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Original Article

Change in redox state and heat shock protein expression in an Indian major carp *Cirrhinus cirrhosus* exposed to zinc and lead

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ABSTRACT — Fish are exposed to different heavy metals that may induce numerous physiological changes. In the present study, we examined the redox state in response to a severe stress resulting from two heavy metals (Zinc and Lead) contamination in carp *Cirrhinus cirrhosus*. Fish were exposed to 1/10th of LC50 of the respective metals [zinc chloride (2.72 mg/L) and lead acetate (2.53 mg/L)] for 30 days and allowed to recover for another 30 days without any metal exposure. Concentration of metals, different enzymatic and non-enzymatic antioxidant agents and expression levels of heat shock protein (HSP) 70 and 90 were measured in the liver and the kidney of fish. The lipid peroxide levels in fish tissues gradually increased with duration of treatment for both metals. After 15 days of treatment, glutathione (GSH) levels had increased, but decreased as the treatment continued for 30 days and returned to basal levels after a 30-day recovery period. Activities of all the anti-oxidant enzymes, except glutathione peroxidase, in stressed fish were significantly increased compared to those in the control at 15 days and continued till the 30th day of treatment, showing a tendency to return to basal levels after the recovery period. Expression levels of HSP70 and HSP90 gradually increased after zinc and lead treatment, respectively. The expression of HSP was higher in the liver. The results suggest that different heavy metals may have differential effects on the redox state and induction of oxidative stress in carp, *in vivo*.

Key words: Heat shock protein, Antioxidant enzymes, Zinc, Lead, *Cirrhinus cirrhosus*

INTRODUCTION

Aquatic organisms are often exposed to an acute change in the heavy metal composition of the environment (Nakano, 2007; Prunet *et al*., 2012). Exposure of fish to such an environment might result in a sequence of biochemical as well as physiological alteration at tissue and cellular levels. Such changes are generally regulated by the variation in the neuro-endocrine system. In addition to neuro-endocrine stress response in the organismal levels, there occurs a cellular stress response following exposure to different heavy metals. These stress responses in organisms might affect their general health status, disease resistance, growth and reproductive activity (Pickering *et al*., 1993; Prunet *et al*., 2012).

Fish growth is known to be genetically regulated but is also affected by both endocrinal and environmental factors. The responses of endocrine secretions are severely affected by the combination of external stimuli with internal cellular signals according to the physiological status of the organism (Nakano, 2011; Deane and Woo, 2009). Fish growth and reproduction could be improved by superior nutrition quality, appropriate aquatic system, availability of physical and biotic factors and finally alteration in the endocrine response of the particular animal (Reinecke, 2010). Accordingly, the major focus of attention should be to decide the effects of stressors such as heavy metals on the expressions of different reproductive and growth-related genes in aquatic animals. Moreover, the functional mechanism and effects of different heavy metals on fish fitness must be studied in order to develop the production rate and health status of the fish. Besides, fish are thought to be an ideal and convenient model to examine the effects of heavy metal contamina-

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tion and other complex stressors on the organism for both short and longer periods. It has been observed that there is a difference in the functional aspect between essential metals such as zinc and non-essential metals such as lead (Serafim *et al*., 2012). However, limited reports are available regarding such functional differences between essential and non-essential metals in fish. The present study aims to identify the differences in response against essential metal zinc and non-essential metal lead on fish.

Elevated amount of heavy metals could be severely harmful due to variation in intracellular protein machinery either directly by denaturation of enzymes and/or indirectly due to production of high amount reactive oxygen species (ROS) (Pourahmad and O'Brien, 2000; Dröge, 2002). ROS might activate the apoptotic pathway and thereby increase cytotoxicity in several tissues. The majority of recent reports have clearly indicated the role of ROS in heavy metal-induced apoptotic pathways in different cell types, including hepatocytes (Ma *et al*., 1998; Pourahmad and O'Brien, 2000; Zhai *et al*., 2000).

Heat shock proteins (HSPs), a family of molecular chaperones, are one of the most important regulators of stress physiology in cellular levels and play a vital role in defending cells from hepatotoxic trauma (Salminen *et al*., 1996), including metal toxicity (Ma *et al*., 1998). The most extensively reported member of the HSP family, particularly during toxic stress response, is the 70 kDa family of the proteins (HSP70) (Hightower, 1991). However, the amount of inducible HSP70 is increasingly synthesized in the stressed cells, especially after heavy metal exposure in fish, compared with their unstressed counterparts (Sanders *et al*., 1995; Boone and Vijayan, 2002). The enhanced levels of HSP70 accumulation in stressed cells shield the destruction of protein machinery, initiate the process of renaturation of denatured proteins, check protein aggregation, target the damaged proteins for breakdown and finally facilitate protein biosynthesis and translocation of new proteins (Feder and Hofmann, 1999; Kregel, 2002). Together, all these studies clearly highlight the significance of HSP stimulation in preventing toxicity in the hepatic tissues (Salminen *et al*., 1996), but the missing link has yet to be established between different fish tissues. HSP90 were generally used as biomarkers against metals and organic compounds' stress in marine diatom *Ditylum brightwellii*. Exposure to different types and doses of copper and nickel compounds was monitored, revealing stimulation of HSP90 gene expression; however the pattern of such stimulation varied (Mahmood *et al*., 2014).

In the present study, we examined the redox state in response to severe stress derived from two important heavy metal contaminations (zinc and lead) in an important edible Indian major carp (*Cirrhinus cirrhosus*). *C. cirrhosus* is one of the important species used in aquaculture worldwide and is known to be susceptible to increase in the level of heavy metal contamination (Ohya *et al*., 1989). The objective of the present study was to examine the effect of heavy metal exposure on HSP70 and HSP90 protein expression and anti-oxidant status in the liver and kidney tissues in carp. As ROS generation is the most important mediator of metal-induced toxicity and stress, our observation might suggest whether HSP response to zinc and lead is self-regulating of the redox status in carp tissues, thus indicating potential mechanism of detoxication in a carp species.

MATERIALS AND METHODS

Experimental design

C. cirrhosus (90-100 g weight and 19-20 cm length) irrespective of sex were collected from water bodies in and around Kolkata, India. Fishes were acclimatized for 15 days to laboratory conditions. The laboratory water was analyzed for different physico-chemical parameters such as temperature, pH, hardness, and salinity, and for concentrations of zinc and lead (APHA, 1999). The water quality parameters were as follows: temperature: 20 ± 1°C, pH: 7.25 ± 0.25, conductivity: 340.6 ± 16.4 µs/cm, total hardness: 135.5 ± 9.3 mg CaCO3/L, alkalinity: 40.7 ± 5.2 mg CaCO3/L. No fish mortality was recorded during acclimatization. The acclimatized fishes showing no signs of stress were selected and divided into three groups of eighteen each for exposure to each toxicant. The first group served as a control and other two groups were exposed to sublethal concentrations of zinc chloride and lead acetate. LC50 values for zinc chloride and lead acetate (Analytical grade, Qualigens) were statistically determined (Finney, 1971). A dose of 2.72 mg/L (1/10th of LC50) of zinc chloride and a dose of 2.53 mg/L (1/10th of LC50) of lead acetate were administered to experimental groups daily for 30 days. The experiment was carried out in triplicate. The fishes were kept in 40-L glass aquaria with proper aeration and the water was renewed once after 24 hr replacing the test solution. After 15 days, 6 fish from each group were anesthetized with phenoxy-ethanol (1:20000, v/v), sacrificed and liver and kidney tissues were collected carefully from both treated and control group. Liver and kidney tissues were collected similarly from another 6 fish from each group after 30 days of treatment.

After completion of 30 days of experimentation, the remaining fish were transferred to fresh glass aquaria with

no trace of either heavy metal. Water quality parameters were maintained as before with proper aeration and the water was renewed regularly. After another 30 days, 6 fish from each group were anesthetized with phenoxyethanol (1:20000, v/v), sacrificed and liver and kidney tissues were collected carefully. All tissues were kept at -20°C till further use.

Estimation of heavy metals in fish tissues

In order to prepare biological samples for the determination of zinc (Zn) and lead (Pb) by AAS, a modified wet digestion procedure was followed (Chernoff, 1975). In brief, 1 g of each tissue was kept for overnight digestion in 5 mL of concentrated HNO_3. The samples were then filtered in Watmann grade 1 filter paper and the filtrate was diluted to 100 mL in volumetric flask and 5 mL conc. HCl was added and the mixture was placed in fume bath for 15 min. After that the samples were cooled, filtered by Millipore filter paper (Grade 0.45 μm) and the filtrates were digested by a proportionate mixture of concentrated HNO_3-$HClO_4$ and finally filtered in Watmann filter paper (grade-42) before detection of heavy metal concentration in AAS.

Sample preparation for antioxidative study

Fraction of each tissue was stored in ice cold phosphate buffer, homogenized and sonicated at 4°C in a homogenizing buffer (50 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA, 100 mM sucrose, 1 mM PMSF, and 1% leupeptin hemisulphate) to prepare 10% tissue homogenate, which was stored at -80°C until used for further study.

Electrophoresis and immunoblotting

The protein content of the samples was measured on 12.5% Laemmli SDS-PAGE and immunoblotted on the PVDF membrane following a wet electroblotting method (Moniruzzaman *et al*., 2016). The primary antibody (1:1000 dilution) was raised against a peptide corresponding to HSP70 / HSP90. The PVDF membranes were incubated separately with the primary antibody, followed by incubation with the respective secondary antibody (dilution 1:500). Densitometric scanning was performed to quantify individual band intensity of each immunoblot using ImageJ Software (Moniruzzaman *et al*., 2016).

Measurement of enzymatic and non-enzymatic antioxidants

Tissues were homogenized with Tris buffered saline (10 mM Tris-HCl, 0.1 mM EDTA-2Na, 10 mM sucrose, 0.8% NaCl, pH 7.4), centrifuged and supernatant was used to measure levels of different enzymatic and non-enzymatic antioxidants such as malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GRd), glutathione peroxidise (GPx), glutathione S-transferase (GST) according to standard methods described earlier (Moniruzzaman *et al*., 2016).

Statistical analysis

Means of intensity of different bands of the same protein was used to calculate the densitometric value of each immunoblot representing a specific protein. The respective band intensity value of each protein (i.e. HSP70 and HSP90) was expressed as relative densitometric units after normalization with the intensity of β-actin. Mean ± S.E. values of such data (n = 6) for respective immunoblots as well as for remaining variables were separately analyzed by one-way ANOVA. Where F values indicated significance, the means were compared by a post hoc multiple range test ($P < 0.05$).

Two separate principal component analyses (PCA) for all the antioxidant enzymes with HSP70 and HSP90 were done to understand the ordination. First, interspecific patterns of stress were examined using a PCA on the correlation matrix of standardized biochemical parameters. This analysis allows comparison of antioxidant parameters with signalling molecules (HSP) within a multivariate morphological space and identification of patterns of correlation among physiological variables. Eigen values of each component of the PCA were considered to interpret results.

RESULTS

Small concentrations of both Zn and Pb were found in liver and kidney tissues of control fish, possibly due to the presence of trace amount of the metals in tap water and fish feed. However, the metal concentration in feed was not measured during the present study. In control fish, the concentration of both the metals was observed to be higher in the liver than in the kidney (Table 1). The concentrations of Zn and Pb in both liver and kidney tissues increased significantly ($P < 0.05$) in metal-exposed fish compared to control fish. Zinc treatment for 30 days resulted in significant increase ($P < 0.05$) in Zn concentration in liver and kidney tissues compared to fish exposed to Zn for 15 days, while Pb treatment for 30 days resulted in significant increase ($P < 0.05$) in Pb concentration in only kidney tissues compared to fish exposed to Pb for 15 days (Table 1). The concentrations of Zn and Pb in both liver and kidney tissues were significantly reduced ($P < 0.05$) after the recovery period compared to that

Table 1. Zinc (Zn) and Lead (Pb) concentration (µg/g) in liver and kidney of *Cirrhinus cirrhosus* after 0, (control), 15 and 30 days of metal treatment, and after 30-day recovery period (n = 6).

TISSUE	DAYS	ZINC (Zn) [µg/g]	LEAD (Pb) [µg/g]
LIVER	CONTROL	0.06 ± 0.01^{gh}	0.05 ± 0.01^{gh}
	15 DAYS	0.45 ± 0.05^{bc}	0.60 ± 0.02^{ab}
	30 DAYS	0.62 ± 0.04^{a}	0.68 ± 0.02^{a}
	RECOVERY	0.19 ± 0.01^{ef}	0.21 ± 0.01^{ef}
KIDNEY	CONTROL	0.03 ± 0.01^{h}	0.03 ± 0.01^{fg}
	15 DAYS	0.40 ± 0.01^{cd}	0.50 ± 0.01^{bc}
	30 DAYS	0.53 ± 0.04^{ab}	0.74 ± 0.02^{a}
	RECOVERY	0.31 ± 0.01^{de}	0.28 ± 0.01^{de}

Note: The letters in superscript denote significant variation within columns ($P < 0.05$).

Table 2. Antioxidative enzymes activity (µ/mg protein/min) in liver and kidney of *Cirrhinus cirrhosus* after 0, (control), 15 and 30 days of Zinc (Zn) treatment, and after 30-day recovery period (n = 6).

TISSUE	DAYS	MDA	GSH	GST	GRd	GPx	SOD	CAT
LIVER	CONTROL	8.43 ± 0.78^{f}	53.06 ± 4.95^{bc}	29.10 ± 3.28^{ef}	10.40 ± 1.08^{ef}	12.96 ± 0.98^{abc}	18.20 ± 2.01^{ef}	18.56 ± 1.43^{def}
	15 DAYS	10.60 ± 0.91^{cde}	59.13 ± 4.94^{a}	32.53 ± 4.8^{bc}	13.30 ± 0.96^{ab}	13.56 ± 1.30^{ab}	22.73 ± 1.51^{ab}	22.56 ± 3.20^{a}
	30 DAYS	12.13 ± 1.01^{bcd}	48.73 ± 5.20^{e}	35.93 ± 2.2^{a}	14.40 ± 2.08^{a}	14.16 ± 1.06^{a}	24.76 ± 1.98^{a}	22.90 ± 1.66^{a}
	RECOVERY	10.53 ± 0.78^{de}	51.36 ± 6.40^{bcde}	34.13 ± 3.08^{ab}	12.33 ± 1.92^{bcd}	13.20 ± 0.88^{abc}	21.56 ± 2.11^{bc}	21.33 ± 2.03^{abc}
KIDNEY	CONTROL	9.19 ± 0.89^{ef}	49.66 ± 3.81^{de}	27.20 ± 4.30^{f}	9.21 ± 1.01^{fg}	11.03 ± 0.80^{e}	15.13 ± 2.02^{g}	15.13 ± 1.21^{g}
	15 DAYS	12.90 ± 0.97^{b}	53.70 ± 3.47^{a}	29.00 ± 2.70^{ef}	11.60 ± 2.40^{cde}	12.80 ± 1.03^{abcd}	18.40 ± 0.95^{fg}	19.23 ± 1.08^{def}
	30 DAYS	14.53 ± 1.86^{a}	45.50 ± 3.95^{f}	32.46 ± 2.90^{bc}	12.86 ± 1.81^{abc}	13.60 ± 0.95^{ab}	20.80 ± 1.08^{bcd}	19.83 ± 1.44^{cde}
	RECOVERY	13.06 ± 0.94^{ab}	50.10 ± 2.89^{cde}	31.46 ± 3.40^{cd}	12.20 ± 0.98^{bcd}	11.93 ± 1.07^{bcde}	18.80 ± 2.14^{de}	18.20 ± 1.33^{efg}

Notes: SOD – Super Oxide Dismutase (u/mg protein/min), CAT – Catalase (u/mg protein/min), GPx – Glutathione Peroxidase (u/mg protein/min), GST – Glutathione S-transferase (u/mg protein/min), GRd – Glutathione reductase (u/mg protein/min), GSH – Reduced Glutathione (u/mg protein/min), MDA – Malondialdehyde (u/mg protein/min). The letters in superscript denote significant variation within columns ($P < 0.05$).

Table 3. Antioxidative enzymes activity (µ/mg protein/min) in liver and kidney of *Cirrhinus cirrhosus* after 0, (control), 15 and 30 days of Lead (Pb) treatment, and after 30-day recovery period (n = 6).

TISSUE	DAYS	MDA	GSH	GST	GRd	GPx	SOD	CAT
LIVER	CONTROL	8.45 ± 0.60^{f}	53.04 ± 4.12^{bc}	29.12 ± 3.66^{d}	10.32 ± 073^{ef}	12.85 ± 1.05^{cd}	18.14 ± 2.20^{c}	18.49 ± 0.92^{bcd}
	15 DAYS	11.43 ± 0.90^{e}	59.36 ± 3.80^{a}	34.20 ± 2.50^{bc}	15.43 ± 2.07^{ab}	13.66 ± 0.90^{bc}	24.06 ± 2.01^{a}	24.90 ± 1.20^{a}
	30 DAYS	13.00 ± 0.81^{abc}	48.13 ± 3.11^{de}	37.93 ± 3.03^{a}	16.13 ± 1.40^{a}	16.50 ± 1.40^{a}	24.80 ± 1.33^{a}	24.39 ± 1.82^{a}
	RECOVERY	11.80 ± 1.02^{cde}	51.70 ± 5.50^{bcd}	37.46 ± 3.70^{ab}	14.00 ± 0.97^{a}	15.86 ± 1.10^{a}	23.60 ± 2.12^{a}	23.33 ± 1.05^{ab}
KIDNEY	CONTROL	9.26 ± 0.80^{f}	49.70 ± 3.29^{cde}	27.29 ± 1.95^{d}	9.26 ± 0.68^{fg}	11.10 ± 1.08^{de}	15.08 ± 2.07^{d}	15.30 ± 2.20^{cd}
	15 DAYS	12.76 ± 1.23^{abcd}	54.70 ± 6.08^{b}	33.10 ± 2.90^{c}	12.43 ± 0.88^{cd}	12.33 ± 0.90^{cde}	19.73 ± 0.96^{bc}	20.90 ± 1.50^{abc}
	30 DAYS	13.93 ± 0.95^{a}	46.40 ± 2.407^{e}	33.60 ± 4.22^{c}	12.63 ± 1.15^{cd}	12.50 ± 0.81^{cde}	20.80 ± 0.92^{b}	20.30 ± 1.10^{abc}
	RECOVERY	13.13 ± 1.61^{ab}	49.33 ± 5.10^{cde}	33.80 ± 4.16^{c}	12.00 ± 1.1^{de}	12.86 ± 1.41^{bcd}	19.23 ± 1.18^{bc}	19.00 ± 0.84^{bcd}

Notes: SOD – Super Oxide Dismutase (u/mg protein/min), CAT – Catalase (u/mg protein/min), GPx – Glutathione Peroxidase (u/mg protein/min), GST – Glutathione S-transferase (u/mg protein/min), GRd – Glutathione reductase (u/mg protein/min), GSH – Reduced Glutathione (u/mg protein/min), MDA – Malondialdehyde (u/mg protein/min). The letters in superscript denote significant variation within columns ($P < 0.05$).

in treated groups. However, Zn and Pb concentrations in both liver and kidney tissues of treated fish after the recovery period were significantly higher ($P < 0.05$) compared to control fish. In both liver and kidney tissues of fish exposed to metal for 15 and 30 days, Pb was found to be present at higher concentrations compared to Zn (Table 1).

MDA levels in fish gradually increased for both heavy metal treatments, and had increased significantly ($P < 0.05$) compared with control fish both at 15 and 30 days post-stress. The level of MDA was higher in the kidney than the liver. After 30 days, MDA level recovery was more evident in the liver than in the kidney, and the extent of recovery was more for Zn treatment in comparison to Pb (Tables 2, 3).

At 15 days post treatment, GSH levels increased sig-

nificantly ($P < 0.05$), but the levels decreased after 30 days. However, an increase was noted at the end of the recovery period, reaching basal levels similar to those in the control fish. GSH levels was found to be significantly higher ($P < 0.05$) in the liver followed by kidney both in control and treated fish (Tables 2, 3).

Activities of both GRd and GST showed significant increase ($P < 0.05$) after 15 days treatment, reaching the peak after 30 days of treatment and decreasing after the recovery period. Activities of both enzymes were found to be comparatively higher in the liver than in the kidney. Both GRd and GST showed higher activity in Pb-treated fish compared to those in Zn-treated fish (Tables 2, 3).

Both SOD and CAT activities were increased significantly ($P < 0.05$) at 15 days treatment and remained unchanged after 30 days of treatment for Zn and Pb. Activities of both enzymes were comparatively higher in Pb-treated fish compared to those in Zn treated fish. SOD activity was found to be significantly higher ($P < 0.05$) in liver tissue than that in kidney tissue for both Zn- and Pb-treated fish (Tables 2, 3).

During Zn treatment, no significant change ($P > 0.05$) was observed for GPx activity in the liver between control, 15-day, 30-day and recovery treatment groups. However, GPx activity in the kidney increased significantly ($P < 0.05$) in 15-day and 30-day Zn-treated fish compared to control (Table 2). Treatment with Pb for 30 days resulted in significant increase ($P < 0.05$) in GPx activity in the liver compared to control, but no significant change ($P > 0.05$) was observed for GPx activity in the kidney between control, 15-day, 30-day and recovery treatment groups for Pb treatment (Table 3).

The HSP90 expression level in the liver of control fish was found to be higher than that of HSP70, while in kidney tissue the expression level of HSP70 was higher (Figs. 1, 2). The expression level of HSP70 showed a gradual and significant increase ($P < 0.05$) in all the tissues with duration of treatment for Pb (Fig. 1). In Pb-treated fish, the HSP70 expression level was observed to be higher in the liver compared to the kidney (Fig. 1). Moreover, the expression level of HSP70 decreased significantly ($P < 0.05$) after the recovery period in liver tissue, while that in the kidney showed no significant differences ($P > 0.05$). The expression level of HSP90 in all tissues of fish increased with Pb treatment for 15 days, but further treatment and recovery period showed no significant change in HSP90 expression (Fig. 1). The expression level of HSP70 showed a significant increase ($P < 0.05$) in liver tissue with treatment duration for Zn treatment (Fig. 2). Treatment with Zn for 15 days was found to significantly increase ($P < 0.05$) the expression level of both HSP70 and HSP90 in kidney tissue, but further treatment and recovery period showed no significant change in HSP70 and HSP90 expression in that tissue (Fig. 2).

All the selected antioxidative enzymes (SOD, CAT, GST, GRd and GPx) showed significant amount of multicollinearity between the values (Figs. 3a and 3b). The stress marker (MDA) showed close correlation with both Pb and Zn (Figs. 3a and 3b). Non-enzymatic anti-oxidant GSH seemed to be correlated with none of the components. In Pb treatment, MDA and level of metal concentration were closely correlated with both HSP90 and HSP70. However, in Zn treatment, MDA and level of metal concentration were not closely correlated with HSPs, and instead remained in similar component with all the antioxidant enzymes (Figs. 3a and 3b).

DISCUSSION

Concentration of heavy metal in the aquatic environment may result in serious damage to a large range of fish species and cause hormonal and physiological stress in the body. The current study has indicated that heavy metal-induced stress might influence several redox-related biomarkers and the redox homeostasis in carp *C. cirrhosus*. Metal accumulation in various tissues in fish varies due to differential metabolic demand between the species. Moreover, such accumulation is often influenced by different environmental factors and the feeding habit, habitat, age, sex and body weight of fish (Authman, 2008). Several studies have indicated tissue-dependent bioaccumulation pattern of heavy metals as observed in the present study (El-Moselhy *et al*., 2014; Malik *et al*., 2014). Relative rates of metal binding and release determine metal accumulation in various tissues. Generally, accumulation of Pb in tissues is proportional to the ambient concentration (Tao *et al*., 1999) but no such report was found for Zn accumulation. The comparative higher amount of lead in fish tissues may be due to its higher solubility in water and corresponding absorption by the organism through the skin and gills (Table 1). Besides, lead does not have any kind of biological essentiality, while zinc is essential for different biological functions (Serafim *et al*., 2012). Moreover, organisms have better mechanism to regulate the concentration of essential metals like zinc but not properly for non-essential metals such as lead (Sanches Filho *et al*., 2017).

The extent of lipid peroxidation in fish tissues changes under various stressful conditions and is known to be a sensitive indicator of damage to various tissues under different environmental conditions (Ho *et al*., 2013; Verlecar

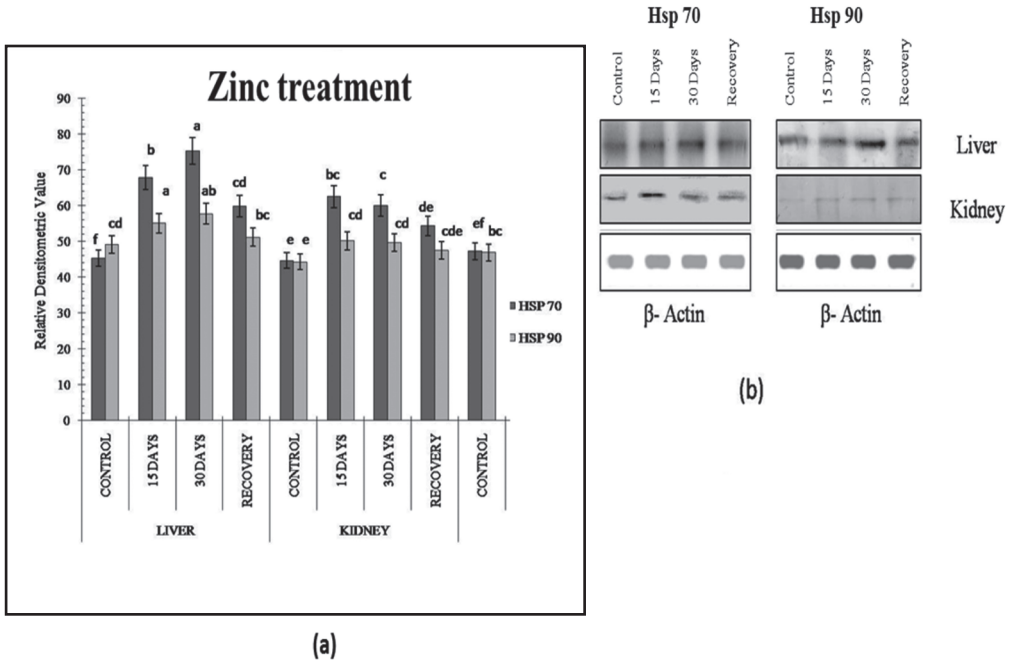

Fig. 1. Graph (a) and corresponding band pattern (b) showing intensity of HSP70 and HSP90 expression in duration-dependent Zinc (Zn) treatment and after recovery period in two different tissues of *Cirrhinus cirrhosus.* The letters over the graph denote significant variation ($P < 0.05$) in protein expression. β-actin was used as internal control, densitometric values are normalised.

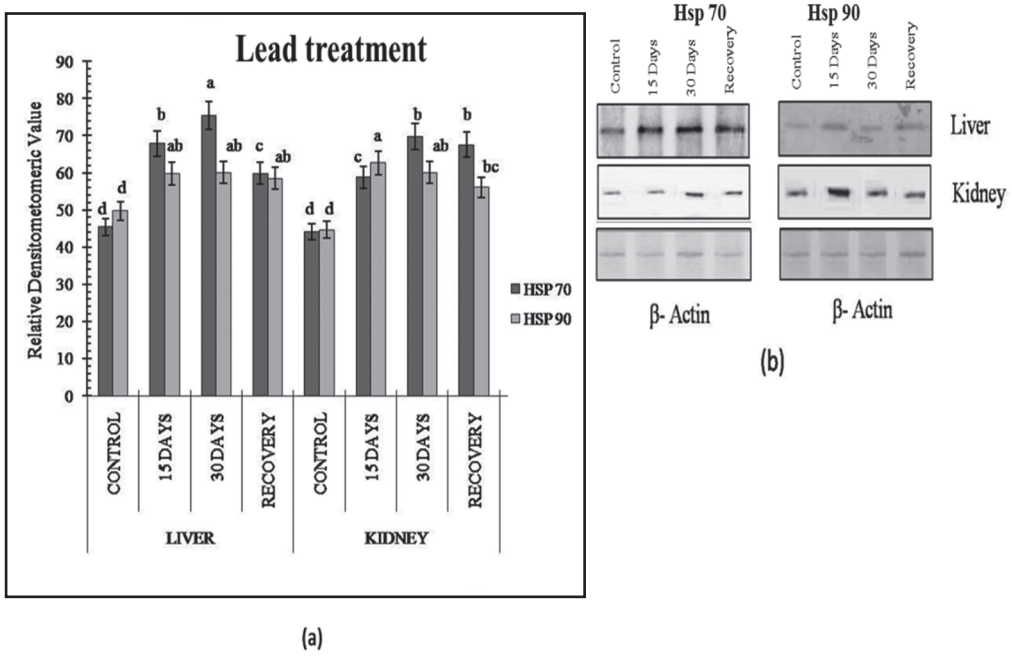

Fig. 2. Graph (a) and corresponding band pattern (b) showing intensity of HSP70 and HSP90 expression in duration-dependent Lead (Pb) treatment and after recovery period in two different tissues of *Cirrhinus cirrhosus.* The letters over the graph denote significant variation ($P < 0.05$) in protein expression. β-actin was used as internal control, densitometric values are normalised.

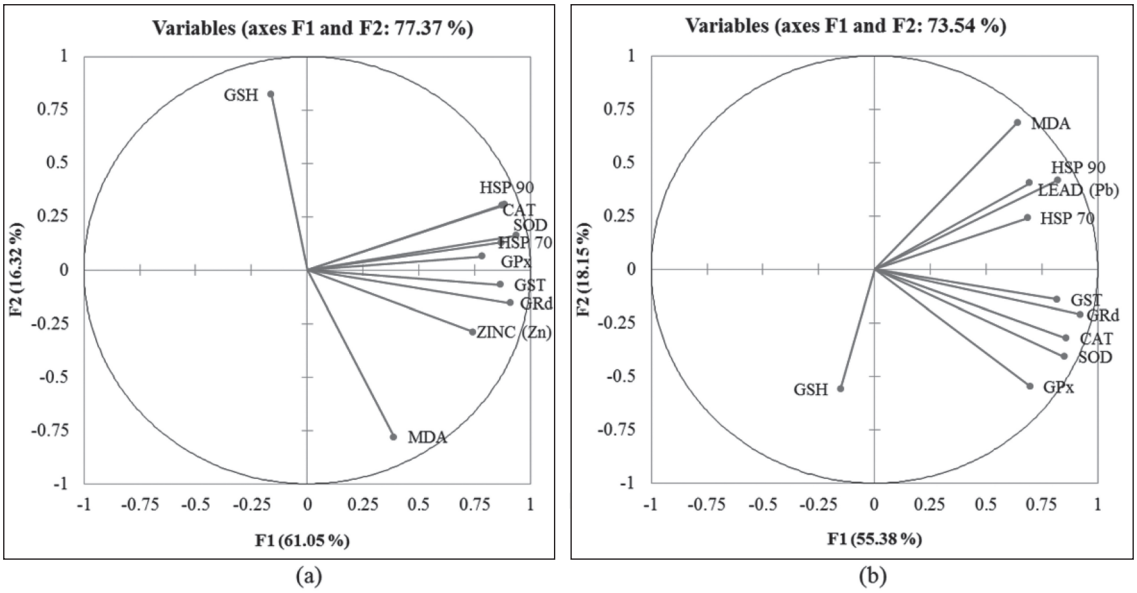

Fig. 3. Ordination diagram of PCA of heavy metals zinc (a) and lead (b) concentrations and all the enzymatic and non-enzymatic parameters and HSP70 and HSP90 measured in the liver and kidney of *Cirrhinus cirrhosus*.

et al., 2007). In this study, high and persistent MDA levels in the kidney tissues might be indicative of the better and quick defensive strategy of kidney tissue and the possible variation of time taken by different tissues to initiate the expression of their protective enzymes. The MDA level in Pb-treated tissues was higher due to the metal's higher accumulation in the carp tissues.

Fish have both enzymatic and non-enzymatic antioxidative defense systems against ROS-related damage caused by heavy metal toxicity (Lesser *et al*., 2006). GSH is one of the major regulators of the intracellular redox state and plays an important role in the non-enzymatic defense system (Arrigo *et al*., 1999). The antioxidative enzyme GPx, which scavenges ROS and lipid peroxides (LPO) generated within cells, uses GSH as substrate (Nakano *et al*., 1992). The changing patterns of GSH levels in all the tissues observed in this study were similar to those in the livers of fish affected by other stressors (Valavanidis *et al*., 2006). At the initial post-stress stage of 15 days, GSH levels increased which may be consumed to eliminate ROS generated in the tissues and decreased thereafter during 30 days post-treatment. After recovery, GSH levels gradually increased again, which suggested enhanced synthesis and transport of GSH in this period. The liver is known to be the major source of GSH in vertebrates; therefore, the response of GSH was most in the liver in the current experiment and it helped liver tissue to acclimatize earlier than the kidney (Ishikawa and Matsuda, 1988).

Stress has often been found to induce changes in the expression of antioxidative enzymes in fish (Moniruzzaman *et al*., 2016). In particular, SOD catalyzes the reaction of dismutation of superoxide to O_2 and H_2O_2, and is considered to play key roles in the first step of the enzymatic antioxidative defense system (Zelko *et al*., 2002). Activity of both SOD and CAT was found to be higher in Pb-treated carp suggesting that Pb has more adverse effect and it might cause more oxidative damage to the carp tissues. As Pb is non-essential environmental metal while Zn is an essential metal (Sanches Filho *et al*., 2017), Pb is capable of producing more superoxide molecule which is reflected though higher level of SOD and CAT activity. Hence, increased SOD and CAT expression might indicate the tendency to neutralize the harmful effects of superoxide radicals for the initial period of stressful conditions in tissues. Different responses of CAT and GPx in the current experiment may indicate diverse mechanisms for regulation of their differentiality in individual expression pattern at mRNA level. GPx is more involved in the elimination of organic peroxides and, only to a much lesser degree, hydrogen peroxide. Both GRd and GST showed higher activity in Pb-treated carp and increased with the duration of stress. However, GSH levels may be suppressed due to loss of adaptive mechanisms and the oxidation of GSH to oxidised glutathione (GSSG) because of a severe oxidative stress. This indicates their role in eliminating the xenobiotic substances causing oxidative damage to the carp tissues and in maintaining the

GSH/GSSG balance in the cells. Whenever the generation of GSSG becomes higher than its reduction back to GSH, GSSG accumulates and is translocated outside the cell to avoid NADPH exhaustion (Kaplowitz *et al*., 1996; Keppler *et al*., 1997). Low GRd activity means the production of GSSG is low and GPx activity is also low. Consequently, this is followed by a depletion of the GSH pool (Eroglu *et al*., 2015). GSSG levels changed along with GSH levels, which indicates a transformation trend from GSH to GSSG during the oxidative stress. Such a correlation is demonstrated here to some extent possibly by the changing trends of GSH/GSSG ratio. Therefore, our current findings confirm the previous hypothesis regarding the crucial role of heavy metals in regulating several oxidation-reduction reactions, resulting in the formation of secondary components such as ROS (Charrier *et al*., 2014).

Organisms maintain a balance between generation and neutralization of ROS under normal physiological conditions. However, stress exposure and enhanced oxygen consumption are considered to promote the generation of excess ROS, such as $O_2^{\cdot}$, H_2O_2, hydroxylradical ($^{\cdot}OH$), and peroxyl radical ($ROO^{\cdot}$). The resulting ROS exceed primary scavenging capacity of an organism and attack different cellular components, such as nucleic acids, cytoplasmic proteins, lipids, and membranes (Dröge, 2002; Valavanidis *et al*., 2006). ROS production in cells, especially in the mitochondria and peroxisome, has been found to be increased in exercised mammalian muscle, stressed bivalve gills, chicken muscles, lugworm and cultured cells as compared with non-stressed control tissue (Keller *et al*., 2004; Heise *et al*., 2003). Increase in oxidative process during high concentration of heavy metal or any other pollution related stress causes ROS accumulation, and cellular protection against such damage is achieved mainly by enzymes such as SOD, CAT, GPx or GRd (Lin *et al*., 2015; Das *et al*., 2017). Non-enzymatic antioxidants (GSH and MDA) play a major role in the regulation of cellular oxidation-reduction cycle and generation of ROS. Radical-scavenging antioxidants like GSH are utilized during the reactions with ROS, and antioxidant status could be used indirectly to assess the free radical activity (Lü *et al*., 2010). In the present study, efforts were made to measure individual antioxidants (e.g., Glutathione or MDA or antioxidant enzymes) in the tissue homogenates to evaluate the interrelation between the total antioxidant capacity and inference of metal accumulation in ROS production and oxidative stress conditions. The status of enzymatic or non-enzymatic antioxidants in the current study might indicate either the rate of ROS production or how long it would take for oxidation to cause the accumulation of ROS. Determination of antioxidative status per se is not the conclusive but an obvious indicator of the formation of ROS, though further experiment on free radical scavenging activity is needed to direct measurement of ROS accumulation and the oxidative damage (Hutcheson and Rocic, 2012). In the current study the lipid peroxide levels in fish tissues gradually increased with duration of treatment for both metals, indicating the generation of ROS. After short-term metal exposure GSH levels increased, but decreased as the treatment continued for 30 days. This indicates that the free radical scavenging activity of GSH decreased with increase in ROS accumulation. However, GSH levels returned to basal levels after 30 days of recovery, signifying a drop in the ROS level. Activities of all the antioxidant enzymes, except GPx, in stressed fish were significantly increased compared to those in control at 15 days and continued till 30 days of treatment, showing an inclination towards an increased ROS levels. Long-term effects of oxidative stress and ROS accumulation occur after antioxidant agents become low at 30 days of treatment and levels of free radicals become high. Thus, oxidative stress should be recognized as ROS imbalance which is ameliorated after the recovery period to prevent free radical-related tissue damage (Poljsak *et al*., 2013). Therefore, all these antioxidative biomarkers used in the current study can predict the state of the affected tissue by reflecting the ROS accumulation levels and the possible recovery time following metal induced oxidative damage. Current study strengthens the fact that diverse heavy metal toxicity implies altered degree of oxidative stress on different tissues in same species and persistency of toxicity also varies with defense mechanism of tissues.

The induction of various HSP families regarding environmental stressors, bacterial pathogens, and pollutants, has been reported in cell lines and various tissues of fish (Basu *et al*., 2002; Gabai *et al*., 1998; Iwama *et al*., 2006). In the current study, a higher magnitude of change was found in HSP70 expression levels in case of treatment with Pb and Zn treatment showed no such specific pattern of HSP expression. Such induction of different HSP for different metal exposure might indicate levels of protein damage, induced by stress due to heavy metal toxicity. The damage might vary for different exposure period and the response and recovery mechanism was diverse and case specific, as well. Increased expression of HSPs in the liver tissue might be responsible for the possible rapid recovery of these tissues than kidney. A redox state such as antioxidative state has already been reported to modulate the synthesis of HSPs as earlier observed in mammalian tissue (Jäättelä *et al*., 1998; Peng *et al*., 2000).

Thus, the current results regarding the expression patterns of multiple redox-related biomarkers in response to heavy metal contamination stressors suggest that severe pollution due to heavy metal contamination induces oxidative stress in carp, which may enhance oxidation in the body and result in damage to tissues. When exposed to oxidative stress, the levels of GSH, CAT and SOD may primarily increase because of their de novo synthesis to protect tissues against oxidative damage. The stress marker (MDA) showed very close correlation both with Pb and Zn (Figs. 1-2 and Tables 1-2), though non-enzymatic anti-oxidant GSH seemed to be correlated with none of the component factors, signifying the presence of an independent signalling mechanism of organism to maintain the internal GSH level up to a certain levels of stress tolerance. In Pb treatment, MDA and concentration of heavy metal were closely correlated with both HSP90 and HSP70. However, in Zn treatment, MDA and concentration of heavy metal were not closely correlated with HSPs, and instead remained in similar component with all the antioxidant enzymes.

Our result on HSP expression pattern clearly suggests the critical role played by both HSPs after heavy metal toxicity, but the mechanism of their induction or the time needed for induction might vary with the levels and specification of toxicity.

In conclusion, shifts in the oxidative status due to metal accumulation in different tissues may result in the up- or down- regulation of the expressions of several antioxidant genes and respective proteins and affects intercellular signalling process. Balance between the enzymatic and non-enzymatic antioxidants might play an important role in the tolerance against oxidative stress imposed by the metal accumulated stress in the kidney and hepatic tissue. The result of this study indicates the interrelation between the induction of HSPs and antioxidant factors in different tissues of carp in response to heavy metal toxicity. However, it is quite evident that HSPs act as molecular chaperones in the stress-affected tissues and activate the synthesis of the antioxidative proteins. Such molecular markers thereby might be of major interest against antioxidative stress in relation to the impact of pollution on natural aquatic ecosystems. Finally, it can be concluded that severe oxidative stress occurs due to metal induced ROS accumulation which ultimately leads to oxidative damage in the major metabolic organs of fish. However, a certain recovery period might overcome the moderate level of oxidative stress through modulation of cellular functions through the induction of HSP. Further studies will be needed to reveal the relationships between the redox state, oxidative stress, antioxidant factors and the fitness in fish affected by heavy metal contamination.

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Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Arrigo, A.P. (1999): Gene expression and the thiol redox state. Free Radic. Bio. Med., **27**, 936-944.

Authman, M.M.N. (2008): *Oreochromis niloticus* as a biomonitor of heavy metal pollution with emphasis on potential risk and relation to some biological aspects. Global Vet., **2**, 104-109.

APHA-AWWA-WPCF (1999): In standard methods for the examination of water and wastewater, 20th edn. American Public Health Association, Washington.

Basu, N., Todgham, A.E., Ackerman, P.A., Bibeau, M.R., Nakano, K., Schulte, P.M. and Iwama, G.K. (2002): Heat shock protein genes and their functional significance in fish. Gene, **295**, 173-183.

Boone, A.N. and Viajay, M.M. (2002): Constitutive heat shock protein 70 (HSC70) expression in rainbow trout hepatocytes: effect of heat shock and heavy metal exposure. Comp. Biochem. Physiol. C., **132**, 223-233.

Charrier, J.G., McFall, A.S., Richards-Henderson, N.K. and Anastasio, C. (2014): Hydrogen peroxide formation in a surrogate lung fluid by transition metals and quinones present in particulate matter. Environ. Sci. Technol., **48**, 7010-7017.

Chernoff, B. (1975): A method for wet digestion of fish tissue for heavy metal analyses. T. Am. Fish. Soc., **104**, 803-804.

Das, D., Moniruzzaman, M., Sarbajna, A. and Chakraborty, S.B. (2017): Effect of heavy metals on tissue-specific antioxidant response in Indian major carps. Environ. Sci. Pollut. Res., **24**, 18010-18024.

Deane, E.E. and Woo, N.Y.S. (2009): Modulation of fish growth hormone levels by salinity, temperature, pollutants and aquaculture related stress: a review. Rev. Fish Biol. Fish., **19**, 97-120.

Dröge, W. (2002): Free radicals in the physiological control of cell function. Physiol. Rev., **82**, 47-95.

El-Moselhy, Kh.M., Othman, A.I., El-Azem, H.Abd. and El-Metwally, M.E.A. (2014): Bioaccumulation of heavy metals in some tissues of fish in the Red Sea, Egypt. EJBAS, **1**, 97-105.

Eroglu, A., Dogan, Z., Kanak, E.G., Atli, G. and Canli, M. (2015): Effects of heavy metals (Cd, Cu, Cr, Pb, Zn) on fish glutathione metabolism. Environ. Sci. Pollut. Res., **22**, 3229-3237.

Feder, M.E. and Hofman, G.E. (1999): Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu. Rev. Physiol., **6**, 243-282.

Finney, D.J. (1971): Probit analysis 3rd ed. Chembridge University Press, London.

Gabai, V.L., Meriin, A.B., Yaglom, J.A., Volloch, V.Z., Sherman and M.Y. (1998): Role of hsp70 in regulation of stress-kinase JNK:

implications in apoptosis and aging. FEBS Lett., **438**, 1-4.
Heise, K., Puntarulo, S., Pörtner, H.O. and Abele, D. (2003): Production of reactive oxygen species by isolated mitochondria of the Antarctic bivalve *Laternula elliptica* (King and Broderip) under heat stress. Comp. Biochem. Physiol. C: Toxico. Pharma., **134**, 79-90.
Hightower, L.E. (1991): Heat shock, stress proteins, chaperones, and proteotoxicity. Cell, **66**, 191-197.
Ho, E., Karimi Galougahi, K., Liu, C.C., Bhindi, R. and Figtree, G.A. (2013): Biological markers of oxidative stress: applications to cardiovascular research and practice. Redox. Biol., **1**, 483-491.
Hutcheson, R. and Rocic, P. (2012): The metabolic syndrome, oxidative stress, environment, and cardiovascular disease: the great exploration. Exp Diabetes Res., **2012**, 271028.
Ishikawa, T. and Matsuda, Y. (1988): Transport of glutathione. Tanpakushitsu Kakusan Koso (Proteins, Nucleic Acids, and Enzymes). **33**, 1450-1458.
Iwama, G.K., Afonso, L.O.B. and Vijayan, M.M. (2006): Stress in fishes. In The Physiology of Fishes (Evans, D.H. and Claiborne, J.B. ed.), pp.319-342, CRC Press, Boca Raton.
Jäättelä, M., Wissing, D., Kokholm, K., Kallunki, T. and Egeblad, M. (1998): Hsp70 exerts its anti-apoptotic function downstream ofcaspase-3-like proteases. EMBO J., **17**, 6124-6134.
Kaplowitz, N., Fernández-Checa, J.C., Kannan, R., Garcia-Ruiz, C., Ookhtens, M. and Yi, J.R. (1996): GSH transporters: molecular characterization and role in GSH homeostasis. Biol.Chem. Hoppe-Seyler, **377**, 267-273.
Keller, M., Sommer, A.M., Pörtner, H.O. and Abele, D. (2004): Seasonality of energetic functioning and production of reactive oxygen species by lugworm (*Arenicola marina*) mitochondria exposed to acute temperature changes. J. Experi. Biol., **207**, 2529-2538.
Keppler, D., Leier, I. and Jedlitschky, G. (1997): Transport of glutathione conjugates and glucuronides by the multidrug resistance proteins MRP1 and MRP2. Biol. Chem., **378**, 787-791.
Kregel, K.C. (2002): Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. J. Appl. Physiol., **92**, 2177-2186.
Lesser, M.P. (2006): Oxidative stress in marine environments: biochemistry and physiological ecology. Annu. Rev. Physiol., **68**, 253-278.
Lin, H., Peng, Y.L., Chen, J. and Liang, L. (2015): Effect of Heavy Metal Stress on Antioxidase Enzymes. ICMSE, **2015**, 871-876.
Lü, J.M., Lin, P.H., Yao, Q. and Chen, C. (2010): Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. J. Cell. Mol. Med., **14**, 840-860.
Ma, Y., Cao, L., Kawabata, T., Yoshino, T., Yang, B.B. and Okada, S. (1998): Cupric nitrilotriacetate induces oxidative DNA damage and apoptosis in human leukemia HL-60 cells. Free Radic. Biol. Med., **25**, 568-575.
Mahmood, K., Jadoon, S., Mahmood, Q., Irshad, M. and Hussain, J. (2014): Synergistic effects of toxic elements on heat shock proteins. BioMed. Res. Int., **2014**, 564136.
Malik, R.N., Hashmi, M.Z. and Huma, Y. (2014): Heavy metal accumulation in edible fish species from Rawal Lake Reservoir, Pakistan. Environ. Sci. Pollut. Res., **21**, 1188-1196.
Moniruzzaman, M., Hasan, N.K. and Maitra, S.K. (2016): Melatonin actions on ovaprim (synthetic GnRH and domperidone)-induced oocyte maturation in carp. Reproduction, **151**, 285-296.
Nakano, T. (2011): Stress in fish. Aqua. Mag., **48**, 64-67.
Nakano, T. (2007): Microorganism. In dietary supplements for the health and quality of cultured fish. (Nakagawa, H., Sato, M. and Gatlin, D.M.III ed.), pp. 86-108, CAB Inter, Oxfordshire.
Nakano, T., Sato, M. and Takeuchi, M. (1992): Glutathione peroxidase of fish. J. Food Sci., **57**, 1116-1119.
Ohya, S., Shimizu, T., Horikawa Y. and Yamamoto, S. (1989): Relation between temperature of rearing water and numbers of dead coho salmon (*Oncorhynchus kisutch*). Bull. Fish. Lab. Kinki Univ., **3**, 73-77.
Peng, J., Jones, G.L. and Watson, K. (2000): Stress proteins as biomarkers of oxidative stress: effects of antioxidant supplements. Free Radic. Biol. Med., **28**, 1598-1606.
Pickering, A.D. (1993): Growth and stress in fish production. Aquaculture, **111**, 51-63.
Pourahmad, J. and O'brien, P.J. (2000): A comparison of hepatocyte cytotoxic mechanisms for Cu^{2+} and Cd^{2+}. Toxicology, **143**, 263-273.
Poljsak, B., Šuput, D. and Milisav, I. (2013): Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants. Oxid. Med. Cell. Longev., **2013**, 956792.
Prunet, P., Øverli, Ø., Douxfils, J., Bernardini, G., Kestemont, P. and Baron, D. (2012): Fish welfare and genomics. Fish Physiol. Biochem., **38**, 43-60.
Reinecke, M. (2010): Influences of the environment on the endocrine and paracrine fish growth hormone-insulin-like growth factor-I system. J. Fish Biol., **76**, 1233-1254.
Sanches Filho, P.J., Caldas, J.S., da Rosa, N.N. and Peres Pereira, F.O. (2017): Toxicity test and Cd, Cr, Pb and Zn bioccumulation in *Phalloceros caudimaculatus*. EJBAS., **4**, 206-211.
Sanders, B.M., Nguyen, J., Martin, L.S., Howe, S.R. and Coventry, S. (1995): Induction and subcellular localization of two major stress proteins in response to copper in the fathead minnow *Pimephales promelas*. Comp. Biochem. Physiol. C, **112**, 335-343.
Salminen, W.F.Jr., Voellmy, R. and Roberts, S.M. (1996): Induction of hsp 70 in HepG2 cells in response to hepatotoxicants. Toxicol. Appl. Pharmacol., **141**, 117-123.
Serafim, A., Company, R., Lopes, B., Rosa, J., Cavaco, A., Castela, G., Castela, E., Olea, N. and Bebianno, M.J. (2012): Assessment of Essential and Nonessential Metals and Different Metal Exposure Biomarkers in the Human Placenta in a Population from the South of Portugal. J. Toxicol. Environ. Health. A, **75**, 867-877.
Tao, S., Liang, T., Cao, J., Dawson, R.W. and Liu, C. (1999): Synergistic effect of Cu and Pb uptake by fish. Ecotoxicol. Environ. Saf., **44**, 190-195.
Valavanidis, A., Vlahogianni, T., Dassenakis, M. and Scoullos, M. (2006): Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicol. Environ. Saf., **64**, 178-189.
Verlecar, X.N., Jena, K.B. and Chainy, G.B.N. (2007): Biochemical markers of oxidative stress in *Perna viridis* exposed to mercury and temperatu.re. Chem. Biol. Interact., **167**, 219-226.
Zelko, I.N., Mariani, T.J. and Folz, R.J. (2002): Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radic. Biol. Med., **33**, 337-349.
Zhai, Q., Ji, H., Zheng, Z., Yu, X., Sun, L. and Liu, X. (2000): Copper induces apoptosis in BA/F3beta cells: Bax, reactive oxygen species, and NFκB are involved. J. Cell. Physiol., **184**, 161-170.