

## Changes in Chloride Cell Abundance, Na<sup>+</sup>, K<sup>+</sup>-ATPase Immunolocalization and Activity in the Gills of Golden Grey Mullet, *Liza aurata*, Fry During Adaptation to Different Salinities

Saber Khodabandeh, Ph.D.\*, Mohsen Shahriari Moghaddam, M.Sc., Behroz Abtahi, Ph.D.

Faculty of Marine Sciences, Tarbiat Modares University, Tehran, Iran

\* Corresponding Address: P.O.Box: 14115-333, Faculty of Marine Sciences, Tarbiat Modares University, Tehran, Iran  
Email: surp78@gmail.com

### Abstract

Received: 21Apr/2008, Accepted: 26/Oct/2008

**Objective:** Changes in chloride cell abundance, Na<sup>+</sup>, K<sup>+</sup>-ATPase immunolocalization and activity were investigated in the gills of the golden grey mullet, *Liza aurata*, fry acclimated to freshwater (FW) and different salinities (12‰, 36‰ and 46‰).

**Materials and Methods:** Na<sup>+</sup>, K<sup>+</sup>-ATPase localization was performed through immunofluorescence light microscopy using a mouse monoclonal antibody IgG<sub>α</sub>. Quantitative analysis of Na<sup>+</sup>, K<sup>+</sup>-ATPase intensity was analyzed using Optima's version 6.51 image analysis software (Media Cybernetics, Silver Spring, MD, USA).

**Results:** In FW, the fluorescent cells (chloride cells) were observed on the epithelia of filaments (mainly in inter-lamellar regions) and on the lamellae. Following transfer to 12‰ salinity, the abundance of Na<sup>+</sup>, K<sup>+</sup>-ATPase immunofluorescence cells on the filaments decreased 1.7-fold, and no immunofluorescence cells were detected on the lamellae. Samples from 36‰ and 46‰ salinity showed a high density of chloride cells on the epithelia of filaments, and a few cells on the lamellae. Na<sup>+</sup>, K<sup>+</sup>-ATPase intensity did not change significantly with an increase in salinity from 36‰ to 46‰ but it was significantly higher (p>0.05) in the FW compared to 12‰ salinity. There was no significant difference between gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity in FW and 12‰ salinity, but it was significantly higher (p>0.05) in the fish acclimated to 36‰ and 46‰ salinity (3.3- and 5.1-fold) compared to 12‰.

**Conclusion:** The capability of *L. aurata* fry to change the number and size of gill chloride cells, as well as their activities indicate the high degree of adaptability of this fish to a wide range of salinity.

**Keywords:** Immunolocalization, Na<sup>+</sup>, K<sup>+</sup>-ATPase, *Liza aurata* (*Mugil auratus*), Osmoregulation

Yakhteh Medical Journal, Vol 11, No 1, Spring 2009, Pages: 49-54

### Introduction

Osmoregulatory activities enable aquatic animals to adapt to external medium salinity fluctuations. In freshwater, the organism is subjected to water uptake and ion loss. In fish, hyper-osmoregulatory mechanisms compensate for these movements, with a low water intake, active absorption of ions by the gills and production of hypotonic urine by kidney (1). In seawater, hypo-osmoregulatory mechanisms compensate for water loss and ionic invasion. Dehydration is avoided by a high rate of drinking. The water is absorbed by the intestine and the gills reject the excess ions (1-3). Gill ion transport is facilitated by chloride cells (ionocytes or mitochondria-rich cells) in the gill epithelium that possess a plasma-membrane-associated Na<sup>+</sup>, K<sup>+</sup>-ATPase enzyme. Chloride cells can individually transform between ion absorption and ion secretion states in response to salin-

ity changes (4-6). Immunohistochemical localization of Na<sup>+</sup>,K<sup>+</sup>-ATPase has been recognized as a useful method for locating the chloride cells in tissues and organs of aquatic invertebrates (7). This enzyme also has been localized in fish gills, intestine and kidney (8-10). Levels of Na<sup>+</sup>, K<sup>+</sup>-ATPase have been used extensively as an index of transport capacity in fish exposed to a variety of conditions including seawater (11). Na<sup>+</sup>, K<sup>+</sup>-ATPase activity has been studied in several teleost fish (2, 5, 12-19). Most euryhaline teleosts exhibit adaptive changes in gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity following salinity changes (16-18). Gill Na<sup>+</sup>, K<sup>+</sup>-ATPase is reported to have a higher level of activation in seawater residing teleosts (4). In contrast, in several euryhaline species of non-estuarine marine teleosts, such as flounder, mullet (18), and milkfish (20), higher Na<sup>+</sup>, K<sup>+</sup>-ATPase activity was found in freshwater-adapted than seawater-adapt-

ed fish.

Golden grey mullet, *Liza aurata* (synonym, *Mugil auratus* Risso, 1810), is a euryhaline fish that occurs both in freshwater and in highly salinated bodies of water (21). It is currently one of the most important and major economic species that reside in the Caspian Sea (salinities ranging from 5 to 13‰). The number of investigations on the effects of different salinities on the osmoregulation of mugilidae fish is limited. To date, no study has been conducted on the influence of different salinities on chloride cell abundance, Na<sup>+</sup>, K<sup>+</sup>-ATPase enzyme immunolocalization and activity in Caspian Sea mullet. Thus the objective of this study was to use immunolocalization to undertake a quantitative analysis of the intensity and activity of the Na<sup>+</sup>, K<sup>+</sup>-ATPase enzyme in the gills of the golden grey mullet, *Liza aurata*, caught from the Iranian coast of the Caspian Sea, and acclimated to freshwater and to three different salinities (12‰, 36‰ and 46‰).

## Materials and Methods

### Animals

Golden grey mullet (*Liza aurata*) fry (1.5 ± 0.5 g) were caught from the Iranian coast of the Caspian Sea (Mazandaran) in April 2005. They were adapted to experimental conditions (aerated Caspian Sea water at 25°C with 12hr Dark: 12hr Light photoperiod) for a period of 10 days. The experiment began with the transfer of 240 fish to 8, 60 Liter aquariums, containing aerated freshwater (FW), Caspian Sea water (12‰ salinity) and artificial seawater (36‰ and 46‰ salinities) at 24-26°C with a daily 12 hrs photoperiod for 5 days. During the experiment the fish were fed with *Gammarus* 2 times a day. In freshwater, 80% of the fish died after 24 hrs and all had died after 4 days, so the remaining samples were investigated after 72 hrs acclimation. Artificial seawater was made of a mixture of synthetic sea salt and Caspian Sea water. Concentrations of some main ions in Caspian Sea water and artificial seawater are shown in Table 1. Fish were anaesthetized in a solution of phenoxy 2 ethanol (0.3 ml/l) prior to any manipulation.

**Table 1: Concentrations (mEq/l) of some of the main ions in Caspian Sea water and artificial seawater. Values are Means ± SE.**

Salinity	Na <sup>+</sup>	K <sup>+</sup>	Ca <sup>2+</sup>	Mg <sup>2+</sup>
12‰	165 ± 2	2.1 ± 0.05	17 ± 0.23	58.12 ± 1.85
36‰	382 ± 4	5.9 ± 0.18	48 ± 1.8	160 ± 2.95
46‰	502 ± 3.4	7.8 ± 0.82	51 ± 2	199 ± 3.6

### Na<sup>+</sup>, K<sup>+</sup>-ATPase immunolocalization

The heads of 6 young golden grey mullet acclimated to each salinity were dissected. They were immersed in Bouin's fixative for 24 hours, washed and dehydrated in an ascending series of ethanol for embedding in Paraplast (Sigma, 060K19271). Following embedment of the samples (whole heads) in Paraplast, transversal and longitudinal sections of 5 µm were cut on a Leitz Wet-

zlar microtome and collected on poly-L-lysine-coated slides. 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<sub>4</sub>Cl in phosphate-buffered saline (PBS), pH=7.3, for 5 min to mask the free aldehyde groups from the fixative. The sections were washed in PBS and incubated for 10 min with a blocking solution (BS) containing 1% bovine serum albumin (BSA) and 0.1% gelatin in PBS (7, 9, 10, 22-24). Immunolocalization of the Na<sup>+</sup>, K<sup>+</sup>-ATPase was performed by immunofluorescence light microscopy using a mouse monoclonal antibody IgGα<sub>5</sub> (Hybridoma Bank, University of Iowa) raised against the α-subunit of chicken Na<sup>+</sup>, K<sup>+</sup>-ATPase (25). This antibody, diluted in PBS to 20 µg/ml<sup>-1</sup>, was placed on the sections and incubated for 2hrs at room temperature in a moist chamber. The slides were rinsed in BS and they were then incubated for 1h in the secondary antibody (Fluorescein Isothiocyanate Conjugated, FITC) under dark conditions. Negative control sections were incubated in BSA-PBS without primary antibody. All slides were rinsed in BS, and were mounted in a medium for fluorescent microscopy (Sigma, ref. 7534) to retard photobleaching. An Olympus digital camera, adapted to the Olympus fluorescent microscope, was used to obtain images from the tissues.

Na<sup>+</sup>, K<sup>+</sup>-ATPase immunofluorescence cells were counted in 16 longitudinal sections of gill from each fish and expressed per 1 mm<sup>2</sup> of tissue. Mean number of immunofluorescence cells on the filaments and on the lamellae for each group were obtained using the means calculated from each fish.

### Quantitative analysis of Na<sup>+</sup>, K<sup>+</sup>-ATPase intensity

Following immunolocalization of Na<sup>+</sup>, K<sup>+</sup>-ATPase in the 6 samples from each salinity, 6 slides from each fish and 9 sections (containing gill filaments and lamellae) from each slide and 3 images from each section were used to quantify the fluorescence intensity using Optima's version 6.51 image analysis software (Media Cybernetics, Silver Spring, MD, USA). The signal intensity was measured as the fluorescent epithelial surface relative to the whole epithelium (9).

### Na<sup>+</sup>, K<sup>+</sup>-ATPase activity

The Na<sup>+</sup>, K<sup>+</sup>-ATPase activity in the gills of fry was determined according to the technique described by Norby (26) and McCormick (27), and recently used by Khodabandeh (7). The gill samples were quickly excised, weighed and homogenized in a cold imidazole buffer (50 mmol.l<sup>-1</sup> imidazole, 250 mmol.l<sup>-1</sup> sucrose, and 5 mmol.l<sup>-1</sup> EDTA (ethylene diamine tetra-acetic acid) at pH = 7.4 with HCl). The cuvette contained 2 ml of reaction mixture with and without 5 mmol.l<sup>-1</sup> ouabain. The composition of the reaction media was: 25 mmol.l<sup>-1</sup> Tris-HCl; 2 mmol.l<sup>-1</sup> MgCl<sub>2</sub>; 0.25 mmol.l<sup>-1</sup> EGTA (ethyleneglycol tetra-acetic acid), pH = 7.4; 100 mmol.l<sup>-1</sup> NaCl, 25 mmol.l<sup>-1</sup> KCl; 1.5 mmol.l<sup>-1</sup> PEP

(phosphoenol-pyruvate); 0.15 mmol.l<sup>-1</sup> NADH (β nicotinamide adenine dinucleotide); 5 mmol.l<sup>-1</sup> ATP; 8.25 U/ml LDH (lactate dehydrogenase); 2.75 U/ml PK (pyruvate kinase) enzymes. Incubation was conducted at 37 °C for 30 min and the Na<sup>+</sup>, K<sup>+</sup>-ATPase reaction was initiated by the homogenate addition. The Na<sup>+</sup>, K<sup>+</sup>-ATPase activity (μ mole Pi/mg protein/hr) was expressed as the activity in the presence of ouabain (a specific inhibitor of Na<sup>+</sup>, K<sup>+</sup>-ATPase) subtracted from the activity obtained in the absence of ouabain.

**Statistical analysis**

Analysis of variance (ANOVA) and student’s t-test were used for statistical comparisons of the Na<sup>+</sup>, K<sup>+</sup>-ATPase mean activity values (p<0.05) for six fish. Student’s t-test was also used for statistical comparison of the mean intensity values in six images from each gill segment.

**Results**

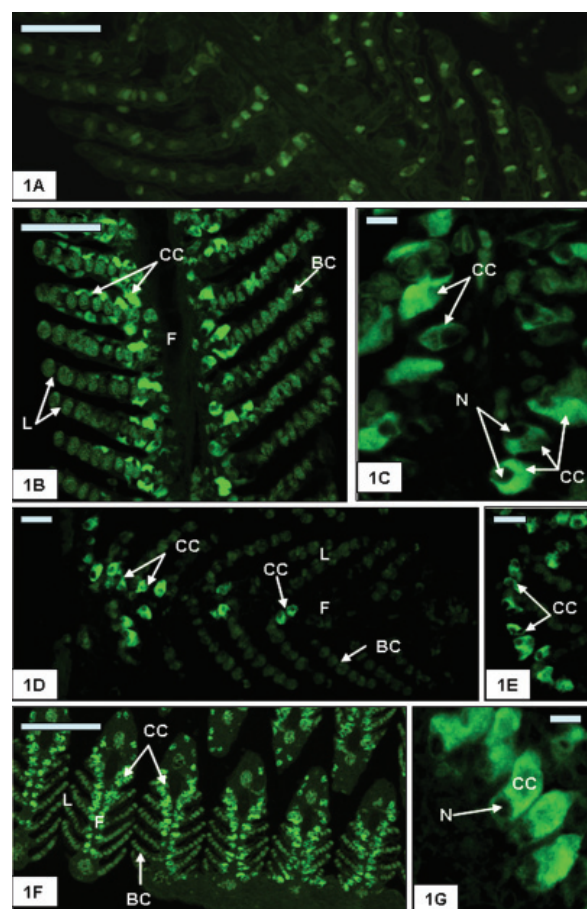
**Na<sup>+</sup>, K<sup>+</sup>-ATPase immunolocalization**

Immunofluorescence microscopy was used for the localization of Na<sup>+</sup>, K<sup>+</sup>-ATPase as the fixation and staining process provides good antigenicity. Positive control *Astacus* gill sections were constantly brightly immunostained (not shown). The negative control sections from the mullet gill samples from freshwater, deprived of primary antibodies, showed no specific immunofluorescence (Fig 1A). A weak auto-fluorescence was observed in the blood cells (Fig 1B, D, F).

Na<sup>+</sup>, K<sup>+</sup>-ATPase enzyme was detected in the samples from all salinities. Immunoreactive staining appeared to be distributed evenly throughout the fluorescent cells (chloride cells), except for the absence of staining in nuclei (Fig 1C, G). In FW samples, the fluorescent cells were observed on the epithelia of filaments (mainly in inter-lamellar regions) and on the lamellae (Fig 1B). The number of fluorescent cells decreased on epithelia of filaments from the Caspian Sea water samples (12‰ salinity) (Fig 1D, Table 2). They were observed mainly in the basal parts of the filaments and were mostly absent from the apical parts (Fig 1D, E). At this salinity, no immunofluorescence cells were detected on the lamellar cells (Fig 1D, Table 2). Samples from the artificial seawater, 36‰ (not shown) and 46‰ (Fig 1F, G), showed a high density of fluorescent cells on the filaments (Table 2). A few fluorescent cells were also observed on the lamellae of samples from these salinities (not shown).

**Table 2: Na<sup>+</sup>, K<sup>+</sup>-ATPase immunofluorescence cells (chloride cells) (cells/1mm<sup>2</sup>) on the filaments and lamellae of mullet fry, after acclimation in different salinities. Values are Means ± SE. N=6 fish for each salinity. Different letters indicate significant difference (p<0.05).**

Immunofluorescence cells (chloride cells) (cells/1mm <sup>2</sup> )	FW	12‰	36‰	46‰
<b>On filaments</b>	32 ± 2 <sup>a</sup>	19 ± 2 <sup>b</sup>	30 ± 1 <sup>a</sup>	34 ± 2 <sup>a</sup>
<b>On lamellae</b>	12 ± 2 <sup>a</sup>	0 <sup>b</sup>	2 <sup>b</sup>	3 ± 1 <sup>b</sup>



**Fig 1: Immunolocalization of Na<sup>+</sup>, K<sup>+</sup>-ATPase in the gills of the golden grey mullet, *Liza aurata*, fry. Negative control (1A). Longitudinal (1B) and transversal (1C) sections of the gill from samples acclimated in FW; the fluorescent cells (chloride cells) were observed on the filaments and lamellae. Longitudinal sections of the gill from samples (1D and 1E) acclimated in Caspian Sea water (12‰); the number of fluorescent cells decreased. Longitudinal section of the gill (1F) from samples acclimated in the 46‰; a high density of fluorescent cells are present in the gills. A high magnification of image from transversal section of the lamella (1G) from samples acclimated in the 46‰; whole cytoplasm of cells show immunofluorescence. Abbreviations: BC, blood cells; CC, chloride cells; F, filament; L, lamellae; N, nucleus. Bars: 60 μm (1A); 80 μm (1B); 5 μm (1C and 1G); 30 μm (1D and 1E), 130 μm (1F).**

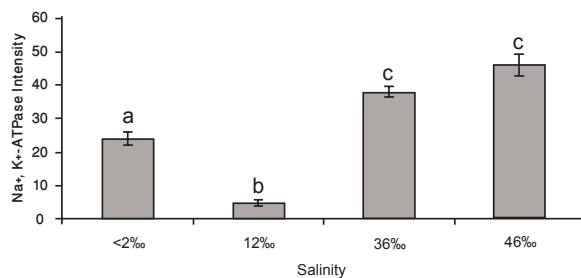
**Quantitative analysis of Na<sup>+</sup>, K<sup>+</sup>-ATPase intensity**

Quantification of Na<sup>+</sup>, K<sup>+</sup>-ATPase intensity in different salinities showed significantly higher intensities in the gills of samples acclimated in artificial seawater (36‰ and 46‰) compared to FW and Caspian Sea water (12‰) (Fig 2). Na<sup>+</sup>, K<sup>+</sup>-ATPase intensity was significantly higher in FW compared to 12‰ salinity but did not differ significantly from the 36‰ and 46‰ salinities (Fig 2).

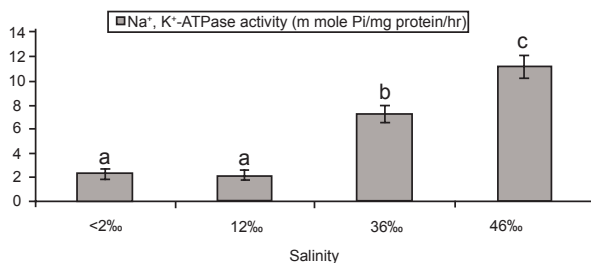
**Na<sup>+</sup>, K<sup>+</sup>-ATPase activity**

Results of the biochemical assay for Na<sup>+</sup>, K<sup>+</sup>-ATPase

activity is shown in Fig 3. The Na<sup>+</sup>, K<sup>+</sup>-ATPase activity was 2.45, 2.23, 7.32 and 11.21 μM Pi mg<sup>-1</sup> protein h<sup>-1</sup> in the gills of samples from FW and the 12‰, 36‰ and 46‰ salinities, respectively. There was no significant difference between gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity of fish from FW and 12‰ salinity. However, significant difference between salinities of 36‰ and 46‰ were found. Na<sup>+</sup>, K<sup>+</sup>-ATPase activity was also significantly higher in fish acclimated to salinities of 36‰ and 46‰ (3.3- and 5.1-fold) compared to 12‰ and FW, indicated by letters in Fig 3.



**Fig 2: Quantitative analysis of the Na<sup>+</sup>, K<sup>+</sup>-ATPase intensity (6 fish/salinity and 162/fish), in the gills of golden grey mullet, *Liza aurata*, fry, after acclimatisation in different salinities (FW, 12‰, 36‰ and 46‰). Data are expressed as Means ± SE. Different letters beside the error bars (A, B, C) indicate significant difference (p<0.05). (Different letters usually represent different levels of significance e.g. <0.05, <0.01 etc)**



**Fig 3: Na<sup>+</sup>,K<sup>+</sup>-ATPase activity (n = 6 fish) in the gills of golden grey mullet, *Liza aurata*, fry, after acclimatisation in different salinities (FW, 12‰, 36‰ and 46‰). Data are expressed as Means ± SE. Different letters beside the error bars (A, B, C) indicate significant difference (p<0.05)**

## Discussion

Na<sup>+</sup>, K<sup>+</sup>-ATPase is a plasma-membrane-associated enzyme which catalyses ubiquitous ATP-driven Na<sup>+</sup>/K<sup>+</sup> transport. This enzyme is crucial to ion and water regulation in both freshwater and seawater fish (1). Salinity-dependent alterations of Na<sup>+</sup>, K<sup>+</sup>-ATPase localization and activity have been found in several species of fish (2, 15, 24, 28).

Immunohistochemical localization of Na<sup>+</sup>, K<sup>+</sup>-ATPase is recognized as a useful marker of mitochondria-rich cells in the different tissues of fish (9, 10, 15, 24, 28, 29). In our study, Na<sup>+</sup>, K<sup>+</sup>-ATPase enzyme was detected in the gills of *L. aurata* from all salinities. Immunoreactive staining appeared to be distributed evenly throughout the fluorescent cells, which correspond to chloride

cells (mitochondria-rich cells or ionocytes) as shown in previous studies (9, 15-17, 24, 28, 30). The abundance and distribution of Na<sup>+</sup>, K<sup>+</sup>-ATPase fluorescent cells (chloride cells) varied across the different salinities. In FW, the chloride cells were observed on the epithelia of filaments (mainly in inter-lamellar regions) and on the lamellae. As observed in mullet in the present study, the number of chloride cells on the lamellae of freshwater-acclimated American shad (*Alosa sapidissima*) is much higher than in seawater-acclimated shad (31). Chloride cells were redistributed on the epithelia of both filaments and lamellae in some freshwater-adapted euryhaline teleosts (5, 30) but not others (32-35). Many investigators have suggested a role for the lamellar chloride cells in ion uptake in hypo-osmotic environments on the basis of observations of cell degeneration following transfer from freshwater to seawater and hypertrophication following transfer to deionized water (16, 36, 37). The number of chloride cells decreased in the filaments of samples from Caspian Sea water (12‰) but no immunofluorescence cells were detected in the lamellae. The occurrence of chloride cells only on gill filaments has already been reported in pufferfish, *Tetraodon nigroviridis*, as well as in tilapia, *Oreochromis mossambicus*, after acclimatisation to freshwater, brackish water and seawater (20, 28, 32). Shikano and Fujio (1998a) reported that lamellar chloride cells degenerated when chum salmon fry were transferred from freshwater to brackish water and reappeared when they were re-introduced to freshwater. In our study, a high density of chloride cells were seen on the filaments in samples from artificial seawater (36‰ and 46‰) and a few were also observed on the lamellae. In the Hawaiian goby (*Stenogobio hawaiiensis*), there were still a large number of chloride cells on the lamellae after seawater exposure, and they had similar Na<sup>+</sup>, K<sup>+</sup>-ATPase immunoreactivity to that of chloride cells on the filaments (15). It is also reported that chloride cells are normally abundant in filament epithelia of both freshwater and seawater teleosts (38), and are effective at secreting ions in hypertonic seawater as well as taking up ions in hypotonic freshwater (19).

In addition to a low density of gill chloride cells, the lowest levels of Na<sup>+</sup>, K<sup>+</sup>-ATPase intensity and activity were observed in the Caspian Sea water samples. The near-iso-osmotic environment of the Caspian Sea is likely subject mullet to very little osmotic stress. Low levels of Na<sup>+</sup>, K<sup>+</sup>-ATPase activity in brackish water acclimated fish have also been reported in sea bass, *Dicentrarchus labrax* (2), milkfish, *Chanos chanos* (35), and pufferfish, *Tetraodon nigroviridis* (28).

Measured levels of gill Na<sup>+</sup>, K<sup>+</sup>-ATPase intensity in FW acclimated fish were significantly higher than in Caspian Sea water acclimated fish, although there was no significant difference in Na<sup>+</sup>, K<sup>+</sup>-ATPase activity between them. All the mullet transferred to freshwater died in less than 4 days, despite an increase in chloride cell numbers and abundant Na-K pump. Possibly the abrupt transfer did not provide enough time for Na<sup>+</sup>, K<sup>+</sup>-

ATPase activity to be established as earlier work on two mullet species indicated higher Na<sup>+</sup>, K<sup>+</sup>-ATPase enzyme activity in freshwater than seawater (37‰) (13, 39).

Na<sup>+</sup>,K<sup>+</sup>-ATPase intensity and activity were at a maximum in the 36‰ and 46‰ acclimated mullet compared to the Caspian Sea water samples. In seawater conditions, Na<sup>+</sup>, K<sup>+</sup>-ATPase still pumps Na<sup>+</sup> from the intracellular compartment of chloride cells into the extracellular space across the basolateral surface. The strong Na<sup>+</sup> gradient drives a secondary active co-transport of Cl<sup>-</sup> into the cell through an Na<sup>+</sup>, K<sup>+</sup>,2Cl<sup>-</sup> co-transporter. This creates an electrochemical gradient that favours diffusion of Cl<sup>-</sup> through a Cl<sup>-</sup> channel on the apical side out to the seawater. It enables the mullet to secrete excess salts efficiently and thus acclimate smoothly to these salinities. A positive correlation between environmental salinity and gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity, several days after transfer of the fish from freshwater to seawater, have been also reported (18). The majority of earlier works have shown a similar positive correlation between environmental salinity and gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity (5), forming the well established 'diadromid paradigm' (18). In contrast, in several species higher or similar levels of gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity have been found in freshwater or low salinity-acclimated fish compared with seawater fish; (2, 4, 6, 13, 14, 35, 39, 40). The majority fish exhibiting this 'alternative' Na<sup>+</sup>, K<sup>+</sup>-ATPase activity response are either marine or estuarine-dependent marine species. This signifies that adaptation of *L. aurata* and other marine fish to low salinity environments can be a response that differs considerably from the diadromid paradigm.

## Conclusion

In conclusion, the present study indicates that: - responses to salinity transfer can differ from one species to another; - changes in Na<sup>+</sup>, K<sup>+</sup>-ATPase intensity are not always matched by changes in Na<sup>+</sup>, K<sup>+</sup>-ATPase activity; - the capability of *L. aurata* fry to change the number and size of gill chloride cells, as well as their activities indicate the high degree of adaptability of these fish to a wide range of salinity, under laboratory conditions.

## Acknowledgments

The monoclonal Na<sup>+</sup>, K<sup>+</sup>-ATPase ( $\alpha$ -subunit) antibody was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa (Iowa City, USA). Thanks are due to the Tarbiat Modares University and the Ministry of Science, Research and Technology, Islamic Republic of Iran for financial aid and support. There is no conflict of interest in this article.

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