovich, The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase, J. Biol. Chem. 247 (1972) 3170–3175.
[30] S. Takahara, B.H. Hamilton, J.V. Nell, T.Y. Kobra, Y. Ogura, E.T. Nishimura, Hypocatalesemia, a new generis Carrier state, J. Clin. Invest. 29 (1960) 610–619.
[31] W.H. Habing, M.N. Pabst, W. Bjacoby, S. Glutathion, Glutathione S-transferases. Transferase, the first enzymatic step in mercatpopunc acid formation, J. Biol. Chem. 249 (1974) 7130–7139.
[32] S. Hestrin, The reaction of acetyl choline and other carboxylic acid derivatives with

hydroxylamine and its analytical application, J. Biol. Chem. 180 (1949) 249–261.
[33] C.J. Secombes, Isolation of Salmonid macrophage and analysis of their killing activity, in: J.S.T.C. Stolen, D.P. Fletcher, B.S. Anderson, W.B. Van Muiswinkel (Eds.), Techniques in Fish Immunology, SOS Publication, Fair Haven (NJ), 1990, pp. 137–152.
[34] A.S. Stasiack, C.P. Bauman, Neutrophil activity as a potent indicator concomitant analysis, Fish Shellfish Immunol. 37 (1996) 539.
[35] B.T. Doumas, W. Watson, H.G. Biggs, Albumin standards and measurement of serum albumin with bromocresol green, Clin. Chim. Acta 31 (1971) 87–96.
[36] M.J. Quade, J.A. Roth, A rapid, direct assay to measure degranulation of bovine neutrophil primary granules, Vet. Immunol. Immunopathol. 58 (1997) 239–248.
[37] P.K. Sahoo, J. Kumari, B.K. Mishra, Non-specific immune responses in juveniles of Indian major carps, J. Appl. Ichthyol. 21 (2005) 151–155.
[38] D.P. Anderson, A.K. Siwicki, Basic haematology and serology for fish health programmes, in: J.R. Shhariff, R.P. Subasinghe (Eds.), Diseases in Asian Aquaculture II. Fish Health Section, Asian Fisheries society, Manila, Philippines, 1995, pp. 185–202.
[39] N. Nelson, A photometric adaptation of the Somogyi method for the determination of glucose, J. Biol. Chem. 153 (1944) 375–380.
[40] M. Somoyogi, A new reagent for the determination of sugars, J. Biol. Chem. 160 (1945) 61–68.
[41] R.A. Nugroho, R. Fotedar, Dietary organic selenium improves growth, survival and resistance to *Vibrio mimicus* in cultured marron, *Cherax cainii* (Austin, 2002), Fish Shellfish Immunol. 35 (1) (2013) 79–85.
[42] Y.H. Lin, Effects of dietary organic and inorganic selenium on the growth, selenium concentration and meat quality of juvenile grouper *Epinephelus malabaricus*, Aquaculture 430 (2014) 114–119.
[43] X. Zhou, Y. Wang, Q. Gu, W. Li, Effects of different dietary selenium sources (selenium nanoparticle and selenomethionine) on growth performance, muscle composition and glutathione peroxidase enzyme activity of crucian carp (*Carassius auratus gibelio*), Aquaculture 291 (2009) 78–81.
[44] N. Kumar, K. Ambasankar, K.K. Krishnani, S. Gupta, P.S. Minhas, Dietary pyridoxine promotes growth and cellular metabolic plasticity of *Chanos chanos* fingerlings exposed to endosulfan induced stress, Aquacult. Res. 55 (2016), https://doi.org/10.1111/are.13042.
[45] N. Kumar, S.B. Jadhao, N.K. Chandan, R.S. Rana, Dietary choline, betaine and lecithin mitigates endosulfan induced stress in *Labeo rohita* fingerlings, Fish Physiol. Biochem. 38 (2011) 989–1000.
[46] N. Kumar, S.B. Jadhao, N.K. Chandan, M.D. Akhlak, R.S. Rana, Methyl donors potentiates growth, metabolic status and neurotransmitter enzyme in *Labeo rohita* fingerlings exposed to endosulfan and temperature, Fish Physiol. Biochem. 38 (5) (2012) 1343–1353.
[47] H.B. Chen, H.Q. Huang, Proteomic analysis of methyl parathion-responsive proteins in Sparus latus liver, Fish Shellfish Immunol. 30 (2011) 800–806.
[48] N. Kumar, A.J. Prabhu, A.K. Pal, S. Remya, M. Aklakur, R.S. Rana, S. Gupta, R.P. Raman, S.B. Jadhao, Anti-oxidative and immuno-hematological status of Tilapia (*Oreochromis mossambicus*) during acute toxicity test of endosulfan, Pestic. Biochem. Physiol. 99 (2011) 45–52.
[49] N. Kumar, K. Ambasankar, K.K. Krishnani, S.K. Gupta, S. Bhushan, P.S. Minhas, Acute toxicity, biochemical and histopathological responses of endosulfan in Chanos chanos, Ecotoxicol. Environ. Saf. 131 (2016) 79–88.
[50] Y.M. Hossein, M. Masoudifar, B. Varastehmoradi, E. Mohammadi, E. Kheradmand, S. Homayouni, A.R. Shahverdi, Effect of oral supplementation of biogenic selenium nanoparticles on white blood cell profile of BALB/c mice and mice exposed to X-ray radiation, Avicenna J. Med. Biotechnol. (AJMB) 5 (3) (2013) 158–167.
[51] S. Roth, S. Zhang, J. Chiu, E.K. Wirth, U. Schweizer, J. Trace Elem. Med. Biol. 24 (2010) 130–137.
[52] Y. Zhang, Y. Zhou, U. Schweizer, N.E. Savaskan, D. Hua, J. Kipnis, D.L. Hatfield, V.N. Gladyshev, J. Biol. Chem. 283 (2008) 2427–2438.
[53] N.A. Muthappa, S. Gupta, S. Yengkokpam, D. Debnath, N. Kumar, A.K. Pal, S.B. Jadhao, Lipotropes Promote immunobiochemical plasticity and protect fish against low-dose pesticide-induced oxidative stress, Cell Stress Chaperones 19 (1) (2014) 61–81.
[54] N. Kumar, R. Sharma, G. Tripathi, K. Kumar, R.S. Dalvi, G. Krishna, Cellular metabolic, stress and histological response on exposure to acute toxicity of endosulfan in Tilapia (*Oreochromis mossambicus*), Environ. Toxicol. 31 (1) (2014) 106–115.
[55] J.C.H. Kim, H.J. Kang, The selenium accumulation and its effect on growth, and haematological parameters in red seabream, *Pagrus major*, exposed to water borne selenium, Ecotoxicol. Environ. Saf. 104 (2014) 96–102.
[56] X.Y. Zhang, M.Z. Zhang, C.J. Zheng, J. Liu, H.J. Hu, Identification of two hsp90 genes from the marine crab, *Portunus trituberculatus* and their specific expression profiles under different environmental conditions, Comp. Biochem. Physiol., C 150 (2009) 465–473.
[57] G.K. Iwama, M.M. Vijayan, R.B. Forsyth, P.A. Ackerman, Heat shock proteins and physiological in fish, Am. Zool. 39 (1999) 901–909.
[58] T. Shinohara, N. Takahashi, T. Ooie, M. Hara, S. Shigematsu, M. Nakagawa, H. Yonemochi, T. Saikawa, H. Yoshimatsu, Phosphatidylinositol 3-kinase-dependent activation of akt, an essential signal for hyperthermia-induced heat-shock protein 72, is attenuated in streptozotocin-induced diabetic heart, Diabetes 55 (2006) 1307–1315.
[59] Y.Z. Fang, S. Yang, G.Y. Wu, Free radicals, antioxidants, and nutrition, Nutrition 18 (2002) 872–879.
[60] J.M. May, S. Mendiratta, K.E. Hill, R.F. Burk, Reduction of dehydroascorbate to ascorbate by the selenoenzyme thioredoxin reductase, J. Biol. Chem. 272 (1997) 22607–22610.
[61] J.M. May, C.E. Cobb, S. Mendiratta, K.E. Hill, R.F. Burk, Reduction of the ascorbyl free radical to ascorbate by thioredoxin reductase, J. Biol. Chem. 273 (1998) 23039–23045.
[62] F. Qin, M. Shi, H. Yuan, L. Yuan, W. Lu, J. Zhang, J. Tong, X. Song, Dietary nano-selenium relieves hypoxia stress and, improves immunity and disease resistance in the Chinese mitten crab (*Eriocheir sinensis*), Fish Shellfish Immunol. 54 (2016) 481–488.
[63] H. Wang, J. Zhang, H. Yu, Elemental selenium at nano size possesses lower toxicity without compromising the fundamental effect on selenoenzymes: comparison with selenomethionine in mice, Free Radic. Biol. Med. 42 (2007) 1524–1533.
[64] N. Kumar, K. Ambasankar, K.K. Krishnani, S. Bhushan, P.S. Minhas, Dietary pyridoxine protects against stress and maintains immune-hematological status in *chanos chanos* exposed to endosulfan, Basic Clin. Pharmacol. Toxicol. 119 (2016) 297–308.
[65] D. Choudhury, A.K. Pal, N.P. Sahu, S. Kumar, S.S. Das, S.C. Mukherjee, Dietary yeast RNA supplementation reduces mortality by *Aeromonas hydrophila* in rohu (*Labeo rohita L.*) juveniles, Fish Shellfish Immunol. 19 (3) (2005) 281–291.
[66] A.K. Jha, A.K. Pal, N.P. Sahu, S. Kumar, S.C. Mukherjee, Haemato-immunological responses to dietary yeast RNA, omega-3 fatty acid and beta-carotene in *Catla catla* juveniles, Fish Shellfish Immunol. 23 (5) (2007) 917–927.
[67] S.K. Nayak, P. Swain, S.C. Mukherjee, Effect of dietary supplementation of probiotic and vitamin C on the immune response of Indian major carp, *Labeo rohita* (Ham.), Fish Shellfish Immunol. 23 (4) (2007) 892–896.
[68] G.F. Wiegertjes, R.J.M. Stet, H.K. Parmentier, W.B. Van Muiswinkel, Immunogenetics of disease resistance in fish; a comparable approach, Dev. Comp. Immunol. 20 (1996) 365–381.
[69] B. Beutler, Innate immunity: an overview, Mol. Immunol. 40 (2004) 845–859.
[70] R.A. Dalmo, K. Ingebrigtsen, J. Bøgwald, Non-specific defence mechanisms infish, with particular reference to the reticuloendothelial system (RES), J. Fish. Dis. 20 (1997) 241–273.
[71] S.T. Chiu, S.L. Hsieh, S.P. Yeh, S.J. Jian, W. Cheng, C.H. Liu, The increase of immunity and disease resistance of the giant freshwater prawn, *Macrobrachium rosenbergii* by feeding with selenium enriched-diet, Fish Shellfish Immunol. 29 (4) (2010) 623–629.
[72] M.C. Holland, J.D. Lambris, The complement system in teleosts, Fish Shellfish Immunol. 12 (5) (2002) 399–420.
[73] B. Puchau, M.A. Zulet, A. Gonzalez de Echavarri, I. Navarro-Blasco, J.A. Martinez, Selenium intake reduces serum C3, an early marker of metabolic syndrome manifestations, in healthy young adults, Eur. J. Clin. Nutr. 63 (7) (2009) 858–864.