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Effects of cortisol and salinity acclimation on $Na^+/K^+/2Cl^-$- cotransporter gene expression and Na^+, K^+-ATPase activity in the gill of Persian sturgeon, *Acipenser persicus*, fry

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SUMMARY: Na^+, K^+-ATPase activity and $Na^+/K^+/2Cl^-$ cotransporter (NKCC) gene expression in the gills of Persian sturgeon, *Acipenser persicus*, fry (2-3 g, 3.30-8.12 cm total body length) in freshwater (control group), diluted Caspian Sea water (5 ppt) and after treatment with cortisol in freshwater were studied. Na^+, K^+-ATPase activity was lower in the 5 ppt–acclimated fish (1.07±0.05 μmol P_i/mg protein/h) than in the control fish (1.19±0.05 μmol P_i/mg protein/h) but this difference was not significant. NKCC gene expression in the 5 ppt–acclimated fish (1.6±0.07) was significantly higher than in the control fish (0.8±0.00). In the cortisol treated fish, Na^+, K^+-ATPase activity (1.91±0.05 μmol P_i/mg protein/h) and NKCC gene expression (3.2±0.1) were significantly higher than in the control group. Our results show that Persian sturgeon fry (2-3 g) can tolerate 5 ppt salinity by changing their enzymatic content and activity, and that exogenous cortisol application can increase the osmoregulatory capacity of fry before release into brackish water and can reduce their mortality.

Keywords: cortisol, Na^+, K^+-ATPase, NKCC, Persian sturgeon, salinity.

RESUMEN: EFECTOS DE LA ACLIMATIZACIÓN AL CORTISOL Y A LA SALINIDAD EN EL $Na^+/K^+/2Cl^-$- COTRANSPORTADOR DE EXPRESIÓN GÉNICA Y EN LA ACTIVIDAD Na^+, K^+-ATPASA EN LAS BRANQUIAS DE JUVENILES DEL ESTURIÓN DE PERSIA. – Se estudió la actividad Na^+, K^+-ATPasa y el cotransportador de expresión génica (NKCC) $Na^+/K^+/2Cl^-$ en las branquias de juveniles de esturión de Persia, *Acipenser persicus*, (2-3 g, 3.30-8.12 cm de longitud total) en agua dulce (grupo control), agua diluida del mar Caspio (5 ppt) y posterior tratamiento con cortisol en agua dulce. La actividad Na^+, K^+-ATPasa fue menor en los peces aclimatados en 5 ppt (1.07±0.05 μmol P_i/mg proteína/h) que en los peces (1.19±0.05 μmol P_i/mg proteína/h), pero esta diferencia no fue significativa. La expresión génica NKCC en peces desde 5 ppt de salinidad (1.6±0.07) fue significativamente más alta que en el grupo (0.8±0.00). En los peces tratados con cortisol la actividad Na^+, K^+-ATPasa (1.91±0.05 μmol P_i/mg proteína/h) y la expresión génica NKCC (3.2±0.1) incrementaron significativamente en comparación con el grupo control. Nuestros resultados mostraron que los juveniles de esturión de Persia (2-3 g) pueden tolerar 5 ppt de salinidad y esta capacidad se consiguió cambiando su contenido y actividad enzimática; -la aplicación de cortisol exógeno puede incrementar la capacidad osmoregulatoria de los juveniles antes de soltarlos a aguas salobres y puede reducir su mortalidad.

Palabras clave: cortisol, Na^+, K^+-ATPasa, NKCC, esturión de Persia, salinidad.

INTRODUCTION

Although ion regulation in fish is mediated by a group of structures including the gastrointestinal epithelium and kidney, the gill is the major site in the balance of ion movement between diffusional gains or losses (Evans, 1993). The branchial epithelium mitochondrion-rich cells (MR cells, i.e. chloride cells or ionocytes) are the main sites for active ion transport. They secrete ions in seawater-

adapted fish and in freshwater-adapted fish absorb ions and maintain the acid-base balance (Wood and Marshall, 1994).

Na^+, K^+-ATPase is a universal membrane-bound enzyme that actively transports Na^+ out of and K^+ into animal cells. It is important not only for sustaining intracellular homeostasis, but also for providing a driving force for many transporting systems in a variety of osmoregulatory epithelia including fish gills (McCormick, 1995). The ubiquitous Na^+/K^+-ATPase, constantly located basolaterally in the cell, is one of the main enzymes involved in primary ion transport by creating an electrochemical gradient. Different transmembrane proteins have been described that are involved in ion (ion channels, cotransporters) and water (aquaporins) exchanges following this gradient (Evans *et al.*, 2005). The specificity, location (apical/basal) and relative abundance and expression of these proteins within the ionocytes result in either ion absorption or excretion (Kultz, 2001). The $Na^+/K^+/2Cl^-$ cotransporter (NKCC) is one of these transmembrane proteins whose pattern of expression at different osmoregulatory sites is ultimately responsible for the adaptation of teleosts to salinity. NKCC, a member of the chloride-cation cotransporter family, is widely distributed among different species of vertebrates (Haas, 1994; Gagnon *et al.*, 2003). The coupled electrically neutral movement of sodium, potassium and chloride ions serves a number of different physiological functions according to the cell type. NKCC is generally recognised as playing a central role in cell volume homeostasis, maintenance of the electrolyte content and transepithelial ion and water movement in polarised cells (Russell, 2000; Cutler and Cramb, 2002). There are two major subclasses of NKCC isoforms identified in invertebrates, the secretory isoform (NKCC1) and the absorptive isoform (NKCC2), located at the basolateral and apical sides of epithelial transporting cells, respectively. Recently, duplicate isoforms have been reported for NKCC1 (NKCC1a and NKCC1b) in European eel (Cutler and Cramb, 2002) and also two NKCC2 homologues have been isolated from Mozambique tilapia (Hiroi *et al.*, 2005). Gill NKCC protein abundance was found to increase in European eels (Cutler and Cramb, 2002) and sea bass (Lorin-Nebel, 2006) and also in salmonids during smoltification (Tipsmark *et al.* 2002; Hiroi and McCormick, 2007). NKCC1 is the most widely distributed isoform whereas NKCC2 appears to be expressed in the kidney (Lytle *et al.*, 1995; Bachmann *et al.*, 1999; Lorin-Nebel *et al.*, 2006).

The endocrine system modulates the osmoregulatory ability of teleost fish (Bern and Madsen, 1992; Hirose *et al.*, 2003). Whereas prolactin has been established as an important hormone for the maintenance of ionic homeostasis in freshwater, growth hormone (GH) and cortisol are recognised as the major seawater-adapting hormones (Madsen, 1990a and 1990b; McCormick, 1995; Kiilerich *et al.*, 2007). Many studies have shown that changes in salinity and hormones such as cortisol, growth hormone (GH) and prolactin (PRL) can stimulate expression and activity of different ion and electrolyte transporters in the ionocytes, such as NKA and NKCC (Pelis and McCormick, 2001; Mancera *et al.*, 2002; Singer *et al.*, 2003; Lin *et al.*, 2004; Tse *et al.*, 2006;).

Persian sturgeon, *Acipenser persicus*, is an anadromous species which lives in the southern part of the Caspian Sea. The stock of this highly precious species has so drastically decreased that it has been listed as an endangered species (IUCN, 1996). Artificial propagation of this valuable and rare species and annual release of over 3 million seedlings (2-3 g) into the Caspian Sea is one of the activities undertaken by the Iranian Shilat centres. High mortality has been observed in seedlings following release into the Caspian Sea, so enhancing of the ability of osmoregulation in *Acipenser persicus* fry can increase their survival during release from freshwater to brackish water in estuaries. Also, although a good amount of information is now available on anadromous fish osmoregulation (Evans, 1993), the number of studies on acipenserids is limited, especially with regard to Caspian Sea autochthons species (Khodabandeh *et al.*, 2009). This study is the first one dealing with effects of cortisol and salinity acclimation on NKCC gene expression (all isoforms together) in this family.

MATERIALS AND METHODS

Animals and experimental design

Acipenser persicus fry were obtained from Shahid Behesti Hatchery, Rasht, Iran in July 2006. Fish were adapted to experimental conditions with well aerated fresh water (25-26°C, 12 h L/12 h D photoperiod) for 7 days in a 600 L stock tank. They were

fed twice daily with commercial fish food (Biomeal, France), approximately 2% of body weight/day.

The fry used in this experiment were from fresh water and ranged from 2 to 3 g in body weight and from 3.30 to 8.12 cm in total body length. To determine the effects of cortisol and salinity on gill Na^+, K^+-ATPase activity and Na^+, K^+, $2Cl^-$-cotransporter gene expression of the fry, three groups of fish (in each group n=30 and each tank was 100 L) were transferred directly from the stock tank to fresh water, diluted Caspian Sea water (5 ppt salinity) and fresh water containing 5 mg l^{-1} cortisol for 24 hours. Over the entire experimental period temperature, dissolved oxygen (6-7 ppm) and pH (7-8) were controlled daily using a GRANT-YSI 3800 data-logger (Grant Instrument Ltd, Cambridge, UK). During the acclimation period (24 hours) fish were not fed.

Na^+, K^+-ATPase Activity

Na^+, K^+-ATPase activity in the gills of *Acipenser persicus* fry was determined according to the technique described by Norby (1998). Following 24-hour acclimation, total gill filaments and lamellae of the 6 fry from each group were quickly excised, weighed, and homogenised in cold imidazol buffer (50 mmol/l imidazol, 250 mmol/l sucrose, and 5 mmol/l EDTA at pH 7.4 with HCl). The cuvette contained 2 ml of the reaction mixture with and without 5 mmol/l ouabain. The composition of the reaction media was 25 mmol/l Tris-HCl, 2 mmol/l MgCl2, 0.25 mmol/l EGTA (pH 7.4), 100 mmol/l NaCl, 25 mmol/l KCl, 1.5 mmol/l PEP, 0.15 mmol/l NADH, 5 mmol/l ATP and LDH/PK enzymes. Incubation was conducted at 37ºC for 30 min, and the Na^+, K^+-ATPase reaction was initiated by the addition of homogenate. The Na^+, K^+-ATPase activity was expressed as the activity in the presence of ouabain (a specific inhibitor of Na^+, K^+-ATPase) subtracted from the activity obtained in its absence. Results are expressed in terms of both milligrams of tissue (wet weight) and milligrams of protein. Protein concentrations were determined according to the modified procedure described by Lowry *et al.* (1951).

Quantification of NKCC expression by real-time PCR

The gill filaments and lamellae of the 6 fry from each group were quickly dissected. Total RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instruction and quantification of RNA was based on the absorbance at 260 nm. After verification of the integrity of the RNA samples on the gel, 2 μg of total RNA were treated with RNase-free DNase (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 (Table 1) were then used to generate a PCR product of 346 bp. The results were normalised 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). The forward (EF1α-F) and reverse (EF1α-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}), 1 μl of reverse primer (0.5 μmol l^{-1}), 2 μl of the Mastermix FastStart DNA MasterPLUS SYBR Green I (Roche Applied Science, Basel, Switzerland). The LightCycler glass capillaries were filled with 9.5 μl of the mix and 0.5 μl of cDNA was added as PCR template. The cycling conditions were: denaturation programme (95°C for 10 min), amplification, hybridisation and elongation programmes 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 LightCycler Software, version 3.5 (Roche Molecular Biochemicals). All samples were analysed 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 in Scott *et al.* (2004), the absolute mRNA expression was semi-quantitatively estimated using the formula E^{-CP}. The results were normalised to the estimated absolute

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

Primer name	Nucleotide sequences (from 5' to 3')
NKCC1 reverse	AGAGAAACCCACATGTTGTA
NKCC5 forward	TCATCACTGCTGGAATCTT

The sequences used standard IUPAC code: W: A/T; D: A/G/T; Y: C/T.

TABLE 2. – Na^+, K^+-ATPase activity (μmol P_i/mg protein/h) and NKCC gene expression in the gills of *Acipenser persicus* fry, following 24 hours acclimated to fresh water (control group), diluted Caspian Sea water (5 ppt salinity) and cortisol (5 mg l^{-1}) (mean±SE).

Treatment	Na^+, K^+-ATPase activity	NKCC
Control	1.19±0.05	0.8±0.00
5 ppt salinity	1.07±0.05	1.6±0.07
Cortisol	1.91±0.05	3.1±0.1

expression of EF1 in order to compare the expression levels between different organs and salinities (Scott and Schulte, 2005; Kiilerich *et al.*, 2007).

Statistical analysis

Gene expression and Na^+, K^+-ATPase activity data are expressed as mean ± SE, and met the assumption of normality and homogeneity of variance. ANOVA was used to determine overall difference, and a Tukey test also was used for comparisons of mean values.

RESULTS

Mortality

Fish survived over the entire period of this experiment, and no mortality was observed during 24-hour acclimation periods.

Na^+, K^+-ATPase activity

Results of the biochemical assay for Na^+, K^+-ATPase activity are illustrated in Table 2. The Na^+, K^+-ATPase activity was lower in the 5 ppt–acclimated group than in the freshwater group, but this decrease was not significant (Fig. 1). In the cortisol-treated group, Na^+, K^+-ATPase activity showed significant increases in comparison with the freshwater group ($p>0.05$, Fig. 1).

NKCC gene expression

The results of the quantification of NKCC gene expression by real-time PCR are shown in Table 2. NKCC gene expression in the 5 ppt–acclimated group and in the cortisol-treated group was significantly higher than in the control group ($p<0.01$). However, between the cortisol-treated group and the 5 ppt–acclimated group the gene expression showed no significant differences ($p>0.05$, Fig. 2).

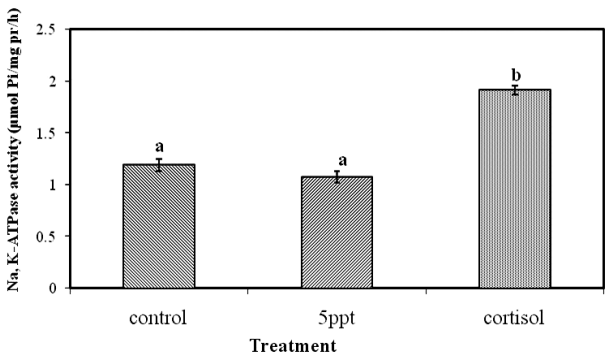

FIG. 1. – Gill Na^+, K^+-ATPase Activity in control group, 5 ppt salinity–acclimated and Cortisol (5 mg l^{-1})-treated groups of *Acipenser persicus* fry. Values are means± SE. Different letter above the columns indicate significant difference between treatments.

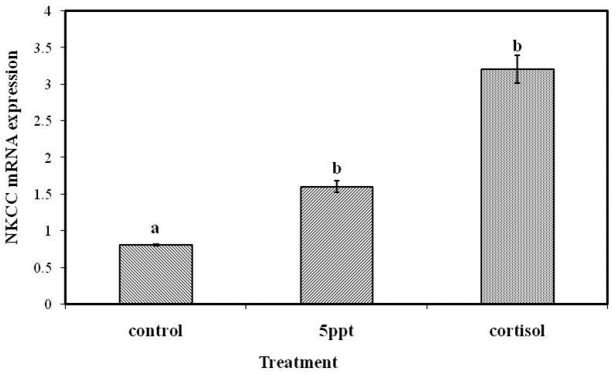

FIG. 2. – Gill NKCC gene expression in control group, 5 ppt salinity–acclimated and cortisol (5 mg l^{-1})-treated groups of *Acipenser persicus* fry. Values are means± SE. Different letter above the columns indicate significant difference between treatments.

DISCUSSION

Na^+, K^+-ATPase is one of the most important enzymes of the fish gill epithelium; it is not only important in cell homeostasis but is also a driving force for other cell transporters (McCormick, 1995). Increases in gill Na^+, K^+-ATPase activity in sturgeons after acclimation to salinity was observed in the Siberian sturgeon, *Acipenser baerii.* In this species, increases in Na^+, K^+-ATPase activity have been reported after acclimation to iso- and hyperosmotic conditions (Rodriguez *et al.*, 2003). McKenzie *et al.* (1999) also observed an increase in Na^+, K^+-ATPase activity in juveniles of *Acipenser naccarii* after direct transfer from fresh water to salt water (23 ppt), whereas Jarvis and Balantyne (2003), testing salinity (20 ppt) effects on shortnose sturgeon, *Acipenser brevirostrum,* observed no significant changes in Na^+, K^+-ATPase activity. The results of our study suggest that Persian sturgeon fry in their natural habitat (freshwater rivers) have high Na^+, K^+-ATPase

activity for active absorption of different ions from hypoosmostic surrounding water. When these fish are suddenly acclimated to water with 5 ppt salinity, if their ionocytes have previous Na^+, K^+-ATPase activity and they are faced with a high amount of incoming ions (because of higher amounts of ions in 5-ppt salinity than freshwater), they will decrease their gill Na^+, K^+-ATPase activity.

Acipenser persicus showed significant increases in gill Na^+, K^+-ATPase activity after treatment with cortisol, and it was shown that this exogenous cortisol can provide these fish with acclimation to salinity. Cortisol is the first and most important hormone that stimulates Na^+, K^+-ATPase activity (Pickford *et al.*, 1970). An increase in gill Na^+, K^+-ATPase activity in response to cortisol treatment was previously reported in different species (*Oncorhynchus mykiss* [Shrimpton and McCormick, 1999]; *Salmo trutta* [Seidelin *et al.*, 1999]; *Anguilla japonica* [Wong and Chan, 2001]; *Salmo salar* [Pelis and McCormick, 2001]; *Sparus aurata* [Mancera *et al.*, 2002]). Some other observations also showed that exogenous cortisol can stimulate Na^+, K^+-ATPase activity and mitochondrion content of the ionocytes and ionocyte distribution in the gill epithelium of freshwater and seawater-adapted fishes (Laurent and Perry, 1990; Madsen, 1990b; McCormick, 1990; Seidelin *et al.*, 1999).

NKCC is localised in the basolateral membrane of the ionocytes in the gill epithelium of both teleosts and elasmobranches (Evans *et al.*, 2005). However, no previous studies have been made of sturgeon gill NKCC cotransporter abundance and gene expression, and the present study is the first. Na^+, K^+-ATPase creates 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 (Na^+/K^+/Cl^-) cotransporter, 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 cotransporter (Pelis and McCormick, 2001). Increasing 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 35 ppt salinity) (Scott *et al.*, 2004; Scott and Schulte, 2005). In our study, gill NKCC gene expression increased significantly in 5 ppt–acclimated fish in comparison with the freshwater group. We propose that in the salinity-acclimated group of *Acipenser persicus* no significant changes in Na^+, K^+-ATPase activity can be compensated by increasing NKCC gene expression and that this cotransporter excreted the additional Na^+, K^+ and Cl^- ions to the external medium.

Pelis and McCormick (2001), and Kiilerich *et al.* (2007) reported that cortisol treatment increases the NKCC abundance in gill of Atlantic salmon, *Salmo salar*. Hirose *et al.* (2003), in a review paper, also stated that cortisol treatment can increase NKCC gene expression and protein in gills of many fish species. As in previous studies, this study showed that cortisol increased gill NKCC gene expression and Na^+, K^+-ATPase activity, and equipped *Acipenser persicus* fry with salinity acclimation to cope with environments with higher salinities.

In conclusion, *Acipenser persicus* fry of 2-3 g body weight responded to exogenous cortisol and can tolerate 5 ppt salinity. This indicates that fry might be able to enter the estuaries with 2-3 g body weight. However, the possibility of tolerating higher salinities at this age (the southern part of the Caspian Sea has salinities of about 10-12 ppt) needs more studies with higher salinities. Because of the biochemical changes caused by dissolved cortisol in these fish, leading to increases in gill Na^+, K^+-ATPase activity and NKCC gene expression, this hormone may be helpful for adaption of fry to higher salinity 24 hours before they are released to the Caspian Sea by hatcheries.

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