CHAPTER-5
DISCUSSION
AND CONCLUSION
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As far as catfish are concerned, literature is scant is on the subject of study undertaken. Much work has been done, however, on carps and other teleosts. The findings of the present study are significant from four considerations: one - the micro and ultrastructural features of the interrenal and chromaffin tissues; two - their distribution pattern; three - their innervation pattern; and four - their stress responses to aquatic hypoxia. These are discussed below.

(I) Micro and Ultrastructural Features of the Interrenal and Chromaffin Tissues

*Carassius auratus* shows the presence of two types of interrenal cells - light and dark cells based upon the electron density of the cytoplasm. Both the cells are endowed with a large heterochromatic nucleus, numerous mitochondria with tubulo - lamellar/vesicular cristae and abundant smooth endoplasmic reticulum (sER) traversing the cytoplasm. The chromaffin cells are also characterized by two types- adrenaline cells and noradrenaline cells depending upon the types of granules present in these cells. The granules in the adrenaline cells are electron - lucent or have moderately electron - dense material where as in the noradrenaline cells, the granules are pleiomorphic and have highly electron - dense material, as has been observed in the chromaffin cells of several teleosts (see Gallo and Civinni, 2003). Kobayashi and Coupland (1993) have attributed the appearance of two different types of granules in these cells to the differential reaction of the catecholaminergic substances stored in the granules to the fixatives glutaraldehyde and osmium.
tetraoxide. Two types of chromaffin cells have been reported for *Pimephales promelas* (Yoakim and Grizzle, 1980), *Salmo gairdneri* (Mastrolia *et al*., 1984), *Gastroesteous aculeatus* (Gallo *et al*., 1993), *Brycon cephalus* (Rocha *et al*., 2001) and *Epinephilus tauvina* (Abdel-Aziz *et al*., 2010).

The ultrastructural findings on the interrenal tissues of the catfish, *Heteropneustes fossilis* reveal only one type of interrenal cells which are similar to those seen in other fishes like *Pimephales promelas* (Yoakim and Grizzle, 1980), *Aphanius* (Mastrolia and Gallo, 1989), stickleback, *Gastroesteous* (Civinni *et al*., 1997) and the characid, *Brycon cephalus* (Rocha *et al*., 2001). Other ultrastructural features found in the interrenal cells of *H. fossilis* such as numerous vesiculated sER, abundant large mitochondria with tubulo-vesicular cristae and free ribosomes are similar to those reported earlier in the only ultrastructural study in the catfish, *Clarias gariepinus* (Vernèclean *et al*., 1995) and in *Pimephales promelas* (Yoakim and Grizzle, 1980). An interesting observation in the present study is that the interrenal cells and the chromaffin cells of *H. fossilis* are in tight contact with each other with processes of both the types of cells interdigitating with each other. This is indicative of a paracrine type of interaction among the cells which is commonly observed in the adrenal gland of other teleosts (Reid *et al*., 1996, 1998) and other higher vertebrates (Ehrhart-Bornstein *et al*., 1998; Mazzocchi *et al*., 1998).

(II) **Distribution pattern**

In the present study, the two carps, *C. auratus* and *C. carpio* show the interrenal and chromaffin tissues present along the cardinal veins - a distributional arrangement similar to that proposed by Nandi (1961) for
members of the cyprinidae family and so demonstrated in *Acanthobrama terrae sanctae* (Yaron, 1970), in *Cyprinus carpio* (Imagawa et al., 1996) and in *C. auratus* (Sampour, 2008). The absence of a separate head kidney is another feature which finds agreement among these studies (in the carps, the head kidney remains a part of the trunk kidney).

On the other hand, in the catfishes - *H. fossilis* and *C. batrachus*, the interrenal and chromaffin tissues are found to lack a definite arrangement. The head kidney which is a separate organ far removed from the main opisthonephros, has chromaffin cells embedded in the wall of the vein as well as interspersed among the interrenal cells. The interrenal tissues, on the other hand, may be present in the vicinity of the cardinal veins as well as distributed as clusters in the haematopoietic tissue. However, the number of chromaffin cells is comparatively much more in the head kidney than in the anterior part of the trunk kidney.

A diffuse distribution of steroidogenic (interrenal) and chromaffin tissues which was reported earlier in Agnatha (Youson, 1972) and in the holostean, *Amia calva* (Youson et al., 1976; Youson and Butler, 1976 and Butler and Youson, 1986) is regarded a primitive position as regards the phylogenetic evolution of the adrenal glands. The distribution of the two tissues in these catfishes, though not strictly of a diffuse nature, occurs not only in variable positions in proximity to cardinal vein but also in the haematopoietic tissue and appear closer to the holostean arrangement as against the more advanced arrangement found in the carps, where the two tissues tend to closely associate with the vein.
It is generally agreed (Bellamy, 1971; Balment et al., 1980; Accordi and Grassi Milano, 1990; and Grassi Milano 1991, 1993,) that there is a general trend towards progressive aggregation of adrenal tissues in the Gnathostomata.

In the catfish, presence of a separate head kidney is a ubiquitous feature and this places the catfish on a possible evolutionary dichotomy- a line that probably diverged from the teleosts paralleling the sarcopterygian dichotomy, which ended in a discrete adrenal gland in tetrapods.

(III) Innervation

Among carps, both in *C. auratus* (Sampour, 2008) and in *C. carpio* (Imagawa et al., 1996) chromaffin cells are found to be innervated by cholinergic nerve fibres, and similar results are obtained in the goldfish in the present study. In teleosts, the chromaffin cells are generally well innervated and the nerve endings show typical presynaptic specializations (Gallo et al., 2001). On the other hand, while in cyclostomes there is a complete lack of extrinsic innervation of the chromaffin tissue (Paiement and McMillan, 1975; Perry et al., 1993; Bernier and Perry, 1996, 1998), in the chondrostean *Huso huso* a sparse innervation has been reported which has been regarded as a primitive condition (Gallo et al., 2004). In the present study, no nerve fibres were located close to the chromaffin cells of the catfish, *H. fossilis*., which again recalls a primitive condition.

Carps Versus Catfish

The detailed studies of the interrenal and chromaffin tissues have revealed some striking differences in the morphological, histological, distribution and innervation pattern of these tissues in these fish species
belonging to the major sub groups of teleosts – carps and catfish. These differences appear to be significant from an evolutionary point of view, as they substantiate the position of catfish close to the more primitive groups as against the more advanced groups. While in catfish, *H. fossilis* and *C. batrachus*, a separate head kidney exists as in all other catfishs, and a rather diffuse arrangement of interrenal and chromaffin tissues with respect to their proximity to cardinal veins is evident, in the carps, *C. auratus* and *C. carpio* there is a total absence of a separate head kidney and the interrenal and chromaffin tissues are always found in close vicinity of the cardinal veins. These features besides dealienating the catfish from the carps also indicate their similarity to primitive fish groups like Agnatha Chondrostei and Holostei.

Catfishes are unique among the teleost fishes, in that they are endowed with a mixture of several primitive characters and advanced teleostean features. They possess such primitive features as-electrorecepters, pined eye/pineal window, Landolts clubs, neurourohypophysis, carotid labyrinth and associated pseudobranchial neurosecretory cells, (see Srivastava, 2005) which bring them close to ancient fishes. The presence of a separate head kidney, lack of innervation of chromaffin cells, and diffuse arrangement of the adrenal tissues in the catfishes can be considered as other additions to the existing list of archaic features.

As far as the ultrastructural characteristics of the interrenal and chromaffin tissues are concerned, while two types of interrenal cells - the light and dark cells and two types of chromaffin cells - the adrenaline and noradrenaline are discernible in the carp, *C. auratus*, only one type of interrenal cells is seen in the catfish, *H. fossilis*. However, the cellular details
of the interrenal cells of both the fishes are same as in both the cases, the interrenal cells show typical characteristics of steroidogenic cells viz, numerous large mitochondria with tubulo vesicular cristae, abundant smooth endoplasmic reticulum and free and grouped ribosomes. Nucleoli are prominent in these cells. In both the fishes, the chromaffin cells are of two types – the noradrenaline and adrenaline cells. In the catfish, H. fossilis, however, the interrenal and chromaffin cells appear in a very closely packed manner indicating a paracrine interaction among them a feature which was not seen in C. auratus. Besides, the chromaffin cells are sparsely innervated in H. fossilis whereas in the goldfish, C. auratus, a cholinergic innervation of the chromaffin cells, typically found in majority of teleosts, is seen.

Thus there appear two distinct patterns of the adrenal tissues – one that belongs to the carps and the other which belongs to the catfishes. Within the general teleostean plan, the differentiating features in such aspect as structural characteristics, distribution, innervations and so on, probably reflect two lines of evolution along which carp and catfish groups have evolved.

(IV) **Stress Responses to Hypoxia**

It is now well known that in fishes, interrenal and chromaffin tissues which are integrally linked with the hypothalamus, the former through the hypothalamus-pituitary-interrenal (HPI) axis and the latter directly, are the sites of synthesis and release of the stress hormones (see / Chester Jones et al., 1969; Reid et al., 1995). A number of factors are known to increase the synthesis and release of corticosteroids (Selye, 1950; Fagerlund, 1967; Hill and Fromm, 1968) and catecholamines (Mazeaud et al., 1977; Nilsson, 1984; Schreck 1990). Structural
modification in the interrenal and chromaffin tissues has been indicated in response to seasonal and reproductive periods (Grassi Milano et al., 1997; Civinni et al., 2001; Rocha et al., 2001 and Gallo et al., 2004), osmotic stress (Mastrolia and Gallo, 1989; Gallo et al., 1997; Crivellato et al., 2006), detergents (Bromage and Fuchs, 1976) and cold stress (Mahon et al., 1962). So far no work has been done to assess the cellular changes in these tissues in response to aquatic hypoxia, although several studies have dealt with cardio respiratory responses (Stecyk and Farrell, 2002), adrenergic responses (Perry et al., 2004), behavioural and physiological responses (Van Raaji et al., 1996) arising due to stress of hypoxia in teleosts.

The results of the present investigation from this consideration are very interesting and discussed below, separately for (i) moderate hypoxia condition and (ii) acute hypoxia condition:

(i) **Responses to Moderate Hypoxia**

Among the three fishes examined: *H. fossilis, C. auratus* and *C. carpio* for experimental purposes, while *C. auratus* showed significant increase (p< 0.05) in the size of interrenal cells coupled with thickened cords of interrenal tissues after prolonged exposure (12 hr) to moderate hypoxia; in the other two fishes, only small increase in the interrenal cell sizes was observed.

Microstructural changes as dense staining nuclei and reduced cytoplasmic area were observed in the interrenal cells of *C. auratus*, whereas no microstructural changes were noticed in the cells of *C. carpio* and *H. fossilis*. In all the three fishes, no change was observed in the chromaffin cells. Short term (5 hr) exposure, however, did not evoke
any discernible change in the interrenal or chromaffin cells of any of the three fish species.

After 12 hr exposure, ultrastructural changes discernible in the interrenal cells of the goldfish are suggestive of interrenal hyperactivity. In the light cell type, increased clumping of nuclear heterochromatin and enormous proliferation of the sER was observed. Long cisternae were found running in close proximity and encircling the mitochondria, and some cisternae even opened at the cell periphery. However in the dark cell types, no change was noticed.

As elevated plasma cortisol levels and increased interrenal cell size are regarded as suggestive of HPI axis stimulation (Hall, 1984), the changes may be indicative of the onset of adaptive responses in the steroidogenic interrenal cells of *C. auratus* which show greater responsiveness as compared to the other carp, *C. carpio*. Alternatively, *C. carpio* appears to exhibit more adaptation to prolonged exposure to moderate levels of dissolved oxygen.

The insignificant responses in the interrenal cells of *C. carpio* and *H. fossilis* to prolonged exposure of moderate hypoxia, can be explained as arising due to their biological requirements for their particular modes of life. The common carp is habitually exposed to hypoxic conditions on a daily (Garey and Rahn, 1970) and seasonal basis (Beamish, 1964; Mc Crimmon, 1968). *Heteropneustes fossilis*, the catfish is a known air breather adapted to living in low dissolved oxygen levels. In *H. fossilis*, at the ultrastructural levels, the interrenal cells have displayed some degree of activity such as: appearance of perinuclear spaces, mitochondrial matrix becoming less dense and activated sER appearing as long cisternae.
traversing the cytoplasm. These changes can be attributed to the onset of stress responses in the form of setting in of steroidogenesis.

(ii) **Responses to Acute Hypoxia**

Exposure to both short term (5 hr) and long term (12 hr) duration of acute hypoxia (< 30% oxygen saturation levels) caused significant hypertrophy of the interrenal cells of all the three fish species examined. In case of *C. auratus*, the cells of interrenal tissues showed approximately 150% increase in size under 12 hr exposure to acute hypoxia as compared to 100% increase in size in *H. fossilis* and 120% increase in size in *C. carpio* under the same experimental condition. On the other hand after exposure to 5 hr acute hypoxia, 65% increase in interrenal cells was observed in goldfish whereas only 50% increase was observed in *H. fossilis* and 55% in *C. carpio*.

It is notable that in the three fishes put to experiments, after 12 hr and 5 hr exposure to acute hypoxia maximum increase in the interrenal cell size was observed in *C. auratus*, as compared to the other two fishes, with the least changes observed in the interrenal cells of *H. fossilis*.

Significant morphological changes included dense staining diffuse nuclei and hypertrophied interrenal cells, whose cytoplasm appeared swollen and spongy. In case of the goldfish, numerous vacuolated interrenal cells were observed in the head kidney at the light microscopic levels. Thickened cords of interrenal tissues were predominantly observed in the head kidney of these fishes. However, no change was observed in the chromaffin cells of these fishes.
The ultrastructural change following exposure to prolonged (12 hr) acute hypoxia in the adrenal tissues in the goldfish, *C. auratus* and the catfish, *H. fossilis* appeared to be degenerative in nature. In both the fishes, the nuclei of the interrenal cells became euchromatic and perinuclear spaces widened. The nucleoli became smaller in size. Plasma membranes appeared frayed and irregular in outline and mitochondria became smaller in size and in dividing states. More of vesiculated sER were also discernible. However, in *C. auratus* vacuolated interrenal cells were discernible which were not seen in *H. fossilis*, a feature which points to comparatively more advanced stage of degeneration in the goldfish. In *C. auratus* some interrenal cells developed large spaces in the cytoplasm filled with electron lucent material, which probably compared with the vacuolated cells seen in the semithin section.

Trump *et al.*, (1980) have reviewed stress induced necrotic changes within cells and considered them as the primary indication of cytotoxicity. These included quantitative changes within specific organelles of stressed cells such as hydropic degeneration (cellular swelling), accumulation of cytoplasmic inclusions and changes in cell and nuclear volume. Accumulation of triglycerides in vacuoles within cytosol of affected cells is considered an indicator of acute, subacute and chronic toxicity (Trump *et al.*, 1980).

In general, in such events cellular hypertrophy is considered a cellular adaptation to stress. It involves an increase in intracellular protein rather than cytosol (intracellular fluid). The interrenal cells enlarge when production of corticosteroids is reduced and negative feed back results in increased secretion of ACTH with hypertrophy of cells in a vain attempt
to produce corticosteroids. Drugs like metapirone, which in mammal
blocks 11β- hydroxylation in adrenal cortical tissue elicits the same effect
(Matty, 1985). Congenital adrenal hyperplasia is a group of disorder in
the biosynthesis of cortisol caused by an enzymatic deficiency in the
conversion of cholesterol to cortisol (Gotoh et al., 1988; New, 1992; Pang
hyperplasia and held that low cortisol production was caused by
dysfunction of the enzyme 17α- hydroxylase/ 17-20 lyase (CYP 17).
Interrenal cells from several fish species subjected to various stressful
conditions displayed such changes as mitochondrial proliferation,
vesiculation and enlargement of sER cisternae, indicating altered cortisol
secretion, on account of HPI activation. Such changes have been
observed in Aphanius in response to osmotic stress (Mastrolia and Gallo,
1989; Crivellato et al., 2006), in Brycon cephalus in response to crowding
(Rocha et al., 2001) and in Gastroesteus aculeatus in response to
reproductive cycles (Civinni et al., 2001). These studies also
demonstrated such changes as increased number of lysosomes, indistinct
plasma membranes and smaller nuclear membranes indicating cell
damage. Results obtained in the case of C. auratus and H. fossilis in
present investigation following exposure to 12 hr of acute hypoxia are of
a similar nature. Interrenal hypertrophy and cellular degeneration which
are seen in response to aquatic hypoxia in this study leads one to
speculate that hypoxia may be causing a dysfunction of the enzyme 17α –
hydroxylase / 17-20 lyase, in accordance with Ruane (2007). No cortisol
estimation was done in this study, and so with this limitation, any
correlation between interrenal hypertrophy and plasma cortisol levels
remains unsupported. However, further studies in this direction can be
fruitful.
Aquatic Hypoxia as a Common Stressor to Fish: Adaptive Abilities of Carp and Catfish to it.

It is significant that in the present study, little/or no significant changes were observed in the chromaffin cells. Small changes were observed in the noradrenaline cells of *H. fossilis* only, such as depletion of dense granules, substantial reduction in the clear halo surrounding these granules, and increase in the number of clear vesicles. Such changes have been explained by the fact that catecholamines stored in the chromaffin cells are released under conditions of sudden or severe stress (Reid *et al.*, 1998; Randall and Perry 1992; Thomas and Perry, 1992; Bonga, 1997) to minimize the detrimental effect of stress on physiological functions. In response to mild or moderate hypoxia stress, fish are not reported to release catecholamines (Boutilier *et al.*, 1988; Perry and Reid, 1992). According to Randall and Perry (1992) and Thomas and Perry (1992) severe hypoxia or prolonged moderate hypoxia can elicit significant increases in plasma catecholamines. However, in such cases hypoxemia rather than aquatic hypoxia is believed to be proximate stimulus causing catecholamine release (Perry *et al.*, 1989; Aota *et al.*, 1990).

Aquatic hypoxia is a commonly occurring condition in fish environments where it acts as a common stressor. As a result of exposure to such natural hypoxic environments, several morphological, physiological and behavioural adaptations aimed at optimising oxygen transfer and blood oxygen transport are developed in fish. However, fish adaption to hypoxia does not necessarily mean only the ability to tolerate hypoxia, it also includes mechanism to bypass hypoxia. Thus there are air breathing fishes which are either obligatory or facultative in nature. They have developed a tendency to skim the surface or reach out there.
intermittently (surfacing activity) to take in air. *Heteropneustes fossilis* and *Clarias batrachus* fall in this category of obligate air breathing fishes. Besides, there are some non air breathing fishes which tolerate hypoxic conditions by remaining in hypoxic environments. The common carp, *Cyprinus carpio* is one such notable example whereas goldfish, *C. auratus* is also tolerant to hypoxia but does not inhabit hypoxic environments.

The responses of the carps and the catfish to hypoxia as revealed in this study appear to fall in line with their adaptive abilities. *C. auratus* appear to be the least tolerant, although it can sustain short period (5 hr) of moderate level of dissolved oxygen concentrations without any stress responses as compared to the other two fishes. Common carp, *C. auratus* and the catfish, *H. fossilis*, notwithstanding the fact that they belong to different taxonomic subgroups of Ostariophysi appear to be at par, as far as, their tolerance to hypoxia is concerned.

The present study throws light on this vexed enigma of how *Cyprinus carpio*, a non air breathing carp and *H. fossilis* an established air breathing catfish have evolved with abilities to encounter hypoxia stress in their environments. It seems that they have solved the same problem in two different ways along their two different lines of evolution. *C. carpio*, the common carp has developed the ability for “avoidance” of the problem by developing tolerance towards it the degree of which may vary from species to species as for example - less tolerance as in case of *C. auratus* (only 5 hr of moderate hypoxia) and greater tolerance as in the case of *C. carpio*. (12 hr of moderate hypoxia). The catfish, *H. fossilis*, on the other hand, does it in an altogether different way, that of “correction”. It can face the stress hand on by delayed stress responses of their adrenal tissues, besides metabolic adjustments and air breathing. While *C. carpio*, and *H. fossilis* are endowed with adaptive abilities to deal with hypoxia,
*C. auratus* totally lacks the ability of any kind, like any other teleost. The interrenal tissues in fish which play important role in stress adaptation thus can be considered as stress biomarker tissues.

From the standpoint of Environment Impact Assessment, the results of the present study are very important. In dealing with different strategies for hypoxia survival or adaptation, hypoxia – tolerant animals can open new avenues for the treatment of hypoxia related diseases. By their different abilities towards dealing with aquatic hypoxia, arising naturally or as a result of anthropogenic activity, these fishes can also serve as good biomarkers for indicating the state of pollution in a water body. *C. auratus, C. carpio* and *H. fossilis* can be used to show levels of weak, moderate and acute hypoxic conditions respectively prevailing in any aquatic environment, particularly in fish culture ponds, arising from either natural or man made pollution.