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Osmoregulatory response of Senegalese sole (*Solea senegalensis*) to changes in environmental salinity

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Abstract

The osmoregulatory response of Senegalese sole (*Solea senegalensis*, Kaup 1858) to 14-day exposure and throughout 17-day exposure to different environmental salinities was investigated. A linear relationship was observed between environmental salinity and gill Na^+,K^+-ATPase activity whereas kidney Na^+,K^+-ATPase activity was unaffected. Two osmoregulatory periods could be distinguished according to variations in plasma osmolality: an adjustment period and a chronic regulatory period. No major changes in plasma osmolality and ions levels were registered at the end of the 14- to 17-day exposure period, indicating an efficient adaptation of the osmoregulatory system. Plasma levels of glucose and lactate were elevated in hypersaline water, indicating the importance of these energy substrates in these environments. Glucose was increased during hyper-osmoregulation but only in the adjustment period. Cortisol proved to be a good indicator of chronic stress and stress induced by transfer to the different osmotic conditions. This work shows that *S. senegalensis* is able to acclimate to different osmotic conditions during short-term exposure.

Keywords: Na^+; K^+-ATPase activity; Cortisol; *S. senegalensis*; Salinity

1. Introduction

The concept of euryhalinity in teleosts refers to those fish that can tolerate a wide range in salinity. More specifically, we can distinguish between fully euryhaline teleosts that are capable of surviving in salinities ranging from freshwater (FW) to high salinity water, and partially euryhaline teleosts that survive in a more limited range of salinities ranging from low to high salinity water.

Fully and partially euryhaline teleosts are able to adjust their osmoregulatory mechanisms in gills, intestine and kidney to cope with changes in environmental salinity. Two different phases in the osmoregulatory response following a change in environmental salinity can be observed: i) an initial adjustment period with changes in osmoregulatory variables (i.e. plasma osmolality and electrolyte concentrations) and ii) a chronic regulatory period, in which these variables reach a new homeostasis (Holmes and Donaldson, 1969; Laiz-Carrión et al., 2005). Nevertheless, the general trend for most marine teleosts, including members of the Bothidae and Pleuronectidae flatfish families, suggests that the short-term tolerance to salinities ranging from as high as 65 to as low as 0–5‰ is high for early larvae. It then decreases dramatically during mid-larval development, and achieves levels of usually relatively high tolerance by the completion of metamorphosis (Schreiber, 2001).

In teleosts acclimated to a hyper-osmotic environment, the Na^+,K^+-ATPase sodium pump in the branchial epithelium is an essential mechanism to remove excess ions from the body (McCormick 2001; Marshall, 2002). In teleosts acclimated to a hypo-osmotic environment, the pivotal mechanisms of ion uptake in the gill are the V-type H^+-ATPase (Lin and Randall, 1993; reviewed by Evans et al., 2005) and a Na^+/H^+ exchanger (NHE). The driving force for ion transport is delivered by transmembrane Na^+- and H^+-gradients that are maintained by Na^+,K^+-ATPase and carbonic anhydrase activities, respectively (Hirose et al., 2003). However, the concept of Na^+ uptake via

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NHE has been questioned on thermodynamic grounds (Kirschner, 2004). Modulation of Na^+,K^+-ATPase activity in gill mitochondria-rich cells (MRC) is essential for acclimation to a changing environmental salinity (Marshall, 2002). Likewise, the acclimation of euryhaline teleosts to different environmental salinities also induces several alterations in kidney, viz. general morphology, capacity of excretion of divalent ions, glomerular filtration rates and urine production including changes in Na^+, K^+-ATPase activity (Beyenbach, 1995; Renfro, 1995).

In euryhaline teleosts, gill Na^+,K^+-ATPase activities correlate with environmental salinity. A linear positive correlation between these parameters is displayed by members of the salmonid group (McCormick, 1995). Conversely, a so-called U-shaped relationship is typical of the mullet-sea bass-goby group (Jensen et al., 1998; Laiz-Carrión et al., 2005; Sangiao-Alvarellos et al., 2005). The time course of changes in gill Na^+,K^+-ATPase activities after transfer to different environmental salinities is species dependent. In general, modifications in the activity in fully and partially euryhaline species are often detected within 2 to 3 days after transfer (Evans and Mallery, 1975; Jacob and Taylor, 1983; Jensen et al., 1998; Laiz-Carrión et al., 2005; Sangiao-Alvarellos et al., 2005) while in anadromous species these appear after 3 to 7 days following transfer (McCormick and Naiman, 1985; Madsen and Naamansen, 1989; Berge et al., 1995).

Cortisol is a multifunctional hormone implicated in hyper-osmotic and hypo-osmotic acclimation (McCormick, 1995, 2001). In addition, cortisol has also an important metabolic role, inducing modifications in protein, carbohydrate and lipid metabolism in osmoregulatory and non-osmoregulatory organs in order to provide the energy necessary to fuel osmoregulatory processes (Mommsen et al., 1999; Laiz-Carrión et al., 2003).

Senegalese sole (*Solea senegalensis*, Kaup 1858) is a marine teleost that inhabits coastal waters and riverine estuaries, and is capable of adapting to substantial changes in environmental salinity and temperature (Imsland et al., 2003). It is a novel species in aquaculture and several studies have focussed on physiological aspects such as reproduction and growth, and pathology (Dinis et al., 1999; Imsland et al., 2003). Since sole aquaculture is mainly restricted to coastal ponds in the southern Iberian Peninsula where they are exposed to a wide salinity variation (Dinis et al., 1999), the understanding of osmoregulatory aspects of this species becomes a valuable tool to improve culture. The aim of this study was to analyze the osmoregulatory response of immature juveniles of *S. senegalensis*. We have employed two different experimental approaches: Solea juveniles were exposed to different environmental salinities, i) followed by the determination, i.e. after 14-days exposure, of selected endpoint parameters, and ii) the time course of changes in osmoregulatory parameters, monitored during 17 days of exposure.

2. Materials and methods

2.1. Experimental protocol

Immature juveniles of Senegalese sole (*S. senegalensis,*, Kaup 1858) were provided by Planta de Cultivos Marinos (CASEM, Universidad de Cádiz, Puerto Real, Cádiz, Spain). Fish were transferred to the wet laboratories at the Faculty of Marine and Environmental Sciences (Puerto Real, Cádiz), where they were acclimated for 14 days to full seawater (SW, 38‰, 1090 mOsm kg^{-1} H_2O) in 400-L tanks in an open system. After this period, fish were used for experiments (see below). The experimental salinities were achieved either by mixing full-strength SW with dechlorinated tap water or by mixing full-strength SW with natural marine salt (Salina de La Tapa, Puerto de Santa María, Cádiz, Spain). The osmolality and electrolyte composition of the water used for the different experimental groups in trials 1 and 2 are shown in Table 1. During the experiments (October–December, 2004) the fish were maintained under natural photoperiod and constant temperature (17.5–18 °C). Fish were fed once daily with commercial dry pellets (Dibaq-Diproteg SA, Segovia, Spain) at a ration of 1% body weight, and were fasted for 24 h before sampling. All experimental procedures complied with the Guidelines of the European Union Council (86/609/EU) and of the University of Cádiz (Spain) for the use of laboratory animals.

Table 1
Osmolality and ionic composition of the water at different salinities used in trials 1 and 2

	Salinity				
	5‰	15‰	25‰	38‰	55‰
Osmolality (mOsm kg^{-1})	140	364	637	1090	1546
Na^+ (mmol L^{-1})	63	169	307	570	780
K^+ (mmol L^{-1})	1.28	3.48	6.27	11.28	15.36
Ca^{2+} (mmol L^{-1})	2.67	5.19	7.88	13	17.72
Mg^{2+} (mmol L^{-1})	6.95	19.46	34.31	57.11	88.65
Cl^- (mmol L^{-1})	77	194	355	588	957

2.2. Preliminary study: short-time survival and lowest salinity tolerance

Immature juveniles of SW-adapted *S. senegalensis* ($n=9$, 44±4 g body mass) were randomly divided into 3 different groups (3 fish per group) and transferred directly to 3 tanks with a 15-L capacity. Each tank contained a different salinity: 0‰ (FW, 0 mOsm kg^{-1} H_2O), 2‰ (52 mOsm kg^{-1} H_2O) and 5‰ (140 mOsm kg^{-1} H_2O). Fish were exposed to a specific environmental salinity for 1 week during which period water recirculated. After 7 days, fish exposed to 2‰ or 5‰ salinity were transferred to 0‰ and 2‰ salinity, respectively, and were maintained kept under these new salinities for another 7 days.

2.3. Trial 1: adaptation to different environmental salinities

Immature juveniles of SW-adapted *S. senegalensis* ($n=50$, 43±2 g body mass) were randomly divided in 5 groups (10 fish per group) and transferred directly to 5 tanks with a 400-L capacity. Each tank contained a different salinity: 5‰ (140 mOsm kg^{-1} H_2O), 15‰ (364 mOsm kg^{-1} H_2O), 25‰ (637 mOsm kg^{-1} H_2O), 38‰ (SW, control, 1090 mOsm kg^{-1} H_2O) and 55‰ (1546 mOsm kg^{-1} H_2O). Fish were anaesthetized with 2-phenoxyethanol (Sigma P1126) at a dose

Table 2
Plasma ion levels in fish acclimated to different environmental salinities for 2 weeks

	Salinity				
	5‰	15‰	25‰	38‰	55‰
Osmolality (mOsm kg^{-1})	312±6	306±5	322±9	327±8	308±5
Na^+ (mmol L^{-1})	161±9	163±4	171±4	156±4	166±6
Cl^- (mmol L^{-1})	153±11	153±9	151±12	140±16	161±12

Data are shown as mean±SEM (n=9–10). Differences between groups are not statistically significant.

of 0.5 mL L^{-1} water and measured for total length (to the nearest mm) and body weight (to the nearest 0.1 g). Fish were exposed to a specific environmental salinity for 2 weeks by recirculating tank water. Water salinity was checked daily and corrected when necessary by the addition of small volumes of either FW or SW. Water samples were collected for the determination of electrolyte composition. During the experimental period, water quality criteria (hardness, oxygen, carbon dioxide, hydrogen sulphide, nitrite, nitrate, ammonia, calcium, chlorine and suspended solids) were assessed: no major changes were observed. At the end of the experimental period fish were anaesthetized with 2-phenoxyethanol (1 mL L^{-1}) and samples were collected as described below.

2.4. Trial 2: time course adaptation to different environmental salinities

Immature juveniles of SW-adapted *S. senegalensis* (n=152, 37±7 g body weight) were randomly divided in 4 groups (n=36 animals per group) and directly transferred to four 400-L tanks with different environmental salinities: 5, 15, 38 (SW, control) and 55‰. Fish were anaesthetized with 2-phenoxyethanol (0.5 mL L^{-1} water) and measured for total length and body weight. Fish were sampled on days 1, 3, 7 and 17 post-transfer. Before transfer, 8 fish were sampled and constituted the pre-transfer control group. At each time point, 9 fish were anaesthetized in 2-phenoxyethanol (1 mL L^{-1}) and samples were collected as described below. Water salinity, electrolyte composition and common water quality criteria were assessed as described in trial 1.

2.5. Sampling procedure

Fish were netted and anaesthetized with 2-phenoxyethanol (1 mL L^{-1}), weighed and sampled. Blood was collected from the caudal peduncle into 1-mL syringes rinsed with a solution containing 25,000 U ammonium heparin per 3 mL 0.9% NaCl. Plasma was separated from cells by centrifugation of whole blood (3 min, 10,000 ×g, 4 °C), snap frozen in liquid N_2 and stored at −80 °C. From each fish, the first gill arch on the dorsal side was excised, dried with absorbent paper, and a biopsy was cut using fine-point scissors. A small biopsy from the posterior portion of the kidney was also taken. Biopsies were placed in 100 µL of ice-cold sucrose-EDTA-imidazole (SEI) buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and frozen at −80°C. The liver was excised and weighed to calculate the hepatosomatic index (HSI).

2.6. Gill and kidney Na^+,K^+-ATPase activities

Gill and kidney Na^+,K^+-ATPase activities were determined using the microassay method of McCormick (1993) with a modification for non-salmonid fish. Gill and kidney tissues were homogenized in 125 µL of SEI buffer to which 0.1% deoxycholic acid was added, and centrifuged at 2000 ×g for 30 s. Duplicate 10 µL homogenate samples were added to 200 µL assay mixture in the presence or absence of 0.5 mmol L^{-1} ouabain in 96-well microplates at 25 °C and read at 340 nm for 10 min with intermittent mixing. Ouabain-sensitive ATPase activity was detected by enzymatic coupling of ATP dephosphorylation to NADH oxidation and expressed as µmol ADP mg $protein^{-1}$ h^{-1}. Total sample protein was measured in triplicate in undiluted samples (Pierce BCA Protein kit, #23225) using bovine serum albumin as a standard. Both assays were run on a microplate reader (EL340i, Bio-Tek Instruments, Winooski, VT, USA) using Delta Soft3 software for Macintosh (BioMetallics Inc., Princeton, NJ, USA).

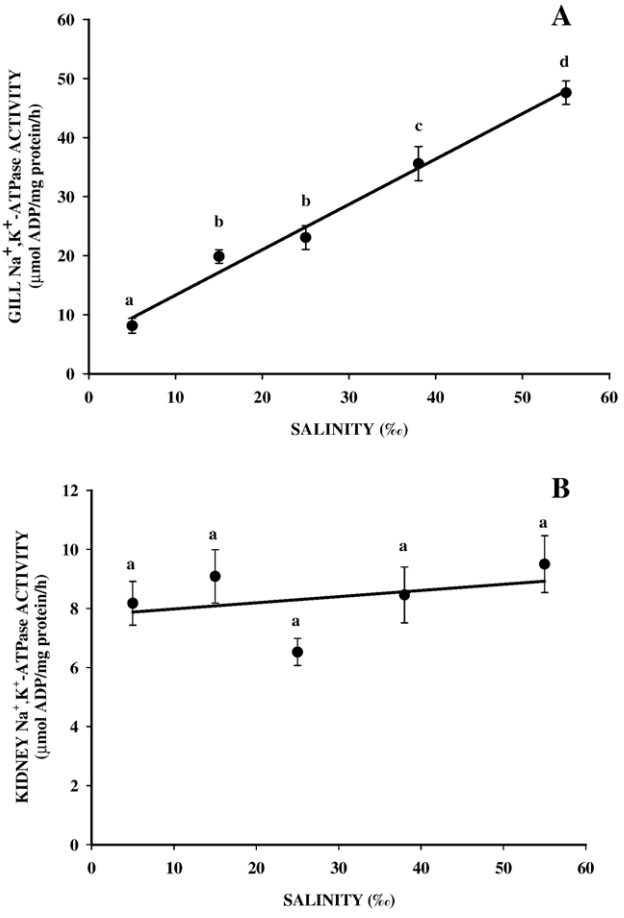

Fig. 1. Effect of different environmental salinities on Na^+,K^+-ATPase activities in gill (A) and kidney (B). Fish were acclimated to different salinities for 2 weeks. Data are expressed as mean±SEM (n=9–10 per group). Different letters indicate significant differences among groups (P<0.05, one-way ANOVA Tukey test).

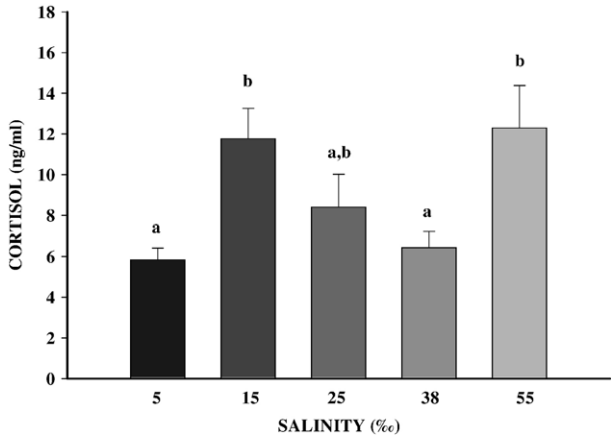

Fig. 2. Effect of different environmental salinities on plasma cortisol levels. See the legend of Fig. 1 for an explanation of the symbols used.

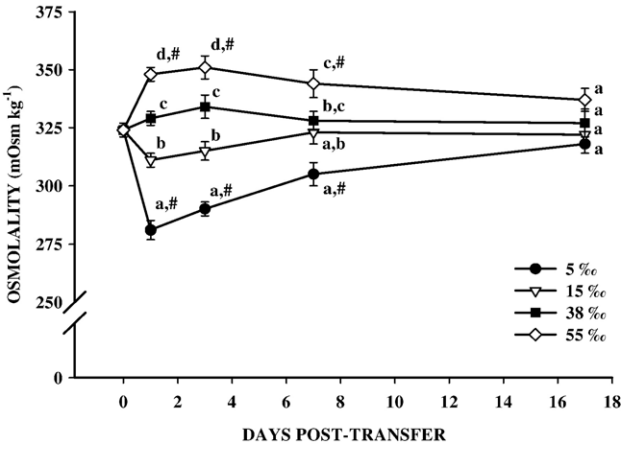

Fig. 3. Time course of changes in plasma osmolality of *S. senegalensis* juveniles after transfer from water of 38 to 38‰ of salinity, hypersaline water (55‰), 15‰-water and low salinity water (5‰). Each point represents mean±SEM of $n=8–9$ fish per group in each sampling time. Different letters indicate significant differences ($P<0.05$) between groups within the same time point. Asterisks indicate significant differences from the previous time point of each treatment. # indicates significant differences from undisturbed fish (pre-transfer control group).

2.7. Blood and water chemistry

Plasma and water osmolality was measured with a vapour pressure osmometer (Fiske One-Ten Osmometer, Fiske, VT, Massachusetts, USA) and expressed as mOsm kg^{-1} H_2O. Water samples were filtered (0.22 μm pore size) prior to analysis. Plasma Na^+ levels were measured using a flame atomic absorption spectrophotometer (UNICAM 939, UNICAM, Cambridge, UK) in samples that were diluted 1:400 (v/v). Water Na^+, K^+, Ca^{2+} and Mg^{2+} levels were measured using atomic plasma emission spectrometry (Iris Intrepid, Thermo Elemental, Franklin, MA, USA) in samples that were diluted 1:1000 (v/v) with double distilled water. Water and plasma Cl^- levels were measured with a chloride Spinreact kit (Ref. 1001360). Plasma glucose and lactate were measured using commercial kits from Spinreact (Glucose-HK Ref. 1001200; Lactate Ref. 1001330) adapted for 96-well microplates. Plasma protein was determined in 1:50 (v/v) diluted plasma samples using the bicinchoninic acid BCA Protein Assay Kit (Pierce #23225). Bovine serum albumin served as a standard. All assays were performed with a Bio Kinetics EL-340i Automated Microplate Reader (Bio-Tek Instruments) using DeltaSoft3 software for Macintosh (BioMetallics Inc.).

2.8. Cortisol radioimmunoassay

Plasma cortisol was measured by radioimmunoassay (RIA) as described by Metz et al. (2005). Briefly, 10 μL 1:5 (v/v) diluted plasma was incubated overnight at 4 °C with 100 μL of first antibody (IgG-F-1; 1:800, Campro Scientific, Veenendaal, The Netherlands), 2000 cpm of ^{125}I-cortisol (Amersham, Buckinghamshire, United Kingdom) and 100 μL of secondary antibody (goat anti-rabbit gamma globulin; 1:320, Campro Scientific, Veenendaal, The Netherlands). All ingredients were dissolved in cortisol-RIA buffer (composition: 0.063 M Na_2HPO_4, 0.013 M Na_2EDTA, 0.02% (w/v) NaN_3, 0.1% (w/v) 8-anilino-1-naphthalene sulfonic acid (Sigma A1028) and 0.1% (w/v) bovine γ-globulin (Sigma G4904)). Immune complexes were precipitated by the addition of 1 mL ice-cold 5% (w/v) polyethylene glycol (PEG-6000, BDH 295774B) and 2% (w/v) bovine serum albumin (Fraction V, Fluka 05475) and subsequently centrifuged (20 min, 2000 ×*g*, 4 °C). Pellets were counted in a gamma counter (1272 Clinigamma, LKB Wallac, Turku, Finland). The antiserum used (first antibody) cross-reacts 100% with cortisol, 5.9% with 11-deoxycortisol, 2.6% with cortisone, 1.7% with corticosterone, 0.16% with cortisone acetate, 0.4% with 17α-OH-progesterone and 0.02% with progesterone.

2.9. Statistics

Differences between groups in trial 1 were tested by one-way analysis of variance (ANOVA) using the environmental salinity

Table 3
Plasma glucose, lactate, and protein levels and hepatosomatic index (HSI) in fish acclimated to different environmental salinities for 2 weeks

	Salinity				
	5‰	15‰	25‰	38‰	55‰
Glucose (mmol L^{-1})	2.61±0.12^a	2.58±0.31^a	2.56±0.13^a	3.01±0.25^a	4.49±0.3^b
Lactate (mmol L^{-1})	0.44±0.04^a	0.54±0.1^a	0.42±0.03^a	0.52±0.07^a	0.77±0.16^a
Proteins (mg mL^{-1})	39.01±1.25^a	39.44±1.48^a	43.89±1.79^a	43.43±1.19^a	43.5±1.54^a
HSI (%)	1.42±0.09^a	1.55±0.18^a	1.58±0.11^a	1.08±0.05^a	1.54±0.05^a

Data are shown as mean±SEM ($n=9–10$). Groups not sharing the same letter are significantly different, $P<0.05$.

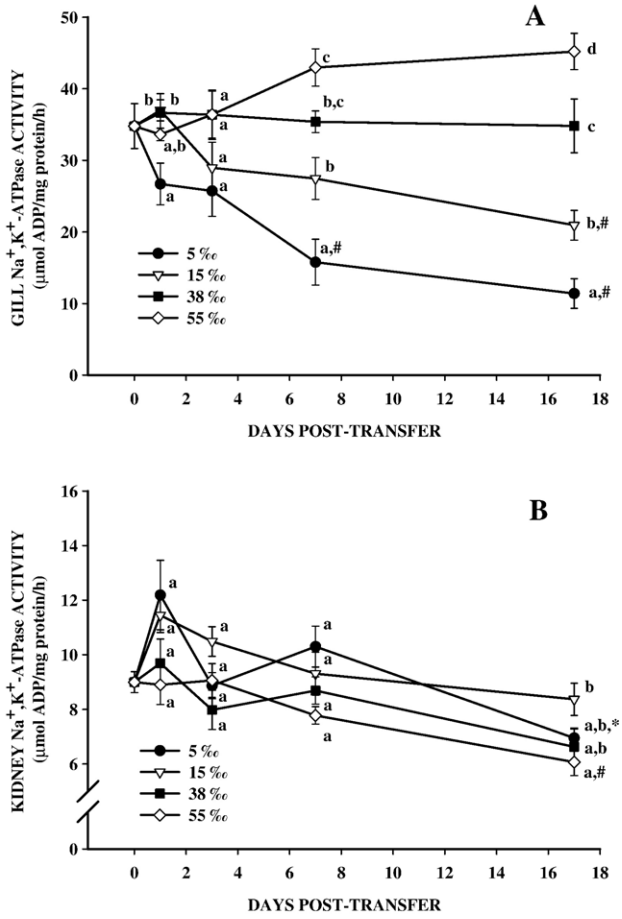

Fig. 4. Time course of changes in gill (A) and kidney (B) Na^+,K^+-ATPase activity of *S. senegalensis* after transfer from water of 38 to 38‰ of salinity, hypersaline water (55‰), 15‰-water and low salinity water (5‰). See the legend in Fig. 3 for an explanation of the symbols used.

as factor of variance. Data from trial 2 were analyzed by a two-way ANOVA using salinity and time as main factors of variance. Tukey's post-test was used to identify significantly different groups. Logarithmic transformations of the data were performed when necessary to fulfil the conditions of the parametric analysis of variance, but data are shown on a linear scale for clarity. When data did not comply with the premises of the parametric ANOVA, data were analyzed using a Kruskal–Wallis ANOVA on ranks, followed by Dunn's multiple comparisons test since group sizes were unequal. Statistical significance was accepted at $P<0.05$.

3. Results

The challenge of juvenile *S. senegalensis* with different external salinities resulted in no mortality either in trial 1 or trial 2.

3.1. Preliminary study — short-time survival and lowest salinity tolerance

Fish transferred from SW to 0‰ (FW) died within 24 h. However, when transferred to 2 and 5‰ salinity, survival was 100%. Fish transferred from 2 to 0‰ died within 24 h, whereas fish transferred from 5 to 2‰ survived for at least 7 days.

3.2. Trial 1 — adaptation to different environmental salinities

Plasma osmolality and electrolyte levels in juveniles of *S. senegalensis* after 14 days of acclimation to different environmental salinities are shown in Table 2. There are no significant effects of salinity on these parameters ($P>0.05$).

Gill Na^+,K^+-ATPase activity correlated linearly and positively with environmental salinity. The correlation is described adequately by the linear regression equation: $y=5.63+0.77x$ ($R^2=0.99$), where y is Na^+,K^+-ATPase activity and x is salinity. The slope is significantly different from zero: $P=7.07\cdot10^{-4}$ (Fig. 1A). Significant differences between groups were observed, except between groups acclimated to 15 and 25‰ of salinity. Kidney Na^+,K^+-ATPase activity did not differ significantly between animals exposed to different salinities. The linear regression equation contains a slope close to that is not significantly different from zero ($P=0.55$): $y=7.77+0.02x$, $R^2=0.13$ (Fig. 1B).

Plasma cortisol was significantly elevated in fish acclimated to 15 and 55‰ compared to fish acclimated to 5 and 38‰ (Fig. 2).

Table 3 shows that plasma glucose levels were significantly higher in fish acclimated to 55‰ compared with those maintained at other salinities. No significant changes were observed in the plasma lactate and protein concentrations, and HSI in fish maintained at different environmental salinities.

3.3. Trial 2 — time course adaptation to different environmental salinities

Transfer from 38 to 38‰ (SW) did not induce significant changes in plasma osmolality (Fig. 3). However, plasma osmolality was significantly elevated in fish transferred to the highest salinity (55‰), whereas those animals at 5 and 15‰

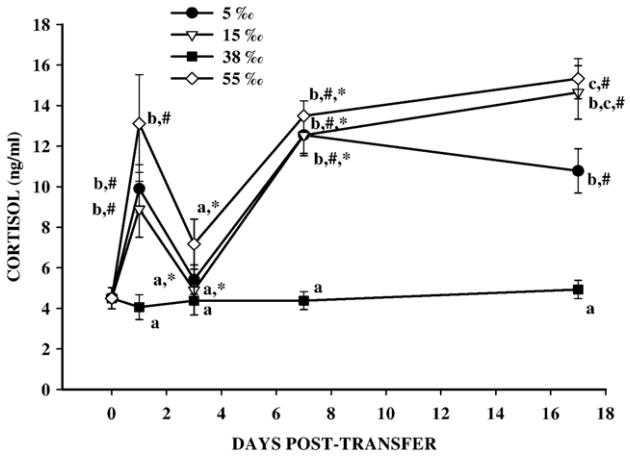

Fig. 5. Time course of changes in plasma cortisol levels of *S. senegalensis* after transfer from water of 38 to 38‰ of salinity, hypersaline water (55‰), 15‰-water and low salinity water (5‰). See the legend in Fig. 3 for an explanation of the symbols used.

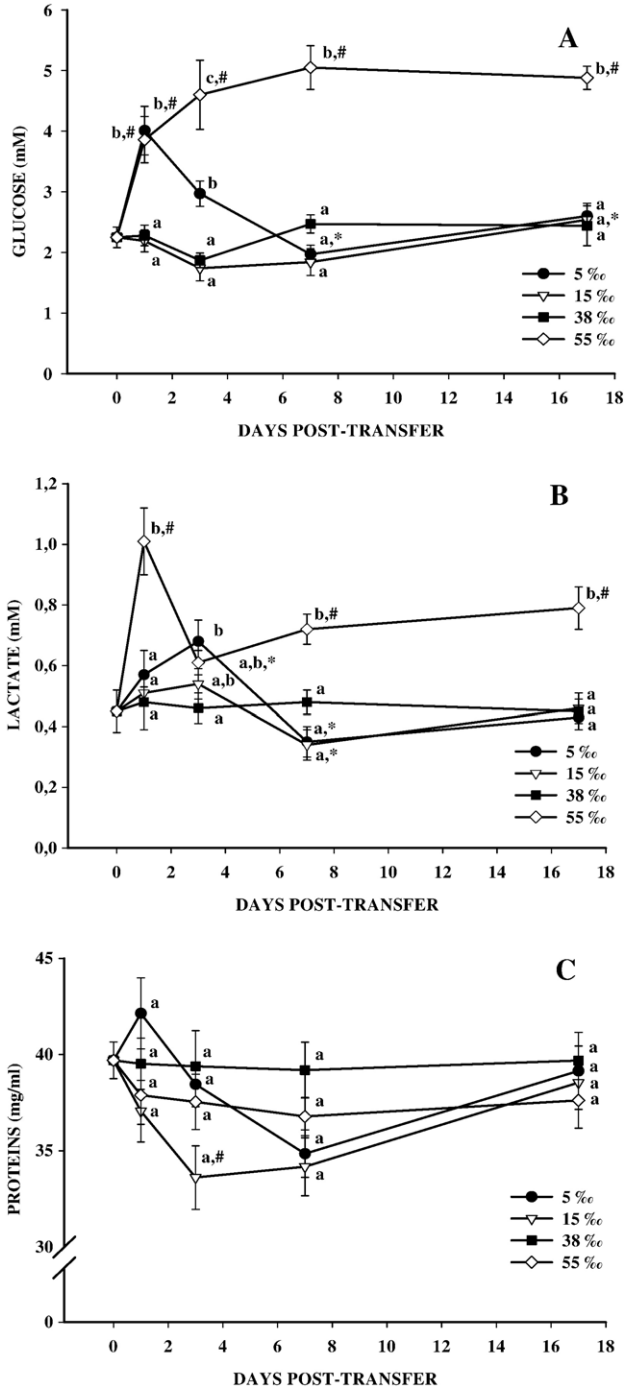

Fig. 6. Time course of changes in plasma glucose (A), lactate (B) and proteins (C) levels of *S. senegalensis* after transfer from water of 38 to 38‰ of salinity, hypersaline water (55‰), 15‰-water and low salinity water (5‰). See the legend in Fig. 3 for an explanation of the symbols used.

showed significantly lowered values compared with SW-transferred fish ($P<0.05$). Changes were transient and differences between groups disappeared at day 17 post-transfer.

Gill Na^+,K^+-ATPase activity did not show any significant changes in fish transferred from 38 to 38‰ during the experimental period (Fig. 4A), but this activity decreased in fish transferred to 5 and 15‰. Statistically significant differences, as compared with undisturbed fish of the pre-transfer control group, were reached at day 7 and 17 post-transfer, respectively ($P<0.05$). Gill Na^+,K^+-ATPase activities in fish transferred to 55‰ were similar compared to animals transferred to 38‰ (SW) until day 3 post-transfer. However, after day 3, these values increased and were higher than those of SW-acclimated fish. Significant differences among groups were observed at day 17 post-transfer showing similar pattern and values to those observed at the end of trial 1 ($P<0.05$).

Kidney Na^+,K^+-ATPase activities did not differ significantly, except at day 17 post-transfer where Na^+,K^+-ATPase activities in fish transferred to 15‰ of salinity were higher than those in fish transferred to 55‰ (Fig. 4B).

Plasma cortisol levels were significantly elevated in fish transferred to 5, 15 and 55‰ at day 1 post-transfer compared with fish transferred to 38‰ and the pre-transfer control group (Fig. 5). At day 3 post-transfer, all groups showed similar plasma cortisol levels but at day 7 cortisol values increased significantly ($P<0.05$) in groups transferred to 5, 15 and 55‰. These values remained significantly higher thereafter.

Plasma glucose levels in fish acclimated to 15 and 38‰ (SW) remained virtually unchanged following transfer (Fig. 6A). This parameter increased in fish transferred to 55‰ and appeared to approach a limiting value of 5 mM. Statistically significant differences between this group and the SW-transferred fish and pre-transfer control group were measured throughout the experimental period ($P<0.05$). On the other hand, fish transferred to 5‰ showed an increase of plasma glucose levels at day 1 post-transfer which was significantly different compared to fish transferred to 38‰ and the pre-transfer control group. From this time point on, glucose levels gradually decreased until reaching basal values at day 7 post-transfer, remaining constant thereafter.

No major changes were observed in plasma lactate levels in fish transferred from 38 to 38‰ (Fig. 6B). Lactate levels increased significantly 2.25-fold at day 1 in fish transferred to highest salinity (55‰). At day 3 and thereafter, lactate levels decreased but remained significantly higher at days 7 and 17 post-transfer compared to the other groups ($P<0.05$). A smaller increase was measured in fish transferred to 5‰ on day 1 and 3 post-transfer, peaking on day 3. However, this time point, lactate levels returned to basal values. No major changes in plasma protein levels were observed between groups during the experimental period (Fig. 6C).

4. Discussion

The main finding of the present study is that immature juveniles of *S. senegalensis* are able to adapt to a wide range of environmental salinities ranging from hypo-osmotic (5‰ salinity) to hyper-osmotic medium (55‰ salinity), in a short-time period. During this process animals are able to adjust osmoregulatory mechanisms reflecting changes in body fluids osmolytes, plasma glucose, lactate, cortisol and gill Na^+,K^+-ATPase activity.

Endpoint plasma osmolality and electrolyte concentrations did not change in different salinities, neither plasma haemo-concentration nor haemodilution was observed, and this points

at the successful achievement of short-term acclimation in ionic regulation. However, transfer of *S. senegalensis* to environmental salinities below 2‰ salinity induced 100% mortality within 24 h, indicating that salinities around 5‰ are at the lowest limit of tolerance for this species. So, *S. senegalensis* is partially euryhaline in juvenile stages, as is the flatfish species *Scophthalmus maximus* in its juvenile phase (Gaumet et al., 1995). However, *Paralichthys orbignyanus*, is capable of surviving a short-term exposure to FW (Sampaio and Bianchini, 2002). This shows that the classification of flatfish as fully or partially euryhaline, at least in the juvenile period, is species dependent and cannot be generalized. In trial 2, the time course of the changes in plasma osmolality are similar to those reported for other teleost species (*Fundulus heteroclitus*: Wood and Marshall, 1994; Marshall et al., 1999; *Sparus sarba*: Kelly and Woo, 1999; *Oreochromis mossambicus*: Lin et al., 2004; *S. auratus*: Mancera et al., 1993; Laiz-Carrión et al., 2005; Sangiao-Alvarellos et al., 2005). From the results obtained in this trial we conclude that the adjustment period in *S. senegalensis* involves approximately 7 days, being the chronic regulatory period registered at day 14 (trial 1) or day 17 (trial 2).

In both trials *S. senegalensis* showed a clear linear relationship between gill Na^+,K^+-ATPase activity and environmental salinity, which agrees with the general pattern found in salmonids and other teleosts (De Renzis and Bornancin, 1984; Marshall, 2002). In hyper-osmotic environments gill Na^+,K^+-ATPase activity drives Na^+ and Cl^- extrusion, which is an essential contribution to the regulation of plasma electrolytes in this environment (Evans et al., 2005). In hypo-osmotic environments, where electrolyte uptake is required, the driving force for Na^+ uptake across the basolateral membrane in branchial MRC is provided by Na^+,K^+-ATPase that, although its activity is down-regulated to basal levels, still plays a pivotal role in Na^+ uptake (Marshall, 2002).

Gill Na^+,K^+-ATPase activity decreased after 1 to 3 days in fish transferred to low salinity waters (5 and 15‰) compared to SW-transferred animals. This decrease reflects a down-regulation of ion extrusion and the development of ion uptake systems in a hypo-osmotic medium (5‰) which require basal levels of gill Na^+,K^+-ATPase activity to carry out this function (Evans et al., 2005). In addition, acclimation to this hypo-osmotic medium requires an up-regulation of the electrolyte uptake system, with driving forces generated by the gill V-type H^+-ATPase pump to restore plasma osmolality during the adjustment period in *S. senegalensis*. However, no consistent measurements of bafilomycin-sensitive ATPase activity, which reflects gill V-type H^+-ATPase activity (Marshall, 2002), have been obtained in this species (personal observation) and we therefore have no indication whether or not *S. senegalensis* is able to actively absorb ions in hypo-osmotic environments. It is very well feasible that this species maintains its ion balance by decreasing ion efflux. Still, kidney Na^+,K^+-ATPase activities were largely unaffected in both trials. Similar observations were reported for *S. auratus* (Sangiao-Alvarellos et al., 2005). In fish kidney, the sodium pump is involved in ion extrusion and uptake in hyper-osmotic environments, the specific mode of action depends on the anatomical location in the nefron (Beyenbach, 1995; Dantzler, 2003). In our study, kidney biopsies were taken to analyze Na^+,K^+-ATPase activity without distinguishing between different areas of the nefron. Thus, in *S. senegalensis* kidney could occur, at least in transfer to 55‰ of salinity, that kidney Na^+,K^+-ATPase activity increase or decrease in different areas of the nefron resulting in no variation of this activity.

Plasma cortisol levels are generally accepted as a good indicator of stress in teleostean fish (Wendelaar Bonga, 1997; Barton, 2002). In this context, environmental salinity can be considered a stressor that induces a primary stress response and the release of cortisol into the circulation (Mommsen et al., 1999; Barton, 2002). Results from a standard handling test (5 min air exposure) followed by blood sampling after 30 min, indicated that plasma cortisol levels in *S. senegalensis* were increased 5.5-fold compared with unstressed fish which showed similar levels to the control group (transfer to 38‰) in trial 1 and 2 of this work (data not shown). In post-larval *S. senegalensis*, plasma cortisol is increased after a disturbance stressor, demonstrating that cortisol is a bona fide stress indicator in this species (Ruane et al., 2005). Our results indicate that, after salinity change, two periods in the cortisol response of *S. senegalensis* can be distinguished: i) an increase within one day associated with the stress imposed by the transfer to different osmotic conditions; and ii) an increase from day 7 onwards associated with a chronic stress situation. Changes in plasma cortisol levels on day 1 induced by transfer to waters of 5, 15 and 55‰ of salinity could be the signal to stimulate the hypothalamic-pituitary-interrenal axis ultimately resulting in the increase of plasma cortisol levels observed in *S. senegalensis* transferred from 38‰ to hypersaline water, as has been reported for other teleosts (*O. mossambicus*: Morgan et al., 1997; *F. heteroclitus*: Marshall et al., 1999; Scott et al., 2004; *Oncorhynchus mykiss*: Richards et al., 2003; *S. auratus*: Laiz-Carrión et al., 2005; Sangiao-Alvarellos et al., 2005) or from 38 to 5 and 15‰ of salinity as is described for other teleosts too (reviewed in McCormick, 2001). The stability of plasma cortisol levels in the control group showed that the effect of salinity was the only source of disturbance to evoke a stress response in *S. senegalensis*. On the other hand, chronic stress situations result in the elevation of plasma cortisol concentrations (Rotllant and Tort, 1997). *S. senegalensis* show from day 7 post-transfer increased plasma cortisol levels in waters different than 38‰, reflecting a chronic stress situation although this fact is not clear in 5‰-water since cortisol levels were similar to control group in trial 1.

Cortisol is the major corticosteroid produced by interrenal gland in fish and functions both as a glucocorticoid and mineralcorticoid (Greenwood et al., 2003). In the acclimation to SW, cortisol promotes salinity tolerance, development and proliferation of MRC, gill Na^+,K^+-ATPase activity, and expression/abundance of Na^+,K^+-ATPase α-subunit and Na^+-K^+-$2Cl^-$ cotransporter (McCormick, 1995, 2001; Sakamoto et al., 2001). Likewise, current evidence suggests that the primary mode of action of cortisol is cytogenic, mediating the growth and differentiation of MRC in the branchial epithelium in both SW and FW. Thus, a rise in plasma cortisol during day 1 could stimulate Na^+,K^+-ATPase activity enhancement observed in the

transfer to water of 55‰ of salinity. These changes are reflected in the chronic regulatory period where an increase in gill Na^+,K^+-ATPase activity is observed (day 7–17 post-transfer) and reflect the time required to let MRC to proliferate. Further studies on MRC proliferation and abundance will be necessary in order to test this fact (see McCormick 1995, Marshall, 2002; Laiz-Carrión et al., 2005).

The high plasma glucose and lactate levels are indicative of a secondary stress response. In the time course study (trial 2) the higher increased glucose levels immediately observed in fish transferred to extreme salinities (5 and 55‰) at day 1 are probably maintained by glycogenolysis stimulated by catecholamines and/or cortisol proper (adjustment period) which could play a role in the immediate production of glucose (Mommsen et al., 1999). In addition, high plasma glucose levels observed in the chronic regulatory period of fish transferred to water of 55‰ of salinity could reflect a high hepatic glyconeogenic activity resulting in elevated plasma glucose levels (van der Boon et al., 1991; Wendelaar Bonga, 1997). The consistently increased glucose levels after transfer to 55‰ suggest a higher energy demand for osmoregulation in this environment. Plasma glucose levels can thus indicate stress in hypersaline environments. Gills are the major osmoregulatory organ in fish acclimated to a high environmental salinity and increased energy expenditure is required to perform osmoregulatory work (Sangiao-Alvarellos et al., 2003, 2005). This also could be the case in *S. senegalensis* where glucose could be used to, ultimately, fuel the energy demand of the increased gill Na^+,K^+-ATPase activities which have been shown at this salinity.

Lactate is a metabolite that can be used in osmoregulatory tissues (i.e. gills and kidney) as an energy substrate (Mommsen, 1984; Mommsen et al., 1985). The increased lactate levels observed in *S. senegalensis* acclimated to 55‰ are in agreement with reported data for *S. auratus* (Sangiao-Alvarellos et al., 2003, 2005) and *O. mossambicus* (Vijayan et al., 1996) that were acclimated to a similar salinity. This suggests that lactate becomes more important during hyper-osmotic acclimation, presumably reflecting its metabolic use in osmoregulatory organs. Therefore, lactate is a good stress indicator in environments with high salinity. The peak in plasma lactate levels measured during the adjustment period could indicate a higher anaerobic metabolic rate caused by osmotic stress, more so than in the chronic regulatory period where plasma osmolality is restored to lower levels. After transfer to low salinity (5‰), a slight increase in plasma lactate levels in the adjustment period was measured which was maximal at day 3 post-transfer. This increase suggests an increase of anaerobic metabolic rate that could be related to osmotic stress in hypo-osmotic environments.

In summary, *S. senegalensis* can be defined as partially euryhaline in the juvenile phase where it displays an effective regulation of gill Na^+,K^+-ATPase activity. Changes in salinity elicited plasma cortisol changes related to stress response as well as metabolic changes reflected in alterations in plasma glucose and lactate, the direction of the effect depending on the salinity, and can be related to the energy repartitioning process occurring in osmotic acclimation.

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