

Modulation of redox regulatory molecules and electron transport chain activity in muscle of air breathing fish *Heteropneustes fossilis* under air exposure stress

Biswaranjan Paital

Received: 26 March 2013/Revised: 11 August 2013/Accepted: 16 August 2013/Published online: 1 September 2013
© Springer-Verlag Berlin Heidelberg 2013

Abstract Responses of redox regulatory system to long-term survival (>18 h) of the catfish *Heteropneustes fossilis* in air are not yet understood. Lipid and protein oxidation level, oxidant (H₂O₂) generation, antioxidative status (levels of superoxide dismutase, catalase, glutathione peroxidase and reductase, ascorbic acid and non-protein sulfhydryl) and activities of respiratory complexes (I, II, III and IV) in mitochondria were investigated in muscle of *H. fossilis* under air exposure condition (0, 3, 6, 12 and 18 h at 25 °C). The increased levels of both H₂O₂ and tissue oxidation were observed due to the decreased activities of antioxidant enzymes in muscle under water deprivation condition. However, ascorbic acid and non-protein thiol groups were the highest at 18 h air exposure time. A linear increase in complex II activity with air exposure time and an increase up to 12 h followed by a decrease in activity of complex I at 18 h were observed. Negative correlation was observed for complex III and V activity with exposure time. Critical time to modulate the above parameters was found to be 3 h air exposure.

Dehydration induced oxidative stress due to modulation of electron transport chain and redox metabolizing enzymes in muscle of *H. fossilis* was clearly observed. Possible contribution of redox regulatory system in muscle tissue of the fish for long-term survival in air is elucidated. Results of the present study may be useful to understand the redox metabolism in muscle of fishes those are exposed to air in general and air breathing fishes in particular.

Keywords Air exposure · Antioxidants · *Heteropneustes fossilis* · Muscle physiology · Oxidative stress · Tissue oxidation

Introduction

Muscle is the principal consumable organ in the teleost fresh water Indian stinging fish *Heteropneustes fossilis*. This fish is well known in India for nutritional as well as medicinal values of its flesh (Bailly 2013; Erfanullah and Jafri 1999). Therefore, this fish represents one of the most important fishery resources in India (Bailly 2013; Menon 1999). The majority of its habitats such as ponds, ditches, marshes and muddy rivers dry in summer season due to prolonged drought (Bailly 2013; Menon 1999). Nevertheless, *H. fossilis* can hide itself in own burrows and live at least for 30 days inside mudflats to avoid total dehydration in summer season (Saha et al. 2001; Talwar and Jhingran 1991). *H. fossilis* can also survive up to 20–70 h under air exposure conditions (Paital 2013; Saha and Ratha 1989). Air exposure can initiate dehydration and hypoxia condition in air breathing marine and fresh water fishes (Bailly 2013; Bridges 2013; Graham 1997; Talwar and Jhingran 1991; Thurston and Gehrke 1993).

Communicated by G. Heldmaier.

Electronic supplementary material The online version of this article (doi:10.1007/s00360-013-0778-8) contains supplementary material, which is available to authorized users.

B. Paital (✉)
Biochemical Adaptation Lab., Department of Zoology, Banaras Hindu University, Varanasi 221005, UP, India
e-mail: biswaranjanpaital@gmail.com;
biswa@mrdg.iisc.ernet.in

Present Address:

B. Paital
Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore 560012, India

During commercial handling or in their natural habitat in summer season (inside mudflats), water running through gills of *H. fossilis* becomes interrupted. This can limit O₂ supply through gills of the fish (Munshi et al. 1978). As a result, the tissues of the fish may respond differentially to oxidant (reactive oxygen species: ROS) generation at compromised O₂ availability condition (Paital 2013). Limited O₂ supply due to change in abiotic environmental factors to alter oxidative stress (OS) pathways in tissues of aquatic animals has already been noticed (Paital and Chainy 2010, 2012, 2013a; Romero et al. 2007). Reactive oxidants or ROS such as superoxide radical (O₂⁻), H₂O₂ and hydroxyl radical (·OH) are generated as by-product in normal oxidative metabolism. Under non-stressed conditions, about 1–2 % of O₂ consumed by mitochondria are incompletely reduced to O₂⁻ by the electrons that are leaked at respiratory complex I and III enzymes of electron transport chain (ETC) (Abele et al. 2007; Halliwell and Gutteridge 2001). Eventually, O₂⁻ becomes responsible for production of the other ROS such as H₂O₂ and the highly reactive ·OH (Halliwell and Gutteridge 2001). All the above molecules have high oxidant activity and if not neutralized, can oxidize biological macromolecules including lipids, proteins and nucleic acids. O₂⁻ is catalyzed to H₂O₂ by superoxide dismutase (SOD, EC 1.15.1.1) (Paital et al. 2013). H₂O₂ is neutralized to H₂O and O₂ by the enzyme catalase (CAT, EC 1.11.1.6) (Paital et al. 2011). H₂O₂ is also broken down by glutathione peroxidase (GPx, EC 1.11.1.9) enzyme in presence of reduced glutathione (GSH). The oxidized glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase (GR) with the help of NADPH. ·OH and other ROS are neutralized by small molecular weight non-enzymatic antioxidants such as ascorbic acid (AA), GSH, non-protein sulfhydryl (–SH), vit-E and vit-C. Under normal physiological conditions, balance is maintained between ROS and antioxidants in cells of aerobic organisms. Lower level of ROS especially H₂O₂ can be also useful for animals due to its role in various signal transduction processes (Takano et al. 2003). Insufficient level of antioxidants can be resulted in increased lipid peroxidation (LPx), protein oxidation (carbonylation: PC), nucleic acid adducts and finally, a disorder condition known as OS (Abele et al. 2011; Halliwell and Gutteridge 2001).

Exploring the mechanisms of air exposure and hypoxia-induced lipid and protein oxidation in consumable aquatic animals can be useful in aquaculture industry. At environmental low oxygen level, species-specific biochemical adaptation mechanisms are proposed for long-term survival in aquatic animals (Welker et al. 2013). For example, the long-term survival of common carp *Cyprinus carpio* in hypoxia condition is correlated with the high glycogen level and exclusively anaerobic glycolysis-mediated ATP

generation in tissues (Lushchak et al. 2005). At certain conditions, induction of metabolic depression also contributes for long-term survival of aquatic animals under low O₂ level or anoxia state (Storey and Storey 2004). On the other hand, metabolic damage by high ROS level at hypoxia and constitutively enhancement of antioxidant defenses associated with hypoxia followed by regeneration to restore the required cellular redox level in tissues are recorded in fishes (Lushchak et al. 2001, 2005; Lushchak and Bagnyukova 2006). This particular physiological response in animals now has been referred as “preparation for oxidative stress” (Welker et al. 2013). Although this is a well-studied phenomenon in non-air breathing fishes such as goldfish *Carassius auratus* and common carp *C. carpio* (Lushchak et al. 2001, 2005; Lushchak and Bagnyukova 2006), it is not yet well established in air breathing fishes such as *H. fossilis*. Theoretically, consistent increase in hypoxia condition induced by constant exposure of air breathing fishes to air may also be associated with dynamic change in their cellular oxidative metabolism. However, studies describing a complete picture of redox metabolism under air exposure conditions are limited in any air breathing fish models in general and *H. fossilis* in particular (Maiti et al. 2010; Zaccane et al. 1985). Recently, oxidative metabolic responses in one of the most metabolically active tissues, i.e., in brain of air breathing fish *H. fossilis* in relation to long-term survival under dehydration state is reported (Paital 2013). The present study aimed to analyze the biochemical level of redox metabolism associated parameters in one of the low metabolically active tissue, i.e., in muscle of *H. fossilis* under air exposure condition. This is also the first report describing a composite picture of ROS, OS and antioxidant parameters along with mitochondrial respiratory complex enzyme activity in muscle tissue of *H. fossilis*. The data can be useful to understand the fish muscle physiology under hypoxia state as air exposure can limit O₂ supply in fishes.

Materials and methods

Chemicals

Chemicals for the present experiment were purchased locally from Sigma Chemical Company, Missouri, USA (cumene hydroperoxide, dithiobisnitrobenzoic acid, homovanilic acid, horse radish peroxidase, GR, thiobarbituric acid and duroquinone), Himedia, Mumbai, India (riboflavin, phenylmethane sulphonyl fluoride, hydroxylamine hydrochloride, L-methionine, GSSG, GSH, NADPH, NADH, butylated hydroxytoluene and trichloro acetic acid) and Sisco Research Laboratory, Mumbai, India (analytical grade of all other chemicals).

Animals, experimental set-up and tissue collection

The tissue for the present study was collected and analyzed from the earlier experiment (Paital 2013). Briefly, *H. fossilis* (15–20 g) was purchased from the local fish market (Chowkaghat, Varanasi, India) and were disinfected by dipping them in 500 ppm potassium permanganate solution for 2–3 min. Fishes were then washed adequately under tap water and were released to plastic tanks containing fresh tap water and were acclimatized in laboratory conditions in 12: 12 h light and dark period. Opaque water tanks were used to make least disturbances to the animals. Water (having dissolved O₂ 7.1 ± 0.45 mg L⁻¹, pH 7.13 ± 0.12 , water hardness 24.5 ± 2.19 mg L⁻¹ and water temperature 25.12 ± 3.21 °C) of the tank was changed in every alternative day. Fishes were fed with minced goat liver at every alternative day but on the day water was not changed. Fishes were acclimatized in the above laboratory conditions for a month. During acclimatization around 10 % mortality rate was observed up to the first 5 days which was arrested thereafter.

Only active male fishes (23.44 ± 1.63 g) were selected for experiment. Fishes were randomly divided into five groups ($n = 10$ in each) and four groups of fish were transferred onto four plastic trays ($40 \times 30 \times 6$ cm³) and were exposed to air at 25 °C. Care was taken to have no significant difference in their body weight among the groups. Tap water was sprayed on them with 3 h interval to avoid total dryness of their skin. Fish were then sacrificed after exposing them to air for 3, 6, 12 and 18 h. After 20 h exposure, fish were started to die and >90 % were died at 24 h exposure time. After 18 h air exposure, the revival of animals was observed when they get back into water. Therefore, maximum air exposure time was set to 18 h. One group of fish ($n = 10$) was sacrificed immediately after collecting them from the water tank. This group of fish was treated as control for the rest of the air-exposed groups. Recovery or reoxygenation (submerging fish into water after air exposure) experiment was out of the objective and scope of this article and, therefore, was not performed. After sacrificing the fishes by cryoanesthesia, muscle tissue was dissected immediately, washed in ice-cold normal saline (0.78 %, w/v) solution, blotted, weighed and flash frozen in liquid nitrogen. Then the tissues were stored at -20 °C for further analyses.

Tissue processing and biochemical analyses

A 10 % (w/v) homogenate of muscle tissue was prepared at 4 °C in homogenizing buffer (50 mM tris, 1 mM EDTA, 0.25 M mannitol and 1 mM phenylmethane sulphonyl fluoride, pH 7.6) with the help of pre-cooled mortar and pestle by keeping on ice. The crude homogenate was

subjected to centrifugation at $1,500 \times g$ for 15 min at 4 °C in a cooling centrifuge (REMI, Mumbai, India, model: CPR-24) to sediment nuclei and tissue debris. The crude supernatant obtained was referred as post-nuclear fraction (PNF). PNF was subjected to centrifugation at $14,000 \times g$ for 15 min at 4 °C to obtain the clear supernatant which was referred as post-mitochondrial fraction (PMF). Succinate dehydrogenase activity was measured in PMF to check its purity (Lambowitz 1979). The mitochondrial pellet (mitochondrial fraction: MF) obtained at the above step was washed twice in homogenizing buffer and was used to assay activities of mitochondrial complex enzymes. Protein concentration in samples was determined according to Bradford (1976) using bovine serum albumin as standard.

Determination of OS and ROS levels

For measurement of LPx, muscle tissue was homogenized in 1.15 % ice-cold KCl to obtain PNF as described above. LPx was measured in the form of thiobarbituric acid reactive substances (TBARS) according to Ohkawa et al. (1979). TBARS (nmol mg⁻¹ pr) were calculated from its molar extinction coefficient as 1.56×10^5 M⁻¹ cm⁻¹. Protein carbonyl content was determined in PNF of the tissue (Levine et al. 1994). Carbonyl content (nmol carbonyl mg⁻¹ pr) was calculated from the molar extinction coefficient of aliphatic hydrazones, i.e., 22×10^3 M⁻¹ cm⁻¹. H₂O₂ content was measured fluorimetrically in PNF samples according to Staniek and Nohl (1999) and modified by Paital and Chainy (2010). Briefly, PNF sample was immediately precipitated with 5 % (w/v) trichloroacetic acid and centrifuged at $12,000 \times g$. The supernatant was neutralized to ~pH 7.4 with 6 M K₂HPO₄. H₂O₂ was measured fluorimetrically in the neutralized supernatant using a method in which horseradish peroxidase-mediated homovanillic acid dimmer formation by H₂O₂ is established. Result was compared with the standard curve of H₂O₂ made in similar way to the above and was expressed as ng of H₂O₂ per g of wet tissue. The methods for the above parameters are described in details earlier (Paital and Chainy 2010).

Antioxidant enzyme assays

Activity of antioxidant enzymes was measured at 25 °C in PMF samples as described in details earlier (Paital and Chainy 2010). SOD (EC 1.15.1.1) activity was measured according to Das et al. (2000) and result was expressed as unit mg⁻¹ protein. CAT (EC 1.11.1.6) activity was measured by monitoring the decrease in absorbance of 25 mM H₂O₂ at 240 nm in 3 mL 50 mM phosphate buffer, pH 7 (Aebi 1974). Enzyme activity was calculated from the

extinction coefficient of H_2O_2 as $43.6 \text{ M}^{-1}\text{cm}^{-1}$ and was expressed as one mole of H_2O_2 consumed $\text{s}^{-1} \text{mg}^{-1}$ protein ($\text{nKat mg}^{-1} \text{pr}$). GPx activity (EC 1.11.1.9) was measured according to Paglia and Valentine (1967) in which cumene hydroperoxide (7.5 mM) was used as substrate. GR (EC 1.6.4.2) activity was measured according to Massey and Williams (1965). For GR, the rate of conversion of GSSG (120 mM) to GSH was estimated by monitoring oxidation of NADPH (4.5 mM) in the assay system. For both GPx and GR, utilization of NADPH (4.5 mM) by glutathione system was recorded at 340 nm. Results of both the enzymes were calculated from the extinction coefficient of NADPH as $6.22 \text{ mM}^{-1} \text{cm}^{-1}$ and expressed as nmol of NADPH oxidized $\text{min}^{-1} \text{mg}^{-1} \text{pr}$.

Determination of non-enzymatic antioxidants

AA and $-\text{SH}$ group were measured in supernatant ($14,000\times g$, 15 min) of trichloroacetic acid (5 %, w/v) precipitated PNF. AA was estimated according to Mitsui and Ohta (1961) by monitoring the reduction of phosphomolybdate by AA in acidic medium at 660 nm. Results were calculated from the standard curve of AA (2.5–50 nmol) and were expressed as nmol of AA g^{-1} wet tissue. Reaction product of $-\text{SH}$ content of sample with Ellman's reagent (0.01 M dithiobisnitrobenzoic) was measured at 412 nm (Sedlak and Lindsay 1968). Results were calculated from the standard curve of GSH (5–50 nmol) and expressed as nmol of $-\text{SH}$ g^{-1} wet tissue.

Assay of electron transport chain enzymes

MF was placed in an ice bath in aliquots and sonicated at a protein concentration of $\sim 1 \text{ mg}/100 \mu\text{l}$. Sonication was done by giving four bursts for 15 s with each burst being followed by an interval of 30 s. Sonicated samples were centrifuged at $10,000\times g$ for 10 min at 4°C to separate unbroken mitochondria. Activities of respiratory chain enzymes were measured in the obtained supernatant at 25°C . The detailed protocols for estimating ETC enzyme (complex I, II, II–III and V) level are described in Paital and Chainy (2012).

Briefly, activity of NADH: duroquinone oxidoreductase (complex I, EC 1.6.5.3) was assayed by monitoring the decrease in absorbance (at 340 nm) due to reduction of NADH (13 mM) in the assay mixture (Gassner et al. 1997). Enzyme activity was calculated using molar extinction coefficient of NADH as $6.22 \times 10^3 \text{ M}^{-1} \text{cm}^{-1}$ and result was expressed as nmol NADH oxidized $\text{min}^{-1} \text{mg}^{-1}$ protein. Activity of succinate: 2, 6-dichlorophenolindophenol oxidoreductase (complex II, EC 1.3.99.1) was assayed by monitoring the reduction rate of dichlorophenolindophenol (1.125 mM) at 600 nm (Lambowitz 1979). Complex II

activity was expressed as unit $\text{min}^{-1} \text{mg}^{-1}$ protein. Succinate: cytochrome c oxidoreductase (complex III, EC 1.3.2.1) activity was assayed by following the reduction rate of oxidized cytochrome c (1 %, w/v) at 550 nm (Tisdale 1967). Enzyme activity was calculated using molar extinction coefficient for cytochrome c ($21 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$) and expressed as nmol cyt-c reduced $\text{min}^{-1} \text{mg}^{-1}$ protein. ATPase (complex V, EC 3.6.1.34) activity was assayed according to Cormier et al. (2001) and Chen et al. (1956). First, externally supplied ATP molecules (50 mM) were hydrolyzed by MF sample and then iP produced were measured using a color reagent (6 N H_2SO_4 , 2.5 % ammonium molybdate and 10 % AA in the ratio of 2:1:1, v/v). Absorbance of the color product was read at 820 nm and KH_2PO_4 was taken as standard. Complex V activity was expressed as $\mu\text{mol iP formed h}^{-1} \text{mg}^{-1}$ protein.

Statistical analyses

Each set of data (mean \pm S.D., $n = 10$) were tested for homogeneity of variance (Levene test) and normality (Lilliefors test). Means were compared and analyzed by one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test for normally distributed data. A non-parametric test (Kruskal–Wallis ANOVA) was used for the data which did not reveal homogeneity of variance and normal distribution (CAT, GPx, AA, and GSH). Differences among the means were considered significant at $p < 0.05$ level. Correlation coefficients (r) between OS parameters and air exposure time were determined at 5 % significant level using Microsoft excel program. Discriminant function analysis (DFA) was performed according to Garson (2008) and Jennrich (1977), to evaluate the contribution of the variables (OS physiology parameters and ETC enzymes) on the groups. Mean values of the biochemical parameters between the groups were compared to calculate the absolute change in percentage. Loss in body weight of the fish was compared by two-tailed paired t test using Microsoft excel program.

Results

Change in body weight under air exposure condition

A significant ($p \leq 0.05$, two-tailed t test) loss of 5, 9, 14 and 15 % in body weight of the fish was recorded after air exposure than before exposure time points at 3, 6, 12 and 18 h, respectively (data not shown). The loss in body weight observed at 3 h and onwards air exposure time in *H. fossilis* indicates induction of dehydration condition in the fish.

Effects of dehydration on levels of OS and ROS indices

TBARS level in muscle of *H. fossilis* was increased after 3 h air exposure (Fig. 1). TBARS value was equally the highest in 6, 12 and 18 h groups in comparison to 0 and 3 h groups. From the control set, 100, 232, 243 and 257 % higher ($p \leq 0.001$, one-way ANOVA, Fig. 1a) TBARS level was recorded at 3, 6, 12 and 18 h exposure groups, respectively. PC value was increased and maintained at least ≥ 7 % higher (one-way ANOVA, $p = 0.05$, Fig. 1b) level than control and 3 h groups in comparison to 6, 12 and 18 air exposure groups. No difference in PC level was observed between 0 and 3 h groups and among 6, 12 and 18 h groups. H_2O_2 was increased in the tissue at 3 h group than control set and then was maintained equally in rest of the groups. At least 120 % higher (one-way ANOVA, $p \leq 0.001$, Fig. 1c) H_2O_2 level was recorded in air exposure groups than control set. The tissue was able to retain equal level of H_2O_2 at 3 h onwards air exposure groups than control group.

Antioxidant enzyme responses to air exposure condition

Air exposure has caused a significant decrease in activities of the studied four antioxidant enzymes in muscle of *H.*

fossilis (Fig. 2). Dehydration up to 18 h was found to responsible to diminish SOD activity to the lowest level (at least 55 % in comparison to the other air-exposed groups, $p \leq 0.001$, one-way ANOVA, Fig. 2a) in the tissue of the teleost fish. On the other hand, SOD had at least 28 % higher ($p \leq 0.05$, one-way ANOVA) activity in the control set in comparison to the other air exposure groups. From the control set, 37, 47, 47 and 55 % lower ($p \leq 0.01$, one-way ANOVA) SOD activity was observed at 3, 6, 12 and 18 h air exposure groups, respectively. CAT activity initially was found to decrease linearly from 0 h group up to 12 h group and then remained unchanged at 18 h group than 12 h group. Its activity was 93, 136, 202 and 228 % higher ($p \leq 0.001$, Kruskal–Wallis ANOVA, Fig. 2b) at 3, 6, 12 and 18 h groups than control set. GPx activity was decreased at least by 39 and by 22 % ($p \leq 0.01$, Kruskal–Wallis ANOVA, Fig. 2c) from the control set at 3, 6 and 12 h and at 18 h air exposure groups, respectively. A mild induction (at least 28 % higher, $p \leq 0.01$, Kruskal–Wallis ANOVA) in GPx activity level was observed at 18 h group in comparison to 3, 6 and 12 h groups. The biochemical activity of GR was found to be reduced due to dehydration (Fig. 2d). Air exposure up to 3, 6 and 12 h and 18 h was responsible to reduce ($p \leq 0.001$, one-way ANOVA) GR activity almost double and triple times than 0 h group, respectively. A 54, 54, 53 and 67 % ($p \leq 0.001$) reduction

Fig. 1 Effects of air exposure on oxidative stress indices and ROS (H_2O_2) level in muscle of the teleost *Heteropneustes fossilis*. **a** Lipid peroxidation level, **b** protein carbonylation level, **c** H_2O_2 level. Data are presented as mean \pm SD. ($n = 10$). Different superscripts indicate statistical (one-way ANOVA followed by Duncan’s post hoc test) difference between the mean values at $p \leq 0.05$

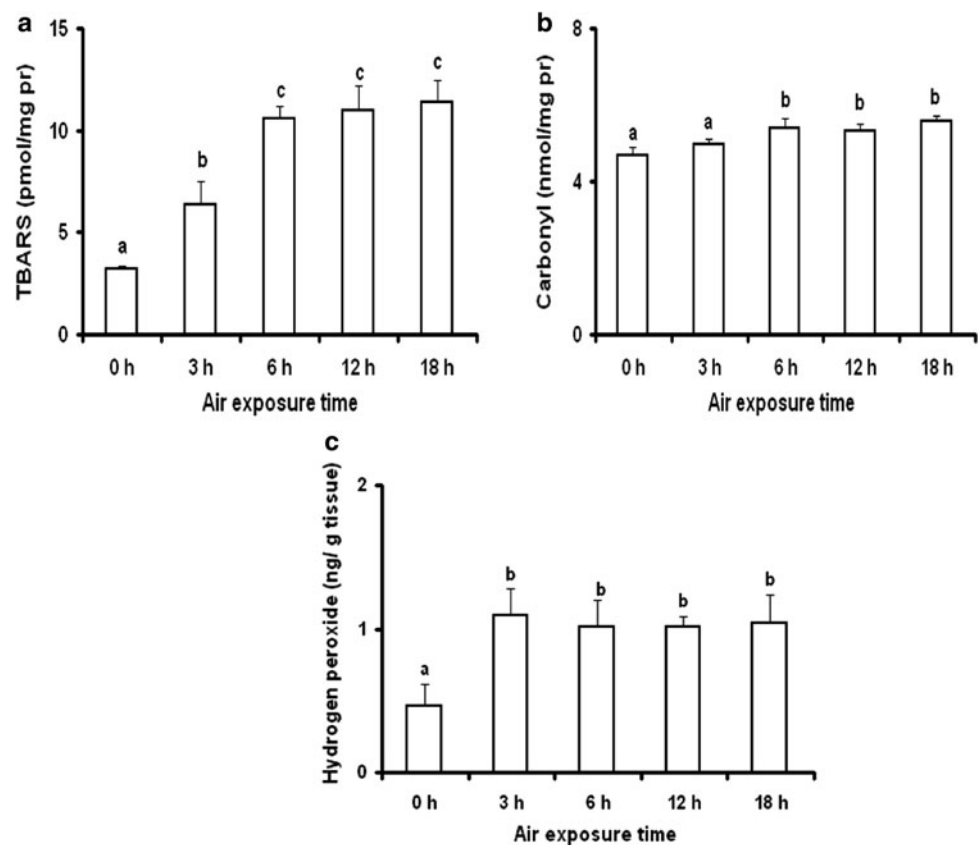
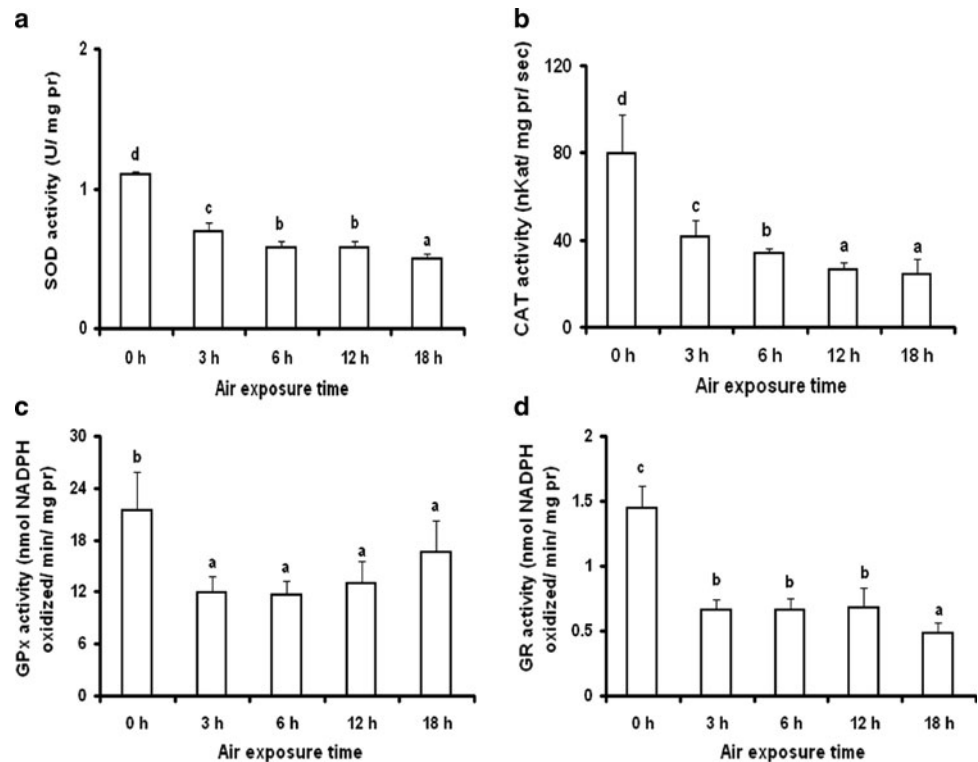


Fig. 2 Activities of antioxidant enzymes in muscle of the teleost *Heteropneustes fossilis* under air exposure condition.

a Superoxide dismutase, **b** catalase, **c** glutathione peroxidase **d** glutathione reductase. Data are presented as mean \pm SD. ($n = 10$). Different superscripts indicate statistical (one-way ANOVA followed by Duncan's post hoc test, Kruskal–Wallis ANOVA for CAT and GPx only) difference between the mean values at $p \leq 0.05$



in GR activity was observed in 3, 6, 12 and 18 h exposure groups than 0 h group, respectively. After reduction of GR activity at 3 h than 0 h group, its level was maintained unchanged up to 6 and 12 h groups then again reduced at 18 h group.

Change in levels of small antioxidant molecules

Dehydration states seem to have no effects on AA level in muscle of *H. fossilis* up to 12 h exposure time (Fig. 3a, $p > 0.05$, Kruskal–Wallis ANOVA). However, ~ 58 % ($p = 0.05$) higher AA level was observed at 18 h group in comparison to 3 and 6 h groups. Free non-protein –SH concentration in the muscle was linearly increased ($p = 0.05$, Kruskal–Wallis ANOVA, Fig. 3b) from 3, 6 and 18 h groups with an insignificant change in its level between 3 and 12 h and between 0 and 12 h groups. Free –SH accumulation in the tissue was observed at 18 h (at least 51 % higher, $p = 0.05$) in comparison to the other groups. It was ~ 156 % higher ($p = 0.01$) at 0 and 6 h groups in comparison to 3 and 12 h groups.

Effects of air exposure on activities ETC enzymes

ETC enzymes and ATPase in muscle tissue of *H. fossilis* have shown significant changes in their level in response to dehydration condition (Fig. 4). Complex I level was increased linearly up to 6 and 12 h groups then again

decreased at 18 h group. Activity of complex I was significantly (one-way ANOVA) increased by 37 ($p = 0.05$) and 74 % ($p = 0.01$) from the control set at 3 and 6 h groups, respectively, followed by no change between 6 and 12 h groups (Fig. 4a). Its activity was reduced by ~ 1.5 times at 18 h group than 6 h group. A linear increase in complex II activity was recorded with respect to time of air exposure (Fig. 4b). It had 31, 70, 93 and 127 % (one-way ANOVA, $p \leq 0.05$) increased activity at 3, 6, 12 and 18 h groups than 0 h group, respectively. Reduction in activity of both complexes III and V was observed in response to air exposure of the fish. Reduction of 28 ($p = 0.05$), 25 ($p = 0.05$), 55 ($p = 0.01$) and 70 % ($p = 0.001$) (one-way ANOVA for all p values) of complex III activity was observed at 3, 6, 12 and 18 h air exposure time than 0 h, respectively (Fig. 4c). Similarly, ATPase activity was diminished linearly (Kruskal–Wallis ANOVA) by 33 ($p = 0.05$), 74 ($p = 0.001$), 81 ($p = 0.003$) and 89 % ($p = 0.0002$) at 3, 6, 12 and 18 h sets than 0 h, respectively (Fig. 4d).

Correlation analysis

Values of the analyzed OS and ROS parameters were positively correlated (correlation coefficient was 0.81 for LPx, 0.76 for PC, 0.49 for H_2O_2) with time period of air exposure in *H. fossilis*. In contrast to the above, negative correlation coefficient values, i.e., -0.73 , -0.73 and -0.68

Fig. 3 Variation of non-enzymatic antioxidant molecules in muscle of the teleost *Heteropneustes fossilis* as a function of air exposure time. **a** Ascorbic acid and **b** non-protein sulfhydryl group (–SH). Data are presented as mean ± SD. (*n* = 10). Different superscripts indicate statistical (Kruskal–Wallis ANOVA) difference between the mean values at *p* < 0.05

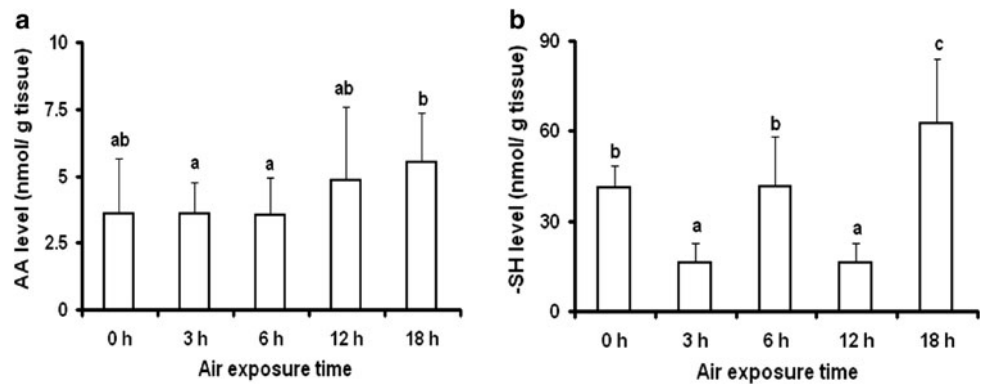
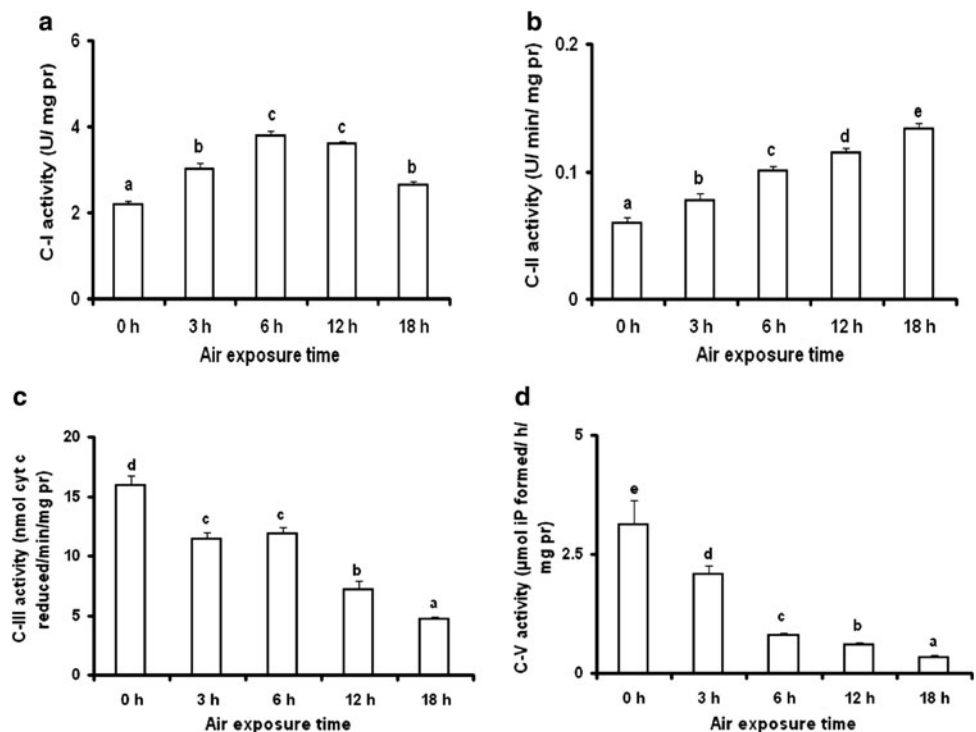


Fig. 4 Activities of respiratory complex enzymes in muscle of the teleost *Heteropneustes fossilis* under air exposure condition. **a** NADH oxidoreductase, **b** succinate dehydrogenase, **c** cytochrome c oxidoreductase and **d** ATPase. Data are presented as mean ± S.E.M. (*n* = 10). Different superscripts indicate statistical (one-way ANOVA followed by Duncan’s post hoc test, Kruskal–Wallis ANOVA for ATPase) difference between the mean values at *p* < 0.05



were observed for SOD, CAT and GR, respectively, with time period of exposure. Although the correlation coefficient values between air exposure time and AA (0.39) and –SH (0.33) level in muscle of *H. fossilis* were low, they were significant and positive. A strong and positive correlation was observed between complex II (*r* = 0.88) activity and air exposure time. Conversely, it was negative between activities of complex III (*r* = –0.88) and V (*r* = –0.82) enzymes and air exposure time (Supplementary Fig. 1).

Discriminant function analysis

OS physiology parameters and ETC enzymes in muscle of *H. fossilis* exposed to air for 0, 3, 6, 12 and 18 h had yielded four roots which revealed discrimination among

five groups (Table 1 and Fig. 5). Figures for root 1 versus root 2 or root 3 are presented in Fig. 5 and data from statistical output (Coefficient for Canonical variables) of all the four roots are presented in Table 1. The contribution of the variables was clearly observed for separation of the groups when all the parameters studied were analyzed together for DFA (Fig. 5d). DFA between any specified roots indicates a clear separation of the groups without overlapping with each other (Fig. 5d for root 1 versus root 3, data for other roots are not given). Taking OS indices into consideration, DFA has revealed that 6, 12 and 18 h groups had overlapping with each other. However, 0 and 3 h groups had strong non-overlapping relation with each other (Fig. 5a). DFA for only antioxidant parameters indicates that only 0 h group was segregated from the other groups which were strongly overlapped with each other

(Fig. 5b). On the other hand, all the groups were separated without any major overlapping when DFA for only respiratory complex enzymes was considered (Fig. 5c). DFA of all antioxidant molecules, OS parameters and complex enzymes taken together exhibited the strongest discriminating function for SOD (via 1st root), –SH (via 4th root), complex III and V (both via 1st root) enzymes. Contribution of GPx and –SH via the second root was also significant. The overall variance was 110 % via the first two roots (Table 1).

Discussion

Tolerance to air exposure or hypoxia-anoxia is an important survival strategy for some specific lower vertebrates, including air breathing fishes. Tissue-specific large glycogen reserves, anaerobic glycolysis, strategy for increased redox status maintenance, metabolic depression with specific biochemical and transductional adjustments and finally reduced ATP generation and energy utilization are few metabolic responses adapted by such fishes for long-term survival under hypoxia state (Lushchak et al. 2001, 2005; Lushchak and Bagnyukova 2006; Welker et al. 2013). Such phenomena are observed in hypoxia

tolerant non-air breathing fish models such as goldfish (*C. auratus*) and common carp *C. carpio* (Lushchak et al. 2005; Van den Thillart and van Waarde 1985). For long-term survival and redox status regulation, expected metabolic responses at such compromised O₂ availability condition in tissues would be the effort to generate low cellular ROS (Turrens 2003). Simultaneously, reduced energy investment not only for the expression of redox status regulating antioxidant molecules but also for the other ATP utilizing pathways would normally happen under such hypoxia state to slow down different metabolic processes (Welker et al. 2013). These responses are highly dependant on metabolic capacity of tissue and species (Lushchak et al. 2005; Welker et al. 2013). The Indian stinging fish *H. fossilis* is a highly air exposure (60–70 h) tolerant teleost fish (Saha and Ratha 1989) and at the present experiment, the critical time of survival period under air exposure induced hypoxia state at 25 °C in this fish weighing 23.44 ± 1.63 g was found to be >18 h. Results of the present study indicate that the dynamic changes in redox metabolism may also be associated with such long-term survival of *H. fossilis* under water deprivation condition.

DFA was used to identify variables that were important to determine oxidative and redox status in muscle tissue of *H. fossilis* under air exposure condition. Altogether, the contribution of the studied variables such as ROS, OS indices, antioxidant enzymes, small antioxidant molecules and respiratory complexes of ETC to separate the five sets, i.e., 0, 3, 6, 12 and 18 h air exposure fish to five groups was distinct. It indicates that air exposure has definite role on the dynamic changes in OS physiology in muscle of the fish. Similar observations were recorded for OS physiology parameter due to seasonal effects in crayfish (Borkovic et al. 2008) and due to seasonal environmental O₂ concentration in mud crabs (Paital and Chainy 2013b). DFA results for only OS indices point out that 6, 12 and 18 h groups were very strongly overlapped with each other whereas 0 and 3 h groups were separated clearly in a non-overlapping manner. It indicates the increase in ROS level and OS state when the fish were kept out of water for 3 h. Once they were out of water, the above states were maintained almost at a steady state at 6, 12 and 18 h groups. Therefore, the critical time observed for OS and ROS generation by dehydration or hypoxia in muscle of *H. fossilis* was 3 (for LPx and H₂O₂) to 6 h (for PC). The unchanged PC level observed in muscle of *H. fossilis* with increased H₂O₂ and TBARS level at 3 h air exposure than 0 h may indicate the imbalance between production of oxidized proteins and removal of the same by proteasomes in favor of the former (Halliwell and Gutteridge 2001). Reduced O₂ supply to the tissue under air-exposed states could be a reason to limit the scope of producing more

Table 1 Standardized Coefficient for Canonical variables of oxidative stress indices, antioxidants and respiratory complex enzyme activities in muscle of the teleost *Heteropneustes fossilis* as a function of dehydration

Parameter	Root 1	Root 2	Root 3	Root 4
Lipid peroxidation	−0.6441	0.188734	−0.398304	0.203426
Protein carbonylation	−0.6680	0.425951	−0.087792	0.136063
H ₂ O ₂ level	−0.1244	−0.266194	0.370895	0.002763
Superoxide dismutase	0.7572	−0.010692	−0.223462	−0.110863
Catalase	−0.1896	0.292030	−0.180490	0.132903
Glutathione peroxidase	0.3600	0.588633	−0.093453	−0.276234
Glutathione reductase	0.2223	0.258620	−0.146785	−0.495911
Ascorbic acid	0.0918	0.328776	0.289557	−0.483918
Non-protein sulfhydryl	−0.3625	0.514057	−0.298406	0.640355
Complex I	−0.5732	−0.165509	−0.879549	−0.200238
Complex II	−0.2049	0.443678	0.088636	−0.234451
Complex III	0.7338	0.190586	−0.385338	0.408494
Complex V	0.6791	−0.167740	0.028270	0.380286
Eigen value	110.9761	5.512569	3.913008	1.515219
Accumulated proportion	0.9103	0.955476	0.987572	1.000000

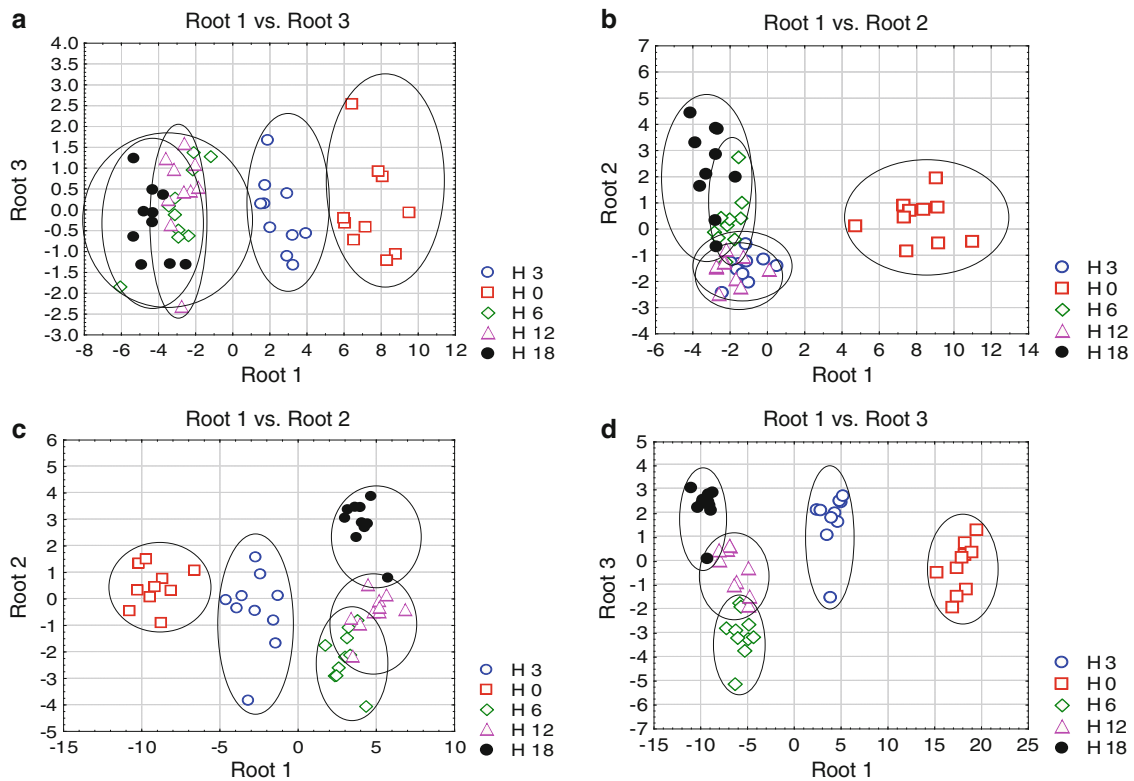


Fig. 5 Discriminant function analysis of oxidative stress indices, antioxidants and respiratory complex enzyme activity levels in muscle of the teleost *Heteropneustes fossilis* as a function of dehydration. Groups were generated by root 1 (x axis) versus root 2 (y axis). **a** Oxidative stress indicators showing overlapping within each other among 0, 3, 6 and 12 h of air exposure groups. **b** Clear group discrimination was observed for antioxidant molecules among 0, 3, 12

and 18 h groups but overlapping between 3 and 6 h exposure group is very distinct. **c** For complex enzymes, 0, 3, 6 and 12 h groups are vividly divided into 5 categories. **d** More distantly 5 groups are obtained when oxidative stress indices, antioxidant molecules and complex enzymes are considered together for discriminate function analysis. H0-control group, H3- 3 h air exposure group, H6- 6 h air exposure group, H12- 12 h air exposure group and H18- 18 h air exposure group

ROS in 3 h onward groups (Turrens 2003). This could be a reason why the tissue maintained equal level of H_2O_2 and its by-products, i.e., TBARS and PC levels at 6, 12 and 18 h air exposure groups and these three groups yielded a non-overlapping DFA result. DFA indicates that both the enzymatic and non-enzymatic redox regulating molecules had the contributions for the observed above pattern for ROS and OS level in the tissue. Other than the control group, 3, 6, 12 and 18 h groups were strongly overlapped with each other in DFA for the studied antioxidant molecules indicating the immediate response (decrease in the present case) of the antioxidant defense in fish to air exposure. Although significant changes within the air exposure groups were observed for all the four antioxidants studied and an inconsistent change were noticed for AA and -SH level, only SOD, GPx and -SH were contributed the highest for discrimination of 0 h groups from the rest four air exposure groups. Overall, DFA suggests that the reduction in antioxidant capacity is responsible for the increase in ROS and OS level in the tissue when the fish were kept out of water. The fact is further supported by the noticed negative correlation coefficients between activities

of the studied enzymes and time of air exposure and positive correlation observed between the levels of ROS and OS indices and time of air exposure. Responses such as slow metabolic rate, initiation of proteolysis and conservative investment strategy for enzyme expression in combination or alone could contribute for the collective dysfunction of the studied four redox state maintaining enzymes in the fish under air exposure condition (Welker et al. 2013). Conversely, a tolerable ROS level and OS state in the tissue at air exposure condition could be also responsible for the lowering of the antioxidant enzyme activity level. Along with the dysfunction of redox regulating enzymes, the increased ROS and OS level observed in the tissue under air exposure stress may also be due to the cumulative action of ETC complex enzymes. DFA revealed that all the five groups were separated without any major overlapping for the four studied respiratory complex enzymes. The consistent increase in complex II activity and increase in complex I activity up to 6 h of air exposure may indicate the facilitation of smooth electron supply to ETC of the fish. The significant contribution of complex II in the above process was also revealed by the higher

standardized coefficient for canonical variables observed for it in DFA. At the same time, the decreased trend of complex III and V enzymes could be an adaptation in the tissue to slow down further electron flow in ETC and ATP production in air, respectively. The high standardized coefficient for canonical value recorded for complex V also supports the latter case. Combined, DFA and correlation analysis show that the lower activities of antioxidant enzymes, reduced ETC activity with possibly alleviated ATP production rate and tolerable ROS and OS could confer the fish to survive for a long period (>18 h) out of water. On the other hand, the redox metabolic responses especially ETC activity in brain of *H. fossilis* were found to be different than muscle under such air exposure condition in *H. fossilis* (Paital 2013).

Interpreting individual set of data such as ROS and OS, redox regulating molecules and ETC activity can make the above picture clearer. The observed positive correlation between the studied ROS and OS indices (TBARS and PC) in muscle of *H. fossilis* and air exposure time undoubtedly indicate signs of OS under hypoxia state. Up to threefold increase in TBARS observed in the liver of *C. carpio* under hypoxia and a similar observation in the budding yeast *Saccharomyces cerevisiae* support the above fact (Dirmeier et al. 2002; Turrens 2003; Lushchak et al. 2005). A concomitant decreased PC level without any change in LPx level was observed in muscle of a common carp *C. carpio* under hypoxia state up to 5.5 h (Lushchak et al. 2005). Although no apoptotic markers were analyzed in the present case, initiation and gradual progression of cell decomposition and damage leading to apoptosis could also contribute to the above fact. Altogether, it could be due to the achievement of hypoxia-induced conservative strategy adapted in muscle of the fish to prevent ROS-induced damage and energy dissipation (Welker et al. 2013). Probably such conservative strategy is one of the adaptation mechanisms which help this teleost fish to survive up to so long period (18 h) out of water. To the contrary, the fish were died after a very prolonged air exposure such as 24 h (data not shown). This may indicate the initiation of reduced protein turn over, increased proteolysis and cellular metabolic collapsing and possibly triggering apoptosis in *H. fossilis* after 3 h air exposure.

The collective loss in activities of SOD, CAT, GPx and GR observed in air exposure groups than control indicates the reduction in redox capacity in the tissue. Lushchak et al. (2005) noticed similar decrease in only GPx activity in muscle of the common carp *C. carpio* under hypoxia stress up to 5.5 h. On the other hand, an increase in activity of antioxidant enzymes such as CAT and GPx was observed by the above author in brain of carp *C. carpio* under hypoxia condition for 5.5 h (Lushchak et al. 2005). At 18 h, the redox state in muscle of *H. fossilis* could be the

lowest due to the lowest level of all studied antioxidant enzymes. This could create a scope for ROS to be accumulated in the tissue at 18 h. However, no such result observed at 18 h could be due to the neutralization of surplus ROS by the increased –SH group at 18 h than the rest of the air exposure groups. On the other hand, a constant pattern was not observed for non-protein –SH groups in response to air exposure. It is reviewed that usually hypoxia has no effects on glutathione level in fishes (Lushchak and Bagnyukova 2006). No change in AA level in the tissue up to 12 h air exposure may indicate about the synthesis or accumulation of this antioxidant in the tissue (Drouin et al. 2011; Halliwell and Gutteridge 2001). This is because, AA level in muscle of *H. fossilis* under OS condition at air exposure condition should be diminished (Parihar and Dubey 1995), if AA is not synthesized in the tissue and the source of accumulated AA level in the tissue is only from dietary supplement (goat liver in the present study). A small but significantly augmented AA level was noticed at 18 h group in comparison to 3 and 6 h groups may indicate the presence of AA synthesizing enzyme, i.e., L-gulonolactone oxidase in muscle or in any other tissue of *H. fossilis* (Paital 2013). However, the presence of L-gulonolactone oxidase in fresh water teleosts including *H. fossilis* is still unclear (Drouin et al. 2011).

The higher ROS level than control could contribute to the decreased activity of complex I enzyme after 6 h onwards air exposure due to the removal of iron from the Fe–S center of complex I by ROS as observed in mammalian cells (Powell and Jackson 2003). The decreased activity of complex III noticed in air exposure groups could be anticipated by the tissue to slow down the flow of electrons to reduce the ETC activity. Finally, the decrease in transport of electrons in ETC could serve as an adaptive response to limit overproduction of ROS during hypoxia and to slow the rate of oxygen depletion to prevent anoxia in cells (William and Chandel 2011). Reduction in complex V activity can be correlated with the reduced ATP synthesis rate in the muscle of the fish under air exposure condition because, air exposure or hypoxia found to diminish ATP production in part by lowering the activity of the ETC enzymes through activation of the transcription factors, for example, hypoxia-inducible factor-1 (William and Chandel 2011). This fact is further supported by the observed decrease in activity of all complex enzymes in mammalian cells to hypoxic state for 24 h (Colleoni et al. 2013). Therefore, altogether, the observed cumulative response of ETC in muscle of *H. fossilis* under air exposure stress could be helpful to adapt the conservative strategy in the tissue to slow down energy production and subsequent utilization (Abele et al. 2011; Brand et al. 1991; Welker et al. 2013). As a result the fish could manage to survive for a longer period out of water.

The critical time of air exposure for the dynamic change in oxidative metabolism in muscle of *H. fossilis* was mostly found to be 3 h. Lethal air exposure stress period was >18 h after which the cellular physiology might be started collapsing in muscle of *H. fossilis* and possibly this arrested protein turnover or synthesis leading to proteolysis and ceasing of cellular physiology in the fish. This could be a reason for the gradual decrease in antioxidant functions in the tissue after 3 h air exposure. Redox status regulation and responses of respiratory complexes are very important to adapt specific strategy for hypoxia tolerance in *H. fossilis* in water deprivation condition. Future studies on the reoxygenation phase should be carried out to better understand antioxidant response in muscle of *H. fossilis*.

Acknowledgments The work was supported by the financial grant from Department of Biotechnology, Govt. of India, New Delhi under DBT-RA program at Biochemical Adaptation Laboratory, Banaras Hindu University, Varanasi India. The laboratory facilities offered by Prof. B.K. Ratha to conduct the entire work at Biochemical Adaptation Laboratory are highly acknowledged. I am also extremely grateful to the Heads, Dept. of Botany (for fluorimetric analysis in central facility) and Dept. of Zoology, BHU for providing necessary laboratory facilities. The inputs given by Prof. G.B.N. Chainy during the work are also duly acknowledged. Helps rendered by Mr. Ashok Yadav, Ms. Suman Mishra and Smita Verma during animal handling and tissue collection are highly acknowledged.

References

- Abele D, Philipp E, Gonzalez PM, Puntarulo S (2007) Marine invertebrate mitochondria and oxidative stress. *Front Biosci* 12:933–946
- Abele D, Vazquez-Medina JP, Zenteno-Savin T (2011) Oxidative stress in aquatic ecosystems, 1st edn. Blackwell and Wiley, USA, p 548
- Aebi H (1974) Catalase. In: Bergeyer HU (ed) *Methods of enzymatic analysis*, vol 2. Academic Press, New York, pp 673–678
- Bailly N (2013) *Heteropneustes fossilis* (Bloch 1794). In: Froese R, Pauly D (eds) *FishBase 2013: concepts, design and data sources*. ICLARM, Los Baños, p 344
- Borkovic SS, Pavlovic SZ, Kovacevic TB, Stajn AS, Petrovic VM, Saicic ZS (2008) Antioxidant defence enzyme activities in hepatopancreas, gills and muscle of Spiny cheek crayfish (*Orconectes limosus*) from the River Danube. *Comp Biochem Physiol C* 147:122–128
- Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Brand MD, Couture P, Else PL, Withers KW, Hulbert AJ (1991) Evolution of energy metabolism proton permeability of the inner membrane of liver mitochondria is greater in a mammal than in a reptile. *Biochem J* 275:81–86
- Bridges R (2013) Air-breathing in Tropical Fresh Water Fishes. Federation of British Aquatic Society. <http://www.fbas.co.uk/index.html> retrieved on 20.03.2013
- Chen PS, Toribara TY, Warner H (1956) Microdetermination of phosphorus. *Anal Chem* 28:1756–1758
- Colleoni F, Padmanabhan N, Yung H, Watson ED, Cetin I, Patot MCT, Burton GJ, Murray AJ (2013) Suppression of mitochondrial electron transport chain function in the hypoxic human placenta: A Role for miRNA-210 and protein synthesis inhibition. *PLOS One* 8:e55194–e55194
- Cormier A, Morin C, Zini R, Tillement JP, Lagrue G (2001) In vitro effects of nicotine on mitochondrial respiration and superoxide anion generation. *Brain Res* 900:72–79
- Das K, Samanta L, Chainy GBN (2000) A modified spectrophotometric assay of superoxide dismutase using nitrite formation by super oxide radicals. *Ind J Biochem Biophys* 37:201–204
- Dirmeier R, O'Brien KM, Engle M, Dodd A, Spears E, Poyton RO (2002) Exposure of yeast cells to anoxia induces transient oxidative stress. Implications for the induction of hypoxic genes. *J Biol Chem* 277:34773–34784
- Drouin G, Godin J, Pagé B (2011) The genetics of vitamin C loss in vertebrates. *Curr Genomics* 12:371–378
- Erfanullah, Jafri AK (1999) Growth, feed conversion, body composition and nutrient retention in fingerling catfish, *Heteropneustes fossilis* (Bloch), fed different sources of dietary carbohydrates. *Aquac Res* 30:43–49
- Garson GD (2008) Statnotes: Topics in multivariate analysis. <http://www2.chass.ncsu.edu/garson/pa765/statnote.htm>. Retrieved on 24 Feb 2013
- Gassner B, Wuthrich A, Scholtysik G, Solioz M (1997) The pyrethroids permethrin and cyhalothrin are potent inhibitors of the mitochondrial complex I. *J Pharmacol Exp Ther* 281:855–860
- Graham JB (1997) Air-breathing fishes: evolution, diversity, and adaptation. Academic Press, USA 299
- Halliwell B, Gutteridge JMC (2001) Free radicals in biology and medicine, 3rd edn. Oxford University Press, New York
- Jennrich RI (1977) Stepwise discriminant analysis. In: Enslein K, Ralston A, Wilf HS (eds) *Statistical methods for digital computers*. Wiley, New York, pp 76–95
- Lambowitz AM (1979) Preparation and analysis of mitochondrial ribosomes. *Method Enzymol* 59:421–433
- Levine RL, Williams JA, Stadtman ER, Shacter E (1994) Carbonyl assays for determination of oxidatively modified proteins. In: Packer L (ed) *Methods Enzymol*, vol 233. Academic press, California, pp 346–357
- Lushchak VI, Bagnyukova TV (2006) Effects of different environmental oxygen levels on free radical processes in fish. *Comp Biochem Physiol B Biochem Mol Biol* 144:283–289
- Lushchak VI, Lushchak LP, Mota AA, Hermes-Lima M (2001) Oxidative stress and antioxidant defenses in goldfish *Carassius auratus* during anoxia and reoxygenation. *Am J Physiol Regul Integr Comp Physiol* 280:R100–R107
- Lushchak VI, Bagnyukova TV, Lushchak OV, Storey JM, Storey KB (2005) Hypoxia and recovery perturb free radical processes and antioxidant potential in common carp (*Cyprinus carpio*) tissues. *Int J Biochem Cell Biol* 37:1319–1330
- Maiti AK, Saha NC, Paul G (2010) Effect of lead on oxidative stress, Na⁺K⁺ATPase activity and mitochondrial electron transport chain activity of the brain of *Clarias batrachus* L. *Bull Environ Contam Toxicol* 84:672–676
- Massey V, Williams CH (1965) On the reaction mechanism of yeast glutathione reductase. *J Biol Chem* 240:4470–4481
- Menon AGK (1999) Check list: fresh water fishes of India. *Rec Zool Surv India, Misc. Publ., Occas. Pap. No. 175*, 366 p
- Mitsui A, Ohta T (1961) Photooxidative consumption and photoreductive formation of ascorbic acid in green leaves. *Plant Cell Physiol* 2:31–44
- Munshi JSD, Pandey BN, Pandey PK, Ojha J (1978) Oxygen uptake through gills and skin in relation to body weight of an air-

- breathing siluroid fish, *Saccobranchus* (= *Heteropneustes*) *fossilis*. J Zool Lond 184:171–180
- Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissue by thiobarbituric acid reaction. Anal Biochem 95:351–358
- Paglia DE, Valentine WN (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 70:158–169
- Paital B (2013) Antioxidant and oxidative stress parameters in brain of *Heteropneustes fossilis* under air exposure condition; role of mitochondrial electron transport chain. Ecotoxicol Environ Saf 95:69–77
- Paital B, Chainy GBN (2010) Antioxidant defenses and oxidative stress parameters in tissues of mud crab (*Scylla serrata*) with reference to changing salinity. Comp Biochem Physiol C 151:142–151
- Paital B, Chainy GBN (2012) Effects of salinity on O₂ consumption, ROS generation and oxidative stress status of gill mitochondria of the mud crab *Scylla serrata*. Comp Biochem Physiol C 155:228–237
- Paital B, Chainy GBN (2013a) Modulation of expression of SOD isoenzymes in mud crab (*Scylla serrata*): effects of inhibitors, salinity and season. J Enz Inhibition Med Chem 28:195–204
- Paital B, Chainy GBN (2013b) Seasonal variability of antioxidant biomarkers in mud crabs (*Scylla serrata*). Ecotoxic Environ Saf 87:33–41
- Paital B, Kumar S, Farmer R, Tripathy NK, Chainy GBN (2011) *In silico* prediction and characterization of 3D structure and binding properties of catalase from the commercially important crab, *Scylla serrata*. Interdiscip Sci Comput Life Sci 3:1913–2751
- Paital B, Kumar S, Farmer R, Tripathy NK, Chainy GBN (2013) *In silico* prediction of 3D structure of superoxide dismutase of *Scylla serrata* and its binding properties with inhibitors. Interdiscip Sci Comput Life Sci 5:69–76
- Parihar MS, Dubey AK (1995) Lipid peroxidation and ascorbic acid status in respiratory organs of male and female freshwater catfish *Heteropneustes fossilis* exposed to temperature increase. Comp Biochem Physiol C 112:309–313
- Powell CS, Jackson RM (2003) Mitochondrial complex I, aconitase, and succinate dehydrogenase during hypoxia-reoxygenation: modulation of enzyme activities by MnSOD. Am J Physiol Lung Cell Mol Physiol 285:L189–L198
- Romero MC, Ansaldo M, Lovrich GA (2007) Effect of aerial exposure on the antioxidant status in the subantarctic stone crab *Paralomis granulosa* (Decapoda: Anomura). Comp Biochem Physiol C 146:54–59
- Saha N, Ratha BK (1989) A comparative study of ureogenesis in freshwater air-breathing teleosts. J Exp Zool 252:1–8
- Saha N, Das L, Dutta S, Goswami UC (2001) Role of ureogenesis in the mud-dwelled singhi catfish (*Heteropneustes fossilis*) under condition of water shortage. Comp Biochem Physiol A 128:137–146
- Sedlak J, Lindsay RH (1968) Estimation of total, protein-bound and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 25:192–205
- Staniek K, Nohl H (1999) H₂O₂ detection from intact mitochondria as a measure for one-electron reduction of oxygen requires a non-invasive assay system. Biochim Biophys Acta 1413:70–80
- Storey KB, Storey JM (2004) Metabolic rate depression in animals: transcriptional and translational controls. Biol Rev 79:207–233
- Takano H, Zou Y, Hasegawa H, Akazawa H, Nagai T, Komuro I (2003) Oxidative stress-induced signal transduction pathways in cardiac myocytes: involvement of ROS in heart diseases. Antioxid Redox Sign 5:789–794
- Talwar PK, Jhingran AG (1991) Inland fishes of India and adjacent countries, vol 2. Oxford and IBH Publishing Company Ltd., New Delhi 1158 pp
- Thurston RV, Gehrke PC (1993) Respiratory oxygen requirements of fishes: description of OXYREF, a data file based on test results reported in the published literature. In: Russo RC, Thurston RV (eds) Fish physiology, toxicology, and water quality management. proceedings of an international symposium, Sacramento, US Environmental Protection Agency EPA/600/R-93/157, California, USA, p 95–108
- Tisdale HD (1967) Preparation and properties of succinic-cytochrome c reductase (Complex II-III). Method Enzymol 10:213–215
- Turrens JF (2003) Mitochondrial formation of reactive oxygen species. J Physiol 552(2):335–344
- Van den Thillart G, van Waarde A (1985) Teleosts in hypoxia: aspects of anaerobic metabolism. Mol Physiol 8:393–409
- Welker AF, Moreira DC, Campos ÉG, Hermes-Lima M (2013) Role of redox metabolism for adaptation of aquatic animals to drastic changes in oxygen availability. Comp Biochem Physiol A Mol Integr Physiol 165:384–404
- William WW, Chandel NS (2011) Hypoxia. 2. Hypoxia regulates cellular metabolism. Am J Physiol Cell Physiol 300:C385–C393
- Zaccane G, Casio PL, Fasulo S, Licata A (1985) The effect of an anionic detergent on complex carbohydrates and enzyme activities in the epidermis of the catfish *Heteropneustes fossilis* (Bloch). Histochem J 17:453–466