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Comparative analysis of branchial ionocytes in Caspian Sea zander, *Sander lucioperca* (Linnaeus, 1758) using Na^+, K^+-ATPase immunolocalisation at different salinities

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ABSTRACT

Gill morphology and branchial ionocyte variability were examined in zander *Sander lucioperca* (Linnaeus, 1758) fingerlings, having two different body weights (1 and 2 g) for a period of ten days following their transfer from freshwater to two different salinities (7 and 12‰ Caspian Sea water) using Na^+, K^+-ATPase (NKA) immunolocalisation. Survival rates and plasma osmolarity levels at the end of each salinity challenge were measured. By the end of the experiment, both weight groups were able to survive and successfully acclimate to salinity change. The number and size of branchial ionocytes in the gills dramatically decreased in both size groups; while, the filament ionocytes in both weight groups reformed at the end of the acclimation period. The results indicated both groups to be in the same osmoregulatory developmental stage, despite difference in their body weight. Also, their blood osmotic pressure and gill ionocyte changes at different salinity were similar when transferred to iso-osmotic condition. Further studies are needed for complete understanding of zander osmoregulatory systems exposed to different salinities.

Keywords: Caspian Sea, Gill, Iso-osmotic, NKA immunolocalisation, Osmoregulation, Salinity, *Sander lucioperca*

Introduction

Zander, *Sander lucioperca* (Linnaeus, 1758), inhabits both freshwater and brackish habitats in western Eurasia and is an economically important fish in the southern parts of Caspian Sea. Due to decline in their wild stock in recent years, fingerlings having two different body weights, 1 and 2 g are released into the rivers by Iran Fisheries Organisation to enhance the local stock (Sayad Bourani, 2002). Despite this measure, the fishery return coefficient of zander is low in the south of Caspian Sea. This reduction in return can be attributed to potential environmental hazards in the rivers (Abdolmalaki and Psuty, 2007); The return could be improved by direct release of fish into the estuaries or Caspian Sea water (CW). Salinity of the Caspian Sea (in particular the south region) is around 12 ppt, which is classified as brackishwater (Dumont, 1998; Zonn, 1999; Bagheri *et al*., 2012). Hence it was felt that understanding the salinity tolerance of zander fingerlings and whether the difference in body weight affect the ability to cope with stress after release are essential for selection of suitable size of these fish for release.

Some aspects of the osmoregulatory processes, such as hematological indices, plasma osmolarity and ion concentration, cortisol and glucose level and Na^+, K^+-ATPase (NKA activity), have been previously investigated in zander at different weight (Neacsu *et al*., 1981; Craciun *et al*., 1982; Brown *et al*., 2001; Ahmadnezhad *et al*., 2013). However, we still lack a thorough understanding of the cellular behaviour in the juveniles exposed to increased salinity.

NKA immunolocalisation is a method that has been successfully used for the detection of ionocytes in the gill of several fish species such as seabass *Dicentrarchus labrax* (Varsamos *et al*., 2002; Nebel *et al*., 2005), salmonids, *Salvelinus namaycush*, *Salvelinus fontinalis* and *Salmo salar* (Hiroi and McCormick, 2007), grey mullet, *Liza aurata* (Khodabandeh *et al*., 2009a), Persian sturgeon, *Acipenser persicus* (Khodabandeh *et al*., 2009b; Shirangi *et al*., 2016) and Caspian trout, *Salmo trutta caspicus* (Rajabi and Khodabandeh, 2013). In some teleost species like chum salmon, *Oncorhynchus keta* (Uchida *et al*., 1996), American shad, *Alosa sapidissimo* (Zydlewski and McCormick, 2001), brown trout, *Salmo trutta* (Seidelin *et al*., 2000), Atlantic salmon, *Salmo salar*

(Pelis and McCormick, 2001), seabass, *Dicentrarchus labrax* (Nebel *et al*., 2005), euryhaline milkfish, *Chanos chanos* (Lin *et al*., 2006) and silver moony, *Monodactylus argenteus* (Kang *et al*., 2012), the number as well as size of branchial (gill) ionocytes and their location on filaments/lamellae are subject to change following transfer to different salinities.

In the present study, monoclonal antibody based immunohistochemical method which offers high accuracy in ionocyte detection was used for zander gill ionocyte detection and to study their morpho-histological changes under sailnity. The effect of rapid transfer to brackishwater on zander branchial ionocytes (the major site of osmoregulation) is not known. Although body weight has been postulated as a key determining factor for salinity tolerance of fish (Krayushkina and Dyubin, 1974; Conte *et al*., 1996; Gracia-Gallego *et al*., 1998), the effect of weight on different osmoregulatory factors of zander fingerling against Caspian seawater and estuarine salinities is yet undetermined. But, our previous study on salinity tolerance of two weight groups of zander fingerlings (1 and 2 g) showed both could endure abrupt transfer to the two salinities (7 and 12%), but their branchial ionocyte variation has not been yet studied at these salinities (Ahmadnezhad, 2013). Therefore, understanding gill ionocytes properties in zander fingerlings with different body weight exposed to different salinities and identifying the stage at which zander fingerlings can survive in Caspian seawater and estuary are key issues investigated in this study. The fingerlings of 1 to 2 g were abruptly transferred from freshwater to estuary (7‰) and Caspian seawater (12‰) salinities. Fish survival rate, plasma osmolarity, ionocyte density, size and distribution *via* NKA immunolocalisation were recorded up to 10 days after transfer.

Materials and methods

Fish acclimation and experimental design

Specimens of zander fingerlings of two different body weights (group 1: weight=1.09±0.01 g, total length = 60.1±0.3 mm and group 2: weight = 2.03±0.03 g, total length=69.7±0.4 mm) reared in freshwater (FW) were procured from Yousefpour Fish Hatchery Center, Siahkal (Guilan, Iran). The experimental studies were performed at Inland Water Aquaculture Institute in Bandar Anzali (Guilan, Iran). Fish were maintained in FW (18±0.5°C) for one week before the experiments began. A total of 1350 fish were distributed among 18 fiberglass tanks (each 100 l). To keep the mass per volume of fish between the two weight groups balanced, 900 and 450 fishes were assigned to group 1 and group 2, respectively. Each weight group was divided into three subgroups, where each subgroup comprised three replicates of identical size. Then the subgroups were exposed, to three different salinities (each subgroup was exposed to only one salinity) for a period of ten days,: FW (FW<0.5‰, 21 m Osmol l^{-1}, Na^+ = 2.7±0.87, Cl^- = 3.5±0.86, Ca^{2+} = 2.17±0.61, Mg^{2+} = 3.33±0.39, K^+ = 0.07±0.01. SO_4^{2-} = 2.8±0.72 $meql^{-1}$), artificial brackishwater (7S = 7‰, 230 m Osmol l^{-1}, Na^+ = 137.96±3.27, Cl^- = 90.22±1.51, Ca^{2+} = 10.31±2.30, Mg^{2+} = 31.75±3.34, K^+ = 1.97±0.59, SO_4^{2-} = 25.60±1.72 $meql^{-1}$) and Caspian seawater (CW =12‰, 331 m Osmol l^{-1}, Na^+ = 178.26±1.88, Cl^- = 141.37±2.43, Ca^{2+} = 16.06±2.00, Mg^{2+} = 43.2±0.52, K^+ = 1.21±0.34, SO_4^{2-} = 34.45±0.80 $meql^{-1}$). Artificial brackishwater was made of a mixture of FW and CW. Salinity of water was tested with a refractometer (ATAGO, Japan). Water temperature, pH and dissolved oxygen recorded were 8±0.17°C, 7.85±0.01 and 6.4±0.1 mg l^{-1}, respectively. The water in each tank was completely replaced gradually every day. The fish were exposed to natural photoperiod and fed twice a day at 2% of their body weight, with live food (*Daphnia magna*) for the whole experimental period of 17 days. Mortality rates were recorded on a daily basis, throughout the experiment, in order to calculate survival rates at the end of the study.

Sampling

After initiating the experiment, fish were randomly netted, anaesthetised and sampled at intervals of 0, 24, 72 and 240 h. The first sampling, at 0 h was done before exposing the fingerlings to the 7 S (7‰) and CW (12‰) environments. To determine the plasma osmolarity of each treatment group at the end of experiment, the caudal peduncles of 10 fish from each replicate treatments were cut with a surgical blade to withdraw and collect blood in capillary tubes coated with heparin. Blood samples from each replicate treatments were then pooled and centrifuged (5 min at 6750 *g*) to collect blood plasma.

Laboratory analysis

Plasma osmolarity, as well as osmolarity of water samples in each treatment, were measured *via* cryoscopy using an osmometer (Roebling, Nr .9610003. Type13: Germany) in duplicates with 100 µl volume.

For histological and immunohistochemical studies, gills of six fish from each treatment group (two from each replicate, at intervals of 0, 24, 72 and 240 h) were dissected out and immediately fixed in Bouin's fixative for 24 h, washed and dehydrated in an ascending series of ethanol and then embedded in Paraffin (Merck, Germany). Longitudinal sections of 4 µm were cut and collected on poly-L-lysine-coated glass slides. Some of them were stained with Eosin-Haematoxylin for histological observation (Khodabandeh *et al*., 2005). For

immunohistochemical studies, the other sections were immersed in Histoclear (histological clearing agent, Agar, R1345) twice for 5 min; for dewaxing in butanol for 5 min and hydrated through a graded series of ethanol. Slide rinse was performed using a solution of 10 mM phosphate-buffered saline (PBS), 150 mM NaCl and 0.01% Tween 20, pH 7.3, for 10 min and then treated with 50 mmol NH_4Cl in PBS, pH 7.3 for 10 min to mask free aldehyde groups of the fixative. The sections were then incubated for 10 min with a blocking solution (BS) containing 1% bovine serum albumin (BSA) and 0.1% gelatin in PBS, followed by incubation for 2 h at room temperature in a wet chamber with the primary monoclonal antibody (α5) to Na^+, K^+-ATPase (Hybridoma Bank, University of Iowa) diluted in PBS at 10 µg ml^{-1} covering the sections. Control sections were incubated in PBS without the primary antibodies. After three extensive 5 min wash in PBS to remove unbound antibody, the sections were incubated for 1 h with 10 µg ml^{-1} of the secondary antibody, donkey anti-mouse Alexa Fluor 488 (Invitrogen, Life technologies). Following extensive washing in PBS (six 5-min washes), sections were mounted in 80% glycerine, 20% PBS plus 2% N-propyl-gallate to retard photobleaching (Immuno Histo Mount, Aqueous-based Media, Santa Cruz Biotechnology, USA). Stained and labeled sections were examined and photographed with an Olympus fluorescent microscope (Olympus) equipped with a digital camera (Olympus, Tokyo, Japan).

The number and size of ionocytes located in the gill filaments and along the base of the lamellae of the gill sections of fingerlings exposed to FW and to different salinities (7 and 12‰) were evaluated (six fish per treatment). Gill sections were photographed and measurements were taken using the freeware Image J (http://rsbweb.nih.gov/ij/). The number of ionocytes per 100 µm^2 filament and the size of 10 ionocytes per section (µm^2) were calculated (only ionocytes sectioned through their nucleus were considered) (Khodabandeh *et al*., 2009a; Shirangi *et al*., 2016).

Statistical analyses

Two weight groups within each salinity treatment and three salinity treatments within each weight group (for survival rate and plasma osmolarity) at 240 h after treatment were compared using one way ANOVA. Also, two-way ANOVA in conjunction with Tukey HSD test was used for statistical comparison of the ionocyte area and abundance mean values ($p<0.05$) of six fish at several time points and under different salinities.

Results and discussion

The estimated survival rate of zander fingerlings after transfer from FW to 7 and 12‰ salinities was high (>95%). There were no significant differences between the survival rates of the fingerlings, with 1 or 2 g body weight, in FW versus abrupt transfer to both salinity treatments during 10 days of acclimation time ($p>0.05$) (Table 1). The survival rate of >95% under different salinity treatments indicates that the fingerlings of both 1 and 2 g weight groups, can tolerate abrupt transfer to CW with a salinity up to 12‰ (the natural salinity of south Caspian Sea). Brown *et al*. (2001) reported similar observations where all fish survived rapid transfer from FW to salinities of 8 and 16‰ but the experiments were conducted on adult zanders from the waters of Great Britain for six days.

The plasma osmolarity mean values of 1g fingerlings in all salinity treatments were significantly higher than 2 g group (Table 1). The results showed that the blood of 2 g fish is more diluted than 1g fish and the plasma osmolarity was related to fish weight. High plasma osmolarity level observed in 1g groups could be attributed to comparatively low blood volume. Our previous study on hematocrit (Hct) analysis of two weight groups of zander fingerling exposed to FW, 7S and CW supports this result. It showed Hct of 2 g fingerlings was less than 1g in all salinities while there were no significant difference among different salinities in each weight group (Ahmadnezhad *et al*., 2013). Total blood volume of teleosts is reported to be 30 to 40 ml kg^{-1} which is influenced by fish body weight (Evans, 1998).

In the present study, the plasma osmolarity levels for 1g fingerlings were also not significantly affected by the three salinities at the end of the experiments and within the range of osmolarity of the treatment waters. Similar patterns were also found for medaka (Kang *et al*., 2008) and silver moony (*Monodactylus argentenus*) acclimated to brackishwater (Kang *et al*., 2012).

However, for the fingerlings with 2 g body weight, the level of plasma osmolarity rose in 7S and CW salinities, when compared to FW, at 240 h of exposure. But, there was no significant difference between the two different salinities (7S and CW) (Table 1). Again, the plasma osmolarity in 7S and CW salinities remained almost near the osmolarity level of the water under these two salinity treatments. Similar results were also reported by Brown *et al*. (2001) for adult zander, which were acclimated to brackishwater for six days. He *et al*. (2009) observed that the serum osmolarity of Chinese sturgeon at 480 h after exposure to brackishwater (10‰) were close to the osmolarity of water under this salinity, while plasma osmolarity levels were significantly higher than those of FW control fish. In different salinity exposure of murray cod (*Maccullochella peelii peelii*) juveniles, physiological parameters (especially blood omolarity) were in equilibrium with the environment in iso-osmotic

Table 1. Survival rate and plasma osmolarity in two weight groups of Caspian Sea zander fingerlings, 10 days after exposure to different salinities

Parameters	Weight groups (g)	Salinity treatment		
		FW (<0.5‰)	7S (7‰)	CW (12‰)
Survival rate (%)	1	97.7 ± 0.3	98.7 ± 0.3	99 ± 0.57
	2	97.7 ± 0.67	99 ± 0.6	98 ± 0.6
Plasma osmolarity	1	345 ± 0.58^{aa}	367 ± 7.51^{aa}	376.7 ± 16.22^{aa}
	2	320 ± 3.21^{ab}	350 ± 4.36^{bc}	355 ± 6.03^{bc}

Values bearing different superscripts indicate significant differences between three salinity treatments and two weight groups ($p<0.05$)

condition (Mellor and Fotedar, 2005). The osmoregulatory mechanisms in osmoregulator fish maintain almost constant plasma osmotic pressure in 280-360 mOsmol kg^{-1} range. Therefore, salinity with this osmotic pressure, approximately 10-12‰, is iso-osmotic for the fish. FW is hypotonic relative to the fish's body fluid and the body is thus exposed to ion loss. One of the compensatory mechanisms for ion loss in FW is active ion uptake in the branchial chambers (mainly in the gills, and in particular in the ionocytes) (Varsamos *et al*., 2005). In FW, it was observed that plasma osmolarity of both weight groups of zander were higher than the osmolarity of freshwater. Thus, it appears that 1 and 2 g fingerlings are able to regulate ion uptake through their gill ionocytes.

In the current study, NKA enzymes were detected in the gills in all salinities. In FW, immunoreactive cells (or ionocytes) were observed in the filaments and lamellae of all samples, mainly in the inter-lamellar regions (Fig. 1a, b, e) in both weight groups, similar to the distribution of ionocytes in the gills of rainbow trout (Olson and Fromm, 1973), milkfish (Lin *et al*., 2003), chum salmon (Uchida *et al*., 1996; Shikano and Fujio, 1998), American shad (Zydlewski and McCormick, 2001), golden grey mullet (Khodabandeh *et al*., 2009a) and silver moony (Kang *et al*., 2012) acclimated to FW. Furthermore, the distribution of ionocytes, following transfer to 7 and 12‰ salinities, was altered. In fact, there were much less ionocytes on the lamellae and only few fluorescent cells on the filaments (Fig. 1d, f). Similar observations have been reported in previous studies in brackishwater challenges (Shikano and Fujio, 1998;

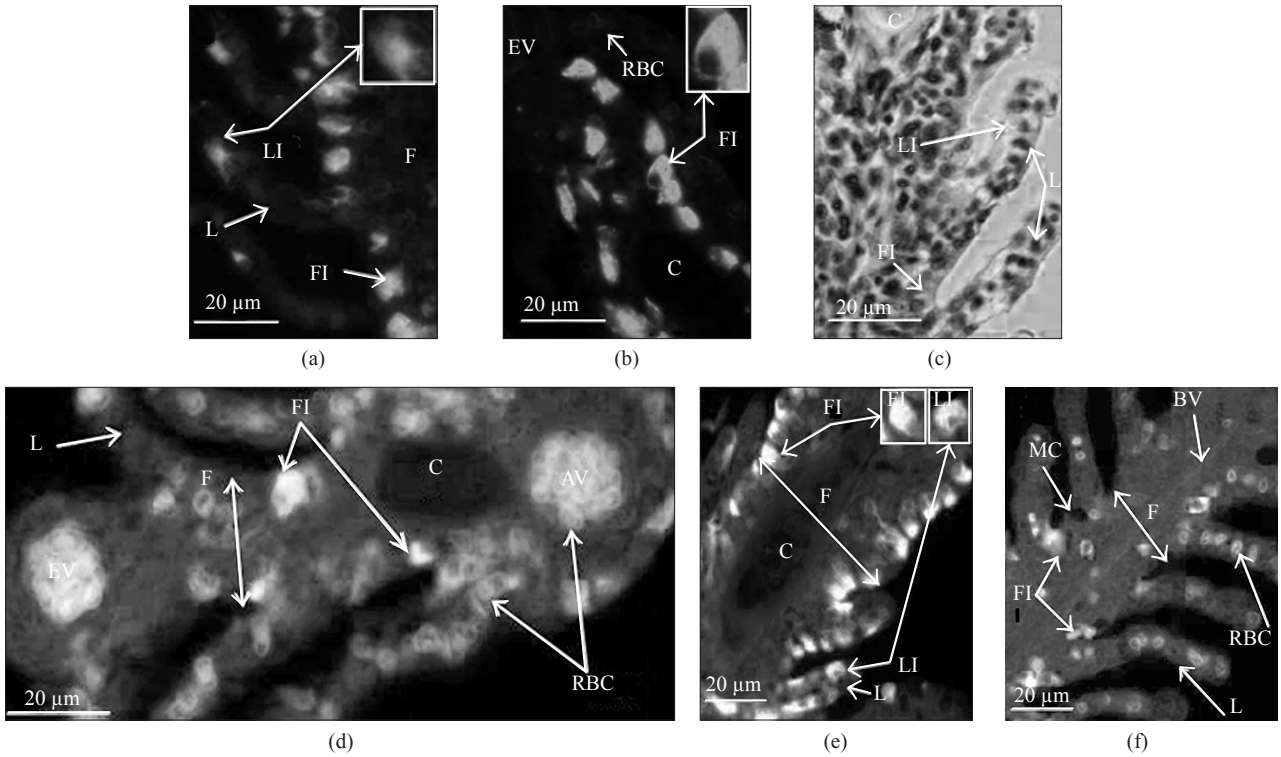

Fig. 1. Immunolocalisation of NKA rich cells in the branchii of zander fingerlings. a, b, c : Longitudinal section of the branchii from 1 g group samples acclimated in freshwater; d: Longitudinal section of the branchii from 1 g group samples acclimated in Caspian seawater; E: Longitudinal section of the branchii from 2 g group samples acclimated in freshwater, f : Longitudinal sections of the branchii from 2 g group samples acclimated in seawater. AV - Afferent vessel, BV - Blood vessel, C - Cartilage, EV - Efferent vessel, F - Filament, FI -Filament ionocyte, L - Lamellae, LI - Lamellar ionocyte, MC - Mucus cell, RBC - Red blood cell

Khodabandeh *et al*., 2009a; Kang *et al*., 2012). A study on chum salmon revealed that lamellar ionocytes degenerated when the fish were transferred from FW to brackishwater and reappeared when they were reintroduced to FW (Shikano and Fujio, 1998). These observations support the hypothesis that lamellar ionocytes in FW stenohaline fish and FW adapted euryhaline fish are responsible for ion uptake in hyposmotic conditions (Lin and Sung, 2003; Hiroi *et al*., 2008).

Significant changes were detected in the number (cell mm^{-2}) and size (μm^2) of ionocytes in the gills of the two weight groups exposed to different salinities. These variables significantly decreased at salinity extremes ($p<0.05$), whereas fish acclimated to FW had more and larger cells. Despite the descending trend in the number of lamellar ionocytes (Figs. 2a, b) and the size of both lamellar and filament ionocytes (Figs. 4a, b and 5a, b), the abundance of ionocytes in filaments had a notably ascending trend over the 24 to 240 h time interval in 7S and CW, for 2 g fingerlings (Fig. 3b) and only in CW for 1g fingerlings (Fig. 3a).

The size of the ionocytes in branchii of both 7S and CW treatments were evidently smaller than those of FW (Figs. 4 and 5), whereas their number did not experience a significant difference in FW for both weight groups ($p>0.05$) (Figs. 2 and 3), particularly in 2 g fingerlings, at the end of the acclimation period. The largest size and number of ionocyte in medaka (Kang *et al*., 2008), silver moony (Kang *et al*., 2012), golden grey mullet (Khodabandeh *et al*., 2009a) and Caspian trout (*Salmo trutta caspicus*) parrs (Rajabi and Khodabandeh, 2013) have been reported in FW. In the present study, fewer and smaller ionocytes, after acclimatisation to 7 and 12‰ salinities (both in the range of brackishwater and near iso-osmotic environment) are in agreement with the observations in gilthead seabream (Laiz-Carrion *et al*., 2005), medaka (Kang *et al*., 2008), silver moony

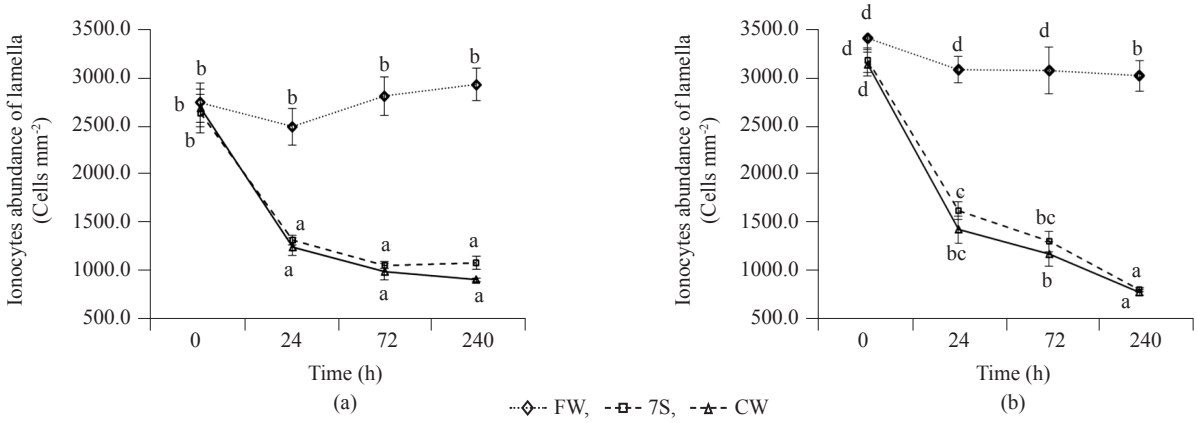

Fig. 2. Change in the abundance of ionocytes (cells mm^{-2}) in the gill lamellae of Caspian Sea zander fingerlings (a: 1g, b: 2g) after acclimation in different salinities over a period of 10 days. Different letters indicate significant difference among combinations of salinity and time ($p<0.05$)

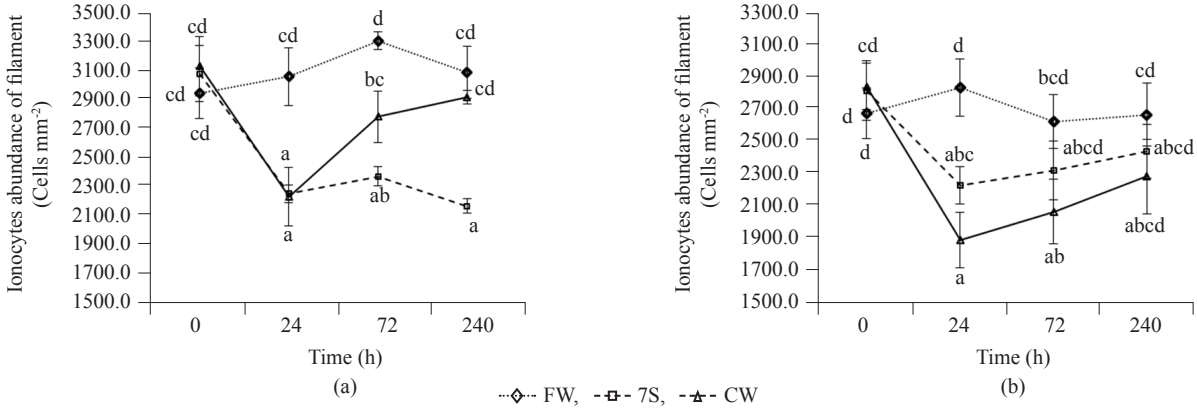

Fig. 3. Change in the abundance of ionocytes (cells mm^{-2}) in the gill filaments of Caspian Sea zander fingerlings (a: 1g, b: 2g) after acclimation in different salinities over a period of 10 days. Different letters indicate significant difference among combinations of salinity and time ($p<0.05$)

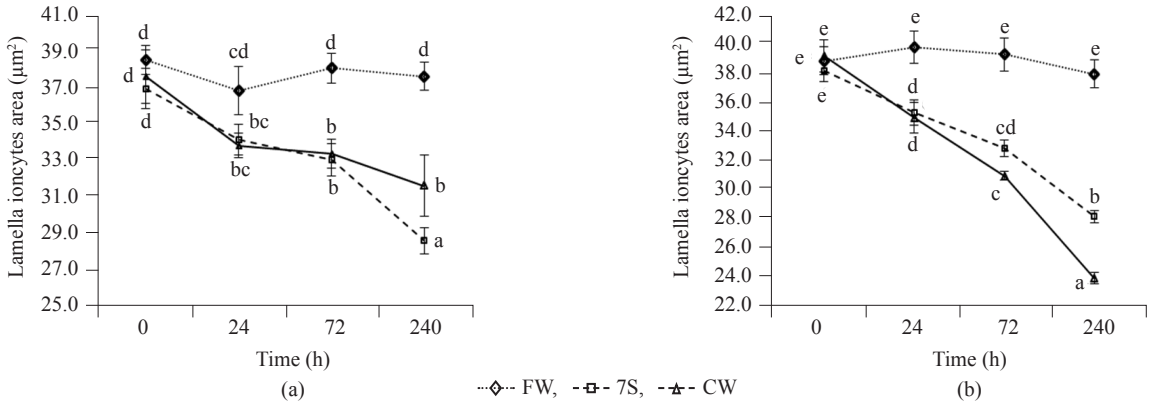

Fig. 4. Change in the area of ionocytes (µm²) on the gill lamellae of Caspian Sea zander fingerlings, (a: 1g, b: 2g) after acclimation in different salinities over a period of 10 days. Different letters indicate significant difference among combinations of salinity and time ($p<0.05$)

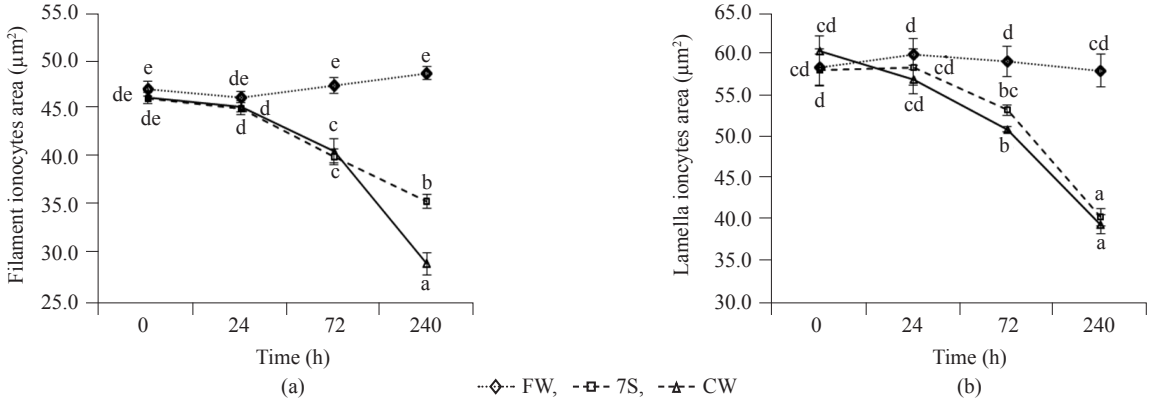

Fig. 5. Change in the area of ionocytes (µm²) in the gill filaments of Caspian Sea zander fingerlings, (a: 1g, b: 2g) after acclimation in different salinities over a period of 10 days. Different letters indicate significant difference among combinations of salinity and time ($p<0.05$)

(Kang *et al*., 2012) and golden grey mullet (Khodabandeh *et al*., 2009a). In brackishwater, the number and size of ionocytes in all of above mentioned species were lower than when the fish were acclimated to very low or very high salinity (Laiz-Carrion *et al*., 2005; Kang *et al*., 2008; Kang *et al*., 2012; Khodabandeh *et al*., 2009a). In some other studies also, the gill NKA activity followed this pattern, as the lowest NKA activity levels were obtained in brackishwater conditions (Laiz-Carrion *et al*., 2005; Kang *et al*., 2008; Kang *et al*., 2012; Khodabandeh *et al*., 2009a). Since NKA is highly expressed in fish gill ionocytes, its activity at the gill level is expected to be reduced with decreased ionocyte size and number (Evans, 1993; Cataldi *et al*., 1995; McCormick, 1996; McKenzie *et al*., 1999; Mancera and McCormick, 2000). Low values of the ionocyte size, number and the gill NKA activity reported previously in the literature for fish acclimated to brackishwater has been confirmed in the current study, especially for 1g fingerlings, by a reduction in the number and size of ionocytes under similar conditions. The near iso-osmotic environment of salinity treatments from the Caspian Sea probably subjected zander fingerlings to a small level of osmotic stress.

Gill ionocyte proliferation due to saline water transfer occurs mainly along the gill lamellae and may also induce lengthening of these lamellae in order to optimise respiration rates. Therefore, a larger respiratory surface with, most probably, higher gas exchange rates, could compensate for the extra cost induced by ionocyte proliferation and activity (Faalk-Petersen, 2005; Rocha *et al*., 2010). However, in iso-osmotic conditions, fish are thought to require less oxygen compared to hyper- or hypo-osmotic conditions (Aristizabal-Abud, 1992; Woo and Kelly, 1995). Therefore, the absence of ionocytes along the gill lamellae of zander fingerlings may have influence on fish oxygen requirements while moving to Caspian seawater.

Multifold ionocyte proliferation particularly along gill filament mainly occurs after salinity exposure compared to hypo-osmotic conditions (Evans *et al*.,

2005). In this study, upward trend in numbers of filament ionocytes in Caspian seawater may be a sign of ionocyte proliferation; nevertheless their number was lower than FW at the end of the experiment. A possible explanation for this might be that increase in ionocyte size and number are not needed in Caspian seawater exposures, in comparison with iso-osmotic condition.

In conclusion, the physiological responses, including the survival rate, changes of plasma osmolarity and gill tissue along with variations in the gill ionocytes of 1 and 2 g weight groups of *Sander lucioperca* fingerling at two levels of salinity revealed both fish groups to be in the same osmoregulatory developmental stage. Also, their osmotic pressure index and gill ionocyte behaviour at the two different salinities were similar when transferred to iso-osmotic condition. These results suggest that gill development of 1 and 2 g zander fingerlings are both suitable for transfer to Caspian seawater. Since, the cost of culture of lighter fish is lower than heavier fish, release of 1g zander in estuaries can be suggested for restocking of zander in Caspian Sea. However, further studies are needed for complete understanding of zander osmoregulatory system at different salinities.

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