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EFFECTS OF SEAWATER ACCLIMATIZATION ON GILL Na^+-K^+-ATPase ACTIVITIES AND CHLORIDE CELLS IN RAINBOW TROUT (*Oncorhynchus mykiss*) AND BROWN TROUT (*Salmo trutta forma fario*)

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ABSTRACT

In this study, rainbow trout (*Oncorhynchus mykiss*) (162.7 ± 3.03 g) and brown trout (*Salmo trutta forma fario*) (160.9 ± 2.94 g) were transferred to full-strength seawater (36.5 $g \cdot l^{-1}$) for directly and gradually (21 days), then changes in gill Na^+-K^+-ATPase activity and size of chloride cells associated with environmental salinity were investigated and also survival of trouts were evaluated in seawater. All fish died when brown trouts were transferred into seawater directly but rainbow trouts survived 50 – 58.3 %. However, significant difference was recorded between brown and rainbow trouts in terms of survival rates by gradual acclimation. Survival of brown trout and rainbow trout that were transferred in seawater gradually, was 66.7 – 75 % and 83.3 – 91.7 %, respectively ($p < 0.05$). Gill chloride cell sizes in both species increased at 36.5 $g \cdot l^{-1}$ salinity. The lowest sectional area of chloride cells was determined at the point of death in brown trouts which were transferred directly. Following direct transfer to seawater, the largest sectional area of chloride cells were determined in rainbow trout. There are significant differences between acclimated and non-acclimated fish in terms of chloride cell sizes and Na^+-K^+-ATPase activities ($p < 0.05$). The highest gill Na^+-K^+-ATPase activity was in brown trouts which were transferred gradually, while the lowest activity was detected in freshwater phase. According to the results, gradually acclimated trouts can perform better adaptation than their directly acclimated counterparts.

Key words: Rainbow trout, brown trout, seawater acclimation, Na^+-K^+-ATPase, chloride cell size.

INTRODUCTION

Mariculture of trout is very important sector in aquaculture industry. As an euryhaline species trouts can be adapted to seawater for cultivation. At this point, getting low mortality during the acclimation period and shortening this duration are very important issues for trout culture. Survival of trout in full-strength seawater (SW) depends on their adaptation capability of gills. Gills are major organ responsible for osmoregulation (Evans, 2008) and euryhaline teleosts normally absorb and secrete ions through gills in order to maintain homeostasis. Euryhaline teleosts have osmoregulatory abilities and can maintain their plasma osmolalities in both hypoosmotic and hyperosmotic environments (Marshall and Grosell, 2006; Kaneko *et al*., 2008).

Gill Na^+-K^+-ATPase (NKA) is an enzyme, located in the gill epithelium cells with a key role in osmoregulation in seawater (SW) and freshwater (FW) teleosts, responsible for active electrolyte uptake and electrolyte secretions from the gill (Ay *et al*., 1999). NKA actively transports Na^+ and K^+ out of and into animal cells, respectively. NKA is crucial for maintaining intracellular homeostasis because it provides a driving force for many other ion-transporting systems (Hirose *et al*., 2003; Hwang and Lee, 2007).

Chloride cell (CC) is defined as mitochondrion-rich cell (Perry, 1997) and CCs are thought to be the ionocytes responsible for ion uptake in FW and ion secretion in SW (Hirose *et al*., 2003; Hwang and Lee, 2007). CCs are significantly modified in euryhaline fish during adaptation to different salinities and show increase in size when adapted to SW (Cioni *et al*., 1991).

Some studies compared the salinity tolerances of euryhaline teleosts in point of survival rates following direct or gradual transfer between different salinity environments (Hiroi and McCormick, 2007; Kang *et al*., 2010). Boeuf (1987) stated that 30 $g \cdot l^{-1}$ salinity is a critical threshold for all trouts reared in SW, hence gradually transfer would provide rather easier osmotic regulation. In our study, the changes in gill NKA activity and CC size were investigated through the gradual and direct transfer of rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta forma fario*) from FW to SW (36.5 $g \cdot l^{-1}$). The adaptation of brown trout, which is considered as an alternative species to trout mariculture, to saline water and survival rates within this adaptation process are compared with commonly produced rainbow trout. This study provide us to understand the osmoregulatory mechanisms of trouts during seawater acclimation and may contribute knowledge to solve problems about adaptation.

MATERIALS AND METHODS

Experiments were carried out at Ege University Fisheries Faculty, Aquaculture Department, Izmir, Turkey. Rainbow trout and brown trout, weighing 162.7 ± 3.03 g and 160.9 ± 2.94 g, respectively (n = 240), were kept in 12 circular tanks with a inflow 10 $L \cdot min^{-1}$. Trials were applied in triplicate.

Trouts were transferred in SW gradually (brown trout = FT, rainbow trout = GT) and directly (brown trout = FD, rainbow trout = GD) for acclimatization to 36.5 $g \cdot l^{-1}$ salinity. Experimental fish were gradually acclimatized to 36.5 $g \cdot l^{-1}$ for 21 days (Figure 1). The other groups were transferred to 36.5 $g \cdot l^{-1}$ directly. Some fish remained in freshwater as a control group.

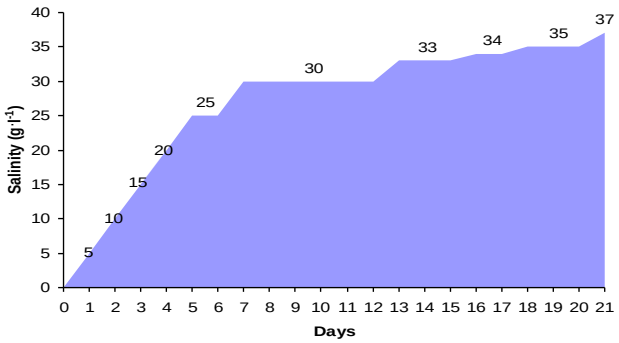

Figure 1. Gradual increasing salinity of external media.

Preparation of gill tissue for enzyme analysis: The fish were anesthetized (0.3 ml phenoxyethanol$\cdot l^{-1}$ for 1 min) and killed immediately. The left gill was removed and filaments were weighed around 200 mg (SCALTEC SBA 31 - 0.0001 g) and homogenized (at +4°C using a glass homogenizer) in 2 ml of buffer containing 250 mmol sucrose, 100 mmol imidazol - pH 7.8, and 5 mmol EDTA (Sigma). Homogenates were centrifuged at 1000 g for 15 min (+4°C). ATPase assays were carried out with supernatants within 1 h.

Determination of gill Na^+-K^+-ATPase (NKA) activity: NKA activity was determined according to the modified methods described by Canli and Stagg (1996) and Ay *et al*. (1999). All assays were carried out in triplicate. The final assay concentrations of the chemicals used here were 135 mmol Tris-HCl (pH 7.4), 100 mmol NaCl, 10 mmol KCl, 6 mmol $MgCl_2$, 0.1 mmol EDTA, 1.5 mmol ouabain, and 6 mmol ATP. Buffer solution (1600 ml) was pre-incubated at 37°C for 5 min and then the reaction was started by adding 200 ml of homogenate and 200 ml of ATP. The reaction was stopped after 30 min by placing the samples on ice and adding a cirrasol acid molybdate mixture. Inorganic phosphate was measured by the determination of the soluble yellow complex of cirrasol acid molybdate at 390 nm (Jenway 6305 UV/Vis spectrophotometer). Samples were compared with standards of KH_2PO_4 phosphate content. The protein content in the samples was determined by the method of Lowry *et al*. (1951). ATPase activity was measured by the determination of the inorganic phosphate (P_i) liberated from the hydrolysis of ATP at 37°C and NKA expressed as milimoles of inorganic phosphate per milligram of protein per hour.

Morphometric analysis of gill Chloride Cell (CC): The right gill of each fish was fixed in buffered formaldehyde (100 ml formaldehyde – 40 %, 4 g $NaH_2PO_4 \cdot H_2O$, 6 g $NaHPO_4$, 900 ml distilled water). Sections of fixed specimens (5 µm) were cut and stained with Alcian blue for light microscopic investigation (Powell *et al*., 2001). CC sizes were estimated by measuring the sectional area of the cells using an ocular micrometer (Olympus) (Cioni *et al*., 1991).

Statistical Analysis: Results are expressed as mean (± SD). Differences among the experimental groups were examined using one-way ANOVA at a significance level of $p < 0.05$ and means were analyzed by the LSD test. Analysis of data was carried out using SPSS. The relationships between NKA activity and MR cell were tested by regression and correlation analyses.

RESULTS

Physico-chemical parameters of experimental media: The water quality parameters for SW and FW are given in

Table 1. Water quality variables for SW and FW conditions (mean ± S.E., n = 5).

Parameters	Seawater	Freshwater
Salinity ($g \cdot l^{-1}$)	36.5 ± 0.3	0.49 ± 0.6
Ca^{+2} ($mg \cdot l^{-1}$)	467.1 ± 24.9	168.8 ± 6.5
Total Hardness ($mg \cdot l^{-1}$)	7376.2 ± 83.1	741.4 ± 31.3
Mg^{+2} ($mg \cdot l^{-1}$)	1501.2 ± 18.9	76.8 ± 5.2
HCO_3 ($mg \cdot l^{-1}$)	161.2 ± 3.1	283.6 ± 27.3
SBV	3.1 ± 0.9	4.9 ± 0.4

Water temperature was measured between 11°C to 12.7°C, while dissolved oxygen was between 8.5 to 9.4 $mg \cdot l^{-1}$ throughout the experiment.

Survival: The mortality in FD groups occurred at 100 % rate. Mortality began from the 3rd day of transfer to SW and continued on 4th to 5th days. At the end of 5th day the mortality rates for FD groups occurred between 25 - 33.3 % ($p > 0.05$). With the final deaths on 23rd, 24th, and 25th days. The mortality rate for FD groups is recorded as 100 %. Increasing the salinity to 30 $g \cdot l^{-1}$ on the 8th day, mortalities occurred on 10th and 11th days and survival rates for FT groups recorded between 75 - 83.3 % ($p > 0.05$). Increasing the salinity to 36.5 $g \cdot l^{-1}$ on 22nd day,

mortalities began on 24th and 25th days and final survival rates for FT groups are determined between 66.7 – 75 % ($p > 0.05$). After the mortalities on 3rd and 4th days with GD groups the survival rates occurred between 50 - 58.3 % ($p > 0.05$). Increasing the salinity to 30 $g \cdot l^{-1}$ on the 8th day in GT groups mortality occurred on 9th and 10th days and the survival rates recorded between 83.3 - 91.7 % ($p > 0.05$). There is significant difference between GT and FT in terms of survival rates ($p < 0.05$).

Gill NKA enzyme activity and CC size: The highest NKA activity was determined in FT groups and the lowest NKA in FW brown trout (Table 2). NKA activity in non-acclimated fish is determined within 0.100 – 0.250 mmol $P_i \cdot$ mg protein$^{-1} \cdot h^{-1}$, while in acclimated in both of species is around 0.500 mmol $P_i \cdot$ mg protein$^{-1} \cdot h^{-1}$. There are important differences between acclimated and non-acclimated fish in terms of NKA activity ($p < 0.05$) (Table 2; 3).

The highest value of CC size is observed in GD groups (Figure 3), while the smallest CC size is recorded in non-acclimated FD groups (Figure 4). There are important differences between acclimated and non-acclimated fish in terms of CC size ($p < 0.05$) (Table 2; 3) (Figure 2; 3; 4; 5). There are no differences between trout species which acclimated to SW by gradually or directly in terms of gill NKA enzyme activities and CC sizes ($p > 0.05$) (Table 2; 3).

Table 2. Gill Na^+-K^+-ATPase activity (mmol $P_i \cdot$ mg protein$^{-1} \cdot h^{-1}$) and size of chloride cells (μm^2) in trouts transfering to seawater gradually.

Day	Salinity ($g \cdot l^{-1}$)	Brown Trout Na^+-K^+- ATPase Activity (mmol $P_i \cdot$ mg protein$^{-1} \cdot h^{-1}$)	Brown Trout Sectional Area of Chloride Cells (μm^2)	Day	Salinity ($g \cdot l^{-1}$)	Rainbow Trout Na^+-K^+-ATPase Activity (mmol P_i mg protein$^{-1} \cdot h^{-1}$)	Rainbow Trout Sectional Area of Chloride Cells (μm^2)
0	0	0.077 ± 0.004 [a]	49.67 ± 2.43 [a,1]	0	0	0.083 ± 0.006 [a]	55.6 ± 2.62 [a,1]
[#] 10	30	0.144 ± 0.006 [b]	45.08 ± 2.02 [b,1]	[#] 10	30	0.184 ± 0.008 [b]	51.52 ± 2.97 [b,1]
[#] 24	37	0.217 ± 0.01	51.14 ± 1.54 [1,2]	-	-	-	-
30	37	0.541 ± 0.015 [c]	64.52 ± 3.51 [c,2]	28	37	0.498 ± 0.011 [c]	62.09 ± 1.97 [c,1]

[*] All data are expressed as mean values ± S.E. (n = 5).
[*] Within the same columns, values with different superscripts with numbers are significantly different ($p < 0.05$).
[*] Within the same lines, values with different superscripts with letters are significantly different ($p < 0.05$).
[#] Data taken from fish just before death.

Table 3. Gill Na^+-K^+-ATPase activity (mmol $P_i \cdot$ mg protein$^{-1} \cdot h^{-1}$) and size of chloride cells (μm^2) in trouts transfering to seawater directly.

Day	Salinity ($g \cdot l^{-1}$)	Brown Trout Na^+-K^+- ATPase Activity (mmol $P_i \cdot$ mg protein$^{-1} \cdot h^{-1}$)	Brown Trout Sectional Area of Chloride Cells (μm^2)	Day	Salinity ($g \cdot l^{-1}$)	Rainbow Trout Na^+-K^+- ATPase Activity (mmol $P_i \cdot$ mg protein$^{-1} \cdot h^{-1}$)	Rainbow Trout Sectional Area of Chloride Cells (μm^2)
0	0	0.077 ± 0.004 [a,1]	49.67 ± 2.43 [a,1]	0	0	0.083 ± 0.006 [a,1]	55.6 ± 2.62 [a,1]
[#] 3	37	0.106 ± 0.007 [b,1]	36.8 ± 3.38 [b]	[#] 3	37	0.107 ± 0.01 [b,1]	42.21 ± 2.19 [b]
[#] 25	37	0.249 ± 0.017 [a]	52.6 ± 1.97 [1,a]	23	37	0.509 ± 0.012 [b]	66.8 ± 2.56 [1,b]

[*] All data are expressed as mean values ± S.E. (n = 5).
[*] Within the same columns, values with different superscripts with numbers are significantly different ($p < 0.05$).
[*] Within the same lines, values with different superscripts with letters are significantly different ($p < 0.05$).
[#] Data taken from fish just before death.

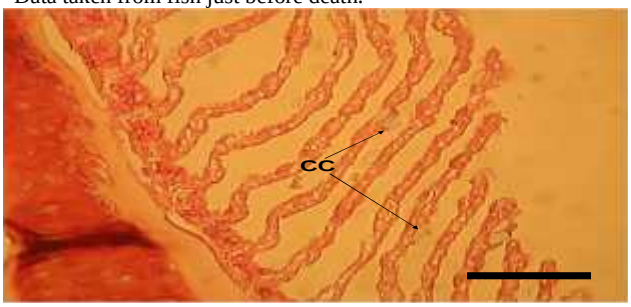

Figure 2. Gill chloride cells of brown trout acclimated to 36.5 $g \cdot l^{-1}$ salinity (Buffered formaldehyde, Alcian blue, x400). CC: Chloride cell, Bar = 50 µm.

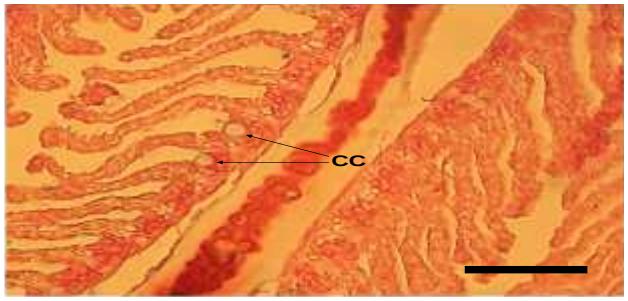

Figure 3. Gill chloride cells of rainbow trout acclimated to 36.5 $g \cdot l^{-1}$ salinity (Buffered formaldehyde, Alcian blue, x400). CC: Chloride cell, Bar = 50 µm.

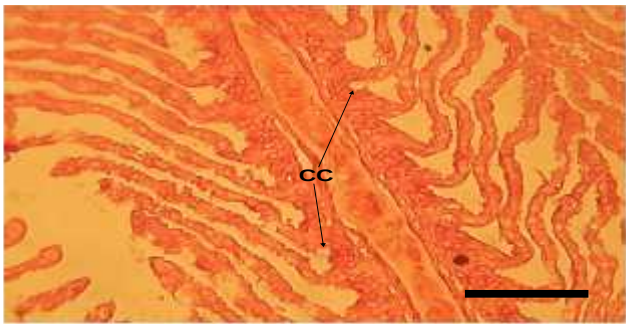

Figure 4. Gill chloride cells of brown trout non-acclimated to 36.5 $g \cdot l^{-1}$ salinity (Buffered formaldehyde, Alcian blue, x400). CC: Chloride cell, Bar = 60 µm.

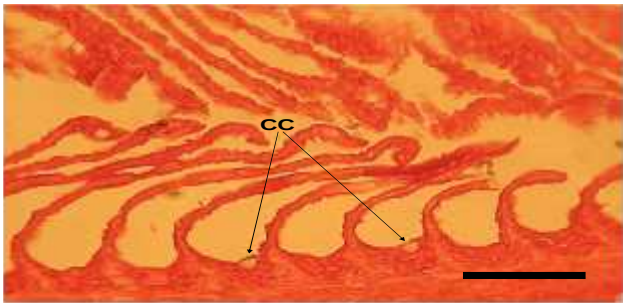

Figure 5. Gill chloride cells of rainbow trout non-acclimated to 36.5 $g \cdot l^{-1}$ salinity (Buffered formaldehyde, Alcian blue, x400). CC: Chloride cell, Bar = 60 µm.

DISCUSSION

Trout can be adapted to SW by different methods. If the adaptation is performed to full strength saline water gradually, survival would be increased especially for small size fish (Quillet *et al*., 1992). In our study, full mortality occured in FD groups while survival rates are recorded in GD groups between 50 - 58.3 % ($p > 0.05$). Acceleration in respiration, irregular movements, departing from shoal and lack of appetite are observed right after direct transfer to SW in both of trout species. Although higher salinity levels have pronounced effect on fish growth which might be due to improved osmoregulation (Iqbal et al., 2012), lack of appetite is an external indicator showing that the osmoregulation mechanism has not precisely performed. Weng *et al*. (2002) has recorded significant decreases in NKA and creatine kinase following direct transfer to 35 $g \cdot l^{-1}$ SW. It is stated that water and ion instability occurred in the cells as a result of severe dehydration after direct transfer to 35 $g \cdot l^{-1}$ salinity might reduce NKA activity and, furthermore, reduction in enzyme activities due to intracellular and hydromineral instability might be the cause of mortalities occurred at 35 $g \cdot l^{-1}$ salinity.

There is significant difference between FT and GT in terms of survival rates ($p < 0.05$). Survival rates are recorded as 66.7 % - 75 % ($p>0.05$) and 83.3 % - 91.7 % ($p>0.05$) in FT and GT, respectively. It has been observed that gradual transfer method has been rather more effective than direct transfer in both trout species. Hwang *et al*. (1989) conducted, the *Oreochromis mossambicus*, when transferred to 20 $g \cdot l^{-1}$ salinity with a 24 hours pre-adaptation as a transfer preparation to 30 $g \cdot l^{-1}$ SW, has exhibited a more swift increase in gill NKA activity and less dehydration compared to the fish directly transferred to 20 $g \cdot l^{-1}$ and 30 $g \cdot l^{-1}$ SW. Depending on the environment salinity, this physiological adaptation explains why gradual adaptation gives better result compared to direct adaptation.

Most euryhaline teleosts exhibit adaptive changes in gill NKA activity following salinity changes (Marshall, 2002; Hwang and Lee, 2007). NKA played a key role in ion excretion through fish gills and therefore the activity increases in various fish species during adaptation to SW (Handeland *et al*., 2000). In our study, the highest NKA activity is determined in FT groups and the lowest NKA activity is observed in FW brown trout. NKA activity of non-acclimated trout is determined in 0.100 – 0.250 mmol $P_i \cdot mg\ protein^{-1} \cdot h^{-1}$ interval, while in acclimated trout is in 0.500 mmol $P_i \cdot mg\ protein^{-1} \cdot h^{-1}$ level. There are important differences between acclimated and non-acclimated fish in terms of gill NKA enzyme activity ($p < 0.05$) (Table 2; 3). Seidelin *et al*. (2000) also recorded increasing in gill NKA activity following the 5th day when they transferred brown trout from fresh water to 25 $g \cdot l^{-1}$ salinity.

The functional and structural differentiation of the gill CC is considerably influenced by environmental salinity (Evans, 2008). In our study, increasing in the size of CC depends on salinity have been noticed. The highest value in terms of CC size is observed in GD groups. The smallest CC size is recorded non-acclimated FD groups. There are important differences between acclimated and non-acclimated fish in terms of CC size ($p < 0.05$) (Figure 2; 3; 4; 5). The gill CC in teleost fish species show difference from each other in terms of density and size (Perry, 1997).

NKA is located in the apical membrane of the CC and particularly in tubular system membranes (Evans *et al*., 1999). The tubular system is continuous with the basolateral membrane and provides a large surface area for the expression of NKA (Marshall, 2002; Evans *et al*., 2005). Therefore, CC hypertrophy is related with the increasing of NKA activity (Cioni *et al*., 1991). There is positively strong relationship between NKA activity and CC size in all of experimental groups; $r = 0.8126$ $p < 0.01$ (FT) , $r = 0.5871$ $p < 0.01$ (GT) , $r = 0.4942$ $p < 0.05$ (FD) , $r = 0.7614$ $p < 0.01$ (GD). According to these results, CC size enlarged along with increasing of NKA activity due to the salinity increase during the adaptation

process. Increase in the cellular size during the adaptation to SW is notified in other species as well (Lee *et al*., 2000).

In conclusion, gradually acclimated trouts can perform better adaptation than their directly acclimated counterparts. On the other hand, rainbow trout has more salinity tolerance than brown trout. Extention of period in gradual transfer can be effective for increasing survival and adaptation capability in both of species. It was also suggested that selective breeding on increasing the salinity tolerance of brown trout could make trout aquaculture more productive in highly saline water.

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