

## Effects of cortisol treatment on the salinity tolerance of Persian sturgeon, *Acipenser persicus* Juveniles

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### ABSTRACT

The freshwater shrimp, *Macrobrachium nipponense* is an invasive species which has recently been reported in Anzali Wetland, Iran. It exhibited good tolerance and adaptation in this wetland ecosystem. This study examined certain aspects of feeding of *M. nipponense* in three habitats of this wetland. Shrimps were randomly sampled from April 2016 to March 2017. The stomach contents were obtained from 367 specimens ranging in length from 4.2 cm to 6.9 cm. The empty stomach index (VI) showed that this shrimp was a voracious ( $0 \leq VI < 20$ ) species in all seasons except winter, when 99% of the specimens had empty stomachs. Fourteen dietary items were categorized in the three habitats of the wetland, with phytoplankton, mollusks and detritus forms being the dominant food items in the western, central and eastern habitats respectively. The feeding precedence index (FP) revealed that the most abundant portion of food was subsidiary one ( $50 \geq FP \geq 10$ ) and the highest proportions of subsidiary food were phytoplankton (24.5%), gastropods (34%) and detritus (29.11%) in the western, central and eastern habitats, respectively. Omnivorous feeding is one of the reasons for the success, high tolerance and adaptation of *M. nipponense* in the Anzali Wetland ecosystem.

**Key words:** Persian sturgeon, cortisol, salinity tolerance, osmotic improvement, chloride cell.

### INTRODUCTION

Persian sturgeon, *Acipenser persicus*, is the most common sturgeon species found along the Southern Caspian Sea coastline (Billard & Leconte 2001; Pourkazemi 2006). This euryhaline fish breeds in freshwater (FW), and its main spawning grounds have been limited along the Iranian coastline (Abdolhay 1997). However, this species is facing serious depletion in the Caspian Sea and its nearby rivers due to overfishing, habitat loss and environmental degradation (Birstein *et al.* 1997; Billard & Leconte 2001). Since the Persian sturgeon has been considered as 'Critically Endangered species (Gesner *et al.* 2010), juveniles are annually produced by hatchery centers of the Iranian Fisheries Organization and released (2-3 g body weight) to the estuaries surrounding the southern parts of the Caspian Sea. However, it does not appear as an efficient way to restock sturgeons. Juveniles cannot withstand the salinity stress when directly transferred from FW to the Caspian Sea water (CSW) and the majority of them released into the estuaries die before entering the sea (Abdolhay & BardaranTahori 2006; Kazemi *et al.* 2006; Mosafer Khorjestan *et al.* 2009; Bakhshalizadeh *et al.* 2011). Among different parameters affecting ion-regulatory system (environmental salinity, feeding, activity, injury, reproductive state and a variety of stressors), the neuroendocrine system is the major means for modulating the osmo-regulatory ability of teleost fish (Bern & Madsen 1992).

Many studies have reported that alterations in the salinity and hormones such as cortisol, growth hormone (GH) and prolactin can stimulate expression and activity of different ion and electrolyte transporters in the chloride cells, such as Na<sup>+</sup>-K<sup>+</sup>-ATPase (NKA) and Na<sup>+</sup>-K<sup>+</sup>-Cl<sup>-</sup> (NKCC) co-transporter (Pelis & McCormick 2001; Mancera *et al.* 2002; Singer *et al.* 2003; Lin *et al.* 2004; Tse *et al.* 2006; Khodabandeh *et al.* 2009).

Cortisol is a major corticosteroid with several physiological roles related to osmoregulation, respiratory and intermediary metabolism, growth, stress and immune function (Wendelaar Bonga 1997; Mommsen *et al.* 1999). In euryhaline teleost this hormone acts on several osmoregulatory organs such as gills, gut, kidney and urinary bladder (Mancera *et al.* 1994). Cortisol has a 'dual osmoregulatory' role. In addition to sea water (SW)-adapting ability in cooperation with GH/IGF-I axis, it improves the FW-adapting ability in cooperation with prolactin in some teleost fish (Bisbal & Specker 1991; McCormick 2001). Cortisol alone or most probably with GH/IGF-I enhance salinity tolerance after transfer from hypo- to hyper-osmotic medium via stimulating ion secretion as well as decreasing plasma ion levels and osmolality in SW-adapted teleosts. At the gill level, this effect is due to increased chloride cells number and size, enhanced NKA activity, NKA  $\alpha$ -subunit and NKCC co-transporter expression induced by cortisol/cortisol with GH treatment (McCormick 1995, 2001; Madsen *et al.* 1995; Pelis & McCormick 2001; Mancera *et al.* 2002; Laiz-Carrión *et al.* 2003). It has been demonstrated that artificially-increased cortisol levels help to counteract osmotic stress when fish are transferred between two different osmotic media by decreasing the plasma ion concentrations in some teleost such as gilthead sea bream, *Sparus aurata* L., Sockeye salmon *Oncorhynchus nerka* and common carp, *Cyprinus carpio* (Mancera *et al.* 1994; Ban 2002; Abo Hegab & Hanke 1984). Also, this hormone induces elevated NKA activity in Sockeye salmon, *O. nerka*, common carp, *C. carpio*, Atlantic salmon, *Salmo salar*, parr and Juvenile Senegalese sole, *Solea senegalensis*, (Ban 2002; Abo Hegab & Hanke 1984; Pelis & McCormick 2001; Arjona *et al.* 2011). Furthermore, exogenous cortisol can affect the branchial chloride cells number and size to improve osmoregulatory ability.

For Atlantic salmon, *S. salar*, Persian sturgeon, *A. persicus*, and beluga sturgeon, *Huso huso*, chloride cells number is increased by cortisol treatment in FW (Pelis & McCormick 2001; Khodabandeh *et al.* 2009; Mazloumi *et al.* 2015). Improvement of the hypo-osmoregulatory ability with cortisol occurred in the coastal cutthroat trout, *Oncorhynchus clarki*, parr (Morgan & Iwama 1996), in the Atlantic salmon, *S. salar* (Bisbal & Specker 1991) and the rainbow trout, *Oncorhynchus mykiss* (Madsen 1990). However, osmoregulation mechanism in some other species such as euryhaline tilapia, *Oreochromis mossambicus*, *Odontesthes bonariensis* and pejerrey, *Odontesthes bonariensis*, are not affected by hormonal treatment either in hypo or hyper-osmotic media (Abo Hegab & Hanke 1984; Tsuzuki *et al.* 2007).

Hence, the objective of the present study is to investigate the role of exogenous hormonal treatment to enhance the osmoregulation of juvenile Persian sturgeon less than 2 g via monitoring daily mortality, plasma osmolality, and changes in the abundance and localization of the NKA within the gill epithelium of Persian sturgeon following treatment with cortisol.

Although some studies have confirmed osmotic improvement of cortisol-treated sturgeon juveniles weighing 2-3 g in Iran (Khodabandeh *et al.* 2009; Khoshnood *et al.* 2010; Fakharzadeh *et al.* 2011; Mazloumi *et al.* 2015) but simultaneous interaction of the cortisol treatment and salinity stress for fish less than 2 g is still needed.

## MATERIALS AND METHODS

### Experimental rearing conditions and fish sampling

In order to improve the salinity acclimation in Persian sturgeon juveniles less than 2 g ( $1.62 \pm 0.27$  g) in weight and  $7.4 \pm 0.58$  cm in length, the fish were treated with cortisol (hydrocortisone, Sigma) bathing method using three different concentrations; 3, 5 and 7 mg L<sup>-1</sup> for 24 h in FW. They were then directly transferred to the CSW (11‰). So that, the fish were randomly distributed into eight fiberglass tanks filled with aerated FW (60 liters). After 72 h adaptation period, fish were transferred to the experimental tanks. Six tanks were used for hormonal treatments with 2 replicates (2 tanks for each treatment) and two tanks for fish abruptly transferred from FW to CSW as control group. 40 individuals were considered for each tank. The fish were then sampled after 1, 4 and 9 days post-transfer to the CSW.

Daily mortality was recorded. Plasma osmolality, alterations in the number and size of the NKA immunoreactive chloride cells within the gill epithelium of Persian sturgeon juveniles were determined following treatment with cortisol and transfer to the CSW.

### Medium and blood osmolality

From each collected fish, blood samples were taken from the caudal vein, just behind the anal fin, with an insulin syringe. On average, 50  $\mu\text{L}$  of blood was collected per fish.

After blood centrifugation (6000g for 10 min), blood plasma was collected. Batches of 10 individuals were pooled in order to determine plasma osmolality. Medium and blood plasma osmolality (triplicates of 15 $\mu\text{L}$  each) were determined using an Osmomat 030 osmometer (GANOTEC, Germany).

### Immunohistochemistry

For fluorescent microscopy, fish gills were excised and fixed in Bouin's fixative solution for 48 hours (4 fish per treatment).

They were then washed in 70° alcohols, dehydrated in an ascending series of ethanol, and processed for embedding in ParaplastX-TRA® (Sigma-Aldrich, P3808).

Sections (4  $\mu\text{m}$ ) cut on a Leitz Wetzlar microtome were collected on glass slides and were stained using the classical Masson's Trichrome staining protocol (Martoja & Martoja-Pierson, 1967) or directly used for immunolabeling.

For immunolabeling, sections were immersed in Histoclear (Histological Clearing Agent, Agar, R1345) twice for 5 min to 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  $\text{NH}_4\text{Cl}$  in PBS, pH 7.3 for 5 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% gelatine in PBS, followed by incubation for 2 hours at room temperature in a wet chamber with the primary monoclonal antibody ( $\alpha 5$ ) to  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase (Hybridoma Bank, University of Iowa) diluted in PBS at 10  $\mu\text{g mL}^{-1}$  covering the sections. Control sections were incubated in PBS without the primary antibodies.

After three extensive 5-min washes in PBS to remove unbound antibody, the sections were incubated for 1 hour with the secondary antibody, donkey-anti mouse; Alexa Fluor® 488 (Invitrogen, Life Technologies), 10  $\mu\text{g mL}^{-1}$ . Following extensive washing in PBS (6 times five-min washes), sections were mounted in 80% glycerine, 20% PBS plus 2% N-propyl-gallate to retard photobleaching (ImmunoHistoMount, Aqueous-based Media, Santa Cruz Bio-technology, USA). Stained and labelled sections were all examined with a Leitz Diaplan microscope equipped with a special filter for fluorescence and associated with a Leica DC 300 F digital camera and its FW 4000 I software (Leica Microsystems, Rueil-Malmaison, France).

### Chloride cell morphometry

The number and size of chloride cells located in the filaments and along the base of the lamellae were evaluated on histological and immunofluorescent gill sections of the juveniles kept in CSW without hormonal treatment and treated with different concentrations of cortisol (4 individuals for each experimental condition). Gill sections were photographed (3 slides for each individual and 5 photographs for each slide) at the same magnification (40x). Morphometrically measures were obtained using the freeware Image J (<http://rsbweb.nih.gov/ij/>). The number of chloride cells /  $\text{mm}^2$  filament and the size of 10 chloride cells per section ( $\mu\text{m}^2$ ) were calculated (only chloride cells sectioned through their nucleus were considered).

### Statistical analysis

Statistical analyses were performed using SPSS (version 23). Data was analyzed by one-way analysis of variance (ANOVA) and post-hoc Tukey's HSD tests ( $P < 0.05$ ) to compare the different concentrations of cortisol treatment to CSW transfer at the different times after transfer to CSW.

## RESULTS

### Mortality rates

Daily mortality rates recorded for fish transferred from FW to CSW differed between control group and fish treated with cortisol bath with three different concentrations (Fig. 1). Higher mortality rate could be observed for untreated fish with cortisol bath compared to treated fish. It seemed that fish treated with higher concentrations of cortisol show higher survivability during the whole experimental period.

Therefore, cortisol treatment with higher concentrations has effect on mortality rates in Persian sturgeon juveniles weighing less than 2 g.

### Blood osmolality

The blood osmolality of fish transferred from FW to CSW, whether treated or untreated with cortisol, significantly increased over the post-transfer hours ( $P < 0.05$ ) (Fig. 2). Compared to the control group, in fish treated with 3 and 5 mg L<sup>-1</sup> cortisol, the highest blood plasma osmolality level was reached 4 days (96 h) after transfer to CSW. However, for the fish treated with 7 mg L<sup>-1</sup> cortisol, blood osmolality was at the maximum level only 1 day (24 h) after transfer to the CSW. Only the fish treated with 3 mg L<sup>-1</sup> cortisol exhibited significantly decreased plasma osmolality level to CSW osmolality 9 days post-transfer. These fish also displayed significantly lower osmolality values compared to the other two groups and control ( $P < 0.05$ ). However, in other groups, it was remained at a higher level than CSW osmolality (40 to 45 mOsm Kg<sup>-1</sup> higher) throughout the entire duration of the experiment.

### Chloride cell morphometry

Chloride cells were identified using NKA immunofluorescent labelling and located along gill filaments and lamellae (Fig. 3). For cortisol treated fish at the all three different concentrations, immunoreactive chloride cells (primary filament or lamellae) number and size were not increased compared to the control group 9 days post-transfer to the CSW (Fig. 4A and 4B) ( $P < 0.05$ ). Almost  $1823 \pm 113$  chloride cells were observed per mm<sup>2</sup> of the gill epithelium and the area of each chloride cell was measured  $\sim 124.78 \pm 13.62$  μm<sup>2</sup> whether in control or in treated groups (Fig. 4A and 4B).

### DISCUSSION

There are different ways of hormonal treatments by corticosteroids including bath, injections and live food enrichment. Injection or hormonal implantation is more acute methods but mostly used in large fish and is especially suitable for broodstocks (Madsen & Korsgaard 2004). Because of small sizes of the Persian sturgeon juveniles in this study and in agreement with the other studies (Khodabandeh *et al.* 2009; Khoshnood *et al.* 2010; Fakharzadeh *et al.* 2011; Mazloumi *et al.* 2015), it is suggested that the osmo or ion-regulatory problems can be solved using cortisol bath method with three different concentrations (3, 5 and 7 mg L<sup>-1</sup>).

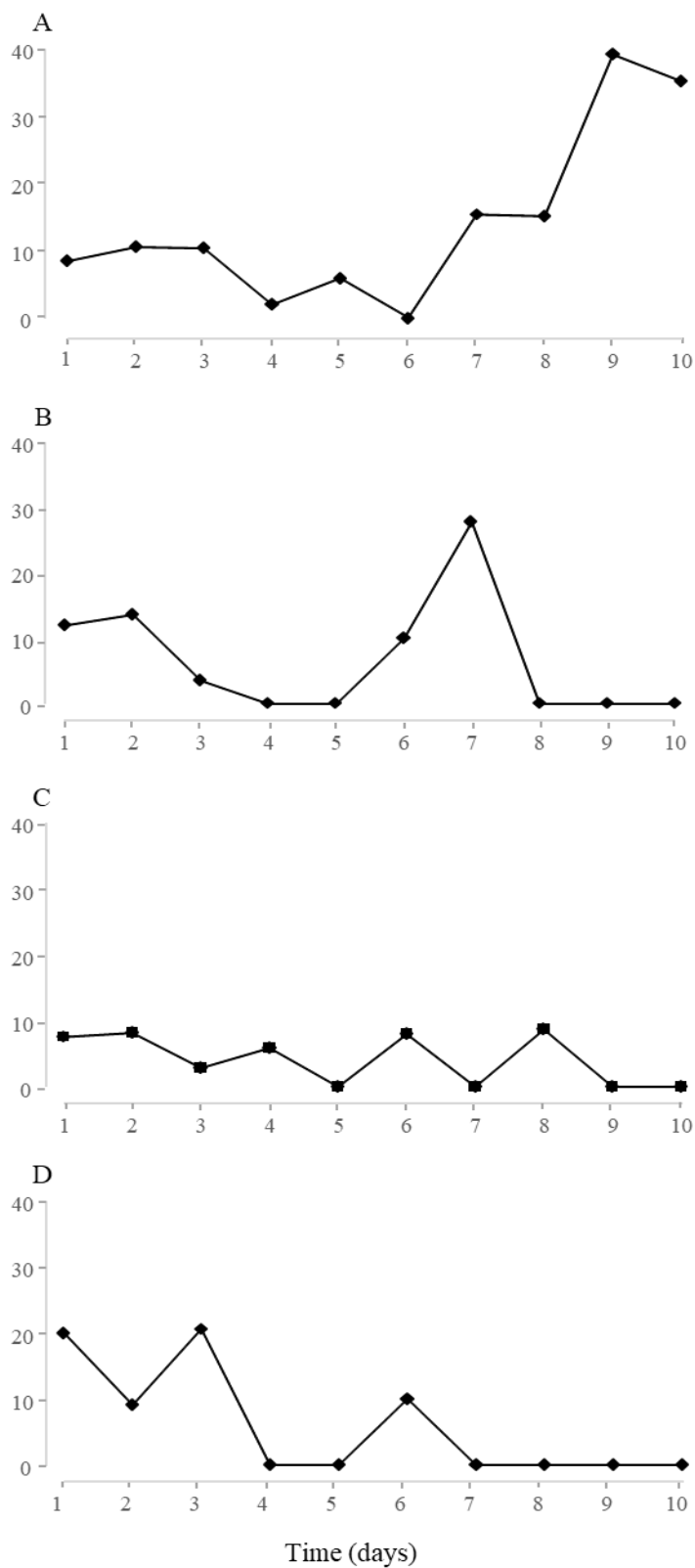
Therefore, in this study, cortisol treatments have been performed with similar concentrations for Persian sturgeon juveniles weighing less than 2 g. In spite of lower mortality in fish treated with 5 and 7 mg L<sup>-1</sup> cortisol, chloride cell number and size in treated fish in general displayed the same trends as the control group after 9-days transfer to the CSW.

Most of the studies have focused on the osmoregulatory readiness of sturgeons either in FW (Khodabandeh *et al.* 2009; Khoshnood *et al.* 2010; Mazloumi *et al.* 2015) or during short-acclimation (24 h) in CSW (maximum 7‰) (Fakharzadeh *et al.* 2011). However, there was no report on simultaneous interaction of hormonal treatment and salinity stress within long-term salinity acclimation (9 days) in the Persian sturgeon juveniles.

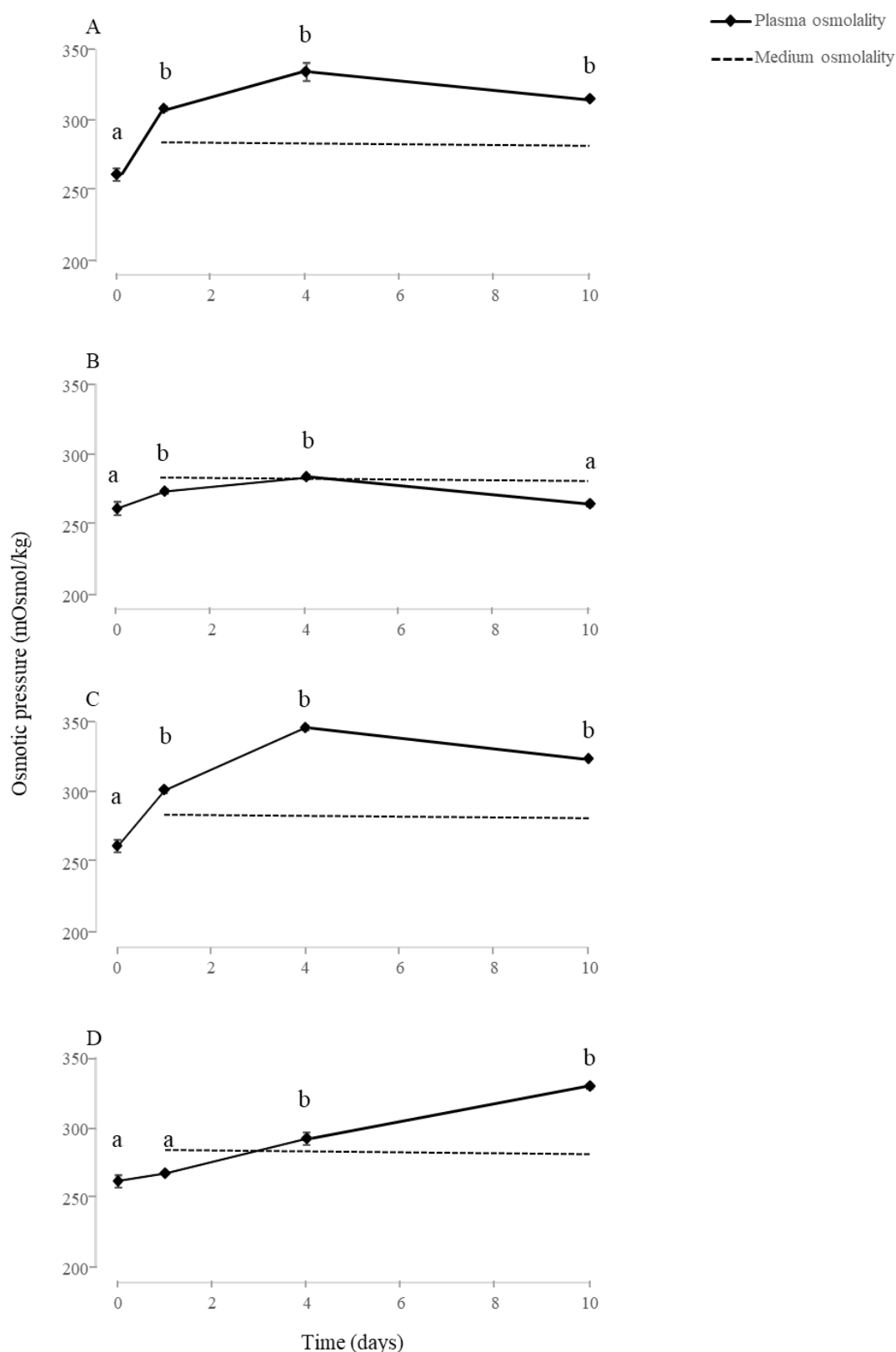
No mortality was observed in juvenile Senegalese sole, *S. senegalensis*, treated with cortisol implantation during 14 days acclimation in SW (38‰) (Arjona *et al.* 2011). Furthermore, for Persian sturgeons, it has been suggested that fish treated with 5 and 7 mg L<sup>-1</sup> cortisol exhibits a lower mortality compared to those treated with 3 mg L<sup>-1</sup> or control group during 24 h acclimation in FW and diluted CSW (7‰) (Khodabandeh *et al.* 2009; Fakharzadeh *et al.* 2011). However, in the present study, mortality rates in both treated and control was lower within the 24 h post-transfer to the CSW, compared to the other studies. Nevertheless, all fish treated with three cortisol concentrations and control group displayed high and similar mortality.

Therefore, result of mortality rates exhibited no effect of cortisol treatment on osmoregulatory enhancement in Persian sturgeon juveniles. In the present study, only fish treated with 3 mg L<sup>-1</sup> cortisol significantly lowered their plasma osmolality levels down to the Caspian Sea osmolality 9 days post-transfer to the CSW. This is similar to results reported from other teleosts exposed to the salinity challenges. However, the other fish (control group and those treated with 5 and 7 mg L<sup>-1</sup>) maintained their plasma osmolality at high level entire the whole experiments. In accordance with the present study, hormonal treatment with cortisol has not displayed influences on the plasma osmolality in gilthead sea bream, *S. aurata* L., Juvenile Senegalese sole, *S. senegalensis*, yearling Coho salmon, *Oncorhynchus kisutch*, channel catfish, *Ictalurus punctatus* and pejerrey, *O. bonariensis*, during transfer from FW to BW/SW (Redding *et al.* 1984; Mancera *et al.* 1994; Eckert *et al.* 2001; Tsuzuki *et al.* 2007; Arjona *et al.* 2011).

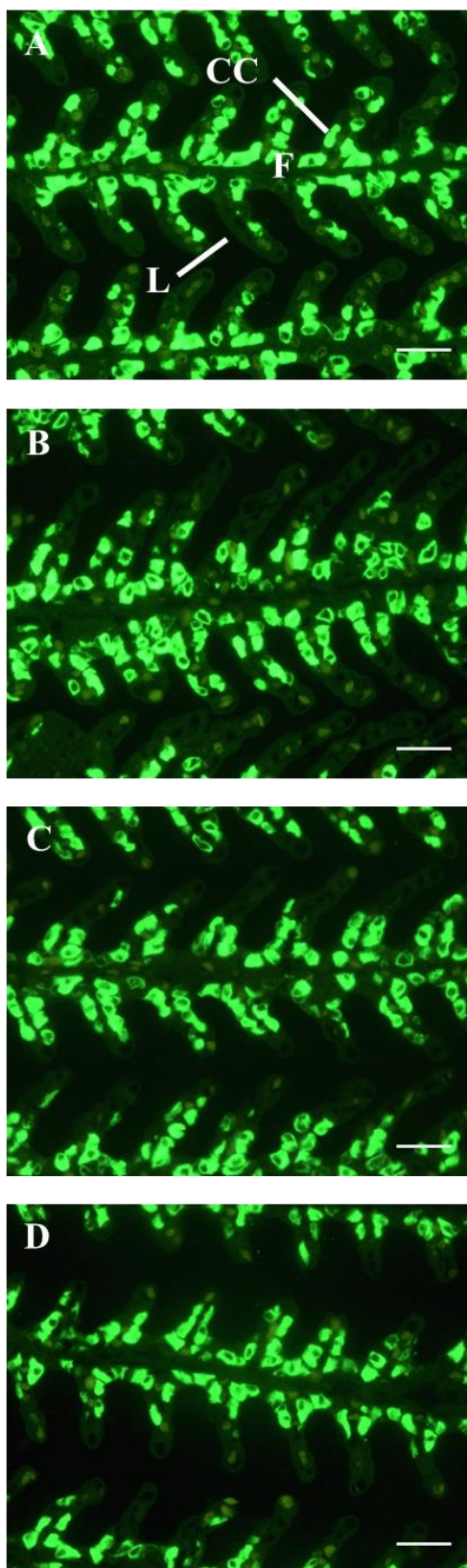
Similar to the result of plasma osmolality, exogenous cortisol treatment alone could not evoke the complete differentiation and maturation of the gill chloride cells in CSW. Chloride cells number and size remained unchanged even 9 days after transfer from FW to the CSW.



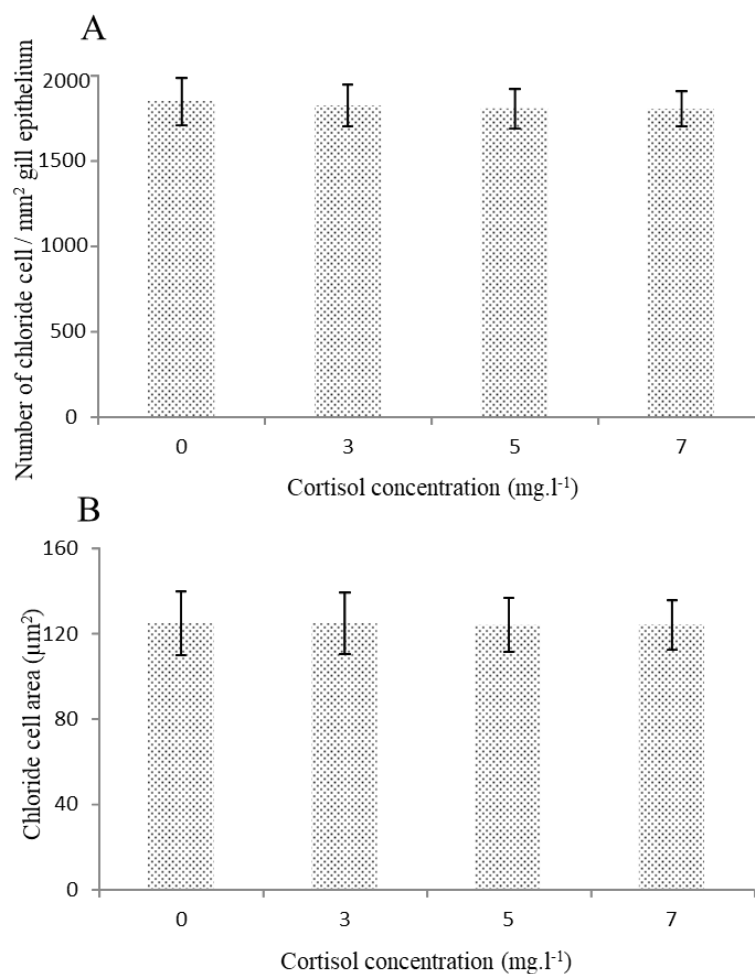
**Fig. 1.** Daily mortality rates for CSW-acclimated Persian sturgeons without cortisol treatment (control) (A), cortisol treated sturgeon with 3 mg L<sup>-1</sup> (B) 5 mg L<sup>-1</sup> (C), 7 mg L<sup>-1</sup> (D) during 9 days in CSW.



**Fig. 2.** Plasma osmolality for CSW-acclimated Persian sturgeons in control group (A), cortisol-treated sturgeon with 3 mg L<sup>-1</sup> (B) 5 mg L<sup>-1</sup> (C), 7 mg L<sup>-1</sup> (D) during 9 days in CSW. The letters are used to indicate significant differences between FW and CSW-acclimated fish at different times post transfer (One-Way ANOVA test and post-hoc Tukey's HSD tests,  $P < 0.05$ ). Data are expressed as mean  $\pm$  SD ( $n = 10$ ).



**Fig. 3.** Immunolocalization of Na<sup>+</sup>, K<sup>+</sup>-ATPase in the gills of CSW-acclimated Persian sturgeons in control group (A), cortisol-treated sturgeon with 3 mg L<sup>-1</sup> (B) 5 mg L<sup>-1</sup> (C), 7 mg L<sup>-1</sup> (D) after 9 days in CSW. CC, chloride cell; F, gill filament; L, lamellae. Scale bar: 50 µm.



**Fig. 4.** Simultaneous effect of abrupt salinity transfer from FW to CSW (11‰) and cortisol treatment on chloride cell number (A) and area (B) in the gills CSW-acclimated Persian sturgeons after 9 days in CSW (One-Way ANOVA test and post-hoc Tukey's HSD tests,  $P < 0.05$ ). Data are expressed as mean  $\pm$  SD ( $n = 4$ ).

This, together with an elevated plasma cortisol in the initial phase of SW adaptation, might synchronously stimulate the complete differentiation and maturation of SW chloride cells (Seidelin & Madsen 1999).

Furthermore, an anti-apoptotic action of IGF-I on gill chloride cells, permits cortisol to affect a greater number of partially or fully differentiated chloride cells to enhance salinity tolerance (Veillette & Young 2005). On the other hand, some other hormones mainly prolactin possesses antagonistic effect on cortisol in SW-acclimated fish (Seidelin & Madsen 1997). In hypoosmotic BW, prolactin reduces chloride cell number and size. Sakamoto & McCormick (2006) have suggested that the control of cell turnover (apoptosis and cell proliferation) in different osmoregulatory epithelia (e.g., gill and gastrointestinal tract) is a critical feature of the control of osmoregulation by prolactin. Therefore, it is hypothesized that the level of prolactin in CSW-acclimated fish remained highly and this hormone possesses inhibitory effect on cortisol-treated fish in CSW.

## CONCLUSION

Plasma osmolality and the chloride cell alterations in general, exhibit similar trends, being affected by salinity, time and the interaction of salinity and time, but not by cortisol. In conclusion, this study show that exogenous cortisol at least alone, is not directly implicated in osmoregulation in Persian sturgeon juveniles less than 2 g in weight. Furthermore, effect of hormonal treatment on osmoregulation enhancement in the teleosts is species-specific. It is suggested that other factors perhaps, other hormones acting in concert with cortisol, may activate ion transport mechanisms or inhibit cortisol effect.



In addition to exogenous hormone treatment affecting salinity tolerance, the level of these hormones, mostly cortisol in plasma might be further considered. Because of salinity stress induced by transfer from FW to CSW in Persian sturgeon juveniles, it is possible that the plasma cortisol level increases autonomously. Therefore, artificially cortisol may not be effective for salinity acclimation.

A reason for this result may be due to endogenous cortisol and GH. Therefore, cortisol treatment conjunction with other hormones, as well as measuring plasma cortisol in treated and untreated fish could be suggested. More detailed research is necessary to understand the sequential roles of hormone treatment in salinity tolerance of sturgeon fish.

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## اثرات تیمار کورتیزول بر مقاومت به شوری بچه تاس ماهیان ایرانی (*Acipenser. Persicus*)

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### چکیده

بچه تاس ماهیان ایرانی (*Acipenser persicus*) هر ساله توسط مراکز تکثیر و پرورش تولید می‌شوند و (در اندازه ۲ تا ۳ گرم) به مصب‌های اطراف بخش‌های جنوبی دریای خزر رها سازی می‌شوند. با این وجود، بچه ماهیان قادر به تحمل استرس شوری نمی‌باشند و بیشتر آنها قبل از ورود به دریای خزر از بین می‌روند. به منظور بهبود سازگاری با شوری بچه تاس ماهیان ایرانی با وزن کمتر از ۲ گرم ( $0.127 \pm 0.0162$  گرم)، ماهیان با سه غلظت متفاوت ۳، ۵ و ۷  $mg.l^{-1}$  به مدت ۲۴ ساعت در آب شیرین تحت تیمار حمام کورتیزول قرار گرفتند. سپس ماهیان، به طور مستقیم از آب شیرین به آب دریای خزر (شوری ۱۱‰) انتقال داده شدند و ۱، ۴ و ۹ روز پس از انتقال به آب دریای خزر نمونه برداری شدند. مرگ و میر روزانه ثبت شد. فشار اسمزی پلاسمای خون، تغییر در تعداد و اندازه سلول‌های کلراید مکان یابی شده با ایمونوهیستوشیمی  $Na^+-K^+-ATP$  در اپیتلیوم بافت آبشش بچه تاس ماهیان ایرانی بررسی شد. با وجود مرگ و میر پایین در ماهیان تحت تیمار با غلظت‌های بالاتر کورتیزول، فشار اسمزی پلاسمای، تعداد و اندازه‌های کلراید در تمام ماهیان تحت تیمار کورتیزول روند مشابهی با ماهیان تیمار کنترل (ماهیان بدون تیمار کورتیزول) طی ۹ روز پس از انتقال نشان می‌دهند. در نتیجه، تیمار کورتیزول به تنهایی و به طور مستقیم در تنظیم اسمزی دخالت ندارد و تاثیری بر بهبود مقاومت به شوری بچه تاس ماهیان ایرانی ندارد.

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