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

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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
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 (Pulis 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, Periophthalmodon 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
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 RNase-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 producet 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\(^{-\frac{1}{slopes}}\)). According to the method described by Scott et al. (2004), the absolute mRNA expression was semi quantitatively estimated using the formula E\(^{-\text{CP}}\). 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).
Table 1 Primer sequences used in this study based on Lorin-Nebel et al. (2006)

<table>
<thead>
<tr>
<th>Target sequence</th>
<th>Forward primer 5′-3′</th>
<th>Reverse primer 5′-3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺,K⁺-ATPase α1a</td>
<td>AAGATCATGGAGTCCTTAAGAATCTG</td>
<td>CACCTCCTCTGCATTGATGT</td>
</tr>
<tr>
<td>Na⁺,K⁺-ATPase α1b</td>
<td>CTGCTACATCTCAACAAACATT -Tm:67/8° GC:40%</td>
<td>ACCATCACAGTGTTCATTGGAT Tm:67/8° GC:43/5%</td>
</tr>
<tr>
<td>NKCC</td>
<td>TCACTCAGCTGGAATCTT</td>
<td>AGAGAAACCCACATGGTA</td>
</tr>
<tr>
<td>Ef1α</td>
<td>GA/GAACCATTGAGAAGTCGAAG</td>
<td>GCACCAGGCATCTGGAAG</td>
</tr>
</tbody>
</table>

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).
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.
Figure 3 Gill Na\(^+\), K\(^+\)-ATPase \(\alpha_1\) 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 \(\alpha_{1b}\) 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.
Figure 5 Gill \( \text{Na}^+, \text{K}^+ \)-ATPase \( \alpha 1b \) 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 \( \text{Na}^+, \text{K}^+ \)-ATPase \( \alpha 1b \) 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 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.
Na^+, K^+-ATPase (α1a and α1b) and NKCC co-transporter genes expression: ECOPERSIA (2014) Vol. 2(1)

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

**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.
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


McCormick, S.D. and Saunders, R.L. Preparatory physiological adaptation for marine life of


بیانرژن ایزوفرم های α1a و α1b آنزیم Na+, K+-ATPase و همچنین پروتئین کناتال هم انتقال Na⁺، K⁺-ATPase در آبشش نکسی در آبشش ماهی آزاد خزر

بچه ماهیان آزاد خزر

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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 181 قطعه بچه ماهی هم سن (دو ساله) ولی با سه وزن مختلف (5، 15 و 25 گرم) انتخاب و به مدت ده روز در آب شیرین و آب لب شور (19 گرم در لیتر) نگهداری شدند. بعد از اتمام دوره، بیانرژن ایزوفرم های α1a و α1b آنزیم Na⁺، K⁺-ATPase و همچنین پروتئین کناتال هم انتقال Na⁺، K⁺-ATPase در آبشش آنها مورد مطالعه قرار گرفت. در هر سه گروه با وزن 5، 15 و 25 گرم، 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 30 و 5 گرم به طور معنی‌داری افزایش یافت. همچنین در بچه یاب ماهیان با وزن 15 و 25 گرم، mRNA کناتال NA⁺، K⁺-ATPase در بیشتر اعداد شده بود. تفاوت بین سه گروه در میزان این mRNA بسیار کم بود. نتایج نشان داد که بچه ماهیان هم سن، انتهاییه وزن کمتر، پس از 15 گرم را داشته بیشتر از وزن 25 گرمی به زدنگی درآب لب شور سارت به وسیله اینکه این گروه از قطعه‌های یاده بیشتری می‌باشد. بچه ماهیان بعد از گذراندن دوره ابستونمکسیون و رسیدن به وزن بالاتر، میزان BLC ممکن است افزایش یابد. بچه ماهیان بعد از غذارندن دوره ابستونمکسیون و رسیدن به وزن بالاتر، میزان BLC ممکن است افزایش یابد.

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