



Synergistic effect of dietary selenium nanoparticles and riboflavin on the enhanced thermal efficiency of fish against multiple stress factors



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ABSTRACT

An experiment was designed to delineate the efficacy of a dietary mixture of selenium nanoparticles (Se-NPs) and riboflavin (RF) on the thermal efficiency/tolerance of *Pangasianodon hypophthalmus* reared under arsenic (2.8 mg/L) and high-temperature (34 °C) stress. A green synthesis method was employed for the synthesis of Se-NPs using fish gills, which are normally discarded as by-products. Four isocaloric and iso-nitrogenous experimental diets were used, namely, a control diet (Se-NPs and RF @ 0 mg/kg diet) and diets containing RF @ 5, 10 or 15 mg/kg diet and Se-NPs @ 0.5 mg/kg diet, and feeding was performed for 95 days. At the end of the feeding trial, the thermal tolerance was evaluated by determination of the following parameters: critical thermal minimum (CTMin), lethal thermal minimum (LTMin), critical thermal maximum (CTMax), and lethal thermal maximum (LTMax). The anti-oxidative status in the form of catalase (CAT), glutathione-s-transferase (GST) and glutathione peroxidase (GPx) activities was significantly ($p < 0.01$) enhanced upon concurrent exposure to arsenic and high temperature at LTMin and LTMax, whereas a non-significant ($p > 0.05$) change in superoxide dismutase (SOD) activity was observed in the brain at LTMin and brain, gill and kidney at LTMax. Supplementation with Se-NPs @ 0.5 mg/kg diet and RF @ 5, 10 or 15 mg/kg diet significantly ($p < 0.01$) improved the anti-oxidative status with or without stressors. AChE activity in the brain was significantly ($p < 0.01$) inhibited upon concurrent exposure to arsenic and high temperature and improved in the treatment group supplemented with Se-NPs and RF. The arsenic concentration in muscle and experimental water and Se concentration in muscle and experimental feed were analysed. Overall, the results indicated that supplementation with RF @ 5 mg/kg diet and Se-NPs @ 0.5 mg/kg diet could confer protection to the fish against arsenic and thermal stress and led to enhanced thermal efficiency/tolerance of *P. hypophthalmus*.

1. Introduction

The mean global temperature has increased by 0.2 °C during the last two decades and is likely to increase by another 1.8–4.0 °C by the year 2100 (IPCC, 2007). The temperature rise due to human activities were also estimated by IPCC, who determined a 1.0 °C rise in global warming above pre-industrial levels with a range of 0.8–1.2 °C, which will further increase to 1.5 °C between 2030 and 2052 (IPCC, 2018). Moreover, temperature variation has a drastic effect on biological communities, particularly on aquatic organisms (Brown et al., 2004). Temperature also influences all the biochemical and physiological processes of aquatic animals, including fish, and has effects on biogeographical

distribution patterns and species interactions (Hochachka and Somero, 2002; Portner, 2002a, 2002b, 2014). Fishes are cold-blooded animals (poikilothermic); therefore, changes in thermal regimes affect their metabolic activities, behaviour, growth, reproduction, survival rate, and life span (Portner, 2001). Moreover, previous experiments on fishes have suggested that the metabolic rates and life spans of these organisms are inter-related (Das et al., 2004; Kumar et al., 2014a, 2016a; 2017a, b; 2018a). Exposure to high temperatures for longer durations can alter the tissue chemistry of aquatic animals (Hazel and Prosser, 1974; Hochachka and Somero, 1971), but specific behavioural and physiological adaptive mechanisms might facilitate minimization of the temperature-related adverse effects (Prosser and Heath, 1991). Arsenic

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is a hazardous metal in aquatic contaminants worldwide, including in India (Mandal and Suzuki, 2002; Ng, 2005). Arsenic disrupts the ecosystem and its wellbeing (Hughes et al., 1988). Dramatic reduction in the thermal efficiency/tolerance of fishes to concurrent exposure to environmental contaminants (arsenic/other metals) and high/low temperatures has been reported (Kumar et al., 2018a, 2017a). Moreover, arsenic is stored, distributed and metabolized in fish tissues by conversion to arsenite via oxidation/reduction reactions, and then, the arsenite binds with unidentified proteins (Kumari et al., 2017). Arsenic plays a deleterious role in the metabolism of ectothermic animals (Sokolova and Lannig, 2008). Consequently, arsenic disturbs cellular processes, altering homeostasis, protein stability and mitochondrial efficiency, leading to elevated costs of maintenance and detoxification (Sokolova and Lannig, 2008). Theoretically, thermal tolerance of aquatic animals is positively associated with oxygen and capacity-limited thermal tolerance (Portner and Knust, 2007; Portner, 2010). According to this concept, the routine daily metabolic rate increases with increasing temperature. The maximum metabolic rate reaches a high threshold, plateaus and then starts to decrease with increasing temperature (Fry, 1947).

To counteract the effect of rising temperature and contamination (arsenic) stress on the thermal tolerance of fishes, concerted efforts by researchers are ongoing to identify remedial measures and possible alternative solutions to address the situation. For this purpose, we formulated a diet supplemented with a mixture of selenium nanoparticles (Se-NPs) and riboflavin (RF) to tackle the arsenic and high-temperature stress. Selenium is an essential micronutrient with the potential to improve the anti-oxidant levels and immunity of aquatic animals, including fishes (Huang et al., 2012). In the present investigation, Se-NPs were used because they are less toxic than inorganic selenium (Forootanfar et al., 2014) and are essential for the maintenance and functioning of selenoproteins, such as proteins containing selenocysteine and selenomethionine (Rao et al., 2013). Furthermore, selenium plays an important role in the activation, proliferation and differentiation of cells that control innate and adaptive immunity in humans and animals (Huang et al., 2012; Kudva et al., 2015). Riboflavin (RF) is an important water-soluble vitamin that is essential for fish nutrition (Deng and Wilson, 2003). RF plays an important role in the physio-metabolic process and serves as a coenzyme for a variety of flavoprotein enzyme reactions. The active forms of RF are flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which participate in the various oxidation-reduction reactions and are required by all animals, including fishes (Deng and Wilson, 2003). The protective ability of riboflavin against lipid peroxidation and consequently against oxidative stress (Marashly and Bohlega, 2017) has been validated in *Pangasianodon hypophthalmus*.

Indian major carps form the mainstay of aquaculture, contributing more than half of the production from the aquaculture sector (Anjani et al., 2003). However, in the last decade, *P. hypophthalmus* has been introduced as a new species to enhance aquaculture production in the wide-ranging agro-climatic conditions in India. This species has not received much research attention worldwide with regard to its abiotic stress and thermal tolerance. The thermal tolerance limit of *P. hypophthalmus* was determined to be approximately 43 °C in our previous study (Kumar et al., 2017a). Moreover, proper nutrition can modulate the immune system to enhance thermal tolerance in fishes, as validated in previous studies (Kumar et al., 2014b, 2018b; 2019a; Tejpal et al., 2014). Concurrent exposure to arsenic and high temperature leads to noticeable alterations in the anti-oxidative status, such as the levels of superoxide dismutase (SOD), catalase (CAT), glutathione-s-transferase (GST), glutathione peroxidase (GPx) and neurotransmitter enzymes. To the best of our knowledge, literature on the effect of a combinatorial mixture of Se-NPs and RF on the enhancement of the thermal tolerance and biochemical status of *P. hypophthalmus* is unavailable to date. Therefore, the present study was carried out to delineate the effect of a combinatorial mixture of Se-NPs and RF to improve the thermal

tolerance and biochemical status of *P. hypophthalmus* reared under arsenic treatment and high temperature (34 °C).

2. Materials and methods

2.1. Ethics statement

The care and treatment of animals were undertaken as per the guidelines of the Committee for 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. Experimental design and conditions

P. hypophthalmus fish were procured from a fish vendor in Kolkata, West Bengal, in healthy conditions and transferred to the wet laboratory of the National Institute of Abiotic Stress Management, Baramati, Pune, India. The fish were treated with 5 g/L salt solution and 2 mg/L KMnO₄ solution and then transferred to fibre-reinforced plastic (FRP) tanks (circular, 500 L) for one month prior to the experiment. The fish were distributed as per a completely randomized design in 24 tanks with 150 L of water for 95 days. Eighteen fish of uniform size (5.35 ± 1.02 g) were stocked in the 8 different experimental groups in triplicate. The fish were fed daily (10:00 a.m. and 5:00 p.m.) until satiation during the experimental period. Aeration was provided throughout the experimental period, and water exchange was carried out every other day. The experimental groups were designed as follows: fed with control diet and treated with normal water (Ctr/Ctr), fed with control diet and treated with concurrent exposure to arsenic and high temperature (As + T/Ctr); fed Se-NPs @ 0.5 mg/kg diet and vitamin B-2 @ 5 mg/kg diet (Se-NPs-RF-5 mg/kg); fed Se-NPs @ 0.5 mg/kg diet and vitamin B-2 @ 10 mg/kg diet (Se-NPs-RF-10 mg/kg); fed Se-NPs @ 0.5 mg/kg diet and vitamin B-2 @ 15 mg/kg diet (Se-NPs-RF-15 mg/kg); treated with concurrent exposure to arsenic and high temperature and fed Se-NPs @ 0.5 mg/kg diet and RF @ 5, 10 or 15 mg. Arsenic was added to the experimental water at 1/10th of LC₅₀ (2.8 mg/L) at 96 h using sodium arsenite (NaAsO₂) (Kumar et al., 2019). The temperature was recorded in the range of 25.4–28.64 °C and 34 ± 0.92 °C in the normal and temperature-treated groups, respectively. The temperature was maintained with the help of a thermostatic rod heater in the group exposed to high temperature. The other water quality parameters were measured as per APHA-AWWA-WEF (1998).

2.3. Preparation of fish tissue extract for green synthesis of Se-NPs

Gill tissue was dissected from live *Labeo rohita* fish and washed thoroughly in running water to remove dust and blood. The dissected gill tissue was cut into small pieces and mixed thoroughly using a mortar and pestle. Tissue lysate was centrifuged at 5000–6000 × g for 15 min at 4 °C, and the supernatant was collected for further processing by double filtration using Whatman filter paper with a pore size of 0.45 μm to obtain the gill extract. The gill extract was mixed with 200 ml of sodium selenite (2 M) in distilled water and then shaken for 96 h using a shaker. The solution was centrifuged at 6000 × g for 15 min at 4 °C. The pellets were harvested and kept in an oven at 60 °C until dry and subsequently stored at room temperature. Before use, the dry pellet was crushed to a fine powder using a mortar and pestle (Kumar et al., 2018b, c; 2017b).

2.4. Characterization of selenium nanoparticles

The synthesized Se-NPs were evaluated by determination of the absorption spectrum at 300–500 nm with a UV–Vis spectrophotometer

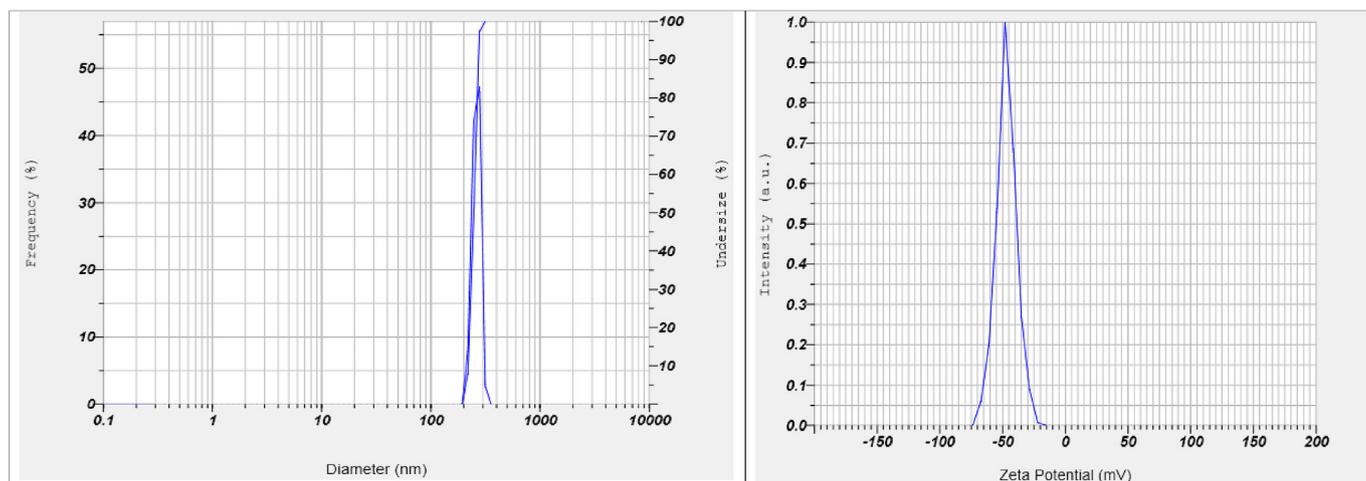


Fig. 1. Particle size (249.4 nm) and zeta potential (-47 mV) of selenium nano-particles.

(Shimadzu, UV-1800, Japan), and the observed peak was obtained in the range of 360–380 nm. The synthesized Se-NP formulations were diluted with Milli-Q water and processed for particle size analysis with a nanosize analyser. The mean particle size of the Se-NPs was 249.4 nm, and the mean zeta potential was -47 mV (Fig. 1); these values were determined using a Horiba Scientific Nanoparticle Analyser (SZ-100 nanoparticle series, Kyoto, Japan) at 25 °C.

2.5. Experimental diet and proximate analysis of feed

Four iso-caloric and iso-nitrogenous diets were prepared: a selenium-free basal diet (control diet) and three diets supplemented with mixtures of Se-NPs and RF, namely, Se-NPs @ 0.5 mg/kg diet + RF @ 5 mg/kg diet, Se-NPs @ 0.5 mg/kg diet + RF @ 10 mg/kg diet, and Se-NPs @ 0.5 mg/kg diet + RF @ 15 mg/kg diet. The pelleted diet was prepared with fish meal, soybean meal, groundnut meal, wheat flour, sunflower oil and cod liver oil. The selenium- and RF-free vitamin-mineral mixtures were prepared manually and mixed after steam cooking. The dough was mixed and baked at 60 °C until dry and subsequently stored at 4 °C until required for feeding (Table 1). The proximate composition of the experimental diets was determined following the standard methods of AOAC (1995) and is presented in Table 1. The moisture content was determined by drying at 105 °C to a constant weight. The nitrogen content was estimated by the Kjeldahl method (2200 Kjeltex Auto Distillation Unit, Foss Tecator, Hogonas, Sweden), and the crude protein (CP) content was estimated by multiplying the nitrogen percentage by 6.25. The ether extract (EE) was measured by the solvent extraction method with a 1045 Soxtec extraction unit (Foss Tecator) using diethyl ether (boiling point, 40–60 °C) as a solvent, and the ash content was determined by incinerating the samples in a muffle furnace at 600 °C for 6 h. The total carbohydrate content was calculated by difference, i.e., total carbohydrate% = 100-(CP% + EE% + ash%). The digestible energy value of the experimental diets was calculated as per Halver (1976).

2.6. Analysis of selenium in the experimental diet

For analysis of selenium (Se) and arsenic (As) in the diet, fish muscle and water were examined by inductively coupled plasma mass spectrometry (ICP-MS). Muscle and feed samples weighing 0.5 g were used for estimation of the Se and arsenic content. The samples were digested in a microwave digestion system (Microwave Digestion System, Model START-D, SN- 135177, Milestone, USA) in acidic conditions ($\text{HNO}_3:\text{H}_2\text{O}_2$ at 5:1). This volume of the digested solution was brought up to 50 ml using triple-distilled water. The digested samples were allowed to cool at room temperature and then filtered with Whatman

filter paper (pore size 0.45 μm). The filtered samples were loaded for Se and arsenic analysis through an ICP-MS instrument (Agilent 7700 series, Agilent Technologies, USA) (Kumar et al., 2017c, d, e).

2.7. Tissue homogenate preparation

Liver, gill, brain, and kidney tissues of the fish were dissected for enzyme analysis. The tissues were homogenized (5% w/v) separately in chilled sucrose solution (0.25 M) in a glass tube using a tissue homogenizer (Omni Tissue Master Homogenizer, Kennesaw, GA). The tube was kept on ice to avoid denaturation of the enzymes during homogenization. The homogenates were centrifuged at 5000 \times g for 20 min at 4 °C in a cooling centrifuge (Eppendorf, Hauppauge, NY 11788). The protein content in the supernatants was quantified following the method of Lowry et al. (1951) using bovine serum albumin as a standard. The supernatants were collected and stored at -20 °C until further analysis.

2.8. Thermal tolerance study

The method applied in the thermal tolerance study was previously described by Beitinger et al. (2000), Dalvi et al. (2009) and Kumar et al., (2014a;2016a; 2017a; 2018a). The study was conducted after a 95-day feeding trial, and before conducting the thermal study, the feeding was stopped 24 h before the start of the experiment. The method was divided into two parts: determination of the critical thermal minimum (CTMin) and critical thermal maximum (CTMax); CTMin represents the minimum scale of temperature, and CTMax represents the maximum scale of temperature. Four fish (two for CTMin and lethal thermal minimum (LTMin) and two for CTMax and lethal thermal maximum (LTMax)) were randomly selected from each treatment and replicates. The fish were shifted to separate thermostatic water bath aquaria (Thermo Scientific Instruments & Equipment, Waltham, MA, USA; sensitivity ± 0.2 °C) for the temperature tolerance study. The temperature and arsenic concentration in the thermostatic aquaria were maintained at values similar to those in the experimental group. The dissolved oxygen concentration was maintained at 6.5 ± 0.5 mg/L throughout the temperature tolerance study by continuous aeration using a 2-HP centralized air blower. The water temperature in the aquaria was increased/decreased at a constant rate of 0.30 °C/min until loss of equilibrium (LOE) was reached, and this temperature was designated as the CTMax/CTMin (Beitinger et al., 2000; Paladino et al., 1980). The LTMax/LTMin were determined by further increasing/decreasing the temperature until opercular movement ceased (Tsuchida, 1995; Kita et al., 1996). LOE indicates a physiological condition in which the brain fails to maintain balance but

Table 1
Ingredients composition of the different experimental diets fed to *Pangasianodon hypophthalmus* during the experimental period of 95 days.

Ingredient	Control diet	Selenium Nanoparticles + Riboflavin diet		
	Selenium and Riboflavin B2 free	0.5 mg/kg + 5 mg/kg diet	0.5 m g/kg + 10 mg/kg diet	0.5 mg/kg+ 15 mg/kg diet
Soybean meal ^a	35.5	35.5	35.5	35.5
Fish meal ^a	20.0	20.0	20.0	20.0
Groundnut meal ^a	10.0	10.0	10.0	10.0
Wheat flour ^a	24.20	24.1945	24.1895	24.1845
Sunflower oil ^a	4.5	4.5	4.5	4.5
Cod liver oil ^a	1.5	1.5	1.5	1.5
CMC ^b	2.0	2.0	2.0	2.0
Vitamin and mineral mix ^{a,c}	2.0	2.0	2.0	2.0
Vitamin C ^d	0.3	0.3	0.3	0.3
Selenium Nanoparticles (Se-NPs)	0	0.0005	0.0005	0.0005
Vitamin B2 ^b	0	0.005	0.010	0.015
Proximate analysis of experimental feed				
CP ¹	35.51 ± 0.57	35.53 ± 0.47	34.71 ± 0.29	35.46 ± 0.35
EE ²	10.57 ± 0.07	11.05 ± 0.28	10.99 ± 0.26	10.51 ± 0.19
Ash	9.35 ± 0.06	9.47 ± 0.14	9.73 ± 0.07	9.56 ± 0.04
TC ³	44.57 ± 0.29	43.95 ± 0.34	44.57 ± 0.19	44.47 ± 0.59
OM ⁴	90.22 ± 0.03	89.90 ± 0.48	91.52 ± 0.60	91.39 ± 1.22
DM ⁵	92.66 ± 0.50	91.99 ± 0.30	91.65 ± 0.15	90.77 ± 0.05
DE ⁶	415.48 ± 1.95	417.37 ± 3.28	416.0 ± 1.06	414.34 ± 0.56

aProcured from local market, bHimedia Ltd, c*Prepared manually and all components from Himedia Ltd, cSD Fine Chemicals Ltd., India. 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 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.
Digestible energy (DE) (K cal/100g) = (% CP x 4) + (% EE x 9) + (TC x 4).
CP1- Crude Protein; EE2- Ether extract; TC3-Total Carbohydrate; OM4-Organic Matter, DM5: Dry matter DE6- Digestible Energy.
Data expressed as mean ± SE, n = 3.

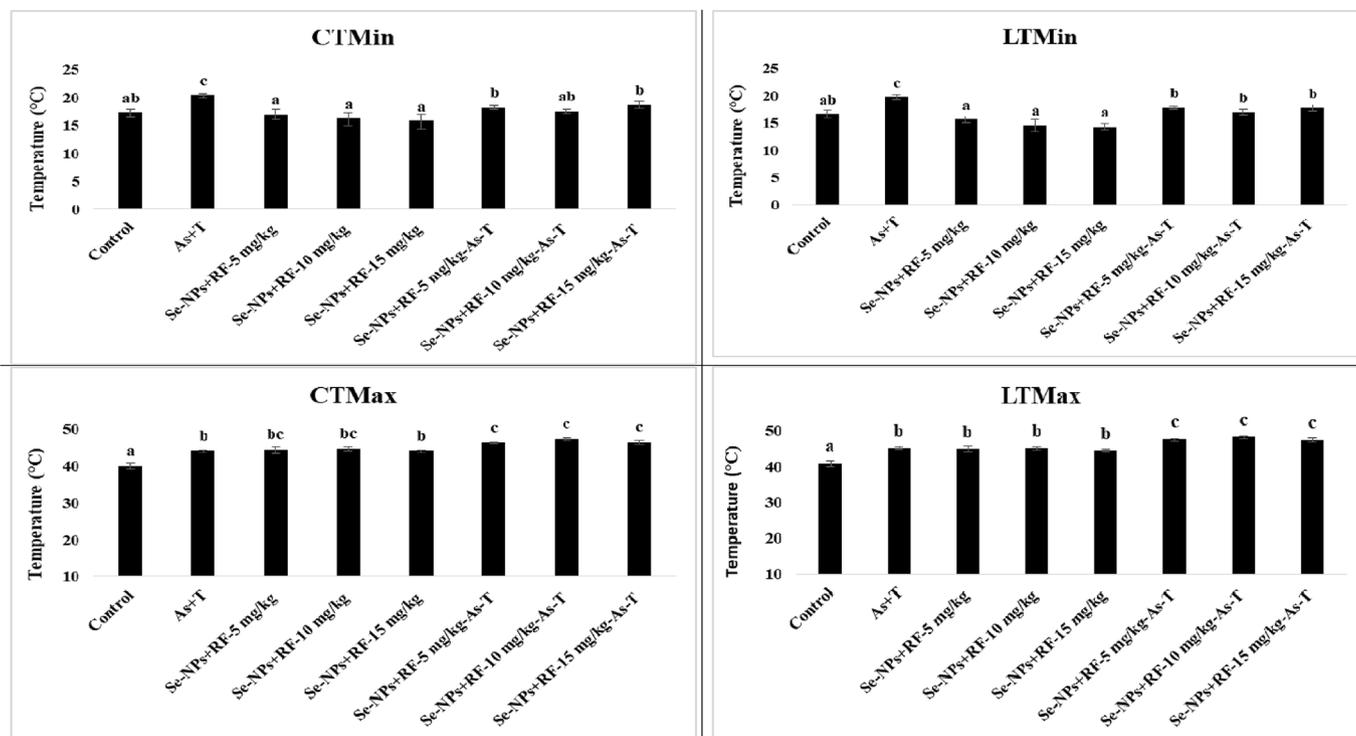


Fig. 2. Effect of combitorial mixture of dietary selenium-nanoparticles (Se-NPs) and Riboflavin on critical thermal minima (CTmin), lethal thermal minima (LTmin), critical thermal maxima (CTmax), lethal thermal maxima (LTmax) of *P. hypophthalmus* under concurrent exposure to arsenic and elevated temperature for 95 days. Values are expressed as mean ± SE, n = 3. Bars bearing different letters (a, b, c) indicate significant differences.

somehow tries to survive by obtaining oxygen through the opercula, whereas loss of opercular movement is a condition in which the fish fails to move the opercula and ultimately dies. Kumar et al. (2014a; 2016a; 2017a; 2018a) critically evaluated this technique and established a tool for studying thermal tolerance in fish (Paladino et al.,

1980; Baker and Heidinger, 1996).

2.9. Measurement of anti-oxidant enzymes

SOD (EC 1.15.1.1) enzyme activity was measured by the method of

Misra and Fridovich (1972); CAT (EC 1.11.1.6) enzyme activity was measured by the method of Takahara et al. (1960); and GST (EC 2.5.1.18) enzyme activity was measured by the method of Habing et al. (1974). GPx (EC 1.11.1.9) activity was measured in the liver and gill tissue by the method of Paglia and Valentine (1967).

2.10. Acetylcholine esterase (AChE)

Acetylcholine esterase (AChE) (EC. 3.1.1.7) activity was measured as the change in OD at 540 nm using the method of Hestrin (1949) modified by Augustinsson (1957).

2.11. Statistical analysis

The data were statistically analysed by Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS, Chicago, IL), for which the data were subjected to a one-way ANOVA followed by Duncan's multiple range tests to determine the significant differences if any between the means. Comparisons were made at a 1% probability level.

3. Results

3.1. Thermal study

In the thermal study, the CTMin, LTMin, CTMax, and LTMax were determined in *P. hypophthalmus* fed Se-NPs (@ 0.5 mg/kg diet) and RF (@ 5, 10, or 15 mg/kg diet) reared under multiple stress conditions (arsenic (As) at 2.8 mg/L and a high temperature of 34 °C), as presented in Fig. 2. The CTMin and LTMin of the group fed Se-NPs @ 0.5 mg/kg diet and RF @ 5, 10, or 15 mg/kg diet without any stressor were noticeably ($p < 0.01$) lower than those of the group fed the control diet with or without exposure to multiple stressors. Similarly, the group fed Se-NPs @ 0.5 mg/kg diet and RF @ 5, 10, or 15 mg/kg diet with exposure to multiple stressors (concurrent exposure to arsenic and high temperature) exhibited significantly ($p < 0.01$) lowered CTmin and LTMin values compared to those of the control group with or without exposure to multiple stressors. The lowest CTMin was observed in the group fed Se-NPs @ 0.5 mg/kg diet and RF @ 5, 10 or 15 mg/kg diet was 16.99 ± 0.87 , 16.19 ± 1.20 or 15.75 ± 1.36 °C, respectively, and the lowest LTMin in the group fed Se-NPs @ 0.5 mg/kg diet and RF @ 5, 10, or 15 mg/kg diet was 15.63 ± 0.58 , 14.56 ± 1.15 or 14.32 ± 0.61 °C, respectively. The highest CTMin and LTMin were observed in the group fed the control diet and exposed to multiple stressors (As and high temperature) (CTMin, 20 ± 0.35 °C; LTMin, 19.75 ± 0.45 °C). Similarly, the highest CTMax and LTMax were observed in the supplemented group fed with Se-NPs @ 0.5 mg/kg diet and RF @ 5, 10 or 15 mg/kg diet with exposure to multiple stressors, followed by the group fed Se-NPs @ 0.5 and RF at 5, 10, or 15 mg/kg diet and the group concurrently exposed to As and high temperature and fed the control diet. The highest CTMax and LTMax were observed in the group supplemented with Se-NPs and RF@ 5, 10 or 15 mg/kg diet, with values of 46.32 ± 0.24 , 47.32 ± 0.39 , or 46.37 ± 0.59 °C, respectively, for CTMin and values of 47.52 ± 0.33 , 48.16 ± 0.27 , or 47.44 ± 0.56 °C, respectively, for CTMax.

3.2. Anti-oxidative status at LTMin and LTMax

The activities of anti-oxidative enzymes such as CAT, SOD, GST and GPx in liver, gill, kidney, and brain tissue were determined during the thermal tolerance study in *P. hypophthalmus* fed Se-NPs @ 0.5 mg/kg diet and RF @ 5, 10 or 15 mg/kg diet (Tables 2–5). The CAT activity in the liver, gill, kidney and brain were noticeably ($p < 0.01$) reduced upon supplementation with a mixture of Se-NPs @ 0.5 mg/kg diet and RF @ 5, 10 or 15 mg/kg diet with or without stressors at LTMin and LTMax in comparison to the control group and the group that was concurrently exposed to arsenic and high temperature and fed the

Table 2
Effect of combinatorial mixture of dietary selenium-nanoparticles (Se-NPs) and Riboflavin on catalase and superoxide dismutase (SOD) of *P. hypophthalmus* under concurrent exposure to arsenic and elevated temperature during LTMin.

Diets	Non-stressors		Stressors (Arsenic and Temperature)		Stressors (Arsenic and Temperature)						P-Value
	Control	Se-NPs + RF-5 mg/kg	Control	Se-NPs + RF-5 mg/kg	Se-NPs + RF-10 mg/kg	Se-NPs + RF-15 mg/kg	Se-NPs + RF-5 mg/kg	Se-NPs + RF-10 mg/kg	Se-NPs + RF-15 mg/kg	Se-NPs + RF-15 mg/kg	
CAT-Liver	8.59b ± 0.87	3.12a ± 0.40	17.52c ± 1.86	3.83a ± 0.30	3.83a ± 0.30	3.28a ± 0.29	3.91a ± 0.31	3.58a ± 0.39	3.98a ± 0.15	3.98a ± 0.15	p < 0.01
CAT-Gill	6.25b ± 1.13	4.62a ± 0.69	13.43c ± 0.97	4.20a ± 0.31	4.20a ± 0.31	5.00a ± 0.60	4.40a ± 0.74	4.34a ± 0.49	4.52a ± 0.38	4.52a ± 0.38	p < 0.01
CAT-Brain	9.66b ± 1.07	5.41a ± 0.81	16.14c ± 1.19	5.75a ± 0.98	5.75a ± 0.98	5.79a ± 1.43	5.84a ± 0.57	5.42a ± 0.36	5.62a ± 0.93	5.62a ± 0.93	p < 0.01
CAT-Kidney	9.41b ± 1.36	6.80a ± 1.02	17.06c ± 1.55	6.93a ± 0.57	6.93a ± 0.57	6.81a ± 1.05	7.16a ± 0.64	6.89a ± 0.83	7.11a ± 0.51	7.11a ± 0.51	p < 0.01
SOD-Liver	55.90b ± 1.02	52.43a ± 0.68	61.14c ± 1.53	53.47a ± 0.62	53.47a ± 0.62	53.59a ± 1.09	52.14a ± 1.65	53.27a ± 2.14	56.40a ± 0.84	56.40a ± 0.84	p < 0.01
SOD-Gill	42.92a ± 1.06	42.57a ± 1.07	44.56b ± 1.31	44.50b ± 1.16	44.50b ± 1.16	42.85a ± 1.49	42.05a ± 1.15	45.55bc ± 1.09	46.14c ± 1.30	46.14c ± 1.30	p < 0.05
SOD-Brain	42.24 ± 0.92	42.33 ± 2.33	50.78 ± 1.33	42.45 ± 2.47	42.45 ± 2.47	44.54 ± 1.70	43.19 ± 0.68	43.72 ± 1.36	42.18 ± 2.58	42.18 ± 2.58	NS
SOD-Kidney	38.23a ± 0.80	39.35a ± 1.42	44.15b ± 1.55	38.19a ± 1.11	38.19a ± 1.11	37.45a ± 1.28	39.12a ± 0.76	39.57a ± 0.45	37.60a ± 1.03	37.60a ± 1.03	p < 0.05

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 3
Effect of combinatorial mixture of dietary selenium-nanoparticles (Se-NPs) and Riboflavin on catalase and superoxide dismutase (SOD) of *P. hypophthalmus* under concurrent exposure to arsenic and elevated temperature during LTM_{max}.

Treatments	Non-stressors		Stressors (Arsenic and Temperature)		Stressors (Arsenic and Temperature)					P-Value
	Control	Se-NPs + RF-5 mg/kg	Se-NPs + RF-10 mg/kg	Se-NPs + RF-15 mg/kg	Se-NPs + RF-5 mg/kg	Se-NPs + RF-10 mg/kg	Se-NPs + RF-15 mg/kg	Se-NPs + RF-15 mg/kg	Se-NPs + RF-15 mg/kg	
CAT-Liver	8.75b ± 0.81	5.44a ± 0.46	4.80a ± 0.21	5.04a ± 0.26	5.27a ± 0.52	5.50a ± 1.03	6.05a ± 0.55	6.05a ± 0.55	6.05a ± 0.55	p < 0.01
CAT-Gill	10.29b ± 1.17	5.38a ± 0.44	5.98a ± 0.82	5.85a ± 0.67	6.20a ± 0.47	5.47a ± 0.61	5.36a ± 0.87	5.36a ± 0.87	5.36a ± 0.87	p < 0.01
CAT-Brain	8.53b ± 1.47	4.43a ± 0.60	4.92a ± 0.54	4.98a ± 0.76	5.40a ± 0.43	5.72a ± 1.28	5.51a ± 0.44	5.51a ± 0.44	5.51a ± 0.44	p < 0.01
CAT-Kidney	11.27b ± 1.47	6.66a ± 1.23	6.07a ± 0.77	7.19a ± 1.26	6.04a ± 0.52	6.18a ± 0.80	6.30a ± 0.85	6.30a ± 0.85	6.30a ± 0.85	p < 0.01
SOD-Liver	50.79b ± 1.72	48.05a ± 0.59	51.89b ± 1.18	52.94b ± 1.16	53.13c ± 1.11	50.42b ± 0.73	51.85b ± 1.28	51.85b ± 1.28	51.85b ± 1.28	p < 0.05
SOD-Gill	38.27 ± 2.47	39.61 ± 1.43	39.85 ± 0.92	40.46 ± 1.65	39.38 ± 1.93	39.94 ± 1.42	39.62 ± 1.63	39.62 ± 1.63	39.62 ± 1.63	NS
SOD-Brain	38.16 ± 0.77	38.31 ± 1.77	38.57 ± 1.19	41.23 ± 0.39	38.98 ± 2.08	40.69 ± 1.48	40.24 ± 0.92	40.24 ± 0.92	40.24 ± 0.92	NS
SOD-Kidney	36.00 ± 2.17	35.82 ± 1.37	36.42 ± 0.44	35.94 ± 0.76	36.38 ± 1.50	36.40 ± 1.03	36.74 ± 0.86	36.74 ± 0.86	36.74 ± 0.86	NS

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 combinatorial mixture of dietary selenium-nanoparticles (Se-NPs) and Riboflavin on glutathione-s-transferase (GST) and glutathione peroxidase (GPx) of *P. hypophthalmus* under concurrent exposure to arsenic and elevated temperature during LTM_{min}.

Treatments	Non-stressors		Stressors (Arsenic and Temperature)		Stressors (Arsenic and Temperature)					P-Value
	Control	Se-NPs + RF-5 mg/kg	Se-NPs + RF-10 mg/kg	Se-NPs + RF-15 mg/kg	Se-NPs + RF-5 mg/kg	Se-NPs + RF-10 mg/kg	Se-NPs + RF-15 mg/kg	Se-NPs + RF-15 mg/kg	Se-NPs + RF-15 mg/kg	
GST-Liver	0.14b ± 0.011	0.10a ± 0.014	0.10a ± 0.01	0.09a ± 0.009	0.11a ± 0.015	0.10a ± 0.005	0.11a ± 0.004	0.11a ± 0.004	0.11a ± 0.004	p < 0.01
GST-Gill	0.20b ± 0.013	0.14a ± 0.020	0.14a ± 0.011	0.14a ± 0.008	0.14a ± 0.005	0.13a ± 0.005	0.13a ± 0.01	0.13a ± 0.01	0.13a ± 0.01	p < 0.01
GST-Brain	0.15b ± 0.01	0.11a ± 0.007	0.11a ± 0.01	0.11a ± 0.008	0.10a ± 0.004	0.11a ± 0.007	0.11a ± 0.008	0.11a ± 0.008	0.11a ± 0.008	p < 0.01
GST-Kidney	0.19b ± 0.011	0.12a ± 0.018	0.11a ± 0.010	0.13a ± 0.006	0.12a ± 0.018	0.13a ± 0.008	0.14a ± 0.028	0.14a ± 0.028	0.14a ± 0.028	p < 0.01
GPx-Liver	6.81b ± 0.78	3.13a ± 0.82	4.01a ± 0.66	4.37a ± 0.63	3.65a ± 0.29	4.12a ± 0.66	3.82a ± 0.48	3.82a ± 0.48	3.82a ± 0.48	p < 0.01
GPx-Gill	6.51b ± 0.86	3.18a ± 0.56	3.10a ± 0.63	3.03a ± 0.30	3.19a ± 0.48	3.55a ± 0.56	3.41a ± 0.51	3.41a ± 0.51	3.41a ± 0.51	p < 0.01
GPx-Brain	8.11b ± 0.69	5.30a ± 0.78	5.35a ± 0.57	5.75a ± 0.47	5.79a ± 0.48	5.66a ± 0.57	5.44a ± 0.51	5.44a ± 0.51	5.44a ± 0.51	p < 0.01
GPx-Kidney	8.87b ± 0.79	5.19a ± 0.73	5.32a ± 0.69	5.97a ± 0.49	5.51a ± 0.63	5.90a ± 0.89	5.75a ± 0.55	5.75a ± 0.55	5.75a ± 0.55	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). GST and GPx: Units/mg protein.

Table 5
Effect of combinatorial mixture of dietary selenium-nanoparticles (Se-NPs) and Riboflavin on glutathione peroxidase (GPx) and glutathione-s-transferase (GST) and glutathione peroxidase (GPx) of *P. hypophthalmus* under concurrent exposure to arsenic and elevated temperature during LTMax.

Treatments	Non-stressors		Stressors (Arsenic and Temperature)		Stressors (Arsenic and Temperature)					P-Value	
	Control	Control	Control	Control	Se-NPs + RF-5 mg/kg	Se-NPs + RF-10 mg/kg	Se-NPs + RF-15 mg/kg	Se-NPs + RF-5 mg/kg	Se-NPs + RF-10 mg/kg		Se-NPs + RF-15 mg/kg
GST-Liver	0.16b ± 0.012	0.23c ± 0.006	0.11a ± 0.007	0.10a ± 0.009	0.12a ± 0.001	0.13a ± 0.005	0.14a ± 0.007	0.13a ± 0.003	0.14a ± 0.007	0.12a ± 0.002	p < 0.01
GST-Gill	0.20b ± 0.012	0.30c ± 0.01	0.13a ± 0.007	0.14a ± 0.013	0.13a ± 0.005	0.13a ± 0.005	0.12a ± 0.007	0.13a ± 0.009	0.12a ± 0.007	0.14a ± 0.009	p < 0.01
GST-Brain	0.15b ± 0.008	0.24c ± 0.016	0.11a ± 0.007	0.12a ± 0.011	0.11a ± 0.007	0.11a ± 0.007	0.11a ± 0.013	0.13a ± 0.016	0.11a ± 0.013	0.12a ± 0.012	p < 0.01
GST-Kidney	0.20b ± 0.018	0.33c ± 0.021	0.13a ± 0.010	0.12a ± 0.013	0.14a ± 0.018	0.14a ± 0.018	0.14a ± 0.014	0.14a ± 0.014	0.13a ± 0.012	0.11a ± 0.015	p < 0.01
GPx-Liver	6.79b ± 0.66	11.39c ± 0.41	3.38a ± 0.62	4.09a ± 0.53	3.83a ± 0.33	3.83a ± 0.33	4.11a ± 0.56	4.11a ± 0.56	4.47a ± 0.62	3.91a ± 0.52	p < 0.01
GPx-Gill	9.36b ± 1.06	14.91c ± 0.56	5.91a ± 0.96	6.29a ± 0.32	6.91a ± 0.52	6.91a ± 0.52	6.44a ± 0.56	6.44a ± 0.56	6.25a ± 0.52	6.66a ± 0.44	p < 0.01
GPx-Brain	6.86b ± 0.69	11.87c ± 0.12	4.03a ± 0.18	4.58a ± 0.58	4.07a ± 0.67	4.07a ± 0.67	4.20a ± 0.36	4.20a ± 0.36	4.22a ± 0.30	4.29a ± 0.86	p < 0.01
GPx-Kidney	10.87b ± 1.45	16.85c ± 0.75	5.25a ± 0.82	5.97a ± 0.36	5.08a ± 0.49	5.08a ± 0.49	5.77a ± 0.34	5.77a ± 0.34	6.14a ± 0.45	5.89a ± 0.57	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). GST and GPx: units/mg protein.

control diet. Moreover, the SOD activity in the liver was significantly lowered (p < 0.01) upon supplementation with Se-NPs and RF at LTMin, but the SOD activity in the gill was significantly reduced upon supplementation with Se-NPs @ 0.5 mg/kg diet and RF @ 5 or 15 mg/kg diet without stressors and in the control group compared to all the other groups. In the case of supplementation with Se-NPs and RF @ 10 or 15 mg/kg diet with concurrent exposure to As and high temperature (p < 0.05), the highest SOD activity was observed at LTMin. The SOD activity in the brain was significantly elevated (p < 0.05) with the group subjected to concurrent exposure to As and high temperature and fed the control diet in comparison to the supplemented and control groups at LTMin (Table 2). Furthermore, the SOD activity determined in the liver was significantly lowered (p < 0.01) upon supplementation with Se-NPs @ 0.5 mg/kg diet and RF @ 5 mg/kg diet without arsenic and high-temperature treatment at LTMax. The highest SOD activity was measured (p < 0.05) in the group concurrently exposed to As and high temperature and fed the control diet followed by supplementation with Se-NPs @ 0.5 mg/kg diet and RF @ 5 mg/kg diet and exposure to multiple stressors at LTMax. The difference in SOD activity in the gill, kidney, and brain was not significant (p > 0.05) among the groups at LTMax (Table 3). The activities of GST and GPx in the liver, gill, kidney, and brain were noticeably reduced (p < 0.01) upon supplementation with Se-NPs and RF @ 5, 10 or 15 mg/kg diet with or without stressors at LTMin and LTMax. Furthermore, the highest activities of GST and GPx in the liver, gill, brain, and kidney were observed (p < 0.01) in the group that was concurrently exposed to As and high temperature and fed the control diet at LTMin and LTMax (Tables 4 and 5).

3.3. Neurotransmitter enzyme

The activity of the neurotransmitter enzyme AChE in the brain of *P. hypophthalmus* fed via supplementation with Se-NPs and RF reared under multiple stressors (As and high temperature) is illustrated in Fig. 3. The brain AChE activity was markedly inhibited (p < 0.01) with concurrent exposure to arsenic and high temperature at LTMin and LTMax. The recovery of the neurotransmitter enzyme (AChE) was noticeably (p < 0.01) enhanced upon supplementation with Se-NPs @ 0.5 mg/kg and RF @ 5, 10 or 15 mg/kg diet with or without stressors (As and high temperature).

3.4. Arsenic and selenium concentrations

The arsenic concentration in muscle and experimental water and selenium concentration in muscle and experimental diet were determined and are presented in Fig. 4. The concentration of arsenic in muscle varied from 0.05 to 0.12 mg/kg (ppm) of muscle, whereas in experimental water, the concentration varied from 5.4 to 797 µg/L (ppb). Similarly, the concentration of selenium in muscle varied from 0.35 to 1 mg/kg, and in feed, the concentration varied from 0.56 to 1.53 mg/kg.

4. Discussion

Temperature is a key indicator for the maintenance of homeostasis in aquatic organisms, especially fishes, which are poikilotherms. Temperature and water contamination are two prime factors that reduce the thermal efficiency of aquatic animals, including fishes (Kumar et al., 2017d). The thermal efficiency (tolerance) of fishes depends on the water quality, fish species, migration, age of the fish, feeding habits and, most importantly, nutrition in the form of feed (Kumar et al., 2016a, 2017a; 2018a). In the present investigation, we attempted to investigate dietary approaches such as Se-NP and RF supplementation to enhance the thermal efficiency (tolerance) of fish exposed to arsenic (As) and high-temperature stress. In our previous study, we claimed that Se-NPs @ 1 and 2 mg/kg diet play an important role in enhancing

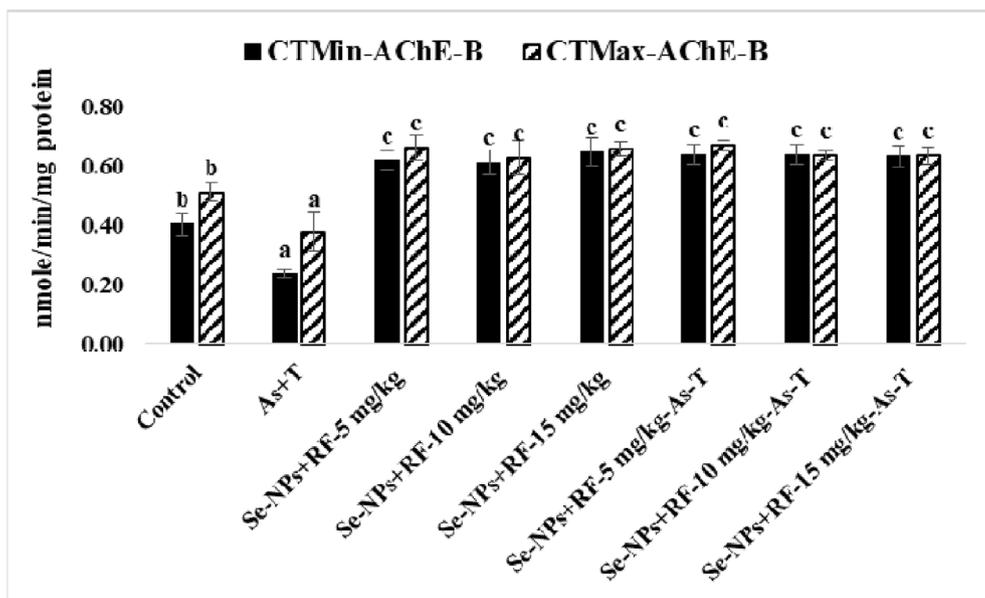


Fig. 3. Effect of combitorial mixture of dietary selenium-nanoparticles (Se-NPs) and Riboflavin on acetylcholine esterase (AChE) during lethal thermal minima (LTmin) and lethal thermal maxima (LTmax) of *P. hypophthalmus* under concurrent exposure to arsenic and elevated temperature during LTmin and LTMax. Values are expressed as mean \pm SE, n = 3. Bars bearing different letters (a, b, c) indicate significant differences.

the thermal tolerance of fish (Kumar et al., 2017b, 2018b); however, the present study describes the effect of a mixture of Se-NPs @ 0.5 mg/kg diet and RF @ 5, 10 or 15 mg/kg diet. Hence, this investigation is the first to claim that a mixture of Se-NPs @ 5 mg/kg diet and RF @ 5, 10 or 15 mg/kg diet has the ability to enhance the thermal tolerance of *P. hypophthalmus*. The CTMax and LTMax were markedly elevated in the group subjected to supplementation with Se-NPs @ 0.5 mg/kg diet and RF @ 5, 10 or 15 mg/kg diet with concurrent exposure to As and high temperature, followed by the control group and the group that was subjected to concurrent exposure to As and high temperature. The results revealed that pre-exposure to temperature in aquatic animals

(fish) leads to increased thermal efficiency in terms of CTMax and LTMax, which is consistent with other reports (Das et al., 2004; Debnath et al., 2006; Dalvi et al., 2009; Kumar et al., 2014a, 2017a; 2017b; Zhou et al., 2019). Aquatic animals (fishes) have a specific response to thermal acclimation, beyond which breakdown occurs, leading to the induction of stress, which hampers production by causing anorexia and disease outbreaks (Dalvi et al., 2009). This factor is directly correlated with pituitary function, such as thermoregulatory mechanism control by the pre-optic region of the anterior hypothalamus to the posterior hypothalamus, which is responsible for heat conservation and heat loss (Myers and Waller, 1973). In the present

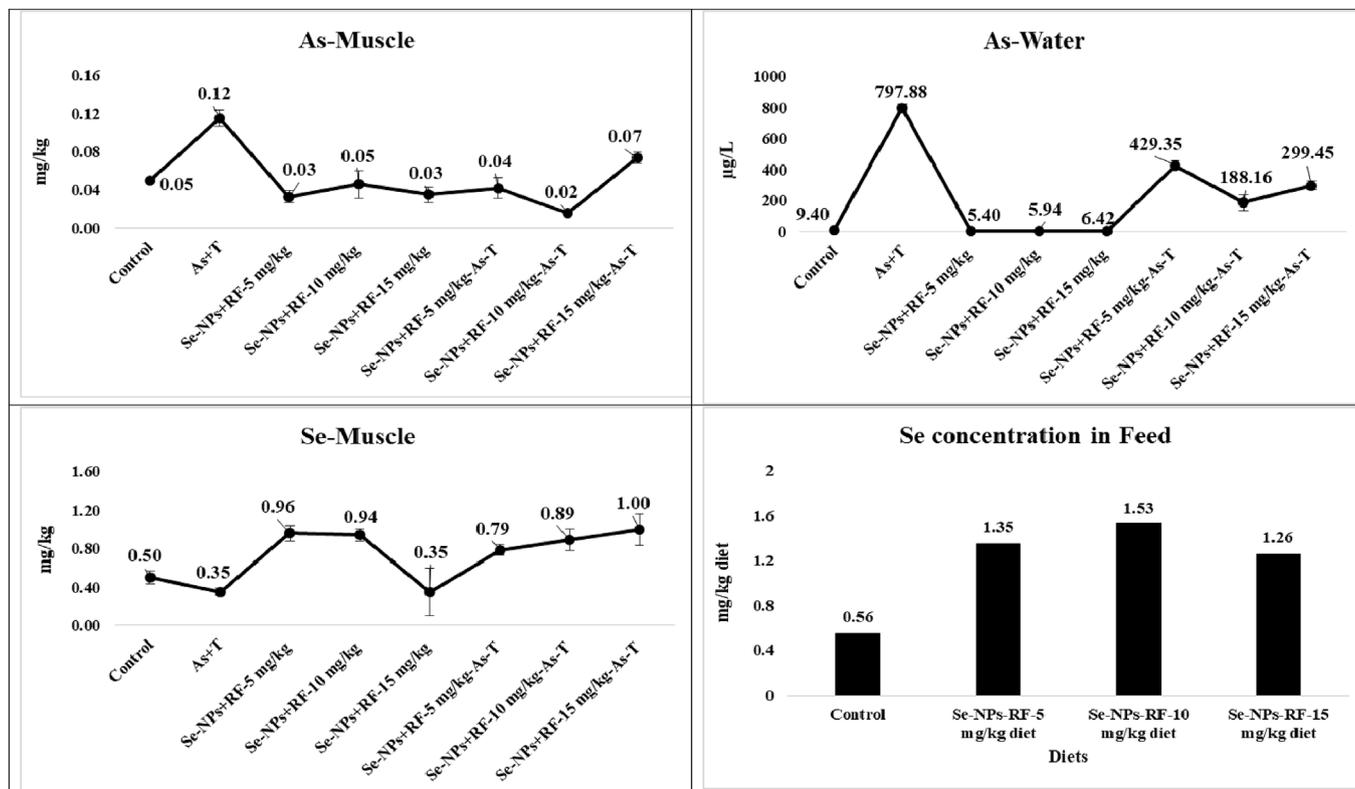


Fig. 4. Arsenic concentration in muscle and experimental water and selenium concentration in muscle and experimental diet.

investigation, supplementation with Se-NPs and RF led to enhancement of thermal tolerance, which might be due to the role of Se-NPs and RF in the thermal regulatory mechanism. This effect might also be due to the expression of heat shock proteins, which are important for the control and repair of cellular damage associated with protein denaturation at low or high temperatures (Nakano and Iwama, 2002). Moreover, Se also plays an important role in the formation of conjugate proteins, such as proteins containing selenocysteine and selenomethionine, to regulate GPx and thioredoxin reductase activities and prevent oxidative damage to body tissues (Rotruck et al., 1973). In the present investigation, feeding with Se-NPs and RF exhibited the potential to improve the thermal tolerance of the fish due to the role of RF in controlling thyroid hormones, which is important for thermal regulation in fishes. Very low temperatures can result in increased levels of circulating thyroid hormones, mitochondrial protein expression and cytochrome c oxidase (COX) in different tissues (Broeders et al., 2016; Zheng et al., 2010). Thyroid hormones play an important role in the regulation of metabolic function and allow the animal to adapt to different ambient temperatures (Decuyper et al., 2005; Liu et al., 2006; Zheng et al., 2013).

In the present investigation, the impact of combined exposure to arsenic and high temperature reduced the thermal tolerance of the fish and drastically altered the enzymatic activities associated with anti-oxidative status (Abele and Puntarulo, 2004; Portner, 2002a, 2002b) in the fish, namely, SOD, GST, and GPx activities, as contamination coupled with high-temperature stress plays a major role in the over-production of reactive oxygen species (ROS), which induce oxidative stress (Kumar et al., 2014a, 2016a, b). Moreover, the membrane fluidity and phospholipid bilayer dynamics are also affected, which leads to improper folding and the production of free radicals (Itri et al., 2014). Temperature and arsenic stress damage the cellular function and biomembrane permeability of DNA. Our previous study revealed that Pb toxicity can induce oxidative damage in animals and humans through the cell membrane, interactions between lead and haemoglobin, auto-oxidation of haemoglobin and auto-oxidation of δ -aminolevulinic acid (Ercal et al., 2001). The combinatorial nutritional supplements, consisting of Se-NPs @ 0.5 mg/kg diet and RF @ 5, 10 or 15 mg/kg diet, reduced the oxidative stress. This effect might be due to the anti-oxidant potential of Se through selenocysteine, which supports the neutralization of ROS through anti-oxidative enzymes such as CAT, SOD, GST and GPx (Reeves and Hoffmann, 2009). The other RF nutrients that play a key role in coenzymes, such as FMN and FAD, might have participated in the various oxidation-reduction reactions essential for all animals, including fishes (Deng and Wilson, 2003).

The neurotransmitter enzyme acetylcholine esterase (AChE) was inhibited upon exposure to arsenic and high temperature, which might be correlated with the adverse effect on neurons and the cranial system mediated through the toxin produced by arsenic contamination. AChE is directly linked to the mode of action of the toxin, and if the inhibition reaches up to 70–80%, mortality of the fish is inevitable. Arsenic is a highly toxic chemical that binds to protein functional groups, such as imidazole, sulfhydryl, and carboxyl (Najimi et al., 1997). In the present investigation, supplementation with Se-NPs and RF led to improvement in AChE activity essentially due to the role of Se in the central nervous system, as selenoproteins mostly appear in the brain cortical and hippocampal neurons (Roth et al., 2010; Zhang et al., 2008). Selenoproteins also play an important role in controlling the brain motor mechanism, coordination, memory, cognition and diverse functions of the CNS.

We also determined the arsenic concentration in fish muscle and experimental water. The arsenic content was elevated in fish muscle (0.12 mg/kg) in the group fed the control diet and concurrently exposed to arsenic and high temperature, while the supplemented group exhibited a decreased arsenic concentration. This finding showed that Se-NPs and RF can detoxify the contaminant inside the body. Similarly, the Se concentration was highest in the supplemented group.

5. Conclusion

Overall, the results showed that the thermal efficiency/tolerance of *P. hypophthalmus* depends upon the acclimation temperature and contamination exposure (arsenic contamination). RF @ 5 mg/kg diet with Se-NPs @ 0.5 mg/kg diet has the potential to enhance thermal efficiency/tolerance and improve the biochemical attributes against multiple stress factors (thermal stress and contamination). Therefore, RF @ 5 mg/kg diet with Se-NPs @ 0.5 mg/kg diet is appropriate for the enhancement of thermal tolerance and reduced cellular metabolic stress in *P. hypophthalmus*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jtherbio.2019.102417>.

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