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Research Article

Quantification and localization of mitochondrial-rich cells in the gills of an anadromous fish, Hilsa shad (*Tenualosa ilisha*)

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Abstract

Tenualosa ilisha has a high economic and valuable marketing in the southern regions of Iran. A recent study was conducted on changes in mitochondrial-rich cells (MRCs) and some blood factors when fish migrated from sea to river. As a model, this research can provide basic information on the osmoregulation functions of this fish and other similar migratory species, and can be useful to other researchers and research centers. Forty mature *T. ilisha* specimens were collected from the Musa Creek and Arvand Rud. In order to examine the distribution, size, and number of MRCs in the gills, histological sections were observed and analyzed by hematoxylins and eosin staining, immunofluorescent staining, and the scanning electron microscope method. For a better understanding of osmoregulation, plasma ion concentrations of each fish were examined. The antibody staining results showed that in the gills, MRCs were found in lamellae, interlamellar, and basolateral in both the sea and river samples but no MRCs were observed in the filament epithelia. The size and numbers of MRCs in the seawater fishes were considerably larger than those from river water. Concentrations of Na^+, Cl-, K^+, Ca^{2+}, Mg^{2+}, and glucose in *T. ilisha* plasma specimens from seawater were significantly higher than the river specimens. According to the study, changes in the gill tissue of *T. ilisha* during migration can be concluded that this fish easily live in both environments with changes in gill structure and some blood biochemical parameters.

Keywords: *Tenualosa ilisha*, Anadromous, Gill, Immunofluorescent, Scanning Electron Microscope, Mitochondrial-rich cells

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Introduction

Hilsa shad, *Tenualosa ilisha*, is anadromous and able to survive in environments with a wide range of salinities. It migrates from the sea to freshwater to spawn (Dastan *et al*., 2017). This animal needs to have a highly effective osmoregulatory system to survive in two different environments. In teleosts, the main organs for the osmoregulatory functions are gills, kidneys, and gut (Choi *et al*., 2011; Sabery *et al*., 2014). Gill is one of the main places for osmoregulatory functions, and in this organ, mitochondrial-rich cells (MRCs) (Na^+/K^+-ATPase-rich cells, ionocytes, or chloride cells) play a crucial role in the ionic and osmotic regulations (Choi *et al*., 2011). These cells have numerous mitochondria and extensive tubular system to provide required energy for osmoregulation. For this function, they are supported by pavement and mucous cells of gill epithelium (Bystriansky *et al*., 2006). A plethora of studies reported that MRCs have been found in gill filaments, lamellae, and opercular membranes of several marines and freshwater species (Pourkhadje *et al*., 2014). In seawater-adapted fish, MRCs secrete and absorb ions and maintain the acid-base balance in such species. When fish is in seawater, Cl- goes out by secondary active transport of branchial MRCs (Kaneko *et al*., 2008). The driving force for Cl^- exit is the Na+ electrochemical gradient established by MRCs in the basolateral membrane. In anadromous fish species, Cl^-/HCO^{3-} the exchange is thought to occur in the apical region of MRCs. Theoretically, the local acidification in the apex of MRCs by H^+-ATPase caused a lower in the activity of HCO^{3-}, for Cl^- uptake. On the other hand, researchers believe that the effects of such environmental changes may result in biochemical and physiological changes in fish's blood (Duffy *et al*., 2011)

The hematological profile is a good indicator of the physiological state of fish and provides information about their health status. Due to the fact that this fish has a high economic and marketing value in Southwest region, especially in Khuzestan province, the present study was conducted on changes in MRCs and some blood factors at time their migrated from sea to river.

Materials and methods

Sampling collection and processing

In July 2018, twenty mature *T. ilisha* (regardless of gender) with a total length of 25.33±1.37 cm (mean length±SEM) and weight of 173.24±11.67 g (mean weight ±SEM) were collected from the seawater of Musa Creek (42.7 PSU; 29.27°C water temperature) situated in the northwest of the Persian Gulf in Khuzestan province of Iran. Additionally, 20 mature *T. ilisha* (female: male ratio 1:1) with a total length of 24.45±1.23 cm (mean length±SEM) and weight of 170.33±12.27 g (mean length±SEM) were caught from the Arvand Rud (2.3

PSU; 21.16°C water temperature) in Khuzestan province (Fig. 1). Animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH).

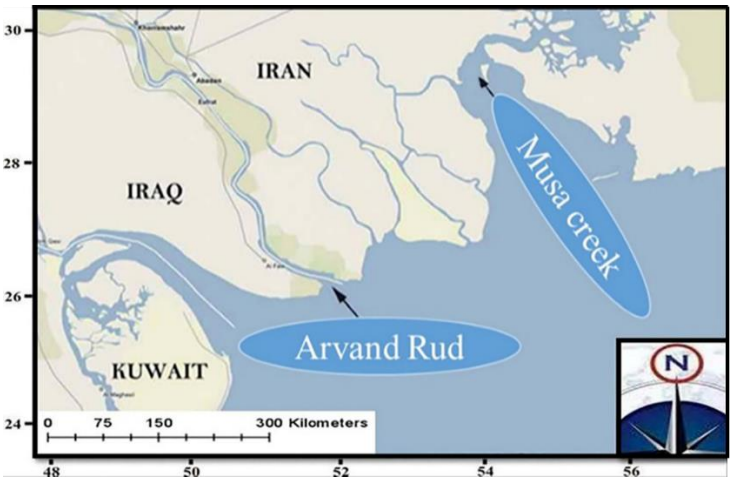

Figure 1: Geographical position and the location of sampling stations in the northern coast of Persian Gulf in Musa Creek (above arrow) and Arvand Rud (lower arrow) in southern of Iran.

The geographic coordinates of sampling stations using GPS devices had got in the Musa Creek longitude (53º 45' 23/13" E), latitude (39º 27' 42/11" N) and Arvand Rud, longitude (52º 24' 15/38" E), latitude (38º 35' 61/31" N). The average of physicochemical amounts was measured of sampling stations in the Persian Gulf and Arvand Rud using (Horiba U- 10, Japan) devices have been as, water salinity (42.7, 2.3) PSU; water temperature (29.27, 21.16) °C; dissolved oxygen (6.34, 7.31) ml/L and pH (7.6, 8). Only healthy-looking specimens were selected for studying.

General histology

For histological studies, specimens were immediately euthanized in a plastic tank (-50 L) using clove powder (75 mg l^{-1}). The middle portion of the 1st gill's arch was immediately immersed into Bouin's fixative for 24 h; then were washed and dehydrated in an ascending series of ethanol for embedding in paraffin (Merck) (Evans *et al*., 2005; Basir and Peyghan, 2020). Following embedment in paraffin, 4-6 µm sections were prepared using a rotatory microtome (RM2245-LEICA, German). Then the sections were stained with hematoxylin and eosin (Merck, German). Finally observed and photographed using a light microscope (Olympus BH-2, Japan) equipped with a Dino-Lite lens (Dino-Eye, Taiwan) with Dino Capture software. The numbers and sizes of gill MRCs of the fish collected from two media were counted from ten different sections of each slide and the mean values were determined (Tang and Lee, 2010; Basir and Peyghan, 2019).

Tests of some blood factors

In this study, some Plasma electrolytes including Na^+, Cl^+, K^+, Mg^{2+}, Ca^{2+}, and glucose were evaluated. For this purpose, along with anesthesia, 3-4 ml blood samples were taken from the caudal vein using a sterile 5 ml plastic syringe that was impregnated with an anticoagulant (Basir and Abdi, 2016). Blood samples were centrifuged for 5 m (5000 rpm) and the isolated plasma were removed and stored at -70°C. Na^+ and K^+ ion concentrations were measured using a flame photometer (Jenway, UK). Plasma Cl^-, Mg^{2+}, and Ca^{2+} were determined with an RA-1000 device (Technicon, USA). In order to measure the plasma glucose, enzymatic methods and an auto analyzer device (Technicon, USA) (Sardella *et al*., 2004) were utilized.

Immunohistochemical staining method

For immunohistochemical studies, the sections were collected on glass slides coated with poly-L-lysine, they were washed in HCl, 90% ethanol, and distillate water, and then finally incubated at the temperature of 37º C for 24 h. Sections were de-paraffinized with xylene (with 100% xylene for 2 min, 3 times) and hydrated with a descending series of ethanol (100%, 90%, 80%, 70%, and 60%) (Moradkhani *et al*., 2020). Sections were preincubated for 10 min in 0.01 mM Tween 20, 150 mM NaCl in 10 mM phosphate buffer, pH=7.3, and then treated with 50 mM NH_4Cl in phosphate-buffered saline (PBS), pH=7.3, for 5 min to mask the free aldehyde groups of the fixative. In the next step, the sections were washed in PBS and incubated for 10 min with a blocking solution (BS) containing 1% bovine plasma albumin (BSA) and 0.1% gelatin in PBS (Tang and Lee, 2010; Fridman *et al*., 2011). Immunolocalization of Na^+/K^+-ATPase was performed through immunofluorescence light microscopy using a mouse monoclonal antibody called IgGα5 (Hybridoma Bank, University of Iowa) raised against the α-subunit of the chicken Na^+/K^+-ATPase (Guo *et al*., 2020). This antibody was diluted in PBS to the concentration of 20 µg/ml and placed on the sections; then they were incubated at room temperature in a moist chamber for 2 h. The slides were rinsed in BS and subsequently incubated for 1 h in the secondary antibody (fluorescein isothiocyanate conjugated, FITC) under dark conditions. All the slides were rinsed in PBS and were mounted on a medium for fluorescent microscopy photobleaching. An Olympus digital camera (Olympus. Japan) adapted to the Olympus fluorescent microscope was used to photograph the tissues.

Scanning electron microscope method

Freshly dissected gills from fishes were fixed for 24 h in a solution of 5% glutaraldehyde buffered at pH 7.4 with 0.2 mol/l sodium cacodylate buffer at 4°C. In the next step, tissues were dehydrated in ascending concentrations of ethanol from 60% to absolute. After

sputter-coating with a gold-palladium complex for 2 min, specimens were examined with an LEO 1455 VP scanning electron microscope (SEM), a common product by (Germany- UK) at the voltage of 20 kV (Elsheikh, 2013). All SEM observations were carried out at the central laboratory of Shahid Chamran University of Ahvaz (Ahvaz, Iran).

Statistical analysis

After data normality was tested with Kolmogorov-Smirnov analysis, all statistical analysis was performed using the Graph Pad Prism (V.8.03. San Diego, California, USA). All parameters were statistically analyzed by One-way analysis of variance (ANOVA) and Tukey multiple-comparison post hoc tests. Finally, the Chi-square test was applied to compare the number of gill MRCs, the results were presented as Mean±SEM, and differences of $p<0.05$ were considered statistically significant

Results

General histology

In the microscopic study, gill epithelium of *T. ilisha* was composed of pavement cells which covered almost the entire gill structure, mucus, or goblet cells which were found on gill arches and MRCs on lamellae, interlamellar, and basolateral of the lamellae in both environments. No MRCs were observed on the filament epithelia. In this staining, there was not much difference in the fish gills between the sea and the river environments (Fig. 2, A-B). The mean size and number of gills' MRCs in specimens caught from the sea water and river water in Fig. 3 demonstrated that the size and number of gills' MRCs in sea water was greater than that river specimens (Fig. 3).

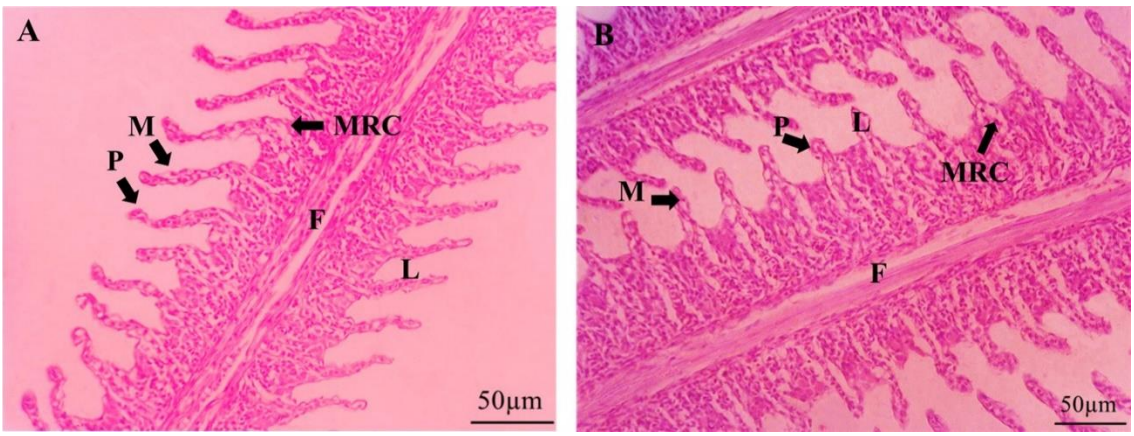

Figure 2: Light microscopic structure of gill tissue in *T. ilisha*, from sea water (A) and river water (B) (H&E.100x). F= filament; L= lamellae; P= pavement cell; M= mucus cell; MRC= mitochondria rich cell.

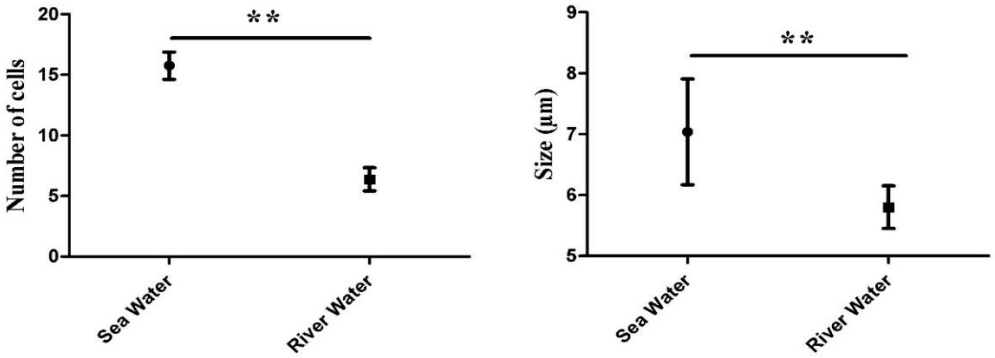

Figure 3: Morphometric parameters of gill MRCs in *T. ilisha*, from seawater (A) and river water (B). Mean ± SEM values with asterisk (**) are different significantly (p<0.01).

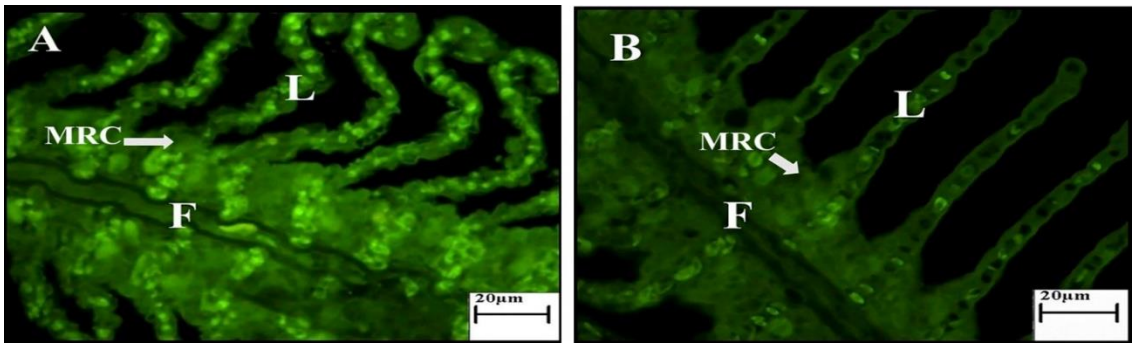

Figure 4: Immunolocalization of Na^+/K^+-ATPase in cross sections of gills of *T. ilisha* from seawater (A) and river water (B) specimens (200x). After immunofluorescent staining, revealed that MRCs were colocalized on the lamellae, interlamellar and basolateral regions membrane of the filamental epithelial cells respectively. A weak immunofluorescence activity is observed in the sea sections (A) and the strong immunofluorescence activity in river (B) specimens. F= filament; L= lamellae; MRC= mitochondria rich cell.

Immunohistochemical staining method

Immunolocalization of Na+/K+-ATPase is shown of *T. ilisha*'s gill at sea water (A) and river water (B) (Fig. 4, A-B). In immunofluorescent staining from the MRCs of gill were identified with greater staining intensity and positive reactions of the Na^+/K^+-ATPase. At Immunofluorescent staining of Na^+/K^+-ATPase α- subunit, demonstrated that epithelial cells are big, oval to round, and located in the lamellae, interlamellar, and basolateral regions.

Scanning electron microscope method

In SEM studies revealed that MRCs were placed between two or more squamous cells and is shown in Fig. 5. The grooves on the apical membrane of the MRCs were mainly microvilli, and the thin edges that were found on the surface of the squamous cells were not seen at MRCs. The apical membrane of MRCs revealed a different morphology of types of ridge openings in these cells including deep hole, shallow basin, and wavy convex. In the deep hole, the apical membrane of MRCs was much lower than the surface of squamous cells and

only their openings were visible among these cells. In the river cases (Fig. 4-B), the depth of these holes of MRCs was low and thus they formed shallow basins. In this type, because of the presence of microvilli, the surface of the membrane was visible.

Plasma biochemical parameters

The plasma electrolytes and glucose in river samples were significantly lower than the seawater one (p<0.05). Regarding sea specimens, the greatest amount (121.83±16.25) and the least amount (3.31±0.27) were reported for glucose and K^+, respectively; however, for river specimens, the greatest amount (108.61±11.25) and the least amount (2.32±0.18) were reported for glucose and K+, respectively (Fig. 6).

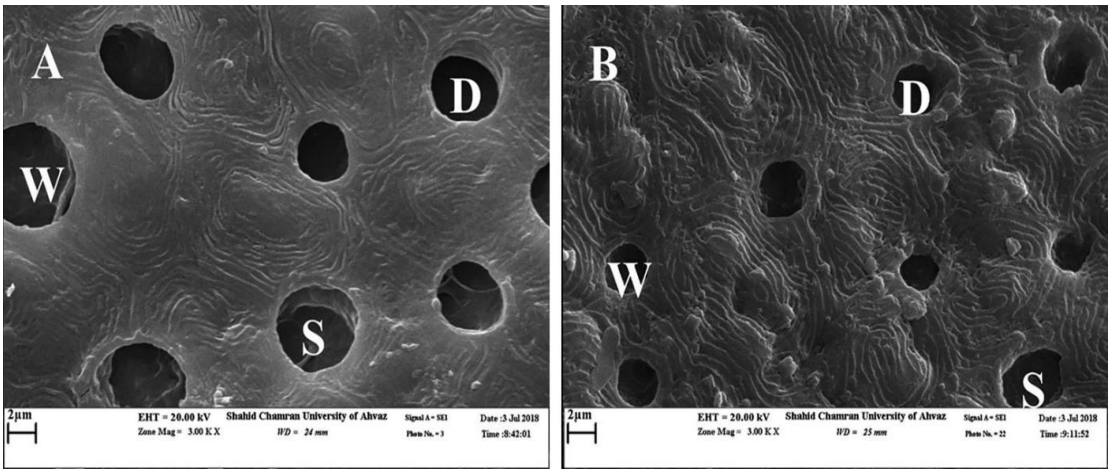

Figure 5: SEM apical region morphology in gill epithelial of *T. ilisha* of sea (A) and river (B) specimens revealed three types of MRCs. D= deep hole MRCs; S= shallow basin MRCs; W= wavy convex MRCs.

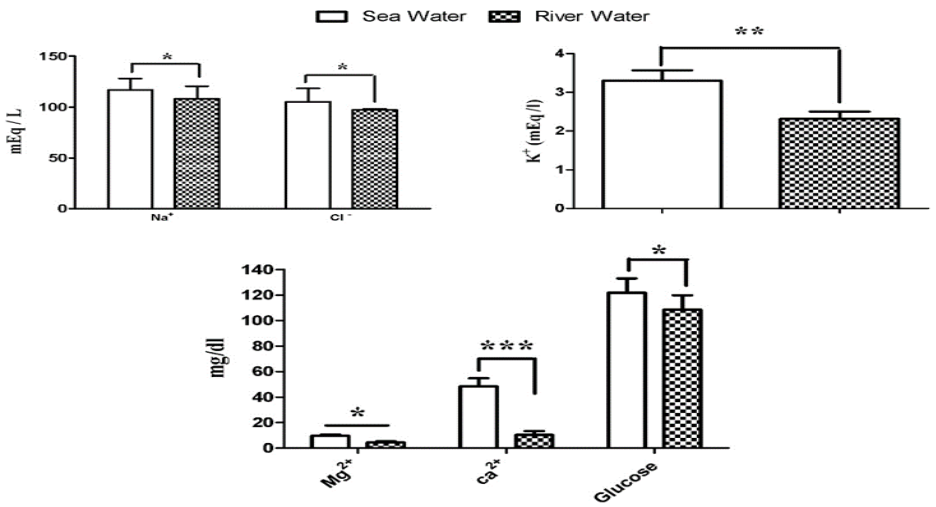

Figure 6: Plasma biochemical parameters of *T. ilisha* in seawater (sw) and river water (rw) specimens. Mean ± SEM values with (* p<0.05, ** p<0.01, and *** p<0.001) are different significantly.

Discussion

Fish physiologists believe that MRCs have different functions in fish adapted to fresh and brackish waters. This adaptation has been investigated in several studies from two different media (Hiroi *et al*., 2005; Blanco *et al*., 2015; Kumar *et al*., 2020). Given their importance, the gills are considered as the major osmoregulatory site in euryhaline teleosts (Evans *et al*., 2005; Boutet *et al*., 2006; Dutta *et al*., 2019). In sea, MRCs are involved in the excretion of excessive ions, while in river, they are responsible for ion uptake in order to compensate for ion loss in hypoosmotic environments (Evans *et al*., 2005). MRCs that their function has been extensively studied in fish may be involved in osmoregulation differently, according to their lamellae, basis of lamellae, lamellar, and filamentary location in some species (Yang *et al*., 2016). Based on histological results, SEM and immunofluorescence, it was found that gill MRCs were round in euryhaline fish and Na^+/K^+-ATPase acidophilic cells were located on the interlamellar, lamellae, and basis of lamellae of gill epithelium. This finding is also reported by other researchers. For example, Hiroi and McCormick (2012), reported that MRCs are acidophilic in the gill epithelium of some euryhaline and diadromous fish. In *Carassius auratus*, epithelial ionocytes are mostly located on the interlamellar region near the afferent side of the filament, but rarely on the trailing edge of the filament, which has been observed in other teleosts (Pourkhadje *et al*., 2011; Bradshaw *et al*., 2012). Researchers have also reported that in juvenile sea-bass *Dicentrarchus labrax*, only filamentary MRCs have been observed (Boutet *et al*., 2006). The results of current study showed that, with variations in environmental salinity, the number and size of MRCs can change as well. The number of MRCs in *T. ilisha* specimens caught in seawater was 2.46 times higher than those were in river water that our results are consistent with those of other scientists (Chang *et al*., 2003; Kumar *et al*., 2020). During migration to a water environment with low salinity, a significant decrease was observed in the number and deep of MRCs. The high number of gill MRCs in specimens from the sea reflects the greater requirement of these individual organs for ion transport. In a study fish were placed in water with a salinity as twice as high as the original salinity, the researchers reported an increase in the number of MRCs (Pourkhadje *et al*., 2014). Of course, there are reports on other fish species such as *Takifugu niphobles*, in which they showed that upon transfer of the fish to saltwater, the number of MRCs were decreased while their size were increased (Tang and Lee, 2013). Researchers believe that reduction in size and number of MRCs following migration to fresh water is a sign of reduction of their requirement for the active excretion of Na^+ and Cl^- in hypoosmotic environments (Hwang *et al*., 2011; Shirangi *et al*., 2016). This

finding is in line with the results of previous studies that showed the large size and the number of MRCs in seawater specimens were necessary for active excretion of salt (Tse *et al*., 2006). In this research, SEM studies of MRCs in the gills of *T. ilisha* were conducted during migration from sea to the river. The results of this study revealed that the morphological changes in MRCs reflect the ionic and osmotic requirements of the animals. In the current study, *T. ilisha* was able to tolerate different ranges of salinities without showing mortality. Three subtypes of MRCs (shallow basin, deep hole, and wavy convex) were considered to describe the ultrastructural characteristics of apical surface of MRCs using scanning electron microscopy. In a previous study on tilapia, *Oreochromis mossambicus* that was adapted to freshwater, MRCs were categorized by the same three subtypes (Pourkhadje *et al*. 2014; Papi *et al*., 2016). The researchers suggested that MRCs with different morphologies are equally active in such environments but exhibit different ion transporting functions (Papi *et al*., 2016; Da Mota Araujo *et al*., 2019). In hypoosmotic environments, the frequency of wavy convex subtype of MRCs increase with numerous microvilli on the apical membrane to be able absorb ions (Huang *et al*., 2011; Morovvati *et al*., 2017). Researchers believe that analysis of biochemical parameters of blood is one of the most valuable methods, since it has been shown that their physiological values are species-specific. The blood parameters varied considerably between the two different environments. According to the results of current study, mean concentrations of Na^+ and Cl^- ions in plasma of *T. ilisha* decreased following migration to river. In a study by Piermarini and Evans (2001), it was reported that concentrations of Na^+ and Cl^- ions in the blood plasma of Atlantic stingray (Dasyatis Sabina) in seawater specimens were significantly higher than those in river. According to Nilsson *et al*. (2012), high concentrations of Na^+ and Cl^- ions in seawater could have resulted in the transfer of these ions into the fish's bodies. Ion concentrations of K^+, Ca^{2+}, and Mg^{2+} in *T. ilisha* blood plasma were significantly reduced following migration to the river. In a study by Lin and Lee (2005) on tilapia, puffer and milkfish, concentrations of Mg^{2+} and Ca^{2+} in seawater were higher than those in freshwater, while the concentration of K^+ in freshwater fish was lower than those in seawater. The pattern of changes in blood plasma magnesium ion content in *T. ilisha* was similar to that of other ions. In a study conducted by Farabi *et al.* (2009) they examined ion concentrations in the plasma of juvenile Russian sturgeon, *Acipenser gueldenstaedtii*, in three different salinities, that were fresh water (5 ppt), estuarine (9.5 ppt), and Caspian Sea water (12.5 ppt), and suggested that plasma concentration in estuarine water was higher than the fresh water and lower than to the Caspian Sea water. Similar to other ions, values for

glucose were decreased during migration to the river. This finding is consistent with the study of Martinez- Alvareztinez (2002) that reported on adriatic sturgeo, *Acipenser naccarii*. They believe that glucose is a carbohydrate that plays a crucial role in generating energy through the production of ATP under stressful conditions, catecholamine and cortisol induce glycolysis and gluconeogenesis, and consequently increase plasma glucose by affecting the liver. In conclusion, the results of the current study provide fundamental information on the osmoregulatory functions and migration biology of *T. ilisha*, which are prerequisites for effective management of this species and other similar migratory species commercially valuable fishery resource in the Iran and other countries.

Acknowledgements

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