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Research Article

VARIABILITY OF Na^+, K^+-ATPASE ACTIVITY AND LOCATION OF CHLORIDE CELLS IN THE GILLS OF *TENUALOSA ILISHA* IN RESPONSE TO DIFFERENT SALINITY ZONES OF BHAGIRATHI-HOOGHLY RIVER SYSTEM, WEST BENGAL, INDIA

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ABSTRACT

The size and location of chloride cells in gill of Hilsa, Tenualosa ilisha, from marine (Digha) and fresh water (Farakka) environments were studied in this paper. Changes in gill and kidney histopathology in terms of Na^+, K^+-ATPase activity were also investigated. Fish samples were collected from fresh water (FW) (salinity ~ 0.1psu) and marine water (MW) (salinity ~ 29 psu) environment. In FW, the chloride cells were observed on the epithelium of the filaments (mainly in inter-lamellar regions) and on the lamellae of Hilsa gill. On the contrary, in the MW samples, the abundance of Na^+,K^+-ATPase increased and few chloride cells were observed on the lamellae. MW samples showed a high density of chloride cells on the epithelium of the filaments, and a few cells on the lamellae. Na^+, K^+-ATPase intensity was significantly differed in two unlike environment (sustainability higher in MW samples compared to FW samples) where kidney samples showed the opposite trend. The capability of T.ilisha to change the number and size of gill chloride cells, as well as their activities indicated that the high degree of adaptability of T.ilisha to a wide range of salinity.

INTRODUCTION

Diadromy is a term used to describe migrations of fishes between fresh waters and the sea; these migrations are regular, physiologically mediated movements which occur at predictable life history phases, typically for breeding and reproduction, in each diadromous species (McDowall, 1997). Tenualosa ilisha is such type of anadromous fish, commonly known as Hilsa (in eastern part of India and Bangladesh). Moreover, Tenualosa ilisha belongs to the clupeidae family and it is one of the most commercially important fish species in a number of countries bordering the Bay of Bengal, Indian Ocean, Persian Gulf and Arabian Sea. It is a large anadromous shad that occurs in the large river systems of southern Asia from the Arabian Gulf to Myanmar and northern Sumatra (McDowall RM 1997).

During migration anadromous fishes developed some unique strategies reguarding their physiology with the evolution of the ion regulating organ like gill, kidney, intestrinal tract etc, for regulating the ion concentration and osmolality of their body fluid at a level that is different from their external environments (Khodabandeh *et al*.2009). Among them gill plays the most important role as the ion regulating organ. Previous researches indicate that active extrusion of NaCl across the gill of sea water teleost's is carried out by specialized mitochondria or ioinocytes rich cells, called chloride cells (Evans *et al*. 1984). These cells are characterized by an elaborate system of intracellular canals (Masoni and Payan, 1974). Chloride cells can individually transform between ion absorption and ion secretion states in response to salinity changes (Uchida *et al*., 2000). Osmoregulatory activities enable aquatic animals to adapt to external medium salinity fluctuations (Evans 1984). In freshwater, the organism is subjected to water uptake and ion loss, as they are hypertonic to the medium and their blood has lower water concentration than the surrounding medium. Thus hyper-osmoregulatory mechanisms compensate with a low water intake, active absorption of ions by the gills and production of hypotonic urine by kidney (Evans 1984). In seawater, hypo-osmoregulatory mechanisms compensate for water loss and ionic invasion. To avoid the dehydration *Tenualosa ilisha* makes up the extra water loss through high rate of drinking sea water. The water is absorbed by intestine and gills reject the excess ions come inside through water (Jensen *et al*., 1998; Hawkings *et al*., 2004; Evans 1984). Ion transport by gill is facilitated by chloride cells present in the gill's epithelium that possess a plasma membrane associated Na^+, K^+-ATPase enzyme. The driving force for the active transport is Na^+, K^+ ATPase, which maintains intracellular Na^+

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concentration at low levels and intracellular K^+ concentration at high levels. The enzyme has been shown to be localised by a chemical named ouabain binding to the basal part of the cells by biochemical precipitate. Ouabain or g-strophanthin is a plant derived toxic substance which is used as an inhibitor that binds to the membrane Na^+, K^+- ATPase close to the site of K-activation (Glynn, 1964). This enzyme also has been localized in fish intestine and kidney (Goss *et al*., 1998; Gaber *et al*., 2014; Khodabandeh *et al*., 2009). Levels of Na^+, K^+-ATPase have been used extensively as an index of migration capacity in fish exposed to a variety of conditions including seawater (Mancera and McCormick, 2000). Na^+, K^+-ATPase activity has been studied in several teleost fish including milk fish, tilapia etc. (Jensen *et al*., 1998; Sakamoto *et al*., 2001). Most of the euryhaline teleosts exhibit adaptive changes in gill Na^+, K^+-ATPase activity following salinity changes (Marshall and Bryson, 1998; Shikano and Fujio, 1998). Gill Na^+, K^+-ATPase is reported to have a higher level of activation in sea water residing teleost's (Uchida *et al*., 2000). Thus another way to test the location and directionality of gill ATPase is to see from which side ouabain binds to and inhibits the enzyme.

However, information on the normal structure of gills and the physiological processes that regulate osmolality and electrolyte concentrations of T .ilisha is rarely documented. Thus the main objective of the study was to describe the morphological changes of specialized cells (chloride cells) in the lamellae of T. ilisha in the two different salinity zone, namely; marine water (Digha) and fresh water (Farakka) through some histopathological and biochemical analysis. This will help to understand about chloride cell function during the transitory life history of T .ilisha.

MATERIALS AND METHODS

Sampling site and sample collection

T ilisha was collected during August 2014 at Digha (21º10′36″N; 87º34′26.6″E) of Bay of Bengal [average salinity range~25-29].Ten different size of the fish (female: male =1:1) (average length 29.5± 2.8 cm) were collected from sampling site, and apparently mature specimens were selected for the study. In addition, ten mature T.ilisha (female: male+1:1; average length 29.5±4.5 cm) were also collected near Farakka Barrage (27°47′53.3″N, 87°56′11.6″E) situated in Bhagirathi - Hooghly River during November 2014 (average salinity~ 0.1-0.5 psu). Gill tissue samples were taken from the second arch of both right and left gills of different sized *T. ilisha*. Tissue samples were preserved in 4% formalin solution and transported to the laboratory for histological examinations.

Gill tissues were dehydrated by a series of ethanol and embedded in paraffin wax (Bernet *et al*. 1999).These samples were then sectioned to a thickness of 5µm with a microtome (Leitz,Germany), and stained with haematoxylin and eosin. Histological slides were examined with the light microscopy method (Made: Zeiss Primo Star and pictures were taken with a Nikon, E-600 camera). Ten microscopic fields were randomly selected on each slide, and measured with the image analyser softer Biocom Visolab.

The kidney is a mixed organ comprising hematopoitic, reticuloendothelial, endocrine and excretory elements. The kidney of fish is usually located in a retroperitoneal position up against the ventral aspect of the vertebral column. The ventral portion of the fishes were dissected, and slowly all the organs were removed, cleaned and kidney was dissected carefully without losing blood. After dissecting, kidney was immediately transferred to 4% para-formaldehyde for 24 hours at room temperature for histopathological analysis. The samples were then sectioned to a thickness of 5µm with a microtome (Leitz, Germany), and stained with haematoxylin and eosin, and the histology analysis was performed same as histology analysis.

Gill Na+,K+-ATPase activity was measured on crude gill homogenates using the methods outlined by McCormick (1993). Gills are covered by a bony operculum and four hollow branches are present in each operculum, of which one side was selected for the study. These sections are cut from each arch on ice, immediately homogenized in ice cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and immediately stored in -80°C until assay. During assay, samples were rapidly thawed, again homogenized and centrifuged at 2,000 rpm at 40°C for 30 seconds. Samples (10-µL) were run in two sets of duplicates, one set containing the assay mixture and the other containing the assay mixture and 0.5 mM ouabain. The resulting ouabain-sensitive ATPase activity is expressed as µg Pi g $protein^{-1}$ $hour^{-1}$. Protein concentrations were determined with a BSA protein assay kit (Pierce). Both assays were run on a THERMOmax microplate reader using SoftMax software (Molecular Devices) at 405 nm and 750 nm respectively. Therefore, kidney Na,K ATPase activity was measured on crude kidney homogenates using by the methods outlined by McCormick (1993) as well as gill sample.

RESULTS

Gill & kidney histopathology studies

Sagittal sections (stained) of the gills of *T. ilisha* collected from different saline zones showed an oval to elongated shape with an epical positioned nucleus (Figure 1). The pink colour stained the chloride cells with nucleus in the middle. In fresh water, lamellar chloride cells appeared at the base close to the filament and expanded proximal to distal part of the lamellae (Figure 1 and Figure 2).Moreover, lamellar chloride cells increased significantly and predominated over filament cells .Chloride cells in the filament have decreased significantly in the freshwater samples (Figure 2**).** In gill filament of *T. ilisha* from both marine and freshwater sources, chloride cells occupied 10 to 15 % out of the total space (Figure 2).

The sagittal section of kidney tissue of *T. ilisha* from different salinity zone was done using hematoxylin and eosin staining procedure. Kidney of fresh water juvenile and adult fish is composed of numerous bowman capsules with well-developed glomeruli and a system of renal tubules like proximal tubules. (Figure 3), whereas in marine water there are few bowman capsules and no proximal tubule shows in the juvenile *T. ilisha* (Figure 3) sample but in case of adult some kidney tubules are followed in the tissue.

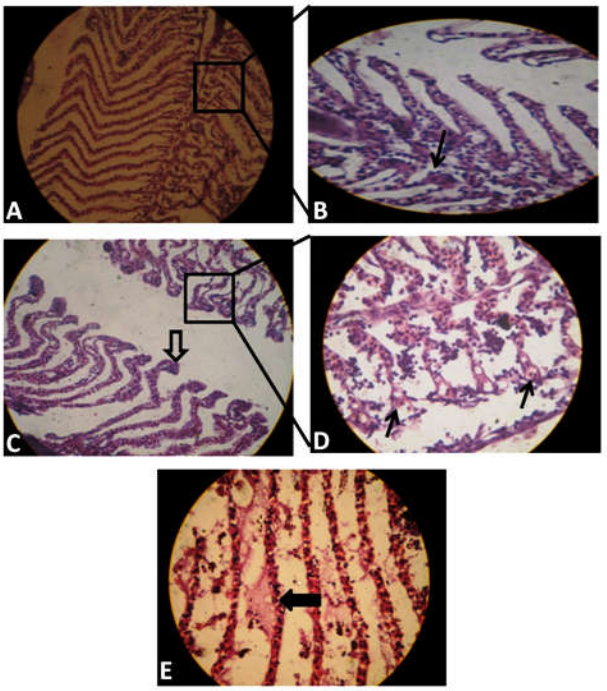

Figure 1 Histopathological analysis of sagittal section of gill tissue section. Figure 1A, shows marine water gill sample in 10 x magnification. B shows marine water gill sample of the same tissue in 40 x magnifications. Higher magnification of the chloride cell accumulation at the junction of gill filament and gill lamellae is also shown. Thin Arrow shows chloride cells (1B). Figure 1C shows fresh water gill sample of Hilsa in 10 x magnification. Figure 1D shows fresh water gill sample of Hilsa (1D) in 40 x magnification showing chloride cell accumulation in the secondary gill lamellae from the proximal part to distal part. Thin arrow shows chloride cells (1D). Figure 1E shows marine water adult Hilsa gill sample in 40 x magnification showing mucous cells in the secondary gill lamellae. Thin arrow shows mucous cell (1E).

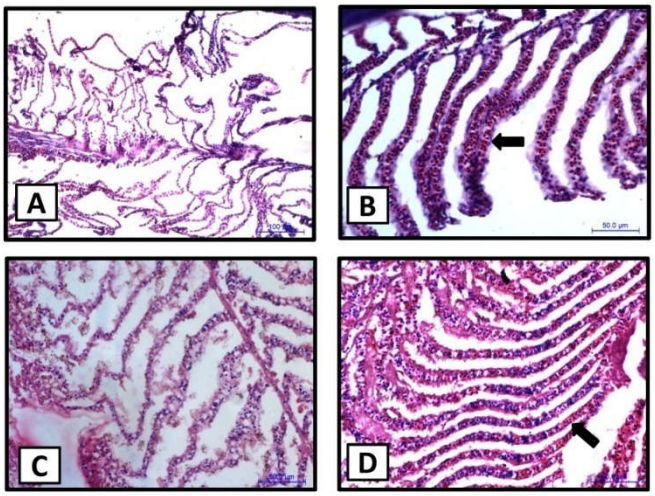

Figure 2 Figure 2A, showing the primary and secondary gill lamellae of fresh water juvenile Hilsa, where the gill lamellae are in developmental stage. Scale bar 200µm. Figure 2B, showing the primary and secondary gill lamellae of fresh water adult Hilsa. In secondary gill lamellae the arrow shows the chloride cells (2B). Scale bar 200µm. Figure 2C, showing the primary and secondary gill lamellae of marine water juvenile Hilsa. Where primary and secondary gill lamellae both in developmental condition. Scale bar200µm. Figure 2D, showing the developed primary and secondary gill lamellae of marine water adult Hilsa, where arrow shows chloride cells in the secondary gill lamellae. Scale bar 200µm.

Quantitative analysis of Na^+, K^+-ATPase intensity

Binding of H^3 -ouabain in seawater hilsa significantly higher than the freshwater adapted fish(4.78± 0.09 vs 3.29± 0.32) pmole.mg-^{1} protein (n=5 of each group).Quantification of Na^+, K^+-ATPase activity of sea water (Digha) as well as freshwater samples (Farakka barrage, Bhagirathi-Hooghly river) showed significantly higher intensities in the gills of different size group of *T. ilisha*. Na^+, K^+-ATPase intensity was significantly higher (Figure 4) in freshwater compared to marine water. In case of kidney showed an opposite trend compared with the results for gill tissues, kidney Na^+, K^+-ATPase protein of fresh water fish proved to be higher than the sea water acclimated fish (Figure 4).

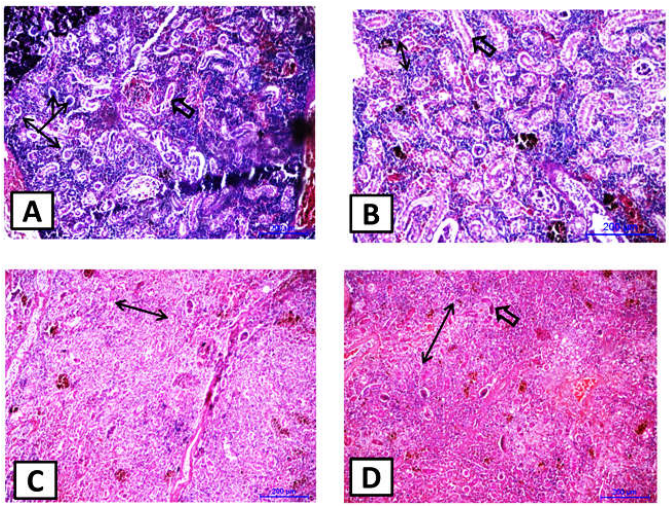

Figure 3 Figure 3A shows fresh water juvenile Hilsa kidney, thin arrows shows bowman capsules; hollow arrow shows the proximal tubule in the Hilsa kidney, scale bar 200µm. Figure 3B shows fresh water adult Hilsa kidney histopathology, where thin arrow shows plenty of bowman capsules and abundance of proximal tubule in the Hilsa kidney; scale bar 200 µm. Figure 3C shows marine water juvenile Hilsa kidney histopathology, where thin arrow shows bowman capsules are less and underdeveloped, no kidney tubules can follows in the tissue; scale bar 200 µm. Figure 3D shows marine water adult Hilsa kidney histopathology, where few bowman capsules are followed but they are also underdeveloped and few undeveloped proximal tubule can follow in the kidney tissue scale bar 200µm.

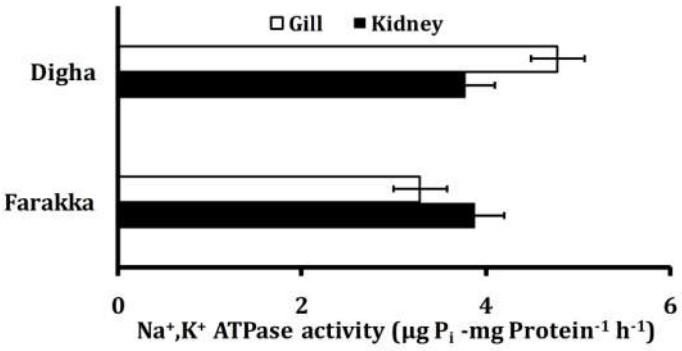

Figure 4 Comparison of Na+, K+ ATPase activity and Ouabain binding in gills and kidneys of T. ilisha adapted to fresh water and sea water environments.

DISCUSSION

Evans (1984) estimated at about 95% of the teleosts are stenohaline, while only 5% are euryhaline, of which *T. ilisha* can tolerate a wide variation in salinity during its life history, and thus provides a very good model for osmo-regulation research. The present study also confirms the same for *T. ilisha*, , the histopathology of gill, kidney changes, as well as, the Na^+, K^+-ATPase activity with increasing salinity. The gills and kidney are the main osmoregulatory organs and are sensitive to environmental factors (i.e salinity) (Uchida *et al*.,2000).The most radical change observed in the present

study is that, the appearance of chloride cells in the lamellae and its abundance in the epithelium of gills during migration of T.ilisha from sea water to fresh water. Previous study showed that most branchial chloride cells were detected in the filament and inter lamellar region of *T. ilisha* from marine environment, whereas lamellar chloride cells become evident in fish from fresh water environment. Chloride cells in *T. ilisha* were abundant on gill filament, but rarely appeared on lamellae (Varsamos *et al*., 2002). The occurrence of lamellar chloride cells is thought to satisfy the physiological demand of ion uptake in some euryhaline teleost's (Uchida *et.al*., 2000; Sasai et.al., 1998) but not in others (Laurent and Perry, 1991). The enzyme that is important in the process of ion exchange, according to previous studies in some fishes like Milk fish (*Chanos chanos*), Tilapia (*Oreochromis mossambicus*) was identified as Na^+, K^+-ATPase. Na^+, K^+-ATPase is a plasma-membrane-associated enzyme which catalyses ubiquitous ATP-driven Na^+/K^+ transport. This enzyme is crucial to ion and water regulation in both freshwater and seawater fish (Evans 1984). In *T. ilisha* the activity of gill Na^+, K^+-ATPase is higher in sea water, while decreased in fresh water, but up to that extent. In seawater conditions, Na^+ ,K^+-ATPase still pumps Na^+ from the intracellular compartment of chloride cells into the extracellular space across the basolateral surface. The strong Na^+ gradient drives a secondary active co-transport of Cl^- into the cell through a Na^+, K^+, $2Cl^-$ co-transporter. This creates an electrochemical gradient that favours diffusion of Cl^- through a Cl^- channel on the apical side out to the seawater. It enables the Hilsa to secrete excess salts efficiently and thus acclimate smoothly to these salinities. A positive correlation between environmental salinity and gill Na^+, K^+-ATPase activity from freshwater to seawater, have been also reported (Marshall and Bryson, 1998).On the contrary it has been see that euryhaline hilsa kidney Na^+, K^+-ATPase protein abundance as well as specific activity in kidneys of the freshwater sample increased significantly (Figure 4), while the pattern of Na^+, K^+-ATPase expression in gills proved to have an opposite response. In FW, the primary function of the kidney is to excrete excess water, while reabsorbing most collecting tubules .Quantification of Na +,K+-ATPase activity of sea water (Digha) as well as freshwater samples (Farkka, Bhagirathi-Hooghly River) showed significantly higher intensities in the gills of different size group of T.hilsa. The majority of earlier works showed a positive correlation between environmental salinity and gill Na^+, K^+-ATPase activity (Uchida *et al*., 2000), forming the well-established 'diadromid paradigm' (Marshall and Bryson, 1998). In contrast, in several species higher or similar levels of gill Na^+, K^+-ATPase activity have been found in freshwater or low salinity-acclimated fish compared with seawater fish; (Jensen *et al*., 1998; Uchida *et.al*., 2000; Kelly *et al*., 1999; Katoh and Kaneko, 2003; Lin and Sung, 2003). Our result confirms, that although fresh water merely a good habitat for *T. ilisha* but not providing it a safest habitat. This signifies that adaptation of *T. ilisha* and other marine teleost fish to low salinity environments can be a response that differs considerably from the diadromid paradigm.

CONCLUSION

Comparing the values obtained for Na^+,K^+-ATPase intensity of Hilsa from the Digha and Farakka, we can conclude that Hilsa have high degree of adaptability in the wide range of salinity during the different phases of its life cycle. This species uses special osmoregulation mechanisms during its migration from marine to freshwater including changes in the size and number of gill chloride cells**.**

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