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Na^+, K^+-ATPase (α1a and α1b) and NKCC co-transporter genes expression in the gills of *Salmo trutta caspus*, parr

Saber Khodabandeh[1]* and Halemeh Rajabi[2]

[1] *Associate Professor, Faculty of Marine Sciences, Tarbiat Modares University, Noor, Iran*
[2] *M.Sc. Student, Faculty of Marine Sciences, Tarbiat Modares University, Noor, Iran*

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ABSTRACT The effects of fish weight on salinity tolerance were studied in Caspian salmon (*Salmo trutta caspius*) parr. 180 fish (all with 2 years old but with three weights; 5, 15, 25g) were selected and they reared in freshwater (FW) and brackish water (BW; 13ppt salinity) for 10 days. The mRNA expression of two α-subunit isoforms of Na^+, K^+-ATPase (α1a and α1b) and NKCC co-transporters were studied in their gill tissue. In all three weight groups, the mRNA levels for the α1a isoforms decreased following BW exposure, whereas α1b levels significantly increased in 15g and 25g groups. In addition, NKCC gene expression were significantly higher in the groups of BW than FW in 15g and 25g weights ($P<0.05$). The reciprocal expression of Na^+, K^+-ATPase isoforms (α1a and α1b) during salinity acclimation suggests that they may have different roles in the gill of FW and BW fishes; ion uptake in FW and ion secretion in BW. In conclusion, in the Caspian salmon, between parrs with the same age, the group with the weight of 15g possesses better compatibility with BW than to other groups. After reaching to 25g, fish passed smoltification and they became more compatible with the FW environment and maybe lost its osmoregulation ability in saline or brackish water.

Key words: *Caspian Salmon, Na^+-K^+ pump, Osmoregulation.*

1 INTRODUCTION

It is well known that, the migration of euryhaline teleost fishes from FW to SW requires a change in the gill from an ion absorbing tissue to an ion secreting one. This reversal of ion pumping is associated with an up regulation of gill Na^+, K^+-ATPase activity (McCormick and Saunders, 1987). It has been discovered that four α-subunit isoforms of Na^+, K^+-ATPase are expressed in rainbow trout gill (Richard *et al.*, 2003). The α-subunit of Na^+, K^+-ATPase is the catalytic portion of the pump that contains ATP, cation and ouabain binding sites (Reuss *et al.*, 1996; Khodabandeh *et al.*, 2009a). It is reported that, in rainbow trout gill, the α1a and α1b isoforms are found at much higher levels than α1c and α3 isoforms and are reciprocally expressed during SW acclimation, as levels of isoform α1a quickly drops while α1b increases following SW exposure (Richards *et al.*, 2003). In addition, as suggested by Bystriansky *et al.* (2006), α1a and α1b isoforms play important roles in osmoregulation ability than other isoforms. They transferred three

* *Corresponding author: Associate Professor, Faculty of Marine Sciences, Tarbiat Modares University, Noor, Iran, Tel: E-mail: surp78@gmail.com, skhoda@modares.ac.ir*

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difference species of salmonid to SW and observed that mRNA levels for the α1a isoform decreased following SW exposure whereas α1b levels increased significantly. In the current model of chloride cells from SW acclimated teleosts, Na^+, K^+-ATPase and NKCC co-transporter are located at the basolateral surface of the cell. Na^+, K^+-ATPase exert a sodium gradient to drive the transport of sodium, potassium and two chloride ions into the cell via the NKCC co-transporter. Furthermore, changes in NKCC co-transporter abundance parallel changes in gill Na^+, K^+-ATPase activity indicate cooperating roles of these proteins in salt secretion by the gill (Pelis *et al.,* 2001). Two major isoforms of NKCC have been found in vertebrates: a secretary form (NKCC1) and an absorptive form (NKCC2). Two isoform of NKCC1 have been found in European eel (*Anguilla anguilla*), and the transcript of NKCC1a (as one of NKCC1) is found in large quantities in the gill and is up regulated after SW exposure (Cutler and Cramb, 2002). Wilson *et al.* (2000) found NKCC in the basolateral membrane and tubular system of chloride cells in the SW acclimated mudskipper, *Periopthalmodon schlosseri*. They secrete ions in sea water-adapted fish by the assistance of NKCC co-transporter as well as absorb ions and maintain the acid-base balance in FW adapted fish (Wood and Marshall, 1994; Khodabandeh *et al.*, 2009b; Frost and Nilsen, 2003; Lin *et al*., 2003).

It is also reported that, fish weight is one of the most important factors in fish following exposure to the sea as the more fish weight when release to the sea, the more survival rate and migrated fish number in the sea (McCormick and Saunders, 1987; Hoar, 1988; Robert, 2000). However, beside fish weight, osmoregulation and acclimation mechanisms to new salinity condition are considerable when fish fries release to the sea (Nordile *et al.,* 1982; Evans *et al.,* 2005). In previous studies, it is also reported that, during parr to smolt transformation in FW, osmoregulation ability was increased in salmonids before they entered to SW (Nance *et al.,* 1990; Arnesen *et al.,* 1998). *Salmo trutta caspius*, is an anadromous, Caspian Sea fish, which they used to breed in freshwater (FW) and spend most of their life in the Caspian Sea waters (CSW). Being mature, they used to migrate towards the river for spawning. Nowadays, this species generation is under extinct due to overfishing, construction in the migration routes of spawners, water pollution and destruction of the spawning locations. Artificial reproduction of the spawners and releasing of parrs to the Caspian Sea environment is a reasonable way to protect these valuable fish species from the extinction (Kiabi *et al.,* 1999; Niksirat and Abdoli, 2009). About millions of the Caspian Salmon parrs with different weights from 5 to 30 g are breed in the north of Iran and then released to the Caspian Sea annually. Although these parrs have the same age, differences in their weight and size would result in different abilities to challenge with the releasing stresses, in particular water salinity. Although several investigations have been studied on osmoregulation in parrs of *Salmo trutta caspius*, while no attention has been paid to the effect of weight in osmoregulation ability in parr fishes with the same age (Rajabi and Khodabandeh, 2013). Therefore, the aim of this study was to investigate the importance of fish weights in osmoregulation ability. Effects of salinity stress have been investigated in parrs with the same age and different weights (5, 15 and 25 g) by using Na^+, K^+-ATPase and NKCC co-transporter gene expression surveys.

2 MATERIALS AND METHODS

2.1 Animals

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Salmo trutta caspius parrs were obtained from Shahid Bahonar Hatchery, Kelardasht, Iran. Fish had different weights ranging from 5-25g in body weight and 8-15cm in total body length. To determine the effects of weight on gill Na^+, K^+-ATPase and NKCC co-transporter gene expression of the parrs, three weight groups of fishes (in each group *n*=30) were transferred directly from the stock tank of FW to BW (13ppt salinity) for 10 days. They were fed twice daily with commercial fish food, approximately 2% of body weight/day. Over the entire experimental period, water quality factors like temperature (18 °C), photoperiod (12 L/12 D), dissolved oxygen (6-7 ppm) and pH (7-8) were controlled daily using a GRANT-YSI 3800 data-logger (Grant Instrument Ltd, Cambridge, UK).

2.2 Tissue sampling

Fish parrs were sampled directly from the holding tank and killed by decapitation. All gill arcs were dissected from the fishes and immediately frozen in liquid N_2. Tissue samples were stored at -80°C until analysis (Khodabandeh *et al*., 2009b).

Quantifications of Na^+, K^+-ATPase and NKCC expressions were conducted by real-time PCR. The gill filament and lamellae of 6 samples from each group were quickly dissected. Total RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instruction and RNA quantification was based on the absorbance at 260 nm. After integrity verification of the RNA samples on the gel, 2µg of total RNA were treated with RNAase-free DNAase (Invitrogen) to remove any genomic DNA contamination. The reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using M-MLV reverse transcriptase (Invitrogen) and an oligo (dT) primer. The NKCC5 (forward) and NKCC1 (reverse) primer were then used to generate a PCR produduct of 346 bp (Table 1). The results were normalized with the elongation factor (EF1). This housekeeping gene has been validated in other species (Frost and Nilsen, 2003; Scott and Schulte, 2005; Kiilerich *et al*., 2007; Khodabandeh *et al*., 2009b). The forward (EF1α-F) and reverse (EFα-R) primers of the elongation factor generated a PCR product of 239 bp. Water was used as negative control in the real-time PCR. A mix of the following reaction components was prepared as follows (final concentrations): 5.5 µl of water, 1 µl of forward primer (0.5 µmol l^{-1}), and 1µl of reverse primer (0.5 µmol l^{-1}), 2µl of the Master mix Fast Start DNA Master PLUS SYBR Green I (Roche Applied Science, Basel, Switzerland). The Light Cycler glass capillaries were filled with 9.5 µl of the mix and 0.5 µl of cDNA was then added as PCR template. The cycling conditions were: denaturation programs (95°C for 10 min), amplification, hybridization and elongation programs repeated 40 times (95°C for 15 s; 60°C for 5 s; 72°C for 10 s). Melting curve analysis was carried out routinely with 30 s for each 1°C interval from 55°C to 95°C. For each reaction, the crossing point (CP) was determined according to the "Fit Point method" of the Light Cycler Software (Ver. 3.5) (Roche Molecular Biochemical) (Frost and Nilsen, 2003; Lin *et al*., 2003). All samples were analyzed in triplicate and the mean CP was calculated. Standard curves were generated for each primer set to calculate the amplification efficiencies (E) from the given slope according to the equation ($E=10^{(-1/slope)}$). According to the method described by Scott *et al.* (2004), the absolute mRNA expression was semi quantitatively estimated using the formula E^{-CP}. The results were normalized to the estimated absolute expression of EF1 in order to compare the expression levels between different organs and salinities (Scott and Schulte, 2005; Kiilerich *et al.*, 2007).

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Table 1 Primer sequences used in this study based on Lorin-Nebel *et al.* (2006)

Target sequence	Forward primer 5′-3′	Reverse primer 5′-3′
Na^+,K^+-ATPase α1a	AAGATCATGGAGTCCT-TTAAGAATCTG	CACCTCCTCTGCATTGATGCT
Na^+,K^+-ATPase α1b	CTGCTACATCTCAACCAACAACATT -Tm:67/8˚ GC:40%	ACCATCACAGTGTTCATTGGAT Tm:67/8˚ GC:43/5%
NKCC	TCATCACTGCTGGAATCTT	AGAGAAACCCACATGTTGTA
Ef1α	GA/GAACCATTGAGAAGTTCGAGAAG Tm:68/3^0 GC:42/9%	GCACCCAGGCATACTTGAAAG Tm:67/1^0 GC:53/2%

2.3 Statistical analysis

Gene expressions data are expressed as mean ± S.E. Data were subjected to the tests of normality and homogeneity of variance. Analysis of variance (ANOVA) was used to determine overall differences among three weight groups. Independent sample T-test were applied for statistical comparison of fry fishes between (FW) and (BW) when α=0.05.

3 RESULTS

3.1 Mortality

The fish entered to FW, survived over the entire period of this experiment. However, mortality was observed in different weight groups after transferring to the BW. The mortality rates in weight groups of 5, 15, and 25g were 25%, 8% and 0%, respectively.

3.2 Gene expression

mRNA gene expression patterns of Na^+, K^+-ATPase α1a and α1b and NKCC genes in the gills of *Salmo trutta caspius* differed between three weight groups in FW and BW. Expression of Na^+, K^+-ATPase α1a mRNA decreased significantly ($P<0.05$) in three weight groups after fry fishes were introduced to BW (Figure 1). By contrast, among weight groups, the highest expression rate of Na^+, K^+-ATPase α1a mRNA was observed in 25 g fish weight than other groups both in FW and BW (Figure 2 and 3). Following their transfer to the BW, expression of Na^+,K^+-ATPase α1b mRNA increased significantly ($p<0.05$) in 15 and 25 g weights as compared to the FW (Figure 4). Also, α1b mRNA was expressed at the highest level in 15 g weight in FW and BW (Figure 5 and 6).

NKCC gene expression in weights groups of 15 and 25g in BW were significantly ($P<0.05$) higher than the FW. However, in 5g weight, the gene expression showed no significant differences ($P>0.05$) between FW and BW (Figure 7). The highest expression rate of NKCC By was observed in 15 g weight group than other groups in both environmental conditions (Figure 8 and 9).

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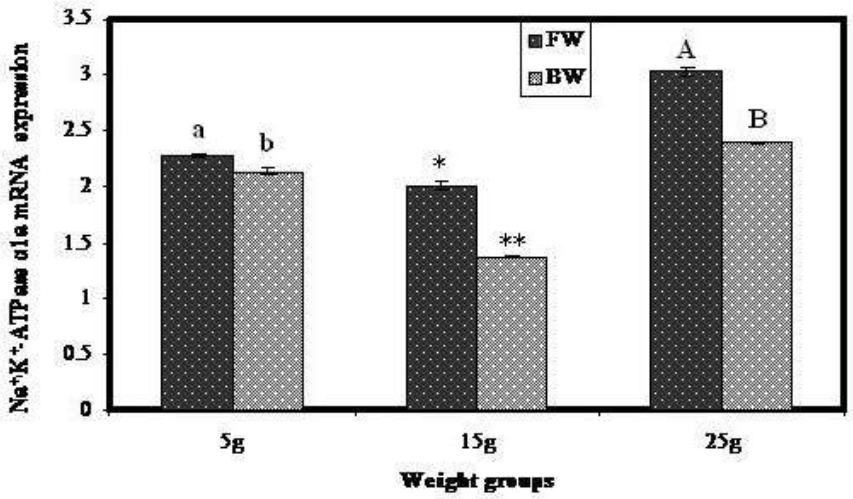

Figure 1 Gill Na^+, K^+-ATPase α1a mRNA expression in 5, 15, 25 g weight groups of *Salmo trutta caspius* in freshwater (FW) and after directly introduction to brackish water (BW) for 10 days. Values are mean ±SE. Different letter above the columns indicate significant difference between two environments

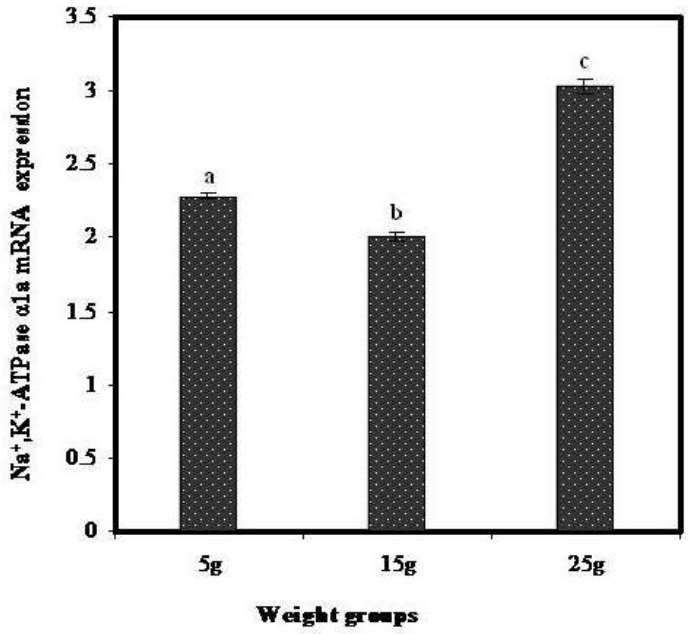

Figure 2 Gill Na^+, K^+-ATPase α1a mRNA expression in *Salmo trutta caspius* after 10 days acclimation in freshwater. Values are mean ±SE. Different letter above the columns indicate significant difference between three weight groups

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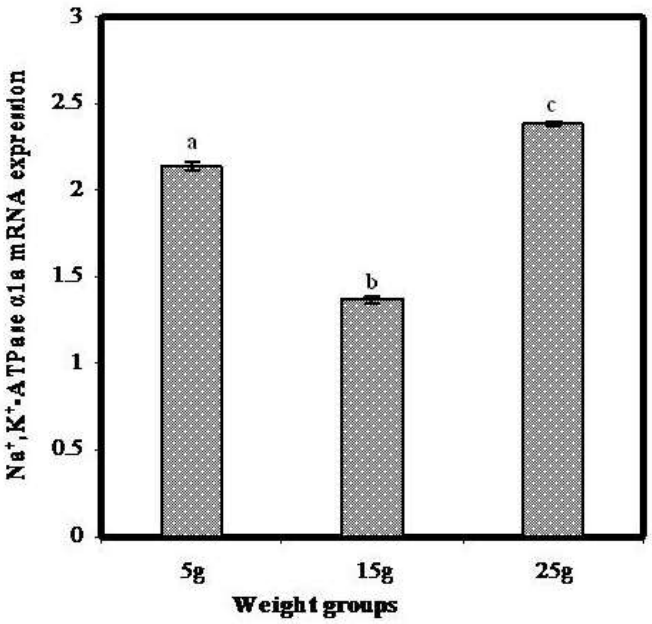

Figure 3 Gill Na^+, K^+-ATPase α1a mRNA expression in *Salmo trutta caspius* after 10 days acclimation in brackish water. Values are mean ±SE. Different letter above the columns indicate significant difference between three weight groups

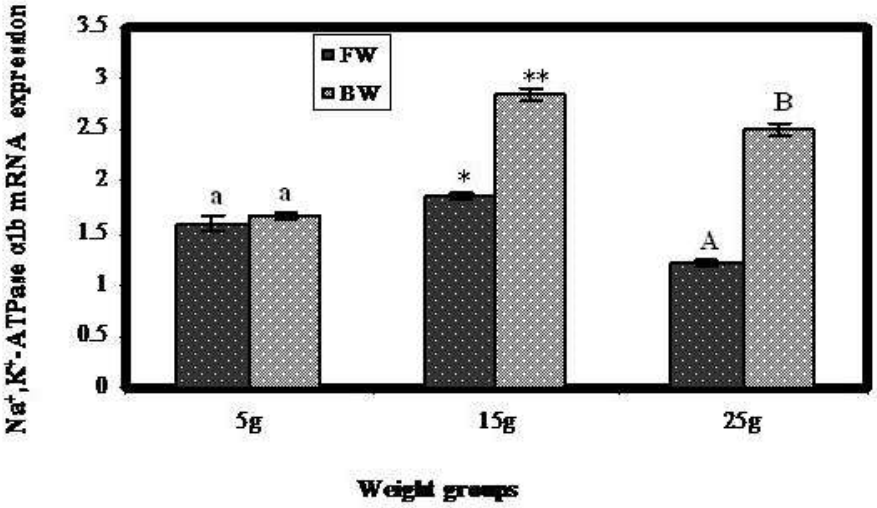

Figure 4 Gill Na^+, K^+-ATPase α1b mRNA expression in 5,15, 25 g weight groups of *Salmo trutta caspius* in freshwater and after directly introduced they to brackish water for 10 days. Values are mean ±SE. Different letter above the columns indicate significant difference between two environments

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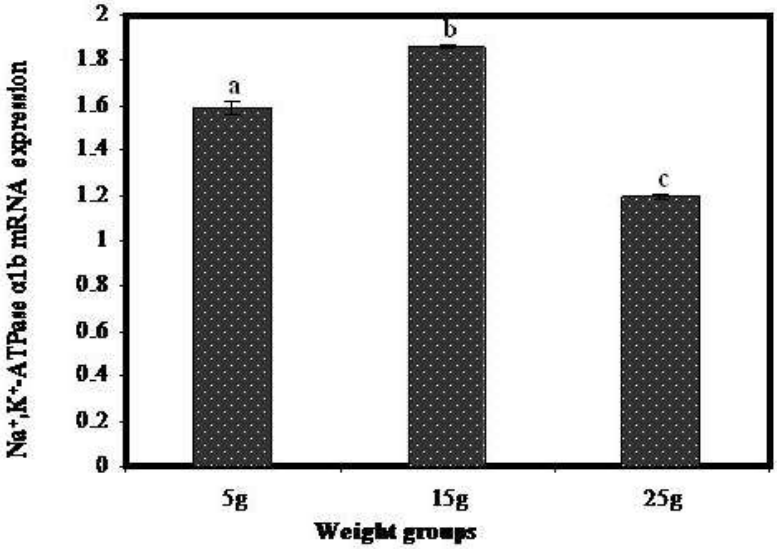

Figure 5 Gill Na^+, K^+-ATPase α1b mRNA expression in *Salmo trutta caspius* after 10 days acclimation in freshwater. Values are mean ±SE. Different letter above the columns indicate significant difference between three weight groups

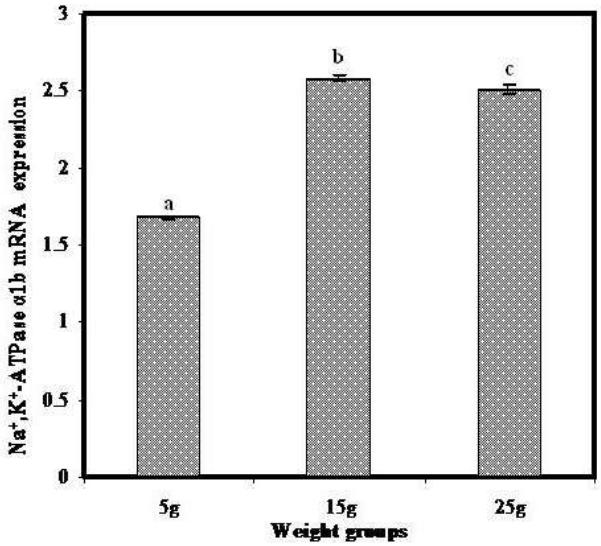

Figure 6 Gill Na^+, K^+-ATPase α1b mRNA expression in *Salmo trutta caspius* after 10 days acclimation in brackish water. Values are mean ±SE. Different letter above the columns indicate significant difference between three weight groups

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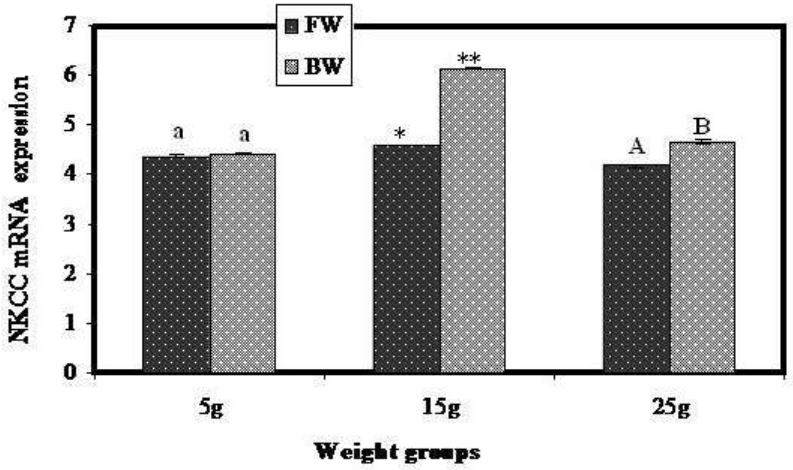

Figure 7 Gill NKCC mRNA expression in 5, 15, 25 g weight groups of *Salmo trutta caspius* in freshwater (FW) and after directly introduced they to brackish water (BW) for 10 days. Values are mean ±SE. Different letter above the columns indicate significant difference between two environments

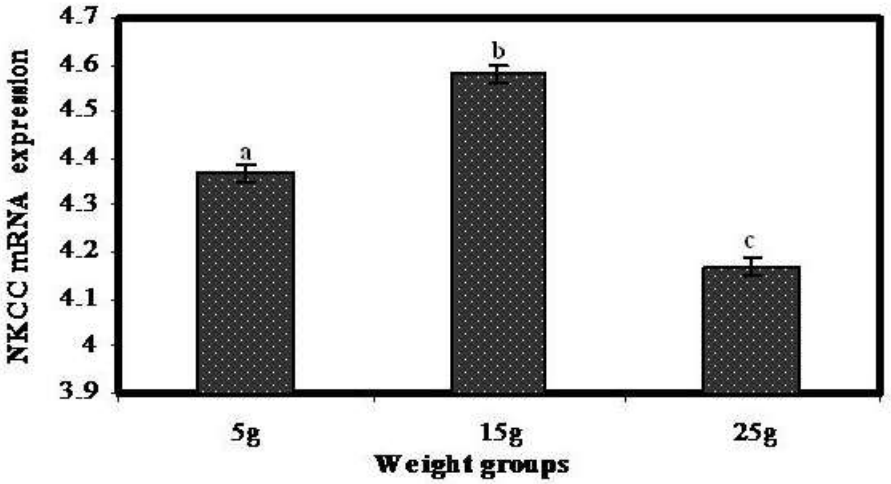

Figure 8 Gill NKCC mRNA expression in *Salmo trutta caspius* after 10 days acclimation in freshwater. Values are mean ±SE. Different letter above the columns indicate significant difference between three weight groups

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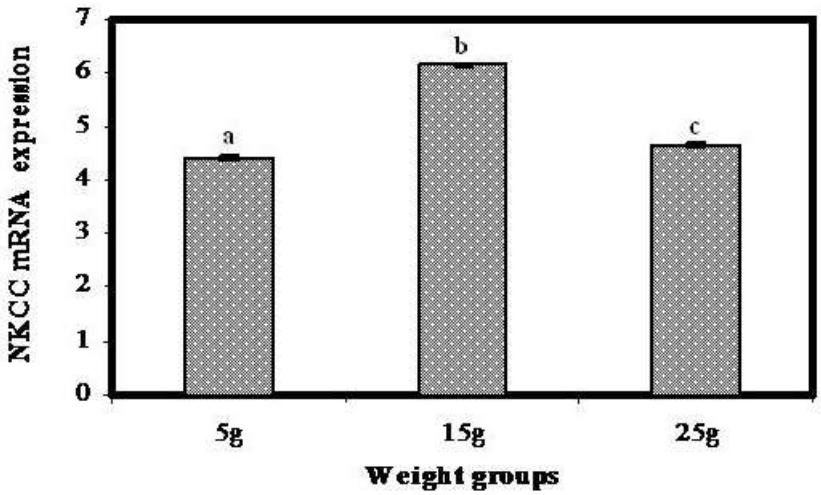

Figure 9 Gill NKCC mRNA expression in *Salmo trutta caspius* after 10 days acclimation in brackish water. Values are mean ±SE. Different letter above the columns indicate significant difference between three weight groups

4 DISCUSSION

Na^+, K^+-ATPase is one of the most important enzymes in gill epithelium of the fish; it is not only important in cell homeostasis but also serves as a driving force for other cell transporters (McCormick, 1995). Most studies involving anadromous salmonids exposure to SW reported that the reciprocal expression of Na^+, K^+-ATPase isoforms α1a and α1b during sea water acclimation may have different roles in the gills of FW and marine fishes; ion uptake in FW fish and ion secretion in marine fishes (Richards *et al.*, 2003; Shirmpton *et al.*, 2005; Bystriansky *et al.*, 2006; Nilsen *et al.*, 2007).

In this study, we observed the most expression of Na^+, K^+-ATPase α1a mRNA in 25 g weight group than other groups in FW and BW as well. Then following exposure to BW, the level of Na^+, K^+-ATPase α1a mRNA reduced in all three weight groups, however increased levels of gill Na^+, K^+-ATPase α1b mRNA was indicated following this exposure in 15 and 25 weight groups of salmonid fish.

The increase in α1b level was more important than the observed decrease in α1a levels following seawater exposure, so it was responsible for the totally observed increase in (α1a + α1b) Na^+, K^+-ATPase levels. Therefore, the observed increase in Na^+, K^+-ATPase α-subunit mRNA expression perceived in other studies (Madsen *et al.*, 1995; D·Cotta *et al.*, 2000; Seidelin *et al.*, 2000; Singer *et al.*, 2002; Timspark *et al.*, 2002) is probably due to a specific increase in α1b isoform expression. In 15 g weight group, α1b mRNA was expressed at the highest level in FW and BW than other weight groups. This suggests that α1b might be the specific Na^+, K^+-ATPase isoform associated with the typical up regulation of gill Na^+, K^+-ATPase seen in salmonids during SW acclimation. The observed changes in gill Na^+, K^+-ATPase mRNA levels explain how best the 15g weight group of *Salmo trutta caspius* performed in BW than other weight groups. Then, it is suggested that these fry fishes show a stronger capacity to challenge with salinity stresses than other weights. Conversely, in 25 g weight group, α1a mRNA was expressed at the highest level in FW and BW than other weight groups. It is likely that α1a isoform to be of less importance in the marine environments. It is appealing to speculate that Na^+, K^+-ATPase α1a

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is the isozyme involving in the gill ion uptake of FW salmonid (Pagliarani *et al.*, 1991). Then, this might be the reason that fry fishes in this weight group were adapted to FW and did not challenge with salinity even with larger size than previous group. The importance of gill Na^+, K^+-ATPase in the regulation of Na^+ uptake in FW fish was also supported by Hirata *et al.*, who provided the evidence that *Osorezan dace* could regulated the internal Na^+ levels following exposure to pH 3.5 by increasing gill Na^+, K^+-ATPase expression, while an increasing in the expression of the apical vacuolar proton ATPase (V-H^+- ATPase) was much more limited (Hirata *et al.*, 2003).

NKCC is localized in the basolateral membrane of the ionocytes in the gill epithelium of both teleosts and elasmobranches (Evans *et al.*, 2005). Na^+, K^+-ATPase makes low intracellular Na^+ and a highly negative charge within the cell. The Na^+ gradient is then used to transport Cl^- into the cell through NKCC co-transporter, and then Cl^- leaves the cell "downhill" on an electrical gradient through an apical Cl^- channel (CFTR). Na^+ is transported through a paracellular pathway down its electrical gradient (McCormick, 1990). This mechanism showed that every change in Na^+, K^+-ATPase activity and abundance can be affected by NKCC co-transporter (Pelis and McCormick, 2001).

In our study, gill NKCC gene expression increased significantly ($P<0.05$) in 15 and 25g weight groups after they were introduced to BW but there was no significant difference ($P>0.05$) in 5g weight group in FW and BW. In addition, 15g weight group had the highest level of NKCC gene expression than other groups. Increased level of NKCC gene expression after acclimation to salinity was previously reported in *Anguilla japonica* (Tse and Wong, 2006), *Dicentrarchus labrax* (acclimated to 36 ppt salinity) (Lorin-Nebel *et al.*, 2006), *Anguilla anguilla* (Cutler and Cramb, 2002) and *Fundulus heteroclitus* (acclimated to 35ppt salinity) (Scott *et al.*, 2004; Scott and Schulte, 2005), *Acipenser persicus* (acclimated to 5ppt salinity) (Khodabandeh *et al.*, 2009b). This might be a response to salinity stress. Then, increased level of NKCC gene expression in 15 g weight might be such a kind of pre-adaptation to tolerate with new environment salinity.

5 CONCLUSION

In conclusion, irregular variations in gene expressions of both subunit α1b and α1a Na^+, K^+-ATPase enzyme and changes in co-transporter gene expression of NKCC in 5 g fish fries were observed more than other weight groups. Therefore, high mortality rate observed in this weight group might be attributed to the fact that these fish fries had not reached to the smolt stage and were not capable of salinity tolerance in new environment. Fish acclimation to the FW in weight group of 25 g is evidenced by high expression of subunit α1a in FW and BW, in spite of lower mortality rate, which might lead to an inability in osmoregulation and resistance to salinity stress. In weight group of 15 g, low mortality rate and regular gene expression of both subunits of α1a and α1b Na^+, K^+-ATPase enzyme and changes in the gene expression of NKCC co-transporter might be considered as a reasonable evidence in efficient osmoregulation when entering to the smolt stage. So, the most suitable weight of these fish parrs to be released to the sea is 15 g weight.

6 REFERENCE

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بیان ژن ایزوفرم های α1a و α1b آنزیم Na^+, K^+-ATPase و همچنین پروتئین کانال هم انتقال NKCC در آبشش بچه ماهیان آزاد خزر

صابر خدابنده[1*] و حلیمه رجبی[2]

۱- دانشیار، دانشکده علوم دریایی، دانشگاه تربیت مدرس، نور، ایران
۲- داشجوی کارشناسی ارشد، دانشکده علوم دریایی، دانشگاه تربیت مدرس، نور، ایران

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چکیده اثر وزن بچه ماهیان روی توان تحمل شوری آنها در ماهی آزاد خزر مورد بررسی قرار گرفت. بدین منظور، تعداد ۱۸۰ قطعه بچه ماهی هم سن (دو ساله) ولی با سه وزن مختلف (۵، ۱۵ و ۲۵ گرم) انتخاب و به مدت ده روز در آب شیرین و آب لب شور (۱۳ گرم در لیتر) نگهداری شدند. بعد از اتمام دوره، بیان ژن ایزوفرم های α1a و α1b آنزیم Na^+, K^+-ATPase و همچنین پروتئین کانال هم انتقال NKCC در آبشش آنها مورد مطالعه قرا گرفت. در هر سه گروه وزنی، میزان mRNA مربوط به ایزوفرم α1a در زمان انتقال به آب لب شور کاهش پیدا کرد و این در حالی بود که میزان mRNA مربوط به ایزوفرم α1b در وزن‌های ۱۵ گرم و ۲۵ گرم به طور معنی‌داری افزایش پیدا کرد. همچنین میزان بیان ژن NKCC در بچه ماهیان ۱۵ و ۲۵ گرمی انتقال داده شده به آب لب شور، بیشتر از آب شیرین بود. تفاوت در بیان ژن دو ایزوفرم α1a و α1b آنزیم Na^+, K^+-ATPase بیانگر نقش متفاوت آنها در آب شیرین و لب شور می باشد. نتایج نشان داد که بین بچه ماهیان هم سن، آنهائیکه وزن کمتر، یعنی ۱۵ گرم را، داشتند بهتر از وزن ۲۵ گرمی به زندگی درآب لب شور سازش پیدا می‌کنند. بچه ماهیان بعد از گذراندن دوره اسمولتیفیکاسیون و رسیدن به وزن بالاتر یعنی ۲۵ گرم، تمایل و توان ماندنشان در آب شیرین تقویت شده و برعکس توانشان برای تحمل آب لب شور و شور کاهش می‌یابد.

کلمات کلیدی: پمپ سدیم– پتاسیم، تنظیم اسمزی، ماهی آزاد دریای خزر.

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