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Ultrastructural Indications of Differential Stress Responses Induced by Experimental Hypoxia in Two Freshwater Fishes - *Carassius auratus* and *Heteropneustes fossilis*

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Abstract

Aquatic organisms exhibit a great deal of sensitivity to changes in their environment to which they respond in very different ways and degrees. The present study was conducted to address this issue. Two freshwater fishes - *Carassius auratus* and *Heteropneustes fossilis* adapted to different ecological habitats were exposed to 12 h of moderate hypoxia (dissolved oxygen concentration 50-30% saturation) and 12 h of acute hypoxia (dissolved oxygen concentration < 30% saturation) conditions. The adrenal tissues of the two fishes were examined electron microscopically for the stress responses in the interrenal and chromaffin tissues - the tissues associated with the production of the stress hormones. The findings indicate that while exposure to moderate hypoxia can be stressful to *C. auratus* eliciting hyperactivity in interrenal cells and stimulation of chromaffin cells, it does not produce any noticeable change in *H. fossilis*. Exposure to acute hypoxia conditions produced degenerative changes in *C. auratus* whereas in *H. fossilis* the stress responses were much reduced. In both the fishes, acute hypoxia induced activation of the chromaffin cells as displayed by the increased presence of adrenaline granules. However, in *H. fossilis*, a paracrine interaction of the interrenal and chromaffin tissues appear to be operating in modifying the stress response. The results highlight low tolerance of *C. auratus* and considerable plasticity of *H. fossilis* to hypoxia stress; capabilities that are in unison with their adaptations to different ecological habitats.

Keywords

Chromaffin; Freshwater Fish; Hypoxia; Interrenal; Stress

Introduction

Ecological sustenance and survival in highly diverse and changing environments is one of the greatest challenges that living organisms face. Sudden or slow changes viz. fluctuations in physico - chemical parameters, ecological disturbances inducing habitat shifts, disturb the environmental homogeneity and present known/unknown challenges to the biological species which they tend to mitigate, resist, adapt or eventually submit to, often becoming a cause of their mortality. As compared to the terrestrial organisms, aquatic species, being in constant and intimate contact with their environs, are far more affected by these challenges. Such changes that create adverse conditions act as stressors and subject the organisms to considerable stress.

Fish, like other animals handle stress through primary and secondary stress responses mediated by the action of stress hormones [1,2]. The interrenal tissues in fish are an important constituent of the hypopthalamic - pituitary axis (HPI) and closely associated with the synthesis and release of the primary stress hormones–the corticosteroids and the catecholamines that are produced by the chromaffin tissues upon sympathetic stimulation [3,4]. However the release of catecholamines is much rapid elevating the circulation levels immediately upon stress [5-7]. The stress hormones trigger a broad spectrum of biochemical and physiological mechanisms aimed at countering the onset of stress and involve metabolic effects like hyperglycaemia, hyperlacticaemia, catabolism, osmotic and ionic imbalances, in blood and rapid mobilization of energy in the tissues [8-10]. Morphological effects of stress responses involving histopathological changes in body tissues have been widely reported in several fishes [11].

Dissolved oxygen (DO) concentration are imperative components of the aquatic systems but are also limiting. They play a critical role in deciding the distribution and abundance of biological communities [12] as the DO concentrations vary considerably between normoxia and severe hypoxia [12,13] and so a major cause of stress. Sudden depletion in the dissolved oxygen concentrations can result either naturally by rise in temperatures, acidification of water, or overgrowth of vegetation in natural or captive environments or due to anthropogenic activities. Fall in concentrations of DO to values 50%-30% saturation can cause moderate hypoxic conditions whereas less than 30% results in acute hypoxia or anoxia. Acute hypoxia is known to induce cardiorespiratory and ventilatory responses in fish [14] whereas chronic hypoxia elicits metabolic and physiological adjustments for greater oxygen extraction and delivery to the tissues [15,16] and biochemical changes that will allow the tissues to function and survive at low oxygen [17-19]. Experimentally induced hypoxia reportedly produce histopathological changes such as necrosis, hyperaemia, haemorrhage, hyperplasia and or hypertrophy in several tissues like liver, spleen, and anterior and posterior kidney [20]. Interrenal hypertrophy and changes in nuclear staining characteristics were reported to be induced in freshwater fishes - *Carassius auratus, Cyprinus carpio* and *Heteropneustes fossilis* in response to hypoxia [21].

Though all fish species are broadly speaking sensitive to hypoxic conditions, differences in their stress responses do surface between species (interspecific variation) or within ecologically distinct populations of the same species (intraspecific variation) [22]. Differences are also seen in the responses of water and air breathers, which display different behavioural and metabolic adjustments to hypoxia stress [23,24]. Hypoxia tolerant fishes possess lower metabolic rates (less oxygen demand) and higher haemoglobin–oxygen affinities than hypoxia tolerant fishes. The present ultrastructural study was undertaken with a purpose to examine and analyse the histological stress responses in the interrenal tissues of two fishes - *Carassius auratus* and *Heteropneustes fossilis* inhabiting ecological habitats of differently oxygenated environments. *C. auratus* inhabits normoxic waters and is a water breather whereas *H. fossilis* survives in hypoxic waters or those with variable oxygen concentrations and is an air breather.

Materials and Methods

Live adult specimens of fresh water species of *Heteropneutes fossilis* (30-50g ± 2.5; 15-25cm), and *Carassius auratus* (10–25g ± 1.5; 9–12cm) were purchased from the local market and maintained in the laboratory. The adult fishes were maintained in aerated glass tank aquarium of standard size 30"x18"x12". Water quality parameters like temperature (25-30 ^{0}C), dissolved oxygen level (7.5 - 11.5 mg/l) and pH (7.03-8.5) were regularly monitored. Fishes were kept in normal photoperiod regimes of 12L:12D. *H. fossilis* were fed with chopped goat liver on alternate days and *C. auratus* were fed with an artificial feed daily. All the fishes were acclimatized for 15-20 days before they were put on experiment.

Experimental Set-up for hypoxia

A simplified version of Beitinger model [25] was designed to create hypoxia conditions in the aquarium using the method of bubbling of nitrogen gas. An airtight aquarium was connected to the nitrogen gas cylinder through a pipe. The pressure of the nitrogen gas to be circulated in the aquarium was constantly monitored through the regulator. Dissolved oxygen level was monitored through the Standard Winkler's method before, during the course and after the experiment. Different levels of hypoxia (moderate and acute) were created by regulating the pressure of the nitrogen gas within the aquarium and monitoring the dissolved oxygen levels in the water. Two fishes were exposed to hypoxia condition at a time and three replicates were performed for each experiment.

Experimental Procedure

Exposure to Moderate Hypoxia: Dissolved oxygen levels of (30-50% oxygen saturation) were maintained for moderate hypoxia, using two fishes per exposure. The exposure period was 12 h.

Exposure to Acute Hypoxia: A dissolved oxygen level less than 30% concentration was maintained for acute hypoxia condition, using two fishes per exposure. The exposure period was 12 h.

Electron Microscopy: Fishes *H. fossilis*, and *C. auratus* (both control and experimental) were gently netted out of the aquarium weighed and measured. They were anaesthetized with ethyl 3 aminobenzoate (MS222) (Across, Germany) at a concentration of 0.35g/l. They were dissected and the head kidney and anterior kidney removed and fixed in 2.5% glutaraldehyde solution for 4 h after which they were washed in 0.1 M phosphate buffer (pH 7.4). The samples were post fixed for 1 h in 1% osmium tetraoxide solution in Millonig buffer, dehydrated, and embedded in an araldite resin and 1 µm thick sections were sliced. Semithin sections were stained with toludine blue and viewed under LM. Ultra-thin sections were stained in aqueous uranyl acetate and lead citrate. Finally the grids were viewed in transmission electron microscope (Model no. TECNAI G^{2} S TWIN (FEI/120KV).

Results

H. fossilis

Control: In semithin sections (Figure 1), the adrenal tissues show clusters of interrenal and chromaffin tissues dispersed in the haemopoetic tissue in close proximity to the post cardinal vein (pcv) and its branches. The interrenal cells are scattered in the haemopoetic tissue whereas the chromaffin cells can be located generally close to the walls of the pcv. The interrenal cells present an appearance typical of steroidogenic cells, cytoplasm rich in free and grouped ribosomes, numerous small dense bodies, electron lucent vesicles, and large number of numerous usually round mitochondria having vesicular cristae and dense matrix and occupying a major part of the cell (Figure 2). The nuclei are large, with prominent eccentrically placed nucleoli and

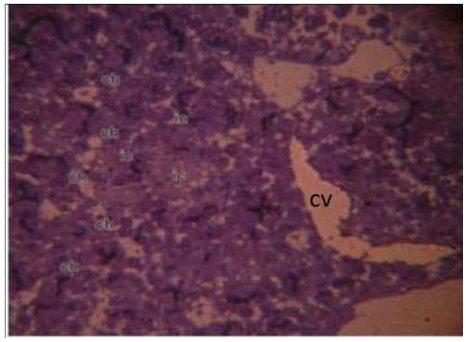

Figure 1: Semithin section of the head kidney of H.fossilis (control) showing interrenal cells(ic) chromaffin cells(ch) and haemopoietic tissue (h) and cardinal vein (CV) × 40.

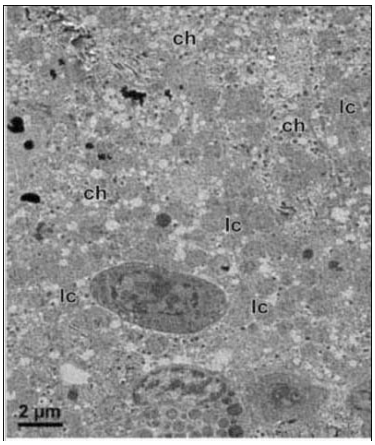

Figure 3: Electron micrograph of the adrenaline tissue of H.fossilis(control) showing chromaffin cells(ch) and interrenal cells (ic) in tight contact.

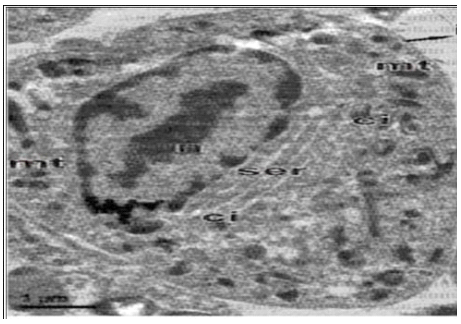

Figure 5: Electron micrograph of interrenal cells of H.fossilis after exposure to 12hrs moderate hypoxia. Showing long cisternae of smooth endoplasmic reticulum (sER) and heterochromatic nuclei (n).

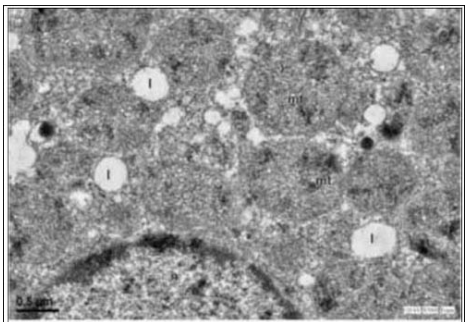

Figure 2: Electron micrograph of interrenal cell of H.fossilis(-control) showing hetero chromatic nucleus (n) tubulo vesicular mitochondria (mt) and lipid droplet (l)

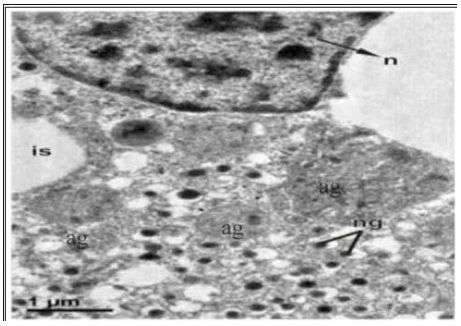

Figure 4: Electron micrograph of chromaffin cell of H.fossilis(-control) showing nucleus (n), intercellular spaces (is) between the cells and numerous noradrenaline granules (ng) and few adrenaline granules (ag).

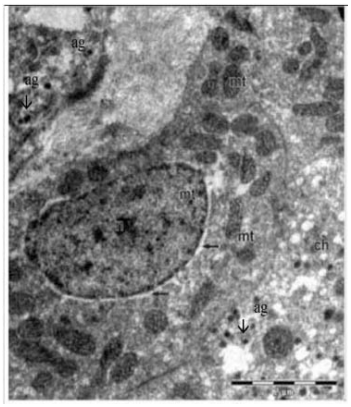

Figure 6: Electron micrograph of interrenal cells of H.fossilis after exposure to 12hrs acute hypoxia. showing numerous, smaller, elongated to dumbbell shaped mitochondria (mt).

Note: The wide perinuclear gap(→) and adjacent chromaffin cell(ch) with dense adrenaline granules (ag) very close to thin cell membrane of interrenal cell (→)

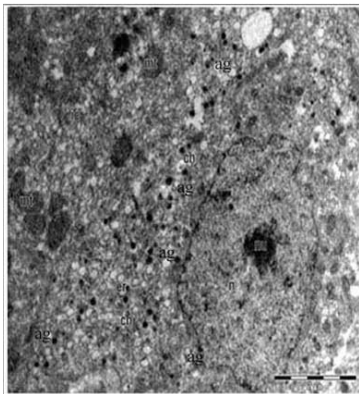

Figure 7: Electron micrograph of chromaffin cell of H.fossilis after exposure to 12hrs acute hypoxia. The nucleus (n) is euchromatic with a prominent nucleolus.

Note: Presence of numerous adrenaline granules (ag) in chromaffin cell.

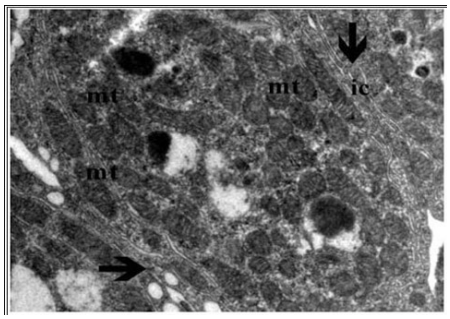

Figure 9: Electron micrograph of head kidney of c.auratus (Control) showing internal cells (ic) with tubulo vesicular mitochondria (mt) and smooth endoplasmic reticulum (sER→)

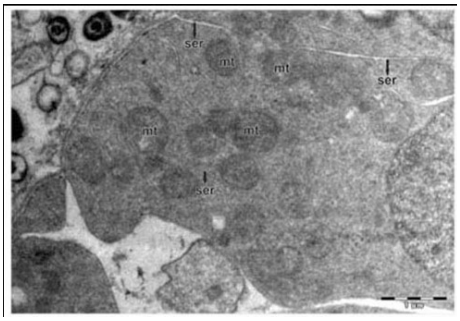

Figure 11: Electron micrograph of internal cells of c.auratus after exposure to 12hr moderate hypoxia. smooth endoplasmic reticulum (sER) were seen traversing the cytoplasm and opening at the cell surface and close to the mitochondria (m).

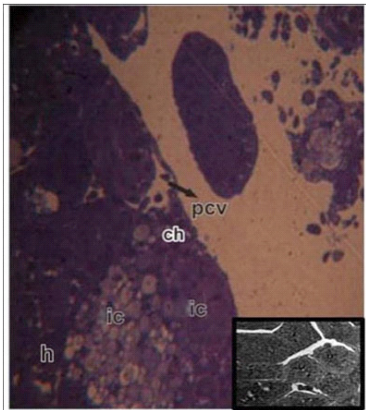

Figure 8: Semithin section of adrenal tissue of c.auratus (control).

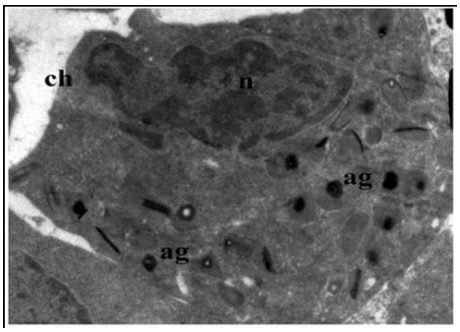

Figure 10: Electron micrograph of c.auratus (Control) showing chromaffin cell (ch), nucleus (n), adrenaline granules (ag).

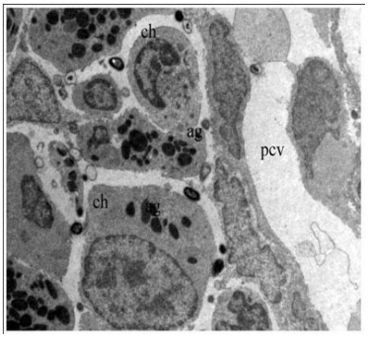

Figure 12: Electron micrograph of c.auratus after exposure to 12hr moderate hypoxia showing chromaffin cell (ch) close to post cardinal vein (pcv)

Note: The increased number of adrenaline granules (ag) in the cells.

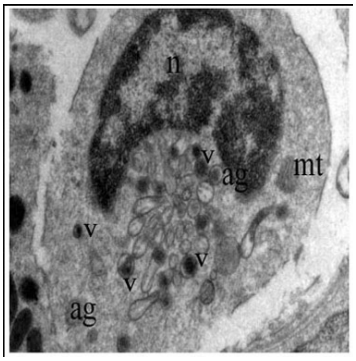

Figure 13: Electron micrograph of c.auratus after exposure to 12hr moderate hypoxia showing nucleus(n), mitochondria (mt) and few adrenaline (ag) and vesicles (v) of varying opacities.

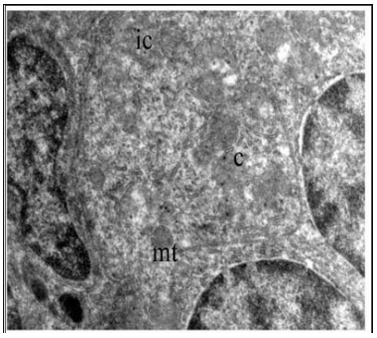

Figure 14: Electron micrograph of c.auratus after exposure to 12hr acute hypoxia showing degenerating interrenal cell (ic) with smaller mitochondria (mt) and broken cisternae of smooth endoplasmic reticulum (c).

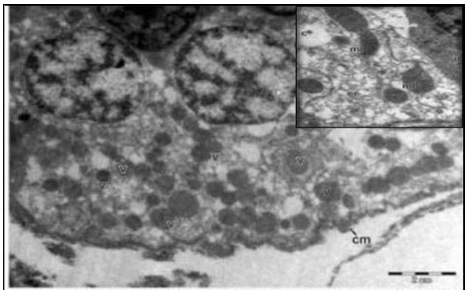

Figure 15: Electron micrograph of c.auratus after exposure to 12hr acute hypoxia showing irregular and frayed peripheral cell membranes (cm) and small dense vesicles (v) close to outer cell membrane.

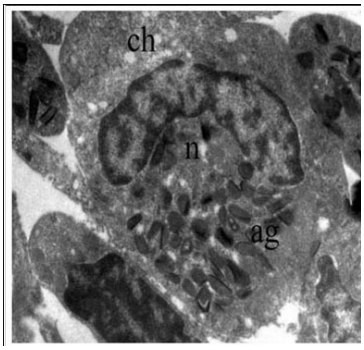

Figure 16: Electron micrograph of chromaffin cell of c.auratus after exposure to 12hr acute hypoxia showing chromaffin cell (ch) with nucleus (and numerous adrenaline granules (ag).

clumped heterochromatin. Numerous intercellular spaces are seen in between the cells. The chromaffin cells where present are always in proximity to interrenal cells and interdigitating with them (Figure 3). They are characterized by a large nucleus with heterochromatin, vesiculate mitochondria and cytoplasm occupied by predominantly noradrenaline granules and few adrenaline granules (Figure 4). The noradrenaline granules are pleiomorphic, small electron dense eccentric granules surrounded by a clear broad halo and the adrenaline granules are large granules of dense electron opacity and a large number of moderately opaque granules were also present in the cytoplasm.

Moderate Hypoxia: The only noticeable change that was seen in the interrenal cells after 12 h of exposure to moderate hypoxia was increased clumping of heterochromatin in the nucleus (figure 5).

Acute Hypoxia: In the interrenal cells, the mitochondria appear ellipsoidal and dumbbell shaped (Figure 6). The plasma membrane appears very thin at some places and adrenaline granules in adjacent chromaffin cell can be seen present close to these spots. Nuclei are euchromatic showing wide perinuclear spaces and nucleoli are not so prominent. The chromaffin cells show a predominance of adrenaline granules that are large, dense, few noradrenaline granules surrounded by narrow clear halo and an increase in the number of vesicles of variable opacities (Figure 7). Nuclei are euchromatic with a prominent nucleoli.

Carassius auratus

Control: In the gold fish, the head kidney containing the adrenal tissues is located in the anterior most part of the opisthonephros. It consists of the interrenal tissues and chromaffin tissues dispersed in between the haematopoetic tissues as seen in the semithin sections (Figure 8). The interrenal cells are columnar to polygonal in shape (figure 8). In both the cell types, the nucleus is large with conspicuous nucleolus and has little heterochromatin. Mitochondria are numerous having dense matrix and tubulo-vesicular cristae (Figure 9), prominent smooth endoplasmic reticulum (sER), and ribosomes - both free and grouped are abundantly present in the cytoplasm. Chromaffin cells are located usually in proximity to cardinal vein and its branches, and characterized

by a large nucleus and adrenaline granules (Figure 10).

Moderate Hypoxia: After exposure to 12 h of moderate hypoxia, in the interrenal cells numerous mitochondria were observed, prominent and several cisternae of sER were seen traversing the cytoplasm usually in close approximation to mitochondria, some of them even extended to the periphery of cells (Figure 11) and a euchromatic nuclei with an inconspicous nucleoli. In the chromaffin cells, the cytoplasm was occupied generally by large moderately opaque adrenaline granules (Figure 12) and some cells possessed large vesicles containing electron lucent material (Figure 13).

Acute Hypoxia: After exposure to 12 h of acute hypoxia, the interrenal cells (Figure 14) presented smaller and more numerous mitochondria which appeared dumb-bell shaped (inset in Fig. 15), broken cisternae of sER, large vesiculated ER and small clear spherical vesicles having electron lucent material and numerous clear spaces. The outer plasma membrane appeared irregular in outline and highly frayed (Figure 15) and the nucleus showed more of condensed heterochromatin. The chromaffin cells showed large heterochromatic nucleus and cytoplasm occupied by several moderately electron opaque granules (Figure 16).

Discussion

The results of the ultrastructural investigation of the adrenal tissues of the two fishes - *C. auratus* and *H. fossilis* show that a separate head kidney exists in *H. fossilis*, a characteristic feature of catfishes unlike in *C. auratus* where it is a part of the opisthonephros. Interrenal and chromaffin cells are present in the adrenal tissues in both the fishes. The interrenal cells of both the fishes show typical steroidogenic characteristics - large and numerous tubular - vesiculo mitochondria occupying a major part of the cell, abundant smooth ER and free or grouped ribosomes. Interrenal cells as observed in *H. fossilis* and *C. auratus* were found to be similar to those reported in many other fishes: *Pimephales promelas* [26], *Aphamus* [27], stickleback *Gastroesteus* [28] in the characid *Bryconcephalus* [29] and in catfish, *Clarias gariepinus* [30]. In the present study, an interesting observation seen for *H. fossilis* is the juxtaposed nature of interrenal and chromaffin cells with their cell processes interdigitating with each other (Figure 14). This is indicative of a paracrine nature of these cells and has been commonly observed in the adrenal gland of other teleosts [31,7] and also in other higher vertebrates [32,33].

Stress Responses to Hypoxia

Structural modifications in the interrenal and chromaffin tissues have been indicated in response to seasonal and reproductive periods [29,34-36], to osmotic stress [27,37,38], to cold stress [39] and to exposure to detergents [40]. Morphometric changes to aquatic hypoxia in the interrenal cells of some tropical freshwater fishes were reported [21] and interrenal hypertrophy was indicated as the first indication of cellular stress in this tissue. This ultrastructural study highlights degenerative changes in the interrenal cells of *C. auratus* and adaptive responses in *H. fossilis* upon long exposures to moderate hypoxia as well as acute hypoxia.

Exposure to 12 h of Moderate Hypoxia

C. auratus displays considerable stress responses in the interrenal cells viz. numerous mitochondria increased clumping of nuclear heterochromatin, enormous proliferation of sER with long cisternae seen in close apposition to mitochondria (figure 11), and a euchromatic nucleus with an inconspicous nucleoli- all indicative of interrenal hyperactivity. Chromaffin cells appeared in secretory mode with the preponderance of large moderately opaque adrenaline granules (figure 12) in various opacities. *H. fossilis* accustomed to habitats of low oxygen levels does not indicate any such cellular responses.

Exposure to 12 h of Acute Hypoxia

Following exposure to 12 h of acute hypoxia, gold fish displays degenerative changes in the interrenal cells as indicated by smaller and more dividing mitochondria, numerous vesiculated sER, broken cisternae of sER and numerous clear vesicles occupying the cytoplasm or those filled with some electron lucent material and the plasma membranes appearing irregular and highly frayed. In *H. fossilis* similar changes were also seen in the interrenal cells but to a much lesser degree. In both the fishes, the nuclei were euchromatic, with smaller nucleoli and showed wide perinuclear spaces indicative of an active nucleus and heightened nuclear activity. Characteristically, the chromaffin cells of both the fishes showed the presence of larger number of adrenaline granules as compared to noradrenaline granules in the control which is suggestive of the synthesis and secretion of the adrenaline stress hormone under stress. The role of adrenaline stress hormones in initiating physiological compensatory mechanisms to restore homeostasis is well known. Stress induced necrotic changes within cells have been described as primary indications of cytotoxicity [41] and those induced by hypoxia have also been widely reported [20,42].

Considering the responses of the two fishes, it may be noted that *Carassius auratus* cannot tolerate long exposure to even moderate hypoxia and shows cellular stress signals whereas *Heteropneustes fossilis* is almost unaffected by long exposure to moderate hypoxia. Exposure to long periods of acute hypoxia produced degenerative changes in the interrenal cells of *C. auratus* whereas in *H. fossilis* the degenerative changes were smaller. In *H. fossilis*, a paracrine effect of the activities of the chromaffin cells and interrenal cells probably appear to modify the stress response towards a slower and adaptive type of response. Vertebrates are known to synthesize and release catecholamine hormones–adrenaline and noradrenaline in response to acute physiological conditions [6,43]. Adaptive responses help organisms to survive stressful conditions which may manifest as increase in lamellar surface area of gills as in cichlids and crucian carp, *Carassius carassius* [16,42,44] or through changes in gene expression/repres-

sion thereby affecting protein biosynthesis and metabolic pathways [42] or through allostasis [45,46].

Variability in stress responses is not an uncommon feature in fishes [22, 47,48], not even in closely related species [9,21]. Differences in tolerance capacities of the gold fish, *C. auratus* and the catfish, *H. fossilis* to hypoxia stress have already been suggested [21] and the present study lends support to it. I, a water breather, surviving in freshwater habitats of rich dissolved oxygen concentrations, is stressed by moderate hypoxia and shows limited tolerance. *H. fossilis*, an air-breather inhabiting low oxygen environment shows a much greater tolerance for hypoxia and still displays cellular stress responses. *H. fossilis* presents a case similar to that of the Atlantic killifish, *Fundulus heteroclitus* that has evolved in highly variable environment and shows considerable ability for short term resistance and long term plasticity to stressors [22]. The capacity of *H. fossilis* to acclimate moderate to acutely low oxygenated waters yet displaying cellular stress responses is suggestive of the effects of local adaptation on stress responses even in broadly tolerant organisms [49,22].

Conclusion

The present study which was conducted to assess stress related histological changes in the adrenal tissues of two ecologically distinct freshwater fishes highlights some very important differences in the tolerance capacities of the two fishes with respect to experimentally induced hypoxia conditions. *C. auratus* – a water breather and adapted to normoxic water, shows stress signals in the interrenal tissues when exposed to moderate hypoxia whereas *H. fossilis* – an air breather and surviving in waters of low dissolved oxygen concentrations remains unaffected by it. Exposure to acute hypoxia conditions produced degenerative changes in the interrenal tissues of both the fishes but they were much more pronounced in *C. auratus* as compared to that in *H. fossilis*, and active stimulation of the chromaffin cells in both the fishes involving probably synthesis and/or secretion of the catecholamine hormones. The close proximity of the interrenal and chromaffin tissues which is far greater in *H. fossilis* than in *C. auratus*, calls for a paracrine nature of interaction between the two tissues. The results clearly indicate a much greater plasticity of *H. fossilis* to hypoxia stress, placing it in the same category of fishes as the Atlantic killifish–that are adapted to variable environments and display short term resistance and plasticity to stressors. The study also highlights the effect of local adaptation on the stress tolerance of fishes.

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