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RESEARCH ARTICLE

Effects of salinity stress on antioxidant status and inflammatory responses in females of a "Near Threatened" economically important fish species *Notopterus chitala*: a mechanistic approach

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Abstract

In the present study, acute stress responses of adult female *Notopterus chitala* were scrutinized by antioxidant status and inflammation reaction in the gill and liver at five different salinity exposures (0, 3, 6, 9, 12 ppt). Oxidative defense was assessed by determining superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase, and glutathione reductase activities, while malondialdehyde (MDA), glutathione, and xanthine oxidase levels were determined as indicators of oxidative load. Pro-inflammatory cytokines (IL-1β, IL-6, IL-10, and TNFα) and caspase 1 levels were also analyzed. Expression levels of transcription factors (NRF2 and NF-κB) and molecular chaperons (HSF, HSP70, and HSP90) were estimated to evaluate their relative contribution to overcome salinity stress. MDA showed a significant ($P < 0.05$) increase (gill, + 25.35–90.14%; liver, + 23.88–80.59%) with salinity; SOD (+ 13.72–45.09%) and CAT (+ 12.73–33.96%) exhibited a sharp increase until 9 ppt, followed by a decrease at the highest salinity (12 ppt) (gill, − 3.92%; liver, − 2.18%). Levels of cytokines were observed to increase (+ 52.8–127.42%) in a parallel pattern with increased salinity. HSP70 and HSP90 expressions were higher in gill tissues than those in liver tissues. NRF2 played pivotal role in reducing salinity-induced oxidative load in both the liver and gills. Serum cortisol and carbonic anhydrase were measured and noted to be significantly ($P < 0.05$) upregulated in salinity stressed groups. Gill Na^+-K^+-ATPase activity decreased significantly ($P < 0.05$) in fish exposed to 6, 9, and 12 ppt compared to control. Present study suggests that a hyperosmotic environment induces acute oxidative stress and inflammation, which in turn causes cellular death and impairs tissue functions in freshwater fish species such as *Notopterus chitala*.

Keywords *Notopterus chitala* · Salinity tolerance · Oxidative stress · Liver · Gill · Cytokines

Introduction

Salinity gradient has gradually been altered in the euryhaline freshwater ecosystems since last few decades (Sinha et al. 2015). Such salinity fluctuations are crucial, affecting fish physiology, reproductive ability, and abundance of different euryhaline freshwater fishes (Schlaff et al. 2014). Several studies have discussed different adaptive measures by the fish body system to adapt themselves with the fluctuating salinities at biochemical and molecular levels (Roessig et al. 2004; Sinha et al. 2015).

Non-enzymatic antioxidants such as malondialdehyde (MDA) and glutathione (GSH), and enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GRd), and glutathione S-tranferase (GST) have often been implicated as effective biomarkers of stress-induced physiological alterations in fish (Moniruzzaman et al. 2018). Studies on teleosts have indicated that stress caused by salinity fluctuations may be a reason for reduced antioxidant defense system (Choi et al. 2008; Yin et al. 2011). Due to the compromised defense system the fish body can no longer neutralise the increased reactive oxygen species (ROS) levels (Lushchak 2011).

Regulation and maintenance of ionic system in aquatic animals depend on numerous proteins such as Na^+/K^+-ATPase and carbonic anhydrase (CA), which regulate

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osmoregulation (Perry et al. 2003; Gilmour and Perry 2009; Pham and Van Phan. 2016). These enzymes are responsible for effective ion transport during osmoregulation (Hwang and Lee 2007; Blondeau-Bidet et al. 2019). CA is a Zn-metalloenzyme which is noted to catalyse the inter-conversion of carbon dioxide and bicarbonate through the reversible reaction, $CO_2 + H_2O \leftarrow \rightarrow H^+ + HCO_3^-$ (Gilmour et al. 2012). CA plays a pivotal role in maintaining pH balance, transporting ions, and gas exchange. Na^+/K^+-ATPase is involved in regulation of ions in freshwater and marine fishes.

Salinity-induced stress also influences immune response; it affects some immune parameters depending on exposure to fluctuating salinities (Gonçalves et al. 2007; Dhabhar 2008; Tort 2011; Schmitz et al. 2017). Along with innate immunity, cytokines and interleukins are also directly affected by salinity fluctuations (Abo-Al-Ela et al. 2017). Heat shock protein (HSP) expression is considered a vital indicator of salinity stress as well (Deane and Woo 2011). HSP expression occurs continuously in the cells, however, when exposed to stress, normal processes get disrupted, and the cells can increase the expression of HSPs for maintaining cellular homeostasis (Puntila-Dodd et al. 2021). Nuclear factor kappa B (NF-κB), nuclear factor E2-related factor 2 (NRF2) are also considered to be crucial factors that mediate mRNA expression of several proteins related to stress physiology including antioxidant proteins in the stressed cells (Kumar et al. 2021; Mukherjee et al. 2019). A few previous reports suggested that many of the antioxidant and inflammatory genes under influence of stress were upregulated by NRF2 to safeguard the cells from ROS-induced apoptosis (Zheng et al. 2016). NRF2, after entering into the nucleus, regulates different antioxidant gene expression (Kumar et al. 2021; Cuadrado et al. 2018). NF-κB plays an important role to regulate the pro-inflammatory and anti-inflammatory cytokines. Caspases play an elementary role in cell immunity as an inflammatory reaction architect. Caspase activation occurs when the cellular architecture is distorted during osmotic stress, carrying out cell death (Rathore et al. 2015). Cortisol mediates a series of physiological changes against different types of abiotic stress (Moniruzzaman et al. 2018).

Gills represent the most important osmoregulatory organ in fish ensuring osmotic homeostasis (Wilson and Laurent, 2002), while the liver is the main site where turnover of glucose occurs. Osmotic adaptation requires increased supply of metabolic fuel to major osmoregulatory organ like the gills for efficient maintenance of internal salt and water balance (Peragón et al. 1998). Thus, the gills and liver have been chosen as the organs of interest in the present study.

Notopterus chitala (Hamilton 1822) (Clown knifefish or featherback fish or commonly known as Chitala) is an economically important fish species having a wide market value as food fish. Besides, the fish is considered "Near Threatened" in the IUCN red list category (Prakash and Singh 2020). *Notopterus* is generally considered euryhaline freshwater species (salinity range varies between 0.01 and 3 ppt for growth and normal physiological activity). However, our earlier observation has indicated that the fish may frequently enter into stagnant brackish water, and survive and maintain its natural activity even in high saline condition (up to 10 ppt) (unpublished data). Thus, it is important to gain information regarding adaptive ability of the fish at fluctuating salinities both in freshwater and estuaries. The present work was designed to determine the effects of salinity on fish physiology involving modulation of antioxidants, gill marker proteins for osmoregulation, molecular chaperones, interleukins, transcription factors, and cortisol. An attempt had also been made to postulate the maximum tolerant salinity level for *Notopterus chitala*.

Materials and methods

Model species and acclimation

Adult female *Notopterus chitala* of the same cohort (weight: 93.4 ± 2.3 gm, length: 21.3 ± 2.4 cm) were collected from a hatchery located at Diamond Harbour, West Bengal, India (22° 11′30″ North, 88° 11′ 5″ East) (salinity: 0.07 ± 0.01; pH: 7.2 ± 0.2). Physiological alterations due to stress often depend on biological factors such as age, sex, and growth, and females of some fish species have been reported in few earlier studies to be more sensitive towards adverse effect of toxicity (Biales et al. 2011; Hansen et al. 2007). Hence, female fish of same age and size was taken for the present study as well. Female *N. chitala* is larger than male, and the abdomen is bulged externally and can be seen prominently disposed as compared to male fish (Chondar 1999; Mitra et al. 2018). During breeding season, adult male *N. chitala* shows diffused but bright red colour at the base of paired and anal fins, while female fish does not show any such marked coloration. Collected fish were first acclimatized for 14 days in aerated cistern (5000 lit) in the laboratory. During acclimatization period, fish were fed commercially available blood worms (Evox freeze dried tubifex worms) twice everyday (at around 11:00 h in the morning and at around 18:00 h in the evening). Water in the cistern was maintained at 28.0 ± 0.5 °C, pH 7.0–7.5, and dissolved oxygen 6.5–7.0 mg/l. Salinity of the acclimatization tank was 0 ppt and levels of ammonia (< 1 ppm), nitrate (< 20 ppm), and nitrite (< 0.1 ppm) were measured daily using aquarium test kits (Ammonium test kit Quantofix®—37,212; Nitrate Test Kit, Aquasol; Nitrite test kit, Bioxen). If levels exceeded the normal limits, 50% water was changed or else 25% of the water was changed every day.

Experimental design

After 14 days of acclimation, 150 healthy fish from the stocking cistern were randomly divided into five experimental categories. Fish in each experimental category was equally distributed in three aquaria (120 × 60 × 50 cm; 10 fish/aquarium). Fish in one category (control) was maintained at 0 (± 0.01) ppt (only tap water), while fish in rest four experimental categories were directly exposed to four different doses of salinity (3, 6, 9, and 12 ppt) for 7 days.

The salinity range selected in this study was based on the environmental salinities. Salinity of the freshwater habitats of *N. chitala* at South and South Western part of Sundarban estuary, West Bengal, may change abruptly due to saltwater intrusion from the Bay of Bengal, and it has recently been observed to increase up to 3–6 ppt in the monsoon and around 9–12 ppt in the dry season (Unpublished data).

The experiment was conducted in semi static water system (Banaee et al. 2015) and water was renewed daily. A stock saline solution of 12 ppt was prepared by dissolving artificial sea salt (SEA SALT, AQUAFOREST, India) in tap water and desired experimental salinity levels (± 0.1 PSU) were obtained by diluting the stock solution with tap water in respective aquaria. Salinity in each treatment aquarium was periodically checked with a digital salinity meter (EUTECH Salt 6 +, Thermo Fisher Scientific, India). During the entire experimental duration, both control and experimental fish were fed with commercially available blood worms (Evox freeze dried tubifex worms) twice everyday (at around 11:00 h in the morning and at around 18:00 h in the evening), and water in each aquarium was maintained at 28.0 ± 0.5 °C, pH 7.0–7.5, dissolved oxygen 6.5–7.0 mg/l, ammonia level < 1 ppm, nitrate level < 20 ppm, and nitrite level < 0.1 ppm. Fish in each aquarium was observed for swimming activity, and mortality was also recorded during the course of the experiment.

Preparation of sample for enzyme assay, immunoassay, and immunoblot analysis

After 7 days of salinity exposure, 18 fish from each treatment category (6 fish/aquarium) were first anesthetized with phenoxy-ethanol (1: 20,000, v/v) and the blood was collected from the caudal vein. Fish were then rapidly decapitated and quick dissection was followed for the collection of gill and liver tissues from each fish. Collected blood was allowed to clot, and the clot was removed by centrifugation at 5000 g for 10 min allowing for blood serum to be isolated. Serum and tissue samples were stored at − 20 °C for further analysis.

Tissue samples were processed for separation of cytosolic and nuclear protein fractions (Kirici et al. 2019). Tissues were first homogenized in lysis buffer [25 mM tris–HCl (pH 7.4), 0.15 M NaCl, 2.0 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycoltetraacetic acid, 23 µM leupeptin, 6.5 µM aprotinin, 1.5 µM pepstatin A, 5 µg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, 1 µM sodium orthovanadate] at a ratio of tissue: buffer = 1: 5. The homogenates were centrifuged at 20,000 g for 30 min at 4 °C. The supernatants were collected as the cytoplasmic fraction, while the pellets were resuspended in the same lysis buffer [with additional 0.5% sodium dodecylsulphate] followed by incubation for 45 min at 4 °C. The samples were then centrifuged at 20,000 g for 30 min at 4 °C, and the supernatants were collected as the nuclear fraction. Both cytoplasmic and nuclear fractions were then stored at − 20 °C for further analysis (Moniruzzaman et al. 2018; Kumar et al. 2021).

Measurement of antioxidants (non-enzymatic and enzymatic)

Non-enzymatic antioxidants Cytoplasmic fraction of gill and liver tissue homogenates of fish ($n = 18$) from each treatment category were used to quantify the level of malondialdehyde (MDA) equivalent, which was derived as a product of lipid peroxidation by TBARS (thiobarbituric acid reactive substances) assay (Draper and Hadley 1990) with slight modifications. Levels of reduced glutathione (GSH) were measured in gill and liver by using Ellman's reagent at 412 nm.

Enzymatic antioxidants Level of superoxide dismutase (SOD) was determined following a spectrophotometric method (Ewing and Janero 1995) based on the $O^{2.-}$ mediated NBT (nitro blue tetrazolium) reduction by a chemical mixture of PMS (phenazine methosulfate) and NADH. Superoxide radicals were generated in Tris–HCl buffer (16 mM, pH 8.0) containing NBT (50 µM) and NADH (78 µM) solution after sample extracts were mixed. PMS solution was added in order to start the reaction followed by incubation at 25 °C for 5 min. The absorbance was measured at 560 nm against the blank. Catalase (CAT) level was monitored at 240 nm up to 90 s at 15-s intervals (Aebi 1984). This assay was validated by treating the gill and liver tissue supernatants with sodium azide, an inhibitor of CAT activity (Aksoy et al. 2004). Glutathione reductase (GRd) activity was measured by assessing the oxidation of NADPH at 340 nm. The reaction mixture contained NADPH (0.15 mM), glutathione (0.5 mM), $MgCl_2$ (3 mM), Tris (pH 7.5), and tissue extract (Pinto and Bartley 1969). Corrections were made for NADPH oxidation in the absence of glutathione. Glutathione S-transferase (GST) activity was assessed spectrophotometrically (Habig et al. 1974) by using GSH (2.4 mM/l) and 1-chloro-2, 4-dinitrobenzene (CDNB, 1 mM/l) as substrate. One hundred-microliter sample was

added into 900 µl of assay cocktail (980 µl of PBS, pH 6.5, 100 mM CDNB, and 100 mM GSH). Phosphate buffer solution (pH 6.5) was used as a control and the absorbance was measured at 340 nm at a regular interval of 60 s for 5 min. Bradford assay kit (abcam, ab102535) was used for the purpose of protein concentration measurement in gill and liver tissues from all treatment groups. The assay protocol used an improved Coomassie blue G reagent which formed a blue complex in the presence of protein. The intensity of the blue complex is proportional to the amount of protein in the sample and it was measured by spectrophotometer at 595 nm.

Activity of gill enzymes

Na^+-K^+-ATPase activity was expressed as micromolars of Pi liberated per mg of protein per hour in the gill tissue of fish ($n = 18$) from each treatment category (Agrahari and Gopal 2008; Mukherjee et al. 2019). Carbonic anhydrase activity in gill tissues was measured using carbonic anhydrase activity assay kit (Biovision; Catalog # K472-100). Samples were mixed with acetazolamide (a CA inhibitor) and OD was obtained at 405 nm. Xanthine oxidase (XO) activity in gill and liver tissues was determined using the standard method of Westerfeld and Richert (1949). Spectrophotometric determination of XO activity was based on determining uric acid production from xanthine or hypoxanthine substrate at around 295 nm.

Immunoassay of cytokines (IL-1β, IL-6, TNFα, and IL-10)

Serum samples of fish ($n = 18$) from each treatment category were used for the measurement of cytokines (IL-1β, IL-6, TNFα, and IL-10) by using standard ELISA (enzyme-linked immunosorbent assay) kit (MyBioSource; Catalog#MBS700230, Catalog#MBS704369, Catalog#MBS175904, and Catalog#MBS282130, respectively). These are ready-to-use microwell, strip plate ELISA kit for analyzing the presence of the cytokines. The kit targets analytes in biological samples. The ELISA analytical biochemical technique of the kit is based on cytokine antibody-cytokine antigen interactions (immunosorbency) and an HRP colorimetric detection system to detect respective cytokine antigen targets in samples. The absorbance was measured by using Tecan-Spectra automatic Microplate reader.

Protein expression analysis

Expression of cytosolic HSF, HSP70, HSP90, and nuclear NF-κB and NRF2 in liver and gill tissues of fish ($n = 18$) from each treatment category were assessed through 12.5% Laemmli SDS-PAGE. A wet electro blotting system (BIO-RAD Trans-Blot) was followed for the immunoblot. Relevant primary Ab (MyBioSource) were used at a standard dilution of 1:1000 whereas secondary at 1:500. Immunoblots were quantified following densitometric method after comparing with the internal control (β-actin) (Moniruzzaman et al. 2018).

Immunoassay of cortisol (SH)

Serum cortisol level in fish ($n = 18$) from each treatment category was measured by standard ELISA method using kit (MyBiosource; Catalog#MBS704055). It is a ready-to-use microwell, strip plate ELISA kit for analyzing the presence of the Cortisol. The kit targets analytes in the biological samples. The concentration gradients of the kit standards or positive controls render a theoretical kit detection range in biological research samples containing cortisol. The ELISA analytical biochemical technique of the MBS704055 kit is based on cortisol antibody-cortisol antigen interactions (immunosorbency) and an HRP colorimetric detection system to detect cortisol antigen targets in samples. (Moniruzzaman et al. 2020).

Statistical analysis

Level of antioxidants, gill enzymes, cytokines, and cortisol and expression of each protein blot in fish were evaluated by univariate ANOVA using SPSS (version 20.0). Significance was calculated at 5% level of $Pr > F$ values.

Results

Behaviour and mortality

Swimming activity was observed to be reduced in fish exposed to 12 ppt salinity. Exposure to 12 ppt salinity also resulted in mortality and a total of 8 fish were found dead after 7 days in this treatment category. No mortality was observed in other treatment categories.

Non-enzymatic and enzymatic antioxidants

MDA level in both gill and liver tissues increased significantly ($P < 0.05$) (+ 25.35–90.14% in gill and + 23.88–80.59% in liver) at all salinity doses than that in control. GSH level in gill and liver tissues increased significantly ($P < 0.05$) (+ 14.55–37.09% in gill and + 11.76–37.74% in liver) from the control with increased salinity doses until 9 ppt but decreased significantly ($P < 0.05$) from there (− 3.92% in gill and − 2.18% in liver) at 12 ppt. Similar pattern was observed for SOD (+ 13.72–45.09%) and CAT (+ 12.73–33.96%) activities in gill tissues. In liver tissues, however, SOD (+ 12.98–50.48%)

and CAT (+15.11–48.44%) activities increased significantly ($P<0.05$) until 9 ppt but no significant changes were noted at 12 ppt. In gill and liver tissues GRd activity increased significantly ($P<0.05$) until 9 ppt but no significant changes were noted at 12 ppt. In both the tissues, GST activity increased significantly ($P<0.05$) from 3–9 ppt while decreased significantly ($P<0.05$) at 12 ppt (Fig. 1).

Protein expression analysis

HSP70 (gill, + 17.4–83.58%; liver, + 13.6–70.42%), HSP90 (gill, + 7.15–76.95%; liver, + 3.86–65.94%), and HSF (gill, + 8.96–83.68%; liver, + 7.11–72.13%) expression level increased significantly ($P<0.05$) from those in control at all the salinities in both tissues. Nuclear NRF2 (gill, + 12.82–57.49%; liver, + 9.94–59.74%), nuclear NF-κB (gill, + 4.11–42.69%; liver, + 8.80–48.27%), and caspase 1 (gill, + 4.28–99.4%; liver, + 3.97–71.19%) expression level also increased significantly ($P<0.05$) at all the salinities in both tissues (Fig. 2).

Activity of gill enzymes

XO level increased significantly ($P<0.05$) (+8.33–145.83%) at all salinities compared to control in gill tissues, while in liver tissues, XO level increased significantly ($P<0.05$) from 3–9 ppt (+9.52–133.33%) but decreased significantly ($P<0.05$) thereafter (− 11.36%) at 12 ppt. Na^+K^+-ATPase level decreased (− 7.41 to 62.07%) significantly ($P<0.05$) at all salinities in a dose dependent manner. On the other hand, carbonic anhydrase level increased (+45.83–232.09%) significantly ($P<0.05$) from the control at all salinities (Fig. 3).

Immunoassay of cytokines (IL-1β, IL-6, TNFα, and IL-10) and cortisol

IL-1β, IL-10, and TNFα levels increased significantly ($P<0.05$) from the control at all salinity exposures. However, IL-6 level increased significantly ($P<0.05$) only at 9 (+52.8%) and 12 ppt (+55.63%) (Fig. 4). Serum cortisol level increased significantly ($P<0.05$) at all salinities (+33.33–311.11%) (Fig. 5).

Discussion

Salinity is one of the most critical abiotic regulators to maintain physiological homeostasis in aquatic ecosystem. Several reports have stated the effects of natural and induced salinity stress on growth performance of fish (Boeuf and Payan 2001). Salinity alteration of 50% could induce a reduction in the growth of sablefish (Kim et al. 2017). When most of the fishes counter a higher osmotic pressure, a primary stress response is always initiated. Cortisol is primarily released which in turn activate the cellular signaling processes that assist in sustaining the homeostasis (Barton and Iwama 1991). In freshwater fishes, body fluid is hyperosmotic to the exterior surroundings. Thus, they should counter the water

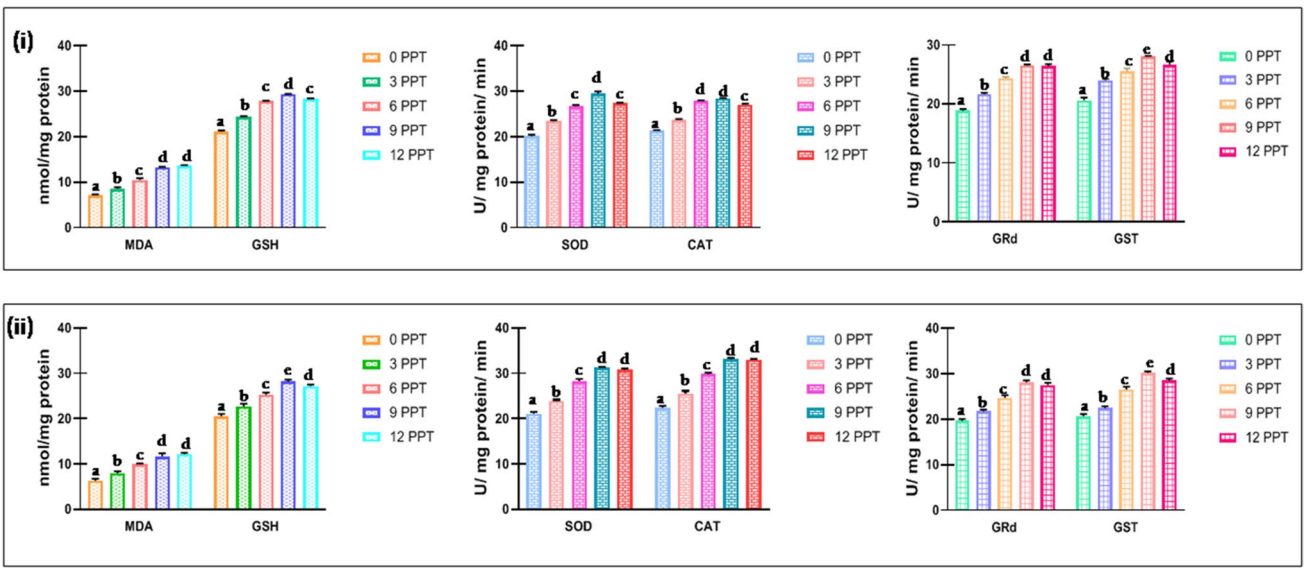

Fig. 1 Bar diagram representing changes in non-enzymatic and enzymatic stress markers in the (i) gill and (ii) liver of *Notopterus chitala* at five different salinity exposures (data = mean ± standard error, $n=18$). Different superscript alphabets denote significant difference ($P<0.05$) in mean values between different treatment groups. Notes: MDA: malondialdehyde (nmol/mg protein), GSH: reduced glutathione (nmol/mg protein), SOD: superoxide dismutase (U/mg protein/min), CAT: catalase (U/mg protein/min), GRd: glutathione reductase (U/mg protein/min), GST: glutathione S-transferase (U/mg protein/min)

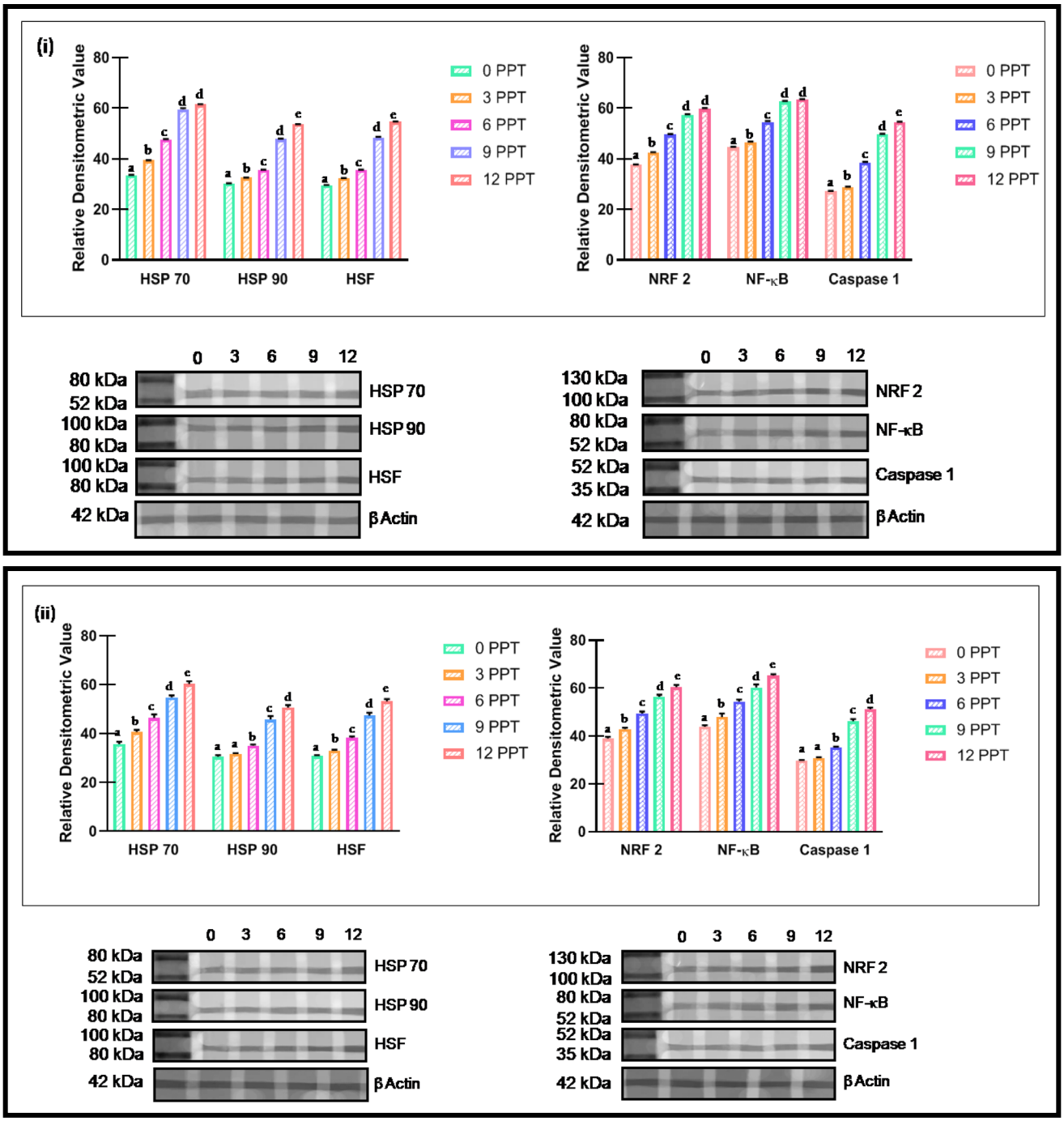

Fig. 2 Bar diagram representing changes in HSP70, HSP90, HSF, NRF2, NF-κB, and caspase 1 expression levels in the (i) gill and (ii) liver of *Notopterus chitala* at five different salinity exposures (data = mean ± standard error, $n = 18$). Different superscript alphabets denote significant difference ($P < 0.05$) in mean values between different treatment groups. Individual band intensity of each immunoblot was quantified by densitometry using β-actin as control

gain and further salt loss by excreting huge quantity of dilute urine and activate salt uptake within their gills. However, in saltwater environment, the body fluids are hyposmotic to the environment. Thus, fish must reduce the loss of water and gain of salt by up taking seawater and excreting little quantity of concentrated urine and secretion of extra salt through the gill epithelium (Evans 2008). However, loss of this osmotic homeostasis leads to reactive oxygen species generation that in turn hampers the prooxidant-antioxidant balances ultimately causing inflammation.

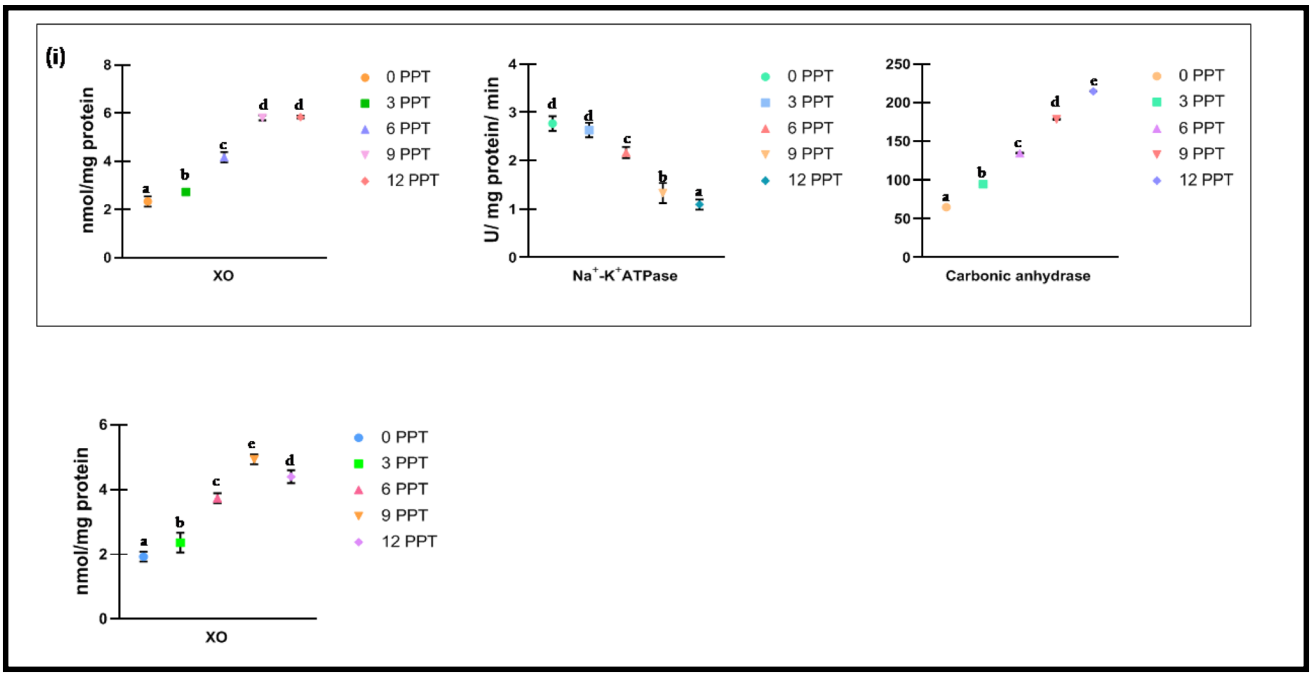

Fig. 3 Box plot representing changes in (i) XO, Na^+-K^+-ATPase and carbonic anhydrase in the gill and (ii) XO in liver of *Notopterus chitala* in four different salinity exposures (data = mean ± standard error, $n = 18$). Different superscript alphabets denote significant difference ($P < 0.05$) in mean values between different treatment groups. Notes: XO: xanthine oxidase (nmol/mg protein), carbonic anhydrase (nmol/mg protein), Na^+-K.$^+$-ATPase (U/mg protein/min)

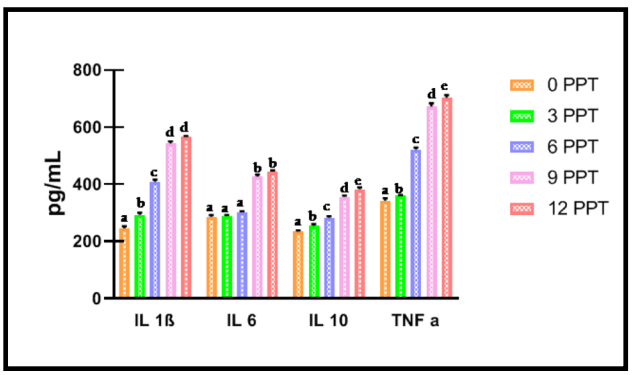

Fig. 4 Bar diagram representing changes in IL-1β, IL-6, IL-10, and TNFα in the serum samples of *Notopterus chitala* at five different salinity exposures (data = mean ± standard error, $n = 18$). Different superscript alphabets denote significant difference ($P < 0.05$) in mean values between different treatment groups. Notes: IL-1β: Interlukin 1β (pg/ml), IL-6: Interlukin 6 (pg/ml), IL-10: Interlukin 10 (pg/ml), TNFα: Tissue Necrosis Factor α (pg/ml)

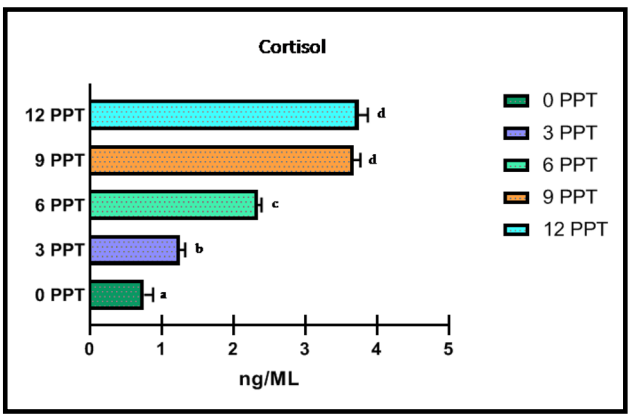

Fig. 5 Bar diagram representing changes in serum cortisol levels (ng/ml) in *Notopterus chitala* at five different salinity exposures (data = mean ± standard error, $n = 18$). Different superscript alphabets denote significant difference ($P < 0.05$) in mean values between different treatment groups

Increase in salinity has previously been shown to induce behavioral alterations and mortality in a number of euryhaline freshwater fish species (Edeline et al. 2005; Tietze and Gerald 2016; Nepal and Fabrizio 2019; Leite et al. 2022). We observed reduced swimming activity and mortality only in fish exposed to the highest salinity level (12 ppt) as well. However, stress caused by salinity up to 9 ppt was not enough to alter behaviour and induce mortality in *N. chitala*. The present results indicate the adaptive ability of *N. chitala* where it can thrive well up to 9 ppt but cannot withstand 12 ppt salinity exposure. However, experiments with longer exposure period will be required to fully comprehend the consequences of salinization on the fish species.

In aquatic animals, fluctuating salinities can induce oxidative impairment by generating ROS (Lushchak 2011). Li

et al. (2007) have suggested that salinity stress is directly related to increased ROS production, which induces oxidative injury. We examined the level of enzymatic and non enzymatic antioxidants along with XO activity as biomarkers to assess effect of salinity. The induced salinity stress demonstrated that oxidative disbalance was generated when *Notopterus* was exposed to increased salinities. MDA, one of the major products of lipid peroxidation increased in response to salinity in the present study. Salinity stress was also reported previously to augment lipid peroxidation in sturgeons (*Acipenser naccarii*) (Martinez-Alvarez et al. 2002). Glutathione always plays a crucial function against oxidative impairment through removal of free radicals (Moniruzzaman et al. 2018). Therefore, discrepancy in GSH level due to salinity exposure signified surplus of free radical in gill and liver tissues. It consequently might decrease the reduced and non-reduced glutathione level ratio (Moniruzzaman et al. 2018). Present results indicated that ROS generation was induced as a result of salinity increase eventually creating a pro-oxidant condition. GST and GRd were found to increase during initial salinity stress up to 9 ppt. This indicated their role in the process of detoxification to eliminate the xenobiotic substances from the tissue (Moniruzzaman et al. 2016). However, significant ($P < 0.05$) drop in GST at high salinity indicated towards alteration in reduced and oxidized glutathione ratio due to accumulation of free radical. Increase in SOD and CAT activity stands as an indicator of surplus of ROS assembly (Wu et al. 2010; Oruc 2010). Significant ($P < 0.05$) increase in activity of SOD and CAT was noted in fish tissues exposed to salinity stress followed by a decrease at the highest salinity. The increase of SOD activity indicates enhancement in superoxide radical assembly and an ensuing elevation in antioxidant system to recompense the associated damage (Oruc 2012). Since these enzymes provide primary line of defense against oxidative impairment (Pandey et al. 2003), the level of SOD and CAT decreases after exposure to higher salinities. XO is also reported to generate ROS which disrupt the cellular architecture of the cell and leads to cell death (Ghosh et al. 2018). Increase in XO level during salinity stress indicates imbalance between ROS and levels of endogenous antioxidants (Murata et al. 2013). Decreasing trend of antioxidants at high salinity indicates promotion of lipid peroxidation and disruption of the membrane lipid bilayer arrangement that may inactivate membrane-bound receptors and enzymes. It may also cause fragmentation of the peptide chain, alteration of electrical charge of proteins, cross-linking of proteins, and oxidation of specific amino acids and therefore may lead to increased susceptibility to proteolysis by degradation. Therefore, it can be concluded that adaptive ability of *N.chitala* to the environment declines when salinity crosses 9 ppt (Yadav et al. 2015).

Cortisol is considered crucial indicator during stress (Barton 2002; Pankhurst and Munday 2011). Cortisol can enhance Na^+-K^+-ATPase activity in gill cells (Dang et al. 2000). Cortisol levels can regulate the plasma ion fluctuations and immune reaction (McCormick 2011). Similar to the results of the present study, Elarabany et al. (2017) noticed increase in cortisol level during salinity exposure in Nile tilapia. Carbonic anhydrase activity plays a putative role in modulating the systemic acid–base equilibrium in the gills (Georgalis et al. 2006). The enzyme catalyzes CO_2 within the branchial cells to produce the acid–base equivalent (Evans et al. 2005; Hirose et al. 2003). Carbonic anhydrase was increased in fish groups exposed to salinity stress. In a previous study, rainbow trout (*Oncorhynchus mykiss*) exposed to hypercarbia showed increased activity of carbonic anhydrase (Georgalis et al. 2006). The homeostasis in fish transferred to a hyperosmotic medium is regulated by gill Na^+-K^+-ATPase activity. In general, gill Na^+-K^+-ATPase activity is positively correlated with salinity of the external environment in most telesosts. However, in *Notopterus chitala*, Na^+-K^+-ATPase activity in gills was observed to be negatively correlated with ambient salinity. Similar observations were noted in few other euryhaline freshwater fish species living in habitats with extreme salinity fluctuations (Tomy et al. 2009). Such change in the enzyme activity plays role in adaptation to fluctuating salinities (Stewart et al. 2016; Zhang et al. 2017) as the fish may take a strategy to save extra energy on ion-/osmoregulation and to channel it for other uses. This observation is consistent with a previous finding that suggests energy saving during osmoregulation enables fish to adapt well to salinity fluctuation (Nordlie 2009).

Molecular chaperones are considered cellular mechanisms to restore the distorted cellular machinery in situation of intense stress environment (Roberts et al. 2010). Expression of HSPs is often regulated by the heat shock factors. HSF1 is crucial for the transcriptional activation of most of the heat shock proteins (Padmini and Usha Rani 2009). HSP70, HSP90 along with HSF expression at salinity exposures were higher than its expression at the control. Deane and Woo (2005) found that HSP70 expression was increased in sea bream gills in response to salinity stress. Salinity stress enhances metabolic activity of osmoregulatory organs which subsequently leads to upregulation of HSP expression (Xu and Liu 2011).

Cytokines and chemokines are signal mediators generated by immune cells in the organism to promote inflammation at the infection site or during stressed environment. It consequently enhances the activity of phagocytic cells to kill the invading pathogenic agents (Secombes et al. 2001; Wang and Secombes 2013). IL-1β is a pro-inflammatory cytokine that performs crucial role during regulation of the inflammatory processes (Bird et al. 2002). IL-10 is an anti-inflammatory cytokine. It is involved with multiple

pleiotropic effects in immunoregulation and inflammation. It can obstruct activity of NF-κB. It is also implicated in the regulation of the JAK-STAT signaling pathway. TNFα is a cell signaling protein (cytokine) involved in systemic inflammation and also makes up the acute phase reaction. It is produced by the cell of immune system in response to inflammation. In the present study, it was noted that during exposure to the salinity stress, there was upregulation of all interleukins.

Our data indicate that salinity stress induces caspase-dependent apoptosis in fish tissue. Caspase 1 plays a pivotal role during inflammatory reactions. Activation of caspase indicates that the cellular architecture is degraded by fluctuating salinities, which means while carrying out cell death there is minimal effect on surrounding tissues (Rathore et al. 2015). Current results suggest that during salinity stress there is activation of apoptotic pathways in the fish body. Once this pathway is activated, it initiates pro-inflammatory response and activation of pro-inflammatory cytokines. NF-κB modulates cell physiology and its response to external stimuli such as oxidative damage is studied previously (Mukherjee et al. 2019). Cellular responses such as survival, apoptosis, and other cell fates during oxidative damage are controlled by NF-κB (Morgan and Liu 2011). Nuclear NF-κB was significantly elevated during salinity stress indicating its translocation upon activation. This finding is similar to previous research, where significant upregulation in nuclear NF-κB has been recognized in the stressed cell (Sarada et al. 2008). Such elevated nuclear NF-κB expression might modulate the SOD-CAT system downstream.

Instigation of oxidative stress response is predominantly coordinated by NRF2, the most crucial and decisive transcription factor in the vertebrate system (Vargas-Mendoza et al. 2019). Under natural and undisturbed condition, NRF2 is remained in quiescent state in the cytosol by Keap1 protein. Under certain oxidative stress condition, intense free radical accumulation steers to the commencement of frequent transducers (kinases), which ultimately phosphorylate both Keap1 and NRF2. These radicals sometime may unswervingly alter the sulfhydryl rich Keap1 protein to finally bring about a conformational change in Keap1 protein. All these sequences of actions during the preliminary phase of the oxidative stress dislodge the Keap1-NRF2 complex and kindle the translocation of sturdy and stable NRF2 protein to the nucleus (Dinkova-Kostova 2012). In the nucleus, NRF2 associates with a number of small proteins and then binds directly to antioxidant response elements (AREs). Thus, NRF2 commences the transcription of most of the antioxidant genes. In the current result as well, a quick augmentation in nuclear NRF2 level was observed in both fish gill and liver exposed to high salinity.

Conclusion

Notopterus chitala is one of the most common euryhaline freshwater fish of tropical estuary and it has been observed to survive and colonize habitats with fluctuating salinity range. Consequently, the fish is expected to gain an advantage over its stenohaline relatives in the foreseeable future. Salinity stress can severely affect specific physiological traits like oxidative balance that may enhance pro-inflammatory cytokines and alter anti-inflammatory cytokines in fish. Therefore, inflammation may be induced by fluctuating salinity. Molecular chaperons and transcription factors were observed to be involved in salinity stress tolerance in both respiratory (gill) and metabolic (liver) organs of *Notopterus chitala* for developing secondary stress response mechanisms that facilitated ion balance by altering the concentration of transport and antioxidant proteins to regulate ion flux rate and metabolic status. However, significant protein function is associated with being highly osmotic stress-tolerant and these may cost high energy to resist osmotically unstable environments. Although a medium salinity range was noted to be nonlethal for *Notopterus chitala*, a higher osmotic challenge might antagonize retention of the physiological capacity for such euryhaline species. Most proteins required for the physiological adaptability appeared to be exhausted at high salinity (12 ppt) and severely affected the immune functions and cellular endurance of the fish. Therefore, high osmotic pressure hampered the biochemical and physiological mechanisms that enabled the fish to cope with limited salinity fluctuations. Finally, keeping in view of the mortality and antioxidant and inflammatory responses as observed in the present study, 9 ppt may be postulated as the maximum salinity tolerance level for survival of *Notopterus chitala.*

Author contribution MM1: conceptualization, visualization, investigation, analysis, writing—original-draft, fund acquisition. MM2: methodology. SK: methodology. SBC: conceptualization, supervision, resources, writing—review and editing.

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Data availability The datasets generated and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Declarations

Ethics approval The investigation was permitted by Institutional Animal Ethics Committee, University of Calcutta (Registration #885/ac/05/CPCSEA).

Consent to participate Manuscript does not contain data from any individual person.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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