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Tertiary stress responses in Senegalese sole (*Solea senegalensis* Kaup, 1858) to osmotic challenge: Implications for osmoregulation, energy metabolism and growth

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ABSTRACT

The effects of three different environmental salinities (15, 25 and 39‰) on growth, osmoregulation and energy metabolism of juvenile Senegalese sole (*Solea senegalensis* Kaup, 1858) after a period of 10 weeks were investigated. Immature sole (n=150, 39±1 g mean initial body weight) were randomly divided in 6 groups of 25 fish and reared under three different environmental salinities in an open system. Growth, weight gain, specific growth rate and estimated feed intake decreased in salinities lower than 39‰, with the most profound effects observed at 15‰. Branchial Na^+,K^+-ATPase activity correlated positively with environmental salinity, while renal Na^+,K^+-ATPase activity was not altered. Plasma electrolyte concentrations did not change in the salinity range tested, indicating that osmoregulatory capacities were unaffected. Plasma cortisol levels were higher in salinities different than 39‰ (15>25>39‰). Plasma glucose, non-esterified fatty acid, triglyceride, and protein levels were decreased in the lower salinities (25 and 15‰), whereas that of lactate was increased two-fold in the 15‰-exposed group only. Measured in tissues (liver, gill, kidney, white muscle), the most profound changes in metabolite levels were generally found in the group exposed to 15‰, compared to the 25‰- and 39‰-groups. A similar pattern was found for the activities of enzymes involved in energy metabolism. Taken together, our data suggest that the poor growth rate of 15‰-exposed fish is caused by decreased feed intake and the subsequent reallocation of energy sources that was non-sufficient to keep similar growth in fish exposed to 15‰ salinity compared with fish exposed to 25 and 39‰ salinity.

1. Introduction

Physiological responses of fishes to environmental stressors such as changes in environmental salinity have been grouped broadly as primary, secondary and tertiary (Barton, 2002). Primary responses involve the activation of the sympathetic nervous system, resulting in the release of catecholamines from chromaffin tissue (Reid et al., 1998), and the stimulation of the HPI axis culminating in the increase of plasma cortisol (Wendelaar Bonga, 1997). Secondary responses usually are defined as the immediate actions and effects of these hormones at the tissue level (Mommsen et al., 1999). Tertiary responses extend to the level of the organism and population and refer to aspects of whole-animal performance such as changes in growth (Wedemeyer et al., 1990).

Fish growth is affected by environmental factors such as salinity, photoperiod and temperature (Brett, 1979; Simensen et al., 2000; Boeuf and Payan, 2001; Imsland et al., 2001). The effects of salinity on growth have been investigated in a wide number of species (reviewed by Boeuf and Payan, 2001) including flatfish (*Scophthalmus maximus*: Gaumet et al., 1995; Imsland et al., 2001; *Paralichthys orbignyanus*: Sampaio and Bianchini, 2002; *Hippoglossus hippoglossus*: Imsland et al., 2008). The effects of salinity on growth rate may be secondary to the increased energy costs of osmotic and ionic regulation that limits the energy available for growth (Kirschner, 1995; Laiz-Carrión et al., 2005). However, alternative explanations that involve the influence of environmental salinity on feed intake (Imsland et al., 2001, 2008), metabolic reorganization (Sangiao-Alvarellos et al., 2003, 2005), and stimulation of osmoregulatory hormones related to growth (McCormick, 2001) should also be considered.

Cortisol is a potent gluco- and mineralocorticoid in teleostean fish (Mommsen et al., 1999; McCormick, 2001), and during long-term exposure to a stressor, plasma cortisol levels can be observed to either return to basal levels (Vijayan and Leatherland, 1990), or to remain

Abbreviations: ACTH, Adrenocorticotropic hormone; Ala-AT, Alanine aminotransferase (EC 2.6.1.2); ANOVA, Analysis of variance; BSA, Bovine serum albumin; CRF, Corticotropin-releasing factor; EDTA, Ethylenediamine tetraacetic acid; FBPase, Fructose 1,6-bisphosphatase (EC 3.1.3.11); G6PDH, Glucose 6-phosphate dehydrogenase (EC 1.1.1.49); GDH, Glutamate dehydrogenase (EC 1.4.1.2); GPase, Glycogen phosphorylase (EC 2.4.1.1); HK, Hexokinase (EC 2.7.1.11); HPI, Hypothalamus-pituitary-interrenal; HSI, Hepatosomatic index; LDH-O, Lactate dehydrogenase-oxidase (EC 1.1.1.27); NEFA, Non-esterified fatty acids; Ppm, Part per million; SEI, Sucrose-EDTA-imidazole; SGR, Specific growth rate; SW, Seawater.

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elevated throughout (Pickering, 1993). The second scenario can convert into the first depending on the type of stressor, intensity and length of exposure. Plasma cortisol levels are modulated by changes in target tissue receptors, tissue uptake and catabolism of the hormone (Mommsen et al., 1999). Exposure to a stressor induces an allostatic state and invokes tertiary stress responses in an animal. The allostatic state is characterized by an altered and sustained activity of primary mediators of the stress response such as cortisol (McEwen and Wingfield, 2003). During the allostatic state, homeostatic imbalance is tolerated for limited periods of time only if feed intake and/or energy stores can fuel homeostatic mechanisms. This is at the cost of the energy budget that is destined for growth, however.

The effects of environmental salinity on energy metabolism in teleosts have recently been reviewed by Soengas et al. (2007). Most studies refer to periods of 2–3 weeks in which effects in growth were not reported. The long-term response to an osmotic challenge and the relationship between changes in energy metabolism rate and growth is still not fully elucidated.

Senegalese sole (*Solea senegalensis* Kaup, 1858) is a member of the Soleidae and is, at least in juvenile phase, partially euryhaline (Arjona et al., 2007). This species is increasingly incorporated into commercial aquaculture production sites in Europe, mainly due to its high acceptance by consumers and its potential as a substitute species for gilthead sea bream (*Sparus auratus*) and sea bass (*Dicentrarchus labrax*) in land-based facilities. Nevertheless, growth and survival from juvenile to market-size fish revealed deficiencies with regard to rearing technology and husbandry conditions (Costas et al., 2008). In the southern Iberian Peninsula, the culture of *S. senegalensis* is mainly restricted to coastal ponds where fishes are exposed to wide natural variations in salinity (Dinis et al., 1999; Imsland et al., 2003). Osmoregulatory as well as primary and secondary stress responses to salinity changes have been assessed previously in juvenile *S. senegalensis* (Arjona et al., 2007) but tertiary responses, reflected in growth rate, have not been addressed yet. The objective of this study is to analyse the influence of three different environmental salinities (15, 25 and 39‰) on osmoregulatory parameters, energy metabolism and growth of *S. senegalensis* juveniles. We selected these environmental salinities following our previous results on the osmoregulatory response of *S. senegalensis* to changes in environmental salinity (Arjona et al., 2007). Insights into the effects of environmental salinity on *S. senegalensis* growth and energy metabolism will provide a valuable tool to improve culture of this species.

2. Materials and methods

2.1. Fish and experimental conditions

Juveniles of *S. senegalensis* (n=150, 39±1 g body weight at the beginning of the experiment) acclimated to SW (39‰ salinity) were provided by ACUINOVA S.L. (San Fernando, Cádiz, Spain) in whose facilities the experiment was undertaken. Three different environmental salinities (15, 25 and 39‰) were used; the 15 and 25‰ salinities were obtained by mixing full-strength SW with dechlorinated tap water. Fish were maintained in 400-l tanks in an open system. Water osmolalities and ionic compositions in the experimental groups are shown in Table 1. Water quality criteria (hardness, and levels of O_2, CO_2, H_2S, NO_2^-, NO_3^-, NH_4^+, Ca^{2+}, Cl_2 and suspended solids) were monitored and no major changes were observed during the experiment. Water salinity was checked daily and, when necessary, adjusted to the nominal salinity by regulation of the flux of dechlorinated tap water or SW. The salinities in the 39, 25 and 15‰ groups varied from 37 to 40‰, 24 to 26‰, and 13 to 16‰, respectively.

The experiment was performed during a period of 71 days (July–September, 2005). Fish were maintained under natural photoperiod and water temperature (21–27 °C). Daily maximum and minimum water temperatures were recorded using a thermometer; no differences in water temperature were observed between experimental tanks. Fish were fed once daily with commercial dry pellets (Dibaq-Diproteg SA, Segovia, Spain) at a ration of 1.5% of the estimated body weight. Feed composition was: 52% crude protein, 20% crude fat, 9.4% nitrogen free extract, 9% humidity, 8% ash, 1.1% total phosphorous, and 0.5% fibre (24.5 MJ/kg of feed). Tanks were cleaned daily with a siphon. The presence of feed pellet remnants on the bottom of the tanks was recorded daily allowing only an estimate of feed intake. Fishes were not fed during the 24 h before sampling for analytical procedures. Twice per week, precautionary formol baths (225 ppm for 1 h) were applied in order to prevent diseases. Vigorous aeration was provided during the formol baths and water levels of O_2 as well as CO_2, H_2S, NO_2^-, NO_3^-, NH_4^+, Ca^{2+}, Cl_2 and suspended solids were checked at the end of the baths, no major changes were registered. The experimental procedures described comply with the Guidelines of the European Union Council (86/609/EU), the Spanish government (RD 1201/2005) and the University of Cádiz (Spain) for the use of laboratory animals.

2.2. Experimental design

SW-acclimated fish were divided randomly into six groups consisting of three duplicates (n=25 animals per group) and transferred directly to 6 open system tanks of 400 l containing SW (39‰). After an initial acclimation period (15 days), salinity was gradually decreased by 2 salinity points per hour to 15‰ or 25‰ in two pair of tanks in open systems, whereas in the remaining two tanks salinity remained at 39‰. Day 1 constitutes the time point on which the desired salinities were attained. Fish were sampled for total length and body weight on day 1 and days 14, 32, 46, 62 and 71. At the end of the experiment (day 71), 10 fish from each tank were randomly selected (20 fish per salinity condition) and sampled for analytical procedures. In order to evaluate the effects of salinity on *S. senegalensis* growth, the following parameters were calculated: weight gain (%) and SGR, calculated as $SGR = 100[\ln(W_f) - \ln(W_i)]/T$ where T is the number of days from the start until the end of the experiment (71 days).

2.3. Sampling

For biometric sampling, which required the recovery from anaesthesia, all fish of each tank were netted, slightly anaesthetized in 0.05% (v/v) 2-phenoxyethanol, weighed and measured for total length after which fish were placed back into their appropriate tanks. At the end of the experiment, fish (20 per salinity condition) were captured and anaesthetized in 0.1% (v/v) 2-phenoxyethanol and sampled for analytical procedures. Mixed arterial and venous blood was collected from the caudal peduncle into 1-ml heparinized syringes. 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 until further analysis. Fish were killed by spinal transection. 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

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

Parameter	Salinity		
	15‰	25‰	39‰
Osmolality (mOsm/kg)	364	690	1068
Na^+ (mM)	227	332	513
K^+ (mM)	5	7	12
Ca^{2+} (mM)	6	8	12
Mg^{2+} (mM)	26	38	59
Cl^- (mM)	204	352	569

Data refer to water sampled at the end of the experiment (day 71).

was taken as well. Biopsies were placed in 100 µl of ice-cold SEI buffer: 150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3 (Zaugg, 1982); frozen in liquid N_2 and stored at −80 °C until the assessment of Na^+,K^+-ATPase activities. Liver, the remaining kidney and branchial arches, and a portion of white muscle from the upper region, between the dorsal and pectoral fins (above the lateral line) of the ocular side, were removed quickly from each fish, frozen in liquid N_2, and stored at −80 °C until assay. Liver was weighed to calculate the HSI ([liver wet weight/body weight]·100).

2.4. Analytical techniques

2.4.1. Water and plasma measurements

Plasma and water osmolalities were measured with a vapour pressure osmometer (Fiske One-Ten Osmometer, Fiske, VT, USA) and expressed as mOsm/kg. Water samples were filtered (0.22 µm of pore size) before water chemistry determination. Plasma Na^+ levels were measured using a flame atomic absorption spectrophotometer (UNICAM 939, UNICAM, Cambridge, UK) in diluted samples 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) after diluting the samples 1:1000 (v/v) with doubly distilled water. Water and plasma Cl^- levels were measured with the chloride Spinreact kit (Spinreact SA, Sant Esteve d'en Bas, Girona, Spain). Plasma glucose, lactate and triglycerides were measured using commercial kits from Spinreact SA (Sant Esteve d'en Bas, Girona, Spain) adapted to 96-well microplates, while plasma NEFA levels were determined via the NEFA-C method (Wako Chemicals, Neuss, Germany), according to the manufacturer's instructions. Plasma proteins were measured in diluted plasma samples 1:50 (v/v) with the bicinchoninic acid method with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) using BSA as a standard. All assays were performed with a PowerWave™ 340 microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA) using KCjunior Data Analysis Software for Microsoft® Windows XP. Plasma cortisol was measured by radioimmunoassay as described by Metz et al. (2005) which method we previously used in *S. senegalensis* (Arjona et al., 2007).

2.4.2. Na^+,K^+-ATPase activity

Gill and kidney Na^+,K^+-ATPase activities were determined in biopsies following the method of McCormick (1993) adapted to microplates. The conditions of the assay used in *S. senegalensis* have been described previously (Arjona et al., 2007).

2.4.3. Tissue preparations

Frozen liver, kidney, gills and white muscle were finely minced on an ice-cold petri dish, vigorously mixed and divided into two aliquots to assess enzyme activities and metabolite levels respectively. The tissues used for the assessment of metabolite levels were homogenized by ultrasonic disruption in 7.5 vol. ice-cold 6% (w/v) perchloric acid, after which the homogenate was neutralized using the same volume of 1 M $KHCO_3$. The homogenates were centrifuged (30 min, 13,000 *g*, 4 °C) and the supernatants were stored in different aliquots at −80 °C until use in the different metabolite assays. The tissues used for the assessment of enzyme activities were homogenized by ultrasonic disruption in 10 vol. of ice-cold stopping-buffer containing 50 mM imidazole-HCl (pH 8.5), 1 mM 2-mercaptoethanol, 50 mM NaF, 4 mM EDTA, 250 mM sucrose, and 0.5 mM p-methyl-sulphonyl-fluoride (Sigma Chemical Co., St. Louis, MO, USA), the latter added as dry crystals immediately before homogenization. The homogenates were centrifuged (30 min, 13,000 *g*, 4 °C) and the supernatants were stored in different aliquots at −80 °C until use in enzymatic assays.

2.4.4. Tissue metabolite levels

Tissue lactate and triglyceride levels were determined spectrophotometrically using commercially available kits (Spinreact SA, Sant Esteve d'en Bas, Girona, Spain) adapted to 96-well microplates. Tissue glycogen concentrations (determined in liver and white muscle) were assessed using the method described by Keppler and Decker (1974). Glucose obtained after glycogen breakdown (after substracting free glucose levels) was determined with commercially available kits (Spinreact SA, Sant Esteve d'en Bas, Girona, Spain); the same kit was used to measure glucose levels in gills and kidney in which glycogen was not determined. Total α-amino acid levels were assessed in liver and white muscle using the nynhidrin method described by Moore (1968) adapted to 96-well microplate format. Spectrophotometric determinations were performed with a PowerWave™ 340 microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA) using KCjunior Data Analysis Software for Microsoft® Windows XP.

2.4.5. Enzymatic activities in tissue homogenates

Enzymatic activities were determined in coupled assays using a PowerWave™ 340 microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA) in a 96-well microplate format. Reaction rates of enzymes were assessed by changes in the absorbance of NAD(P)H at 340 nm. The reactions were started by the addition of homogenates (15 µl) in duplicate at a pre-established protein concentration to 250 µl assay mixture. Substrate for the different reactions assayed was omitted in control incubations. All assays were performed in duplicate at 37 °C and readings were monitored during pre-established times (5–15 min), with intermittent stirring between each reading. The calculation of reaction rates was performed using KCjunior Data Analysis Software for Microsoft® Windows XP. Rates are expressed as U/mg protein where U (unit of enzyme) is defined as µmol of substrate utilized or produced per min. Homogenate protein was assayed in triplicate with the Pierce BCA Protein kit (Pierce, Rockford, IL, USA) using BSA as a standard. Measurements of enzyme activities were all carried under optimal conditions, as defined in preliminary tests, so as to achieve maximum rates. The specific conditions for enzymatic assays were adapted to *S. senegalensis* from methods described for salmonids (Soengas et al., 1996, 1998) and gilthead seabream (Laiz-Carrión et al., 2003; Polakof et al., 2006), and were as follows:

Hepatic Ala-AT was assessed in 50 mM imidazole-HCl (pH 7.8), 0.025 mM pyridoxal 5′-phosphate, 10 mM α-ketoglutaric acid and 0.2 mM NADH; excess LDH (Sigma Chemical Co., St. Louis, MO, USA) and 2.36 mM L-alanine as substrate.

Hepatic FBPase was determined in 85 mM imidazole-HCl (pH 7.7), 0.5 mM NADP and 5 mM $MgCl_2$; excess phosphoglucose isomerase and G6PDH (Sigma Chemical Co., St. Louis, MO, USA); 0.1 mM fructose 1,6-bisphosphate served as the substrate.

Hepatic and branchial G6PDH was assayed in 78 mM imidazole-HCl (pH 7.7), 5 mM $MgCl_2$ and 0.5 mM NADP, and 1 mM glucose 6-phosphate as substrate.

GDH activities in liver and white muscle were assessed in 50 mM imidazole-HCl (pH 7.8), 250 mM ammonium acetate, 0.1 mM NADH and 1 mM ADP, and 0.5 mM (for liver) or 2 mM α-ketoglutaric acid (for white muscle) as the substrate.

Total GPase activities in liver and white muscle were measured in 50 mM phosphate buffer (pH 7), 27 mM $MgSO_4$, 1.61 mM EDTA, 0.5 mM NADP and 2.5 mM AMP; excess phosphoglucomutase and G6PDH (Sigma Chemical Co., St. Louis, MO, USA), 0.1 mM glucose 1,6-bisphosphate as cofactor (Sigma Chemical Co., St. Louis, MO, USA) and 170 µg/ml glycogen as substrate for the reaction.

HK in gills, kidney and white muscle was assayed in 50 mM imidazole-HCl (pH 8), 5 mM $MgCl_2$, 0.15 mM NADP and 1 mM ATP; excess G6PDH (Sigma Chemical Co., St. Louis, MO, USA) and 5 mM glucose as substrate.

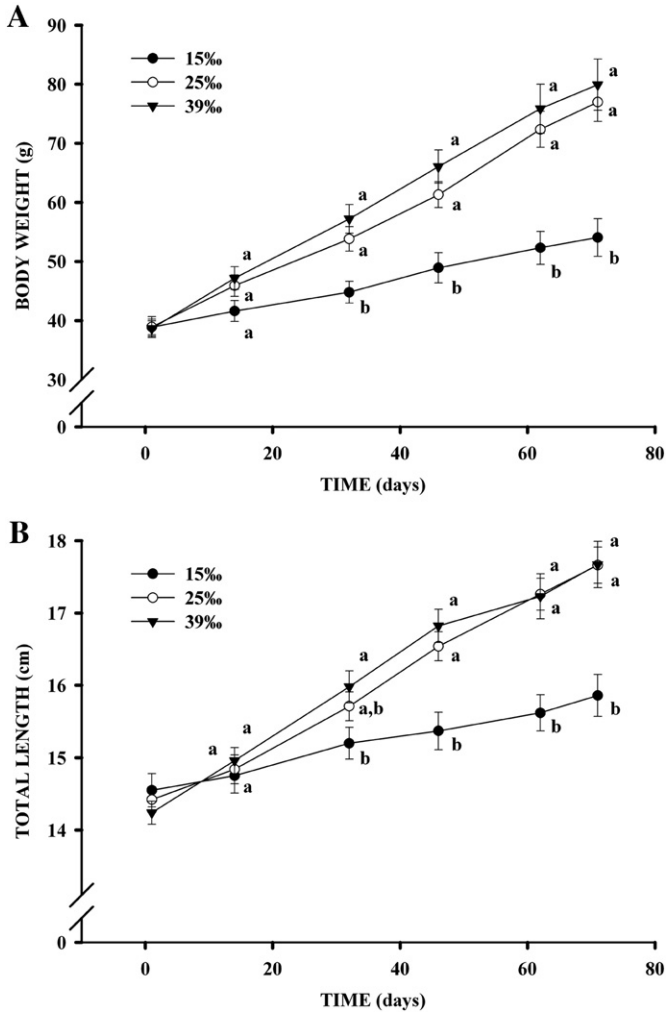

Fig. 1. Changes in body weight (A) and total length (B) over 71 days in *S. senegalensis* juveniles exposed to 3 different salinities. Data points represent mean±S.E.M. (n=50 per group at each sampling time). Different letters indicate significant differences among groups at each time (P<0.05, one-way ANOVA Tukey test).

LDH-O was determined in gills, white muscle and kidney in 50 mM imidazole-HCl (pH 8.5) and 2.5 mM NADP; and 2.35 mM (for white muscle) or 2.95 mM lactic acid (for gills and kidney) as substrate for the reaction.

2.5. Statistics

Differences between groups were tested by one-way ANOVA using salinity as a factor of variance. When appropriate, data were logarithmically transformed to fulfil the requirements for ANOVA but data are shown in their decimal values for clarity. When ANOVA yielded significant differences, Tukey's post-test was used to identify significantly different groups. When data did not comply with the premises of the parametric ANOVA, data were analysed using a Kruskal–Wallis ANOVA on ranks. Statistical significance was accepted at P<0.05. Since no significant differences were found between duplicate experimental tanks at similar sampling points (as judged from Student's t-test), duplicate data were pooled.

3. Results

No mortality and pathologies were observed in any group throughout the experimental period. Changes in water temperature among salinities were not observed (data not shown).

S. senegalensis juveniles exposed to 15‰ salinity presented lower growth compared to animals kept at 39 and 25‰ (Fig. 1A and B). Differences between groups became statistically significant on day 32 and remained so until the end of the experiment (P<0.05). In addition, fish maintained at 15‰ salinity had a 60% reduction in weight gain and SGR than fish exposed to 39 and 25‰. Remarkably, animals kept at 15‰ salinity eat the entire daily ration on 19 out of 71 days. In animals kept at 25 or 39‰ this figure was 61/71 and 70/71, respectively. These observations allowed an estimation of the feed intake in the different salinities that was decreased overall in fish exposed to 15‰. No significant differences between HSI were observed (Table 2).

Plasma osmoregulatory parameters were similar in all groups except for Cl^- which was significantly lowered in 25‰-exposed fish (Table 3). Gill Na^+,K^+-ATPase activity increased concomitantly with environmental salinity, whereas kidney Na^+,K^+-ATPase activity did not differ between groups (P>0.05). Plasma cortisol levels were higher in 15‰- and 25‰-exposed fish compared with 39‰-group, with the highest levels in 15‰-exposed fish (Fig. 2).

Plasma metabolite concentrations are presented in Table 4. Plasma glucose was significantly lower in fish exposed to environmental salinities lower than 39‰ (P<0.05). Plasma lactate was increased approximately 2-fold in 15‰-exposed fish compared to those kept at 25 and 39‰. Plasma triglyceride levels decreased linearly with decreasing environmental salinity, the same as NEFA levels. Finally, plasma protein concentration was significantly lowest in 15‰-exposed fish.

Liver metabolite concentrations and enzyme activities are shown in Table 5. Glycogen levels decreased significantly in 15‰-exposed fish whereas glucose concentrations increased in fishes exposed to salinities of 15 and 25‰. Triglycerides, proteins and total α-amino acids were not affected by environmental salinity. All enzyme activities assessed were altered by environmental salinity except G6PDH. FBPase activity enhanced by 27 to 48% in the 15‰-exposed fish compared to the other groups. Total GPase activities had increased in salinities lower than 39‰. Finally, hepatic Ala-AT activities were higher in fishes exposed to salinities different than 39‰ (15>25>39‰), whereas GDH activity significantly increased in 15‰-exposed fish only (P<0.05).

Branchial metabolite concentrations and enzyme activities are presented in Table 6. Glucose and lactate levels were highest in fishes exposed to 15‰ salinity, but triglyceride and protein values were not affected notably by environmental salinity. HK and LDH-O activities increased by 11 to 19% in fish exposed to 15‰ salinity compared with 25‰- and 39‰-exposed fish. G6PDH activities were similar in 15‰- and 25‰-exposed fish, and enhanced more than 2-fold compared with the 39‰-exposed group (P<0.05).

Table 7 shows metabolite levels and enzyme activities in kidney. Lactate and triglyceride concentrations were highest in 15‰-exposed fish. Protein levels varied with salinity (15<25<39‰). Glucose levels, HK and LDH-O activities were similar between the three groups.

Table 2
Estimation of feed intake, productive parameters, and HSI in *S. senegalensis* juveniles exposed to three different salinities over a period of 71 days

Parameter	Salinity		
	15‰	25‰	39‰
Estimated feed intake (number of days where fish eat the entire daily ration)	19/71	61/71	70/71
Weight gain (%)	39.0±8.3[a]	97.9±8.5[b]	106.9±11.2[b]
SGR (g/day)	0.3±0.09[a]	0.9±0.06[b]	0.9±0.08[b]
HSI (%)	1.0±0.07[a]	1.1±0.05[a]	1.1±0.05[a]

Data (except estimation of food intake) are expressed as mean±S.E.M. (n=50 per group). Different letters indicate significant differences among groups (P<0.05, one-way ANOVA Tukey test).

Table 3
Osmoregulatory parameters in *S. senegalensis* juveniles acclimated to three different salinities for 71 days

Parameter	Salinity		
	15‰	25‰	39‰
Plasma osmolality (mOsm/kg)	310±3[a]	315±2[a]	317±2[a]
Plasma Na^+ (mM)	166±3[a]	167±1[a]	164±1[a]
Plasma Cl^- (mM)	170±3[a]	149±3[b]	176±7[a]
Gill Na^+,K^+-ATPase activity (μmol ADP/mg protein/h)	10.7±0.7[a]	13.4±0.6[b]	18.8±0.9[c]
Kidney Na^+,K^+-ATPase activity (μmol ADP/mg protein/h)	8.5±0.5[a]	7.6±0.4[a]	8.0±0.3[a]

Data are expressed as mean±S.E.M. (n=16–20 per group). Further details are in legend of Table 2.

In white muscle from 25‰-exposed animals, glycogen levels increased compared to animals exposed to 15 and 39‰ (Table 8). Lactate concentrations enhanced in 15‰-exposed fish; protein levels correlated positively with salinity (15<25<39‰). Glucose, triglyceride and total α-amino acid concentrations were similar in all groups (P>0.05). Total GPase activity diminished linearly with environmental salinity. GDH activity was highest in 15‰-exposed fish compared with 25‰- and 39‰-exposed animals that showed similar levels. HK and LDH-O activities were not altered by exposure to different environmental salinities.

4. Discussion

Time-course of osmoregulatory response as well as primary and secondary stress responses of *S. senegalensis* to changes in a wide range of environmental salinities (from 5 to 55‰) have been characterized previously and our results agree with those reported (Arjona et al., 2007). Osmoregulatory balance was achieved through efficient regulation of gill Na^+,K^+-ATPase activity and consequently *S. senegalensis* is able to ionoregulate and maintain its plasma osmolality and Na^+ concentration. Most likely gill Na^+,K^+-ATPase activities confer the osmoregulatory capacity to *S. senegalensis*, since these increased positively with environmental salinity (Table 3). Clasically, cortisol within its mineralocorticoid function is characterized by increasing gill Na^+,K^+-ATPase activity (McCormick, 1995). In this study, gill Na^+,K^+-ATPase activities increase in parallel to salinity (Table 3), conversely to plasma cortisol levels that are elevated in salinities different than 39‰ (Fig. 2). As consequence, mineralocorticoid action of cortisol is not observed. On the other hand, the gluconeogenic pathway and flux of gluconeogenic substrates from amino acids in liver are activated as judged from the increased FBPase, Ala-AT, and GDH activities in the lowest salinity (Table 5). Both routes are stimulated by cortisol in teleosts (Mommsen et al., 1999), supporting the glucocorticoid role for cortisol in this context.

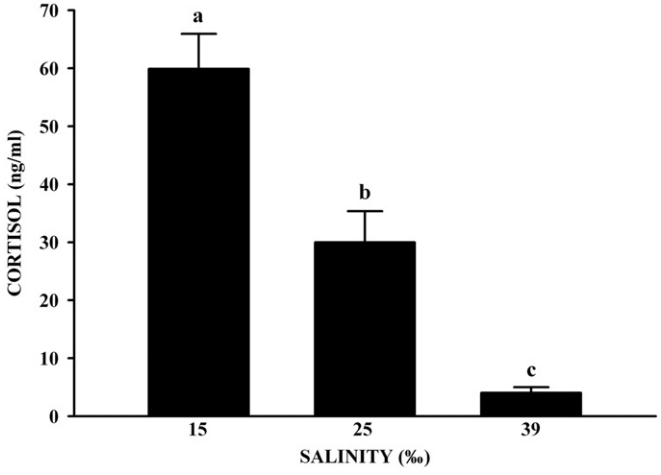

Fig. 2. Plasma cortisol levels in *S. senegalensis* juveniles exposed to 3 different salinities for 71 days. Data are expressed as mean±S.E.M. (n=16–20 per group). Different letters indicate significant differences among groups (P<0.05, one-way ANOVA Tukey test).

Growth, weight gain and SGR of *S. senegalensis* juveniles were reduced in animals kept in 15‰ salinity (Table 2) reflecting tertiary stress responses in this salinity (effects in growth). The effect of decreased salinity on growth in a euryhaline animal can be related to a decrease in the energy cost of osmotic and ionic regulation. However, some experiments indicate that the true energetic cost of osmoregulation remains under debate constituting less than 4% of the total energy budget (Morgan and Iwama, 1999). Therefore, this effect seems to vary among different species, which show different and specific optimal salinities for growth (reviewed by Boeuf and Payan, 2001; see Imsland et al., 2001, 2008; Laiz-Carrión et al., 2005). An optimal growth rate is generally observed in iso-osmotic salinities and is often, but not always, correlated with a lower standard metabolic rate. In flatfish species such as *S. maximus* and *H. hippoglossus*, maximal growth occurs at 10–15‰ (Gaumet et al., 1995; Imsland et al., 2001, 2008). In the case of *S. senegalensis*, optimal growth salinity is 25–39‰ (in both salinities fishes showed the same SGR) and physiological adjustments such as in energy metabolism occurred at 15 and 25‰. Energy for growth is derived from body energy stores and/or directly from ingested/digested feed. Energy enzyme rates increased and feed intake (estimated as the number of days fish eat the entire daily feed ration provided) decreased in those salinities different than 39‰, overall in 15‰, and cover up the effect of lower energy cost for osmoregulation as consequence of reduced ion influx into the animal. The combination of body energy stores mobilization and ingested/digested feed in fish exposed to 15 and 25‰ is a predominant process over the save of energy for osmoregulation in determining the growth in salinities different than 39‰ and expressed in low growth in fish exposed to 15‰.

S. senegalensis is stressed as high plasma cortisol levels indicated by fish exposed to salinities different than 39‰ (Fig. 2). Since the circulating level of cortisol can be interpreted as an indicator of the degree of stress experienced by fish (Wendelaar Bonga, 1997), fishes exposed to 15‰ salinity appear more stressed than those exposed to 25‰. This excessive production of a primary mediator of stress response (cortisol) suggests an allostatic state in *S. senegalensis* that allow the homeostasis in low salinities (allostasis: achieving stability through change; McEwen and Wingfield, 2003).

Salinity is an environmental factor known to affect feeding and macronutrient selection in fish (De Boeck et al., 2000; Rubio et al., 2005). Although we did not accurately measure feed intake in our animals, we observed that fishes exposed to 15‰ salinity did eat their entire daily rations on 19 of the 71 days, and this correlates well with the increased plasma cortisol levels. It is widely accepted that cortisol is a primary mediator of the growth-suppresing effects of stress (Pickering, 1993; Pankhurst and Van Der Kraak, 1997). There is evidence that centrally produced CRF not only steers the pituitary ACTH cells, but also acts as anorexigenic signal in feeding centres (Bernier and Peter, 2001; Bernier, 2006). A matter unsolved yet is whether this concerns one and the same population of CRF neurons or

Table 4
Metabolic parameters in the plasma of *S. senegalensis* juveniles acclimated to three different salinities for 71 days

Parameter	Salinity		
	15‰	25‰	39‰
Glucose (mM)	3.2±0.2[a]	3.2±0.2[a]	5.0±0.3[b]
Lactate (mM)	0.8±0.1[a]	0.3±0.04[b]	0.4±0.03[c]
NEFA (mM)	0.37±0.07[a]	0.62±0.10[a]	1.32±0.18[b]
Triglycerides (mM)	2.8±0.4[a]	6.1±0.5[b]	10.7±1.0[c]
Proteins (mg/ml)	36.3±1.0[a]	42.6±0.7[b]	43.6±0.7[b]

Data are expressed as mean±S.E.M. (n=16–20 per group). Further details are in legend of Table 2.

Table 5
Liver metabolite levels and activities of FBPase, G6PDH, total GPase, Ala-AT and GDH in *S. senegalensis* juveniles acclimated to three different salinities for 71 days

Parameter	Salinity		
	15‰	25‰	39‰
Metabolites			
Glycogen (μmol glycosyl units/g wet weight)	94.8±13.4[a]	161.3±8.3[b]	173.8±7.4[b]
Glucose (μmol/g wet weight)	62.6±2.7[a]	62.0±2.0[a]	54.6±1.0[b]
Lactate (μmol/g wet weight)	Not detected	Not detected	Not detected
Triglycerides (μmol/g wet weight)	12.2±2.4[a]	9.3±1.0[a]	9.9±1.6[a]
Proteins (μmol/g wet weight)	56.1±2.5[a]	60.6±1.7[a]	59.3±2.5[a]
Total α-amino acids (μmol/g wet weight)	28.9±1.8[a]	33.9±1.3[a]	30.9±2.2[a]
Enzyme activities			
Carbohydrate metabolism			
FBPase activity (U/mg protein)	1.18±0.08[a]	0.93±0.06[b]	0.80±0.06[b]
G6PDH activity (U/mg protein)	0.54±0.03[a]	0.53±0.04[a]	0.53±0.03[a]
Total GPase activity (U/mg protein)	0.99±0.07[a]	0.86±0.08[a]	0.59±0.04[b]
Amino acid metabolism			
Ala-AT activity (U/mg protein)	2.66±0.39[a]	1.45±0.17[b]	0.52±0.05[c]
GDH activity (U/mg protein)	6.38±0.39[a]	3.48±0.18[b]	3.51±0.12[b]

Data are expressed as mean±S.E.M. (n=16–20 per group). Further details are in legend of Table 2.

separate populations and, if separate, whether they become both activated during stress. Therefore, lower growth in animals kept in 15‰ of salinity could be produced by decreased appetite, and the stimulation of HPI axis indicated by increased plasma cortisol levels may interact with the control of appetite through the anorexigenic functions of CRF. In this case (fish exposed to 15‰) energy reallocation is not enough to allow a similar growth compared to fishes exposed to 25 or 39‰.

Glucose and NEFA are substrates that fuel energy metabolism processes in fishes (Mommsen et al., 1999). Plasma glucose, NEFA and triglyceride concentrations are derived from ingested/digested feed, and/or gluconeogenesis and glycogenolysis processes (case of glucose) or mobilization of body fat stores (case of NEFA and triglycerides). Processes determining the clearance of these metabolites from the blood are excretion and/or tissue intake. An increased need in tissues for energy substrate could explain the decrease in plasma glucose observed in the 15 and 25‰ groups (Table 4). So, an enhanced cell energy demand could cause a faster tissue uptake of glucose from blood, and induce the observed depletion in plasma glucose levels, despite of the enhancement of glucose production routes to keep glycaemia as glycogenolytic/gluconeogenic pathways in liver. HK activity that refers to the use of exogenous glucose (by phosphorylation), was increased in gills of 15‰-exposed sole (Table 6); and together with other tissues as liver (where the capacity of using exogenous glucose was not assessed) and brain, which is a potent gluco-active tissue in fish (Soengas et al., 2007), could cause the elevated tissue uptake of glucose observed in lowered salinities (15 and 25‰). One of the processes fuelled by glucose in gills is the pentose shunt (indicated by G6PDH activities), that was increased in salinities different than 39‰ (Table 6). An activated pentose–phosphate pathway suggests an increase for reducing capacity in the gills, which may be related to an elevation in lipid synthesis (Laiz-Carrión et al., 2005; Sangiao-Alvarellos et al., 2005). Plasma NEFA, as well as plasma triglycerides decreased in parallel to estimated feed intake (Tables 2 and 4). This correlation suggests plasma NEFA and triglyceride levels are determined mainly by feed intake more than body fat stores mobilization and/or use in the different tissues, and reflect the alterations in appetite by the osmotic stress (De Boeck et al., 2000; Rubio et al., 2005). A similar situation is observed in other teleosts under feed deprivation conditions (Polakof et al., 2006). Breakdown of triglycerides in the blood could be the source of NEFA since levels of both metabolites showed the same trend with salinity (and consequently feed intake), more than production from hepatic tissue.

The liver is the main site of glycogen/glucose turnover, ammoniogenesis, fatty acid synthesis, and gluconeogenesis in teleosts (Peragón et al., 1998; Mommsen et al., 1999). In the present study, liver glycogen levels diminished with decreasing salinities, suggesting that osmotic exposure of *S. senegalensis* to lowered salinities enhances energy requirements which are met by increased glycogenolysis. Indeed, the activation of the glycogenolytic pathway is reflected in the increased hepatic activity of total GPase in decreased salinities (Table 5). FBPase is a key gluconeogenic enzyme that enhances liver glucose production. Our results show an enhanced FBPase activity when salinity decreased. Taken together, the increased total GPase and FBPase activities indicate enhanced glycogenolytic and gluconeogenic potentials respectively. Glycogenolysis produces glucose-1-phosphate that is converted in glucose-6-phosphate by phosphoglucomutase and used in glycolysis and/or exportation to peripheral tissues (by dephosphorylation). Gluconeogenesis yields glucose that explains the increased liver glucose levels observed in *S. senegalensis* exposed to 15‰ and 25‰ salinity.

Gluconeogenesis in liver is fuelled by pyruvate generated by different precursors: one of these are amino acids converted to pyruvate by transdeamination (Mommsen et al., 1999). Protein content in white muscle and kidney correlated positively with salinity, suggesting a proteolytic process in these organs, mostly in 15‰-exposed fish. In white muscle, fish exposed to 15‰ showed increased GDH activities supporting the idea that *S. senegalensis* juveniles are metabolizing amino acids that could be derived from proteolysis. The absence of changes in HSI and liver protein levels in 15‰- and 25‰-exposed specimens compared with 39‰ point to the use of exogenous amino acids to be used as fuel for liver gluconeogenesis. Therefore, these amino acids can come from white muscle and kidney proteolytic

Table 6
Gill metabolite levels and activities of HK, G6PDH and LDH-O in *S. senegalensis* juveniles acclimated to three different salinities for 71 days

Parameter	Salinity		
	15‰	25‰	39‰
Metabolites			
Glucose (μmol/g wet weight)	1.5±0.1[a]	1.0±0.1[b]	1.2±0.1[a,b]
Lactate (μmol/g wet weight)	3.0±0.3[a]	1.7±0.2[b]	1.9±0.2[b]
Triglycerides (μmol/g wet weight)	0.9±0.1[a]	0.8±0.1[a]	1.1±0.1[a]
Proteins (μmol/g wet weight)	37.8±1.2[a]	40.3±0.9[a]	38.5±0.8[a]
Enzyme activities			
Carbohydrate metabolism			
HK activity (U/mg protein)	1.94±0.11[a]	1.67±0.10[a,b]	1.63±0.04[b]
G6PDH activity (U/mg protein)	5.32±0.15[a]	5.22±0.20[a]	2.05±0.08[b]
Lactate metabolism			
LDH-O activity (U/mg protein)	0.49±0.02[a]	0.44±0.01[a,b]	0.43±0.02[b]

Data are expressed as mean±S.E.M. (n=16–20 per group). Further details are in legend of Table 2.

Table 7
Kidney metabolite levels and activities of HK and LDH-O in *S. senegalensis* juveniles acclimated to three different salinities for 71 days

Parameter	Salinity		
	15‰	25‰	39‰
Metabolites			
Glucose (μmol/g wet weight)	4.3±0.2[a]	4.0±0.3[a]	4.4±0.4[a]
Lactate (μmol/g wet weight)	2.5±0.3[a]	1.7±0.2[a,b]	1.5±0.2[b]
Triglycerides (μmol/g wet weight)	0.9±0.1[a]	0.7±0.1[a,b]	0.6±0.1[b]
Proteins (μmol/g wet weight)	55.7±1.5[a]	58.8±1.3[a,b]	62.3±0.9[b]
Enzyme activities			
Carbohydrate metabolism			
HK activity (U/mg protein)	0.61±0.03[a]	0.61±0.03[a]	0.54±0.02[a]
Lactate metabolism			
LDH-O activity (U/mg protein)	0.19±0.02[a]	0.18±0.02[a]	0.19±0.01[a]

Data are expressed as mean±S.E.M. (n=16–20 per group). Further details are in legend of Table 2.

Table 8
White muscle metabolite levels and activities of HK, total GPase, GDH, and LDH-O in *S. senegalensis* juveniles acclimated to three different salinities for 71 days

Parameter	Salinity		
	15‰	25‰	39‰
Metabolites			
Glycogen (μmol glycosyl units/g wet weight)	0.9±0.1[a,b]	1.3±0.2[a]	0.8±0.1[b]
Glucose (μmol/g wet weight)	7.0±0.3[a]	6.8±0.3[a]	6.4±0.2[a]
Lactate (μmol/g wet weight)	36.5±0.8[a]	32.9±1.2[b]	32.8±1.1[b]
Triglycerides (μmol/g wet weight)	0.9±0.1[a]	0.8±0.03[a]	0.9±0.1[a]
Proteins (μmol/g wet weight)	38.0±0.9[a]	40.0±0.8[a,b]	42.3±0.9[b]
Total α-amino acids (μmol/g wet weight)	131.8±9.6[a]	132.7±7.7[a]	128.4±8.2[a]
Enzyme activities			
Carbohydrate metabolism			
HK activity (mU/mg protein)	40.49±4.34[a]	45.78±4.30[a]	35.02±3.73[a]
Total GPase activity (U/mg protein)	3.97±0.14[a]	3.63±0.09[a,b]	3.32±0.09[b]
Amino acid metabolism			
GDH activity (U/mg protein)	0.38±0.03[a]	0.28±0.03[b]	0.26±0.02[b]
Lactate metabolism			
LDH-O activity (U/mg protein)	0.24±0.05[a]	0.29±0.04[a]	0.24±0.05[a]

Data are expressed as mean±S.E.M. (n=16–20 per group). Further details are in legend of Table 2.

processes. General agreement seems to have developed that as part of its widespread catabolic activity, cortisol exerts a proteolytic action (Barton et al., 1987; Vijayan et al., 1997). In the present study, the increased peripheral proteolytic activity seems to be accompanied by substantial adjustments in liver amino acid metabolism, including higher titres of Ala-AT (in 15‰- and 25‰-exposed specimens) and GDH (in 15‰-exposed specimens) that potentially increase the flux of alanine through the transdeamination route enhancing the availability of amino acid-derived carbons (pyruvate) for oxidation or anabolic pathways such as gluconeogenesis. Besides, cortisol enhances alanine oxidation (Vijayan et al., 1993) and alanine gluconeogenesis (Vijayan et al., 1994) and thus, plasma cortisol levels in 15‰- and 25‰-exposed fish could be involved in the up-regulation of these pathways. The lack of changes in liver and white muscle total α-amino acid concentrations between the different environmental salinities assessed indicates a rapid amino acid turnover to be used in oxidation/gluconeogenesis in liver or to be used in oxidation/exportation in white muscle.

A general increase of lactate levels was observed in gills, kidney and white muscle of 15‰-exposed fish. Moreover, this increase was also present in plasma. Previous studies in *S. senegalensis* did not show elevated plasma lactate levels in sole transferred to 15‰ (Arjona et al., 2007) after 17 days, pointing to the reach of increased plasma lactate when the tertiary stress response (effects in growth) is present. Considering increased LDH-O in gills of 15‰-exposed *S. senegalensis*, we can hypothesize that an increase in lactate oxidation rates by those tissues involved in osmotic work and able to use lactate as fuel, such as gills (Mommsen, 1984; Perry and Walsh, 1989), may take place when fish are exposed to different osmotic conditions (Soengas et al., 2007). Increased total GPase activity in white muscle of 15‰-exposed fish indicated a high use of glycogen to provide glycosyl units ready to be used to fuel endogenous pathways such as glycolysis (Table 8). The exogenous source of glucose was not important, as judged by the absence in HK activity. Of the energy stored as glycogen in white muscle, a very large fraction can be provided in the form of lactate that besides being re-converted to glycogen *in situ* (Schulte et al., 1992), it can also be provided to oxidative tissues via the blood stream (Weber, 1992).

In conclusion, the present findings demonstrate that environmental salinity affects the growth in juveniles of *S. senegalensis* in the summer season. In particular the animals exposed to the lowest salinity of 15‰ were affected, displaying a SGR that is approximately one-third of that of fishes kept at higher salinities. The changes that followed exposure to lower salinities (15 and 25‰) measured in metabolite concentrations and enzyme activities in liver, kidney, gills and white muscle as well as the decrease in estimated feed intake suggests the reallocation of energy resources as response to decreased feed intake-derived energy, and explains in reduced growth rates in 15‰-exposed specimens.

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