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ORIGINAL ARTICLE

Changes in plasma amino acid levels in a euryhaline fish exposed to different environmental salinities

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Abstract Previous studies have shown that Senegalese sole is partially euryhaline in the juvenile phase, being able to adapt to a wide range of salinities in a short-time period, due to changes at the osmoregulatory and metabolic level. This study aimed to assess the effects of acclimation of sole to a wide range of salinities, with a special emphasis on the role of plasma amino acids during this process. Sole juveniles were acclimated for 2 weeks to different salinities: 5, 15, 25, 38, and 55 g L^{-1}. Plasma levels of cortisol, glucose, osmolality, and free amino acids were assessed at the end. Changes in plasma levels of cortisol, glucose, and amino acids indicate that fish reared at 5 and 55 g L^{-1} were facing extra energy costs. Amino acids seem to play an important role during salinity acclimation, either as energy sources or as important osmolytes for cell volume regulation.

Keywords Salinity acclimation · Stress · Amino acids · Osmoregulation · *Solea senegalensis*

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Introduction

Senegalese sole (*Solea senegalensis* Kaup 1858) is a Pleuronectiform that inhabits coastal waters and estuaries (Bauchot 1987; Quéro et al. 1986). Therefore, it is expected that this species can tolerate a wide range of salinities. In previous studies it has been shown that Senegalese sole is partially euryhaline in the juvenile phase, being able to adapt to a wide range of salinities (5–55 g L^{-1}) in a short-time period. The osmotic acclimation to different environmental salinities induced changes at osmoregulatory and metabolic levels, as well as the activation of the stress system (Arjona et al. 2007, 2008).

During osmotic acclimation of euryhaline fish species an enhanced energy requirement in osmoregulatory (as gills and kidney) and non-osmoregulatory (as liver and brain) tissues, involved directly or indirectly on osmoregulatory work, is observed. These increased energy requirements must be fuelled by metabolites as glucose, lactate, or amino acids (Sangiao-Alvarellos et al. 2003, 2005; Soengas et al. 2008). Cortisol is considered to be an important hormone in osmotic acclimation and stress response in teleosts (Laiz-Carrión et al. 2003; McCormick 2001; Mommsen et al. 1999), including Senegalese sole (Arjona et al. 2007, 2008; Costas et al. 2008). Among other functions, cortisol may regulate energy metabolism by affecting important metabolic pathways as gluconeogenesis, glycogenesis, lipogenesis, glycolysis, glycogenolysis, or amino acid catabolism (Laiz-Carrión et al. 2002, 2003; Mommsen et al. 1999).

Cortisol administration affects plasma concentration of some indispensable amino acids in fish (Vijayan et al. 1997). Moreover, stress conditions that induced high plasma cortisol levels modified fish amino acid metabolism in several teleost species (Milligan 1997; Vijayan et al.

1997), including Senegalese sole (Aragão et al. 2008; Costas et al. 2008; Pinto et al. 2007). It has been suggested that fish under stressful conditions have additional amino acid requirements, due to higher energetic requirements or for the synthesis of stress-related proteins (Aragão et al. 2008; Costas et al. 2008; Pinto et al. 2007). Therefore, the possible changes in cortisol due to osmotic acclimation and the increased energy requirements during this process are expected to have a great impact on amino acid metabolism in Senegalese sole.

During osmotic acclimation amino acids present a role not only as energetic substrates, but also as plasma and cell osmolytes. Osmotic pressures in teleost fish are regulated at nearly constant levels, even in euryhaline species (Yancey 2001a). In order to maintain cell osmolality and volume, marine fish rely on the use of organic osmolytes, since the use of inorganic ions may be incompatible with long-term protein functions (Yancey 2001b). These organic osmolytes are essentially nitrogen-based compounds, in which several amino acids as glycine, β-alanine, or taurine are included (Yancey 2001a). Dispensable amino acids seem to be preferentially used for osmoregulatory proposes, rather than the ten amino acids considered indispensable for the fish, as they cannot synthesise them (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine—Wilson 2002).

Although amino acids seem to assume a significant importance during osmotic acclimation, few studies with fish have focussed on this subject. Therefore, the purpose of this study was to assess the effects of acclimation of the euryhaline Senegalese sole to a wide range of environmental salinities. The study focused on modifications in plasma amino acid levels and stress-related plasma indicators. The results will be discussed in relation to the role of plasma amino acids during osmotic acclimation in teleosts.

Materials and methods

Experimental procedures

The experiment was carried out in the wet laboratories at the Faculty of Marine and Environmental Sciences (Puerto Real, Cadiz, Spain), following an experimental protocol of acclimation to different environmental salinities used previously with Senegalese sole (*Solea senegalensis*; Arjona et al. 2007, 2008). Immature juveniles of Senegalese sole (60 ± 15 g wet weight) acclimated to full-strength seawater (38 g L^{-1}) were randomly divided into five groups ($n = 10$ for each group) and transferred into 400-L tanks. 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 (1,090 mOsm kg^{-1} H_2O), and 55 g L^{-1} (1,546 mOsm kg^{-1} H_2O), maintained by recirculating the tank water. The experimental salinities were achieved either by mixing full-strength seawater (38 g L^{-1}) with dechlorinated tap water or by mixing full-strength seawater with natural marine salt (Salina de La Tapa, Puerto de Santa María, Cadiz, Spain). Water salinity was checked daily and corrected when necessary by the addition of small volumes of either freshwater or seawater. During the experimental period, water quality criteria (hardness, oxygen, carbon dioxide, hydrogen sulphide, nitrite, nitrate, ammonia, calcium, chlorine, and suspended solids) were assessed with commercial available kits and standard protocols; no major changes were observed. During the experiment the fish were maintained under natural photoperiod (November 2006) and constant temperature (16–17°C). Fish were fed once daily with commercial dry pellets (Dibaq-Diproteg SA, Segovia, Spain) at a ration of 1% body weight. All experimental procedures complied with the Guidelines of the European Union Council (86/609/EU) and of the University of Cadiz for the use of laboratory animals.

Fish were exposed to the different environmental salinities for 2 weeks. At the end of the experimental period, they were fasted for 24 h before sampling, in order to avoid any influence of feeding on cortisol and glucose levels (Arends et al. 1999). Fish were anaesthetised with 2-phenoxyethanol (1,000 ppm; Sigma-Aldrich, Germany). Blood was withdrawn individually from the caudal vein of the fish using 1-mL syringes rinsed with a solution containing 25,000 U ammonium heparin per 3 mL NaCl 0.9%. After each tank sampling, which took 10–15 min, blood was centrifuged at 10,000×*g* for 3 min at 4°C. The collected plasma was frozen in liquid nitrogen for further analysis of cortisol, glucose, osmolality, and free amino acid levels. Analytical procedures were carried out at the Centro de Ciências do Mar do Algarve (CCMAR, Faro, Portugal) facilities and the samples were stored at −25°C until analysis.

Analytical procedures

Although ten fish per treatment were sampled, due to the small size of fish, the plasma volume obtained was not enough to analyse all the parameters in all individuals. Therefore, for each of the parameters determined (cortisol, glucose, osmolality, and free amino acids), plasma samples from six individual fish were analysed.

Plasma cortisol was determined by radioimmunoassay (RIA) as described by Rotllant et al. (2006). Briefly, 50 µL of plasma samples was diluted in 950 µL phosphate buffer containing 1 g L^{-1} gelatin, pH 7.6, and denatured at 80°C for 1 h. Duplicate aliquots (100 µL) of diluted denatured plasma were then used in the assay.

Plasma glucose was assayed using a commercially available kit (Boehringer Mannheim, R-Biopharm AG., Darmstadt, Germany). Plasma osmolality was determined using a cryo-osmometer (Osmomat 030, Gonotec, Berlin) and expressed as mOsm kg^{-1} H_2O.

Plasma samples for free amino acid analysis were deproteinised by centrifugal ultrafiltration (10 kDa cut-off, 2,500×*g*, 20 min, 4°C). After deproteinisation, samples were pre-column derivatized with phenylisothiocyanate (PITC, Pierce), using the PicoTag method (Waters, USA) described by Cohen et al. (1989). External standards were prepared along with the samples, using physiological amino acid standard solutions (acid/neutral and basics from Sigma) and a glutamine solution. Norleucine was used as an internal standard. Samples and standards were analysed by high pressure liquid chromatography (HPLC) in a Waters Reversed-Phase Amino Acid Analysis System, equipped with a PicoTag column (3.9 × 300 mm), a column heater (set at 46°C), a binary pump, an autosampler, and an UV/Vis detector (at 254 nm), using the conditions described by Cohen et al. (1989). Resulting peaks were analysed with the Breeze software (Waters).

Data analysis

For each plasma parameter analysed, results were expressed as mean ± standard deviation (SD) of six individual fish samples. Data were analysed by one-way analysis of variance (ANOVA) using the computer package SPSS for Windows 15.0. When significant differences were obtained from the ANOVA, Tukey's post hoc tests were used to identify significantly different groups. The level of significance used was $P \leq 0.05$ for all statistical tests.

Results

Acclimation of Senegalese sole juveniles from full-strength seawater (38 g L^{-1}) to different environmental salinities affected some plasma parameters, as shown in Table 1. In fish acclimated to 55 g L^{-1} a significant increase in cortisol and osmolality levels was found compared with the other salinities tested. A significant effect of the environmental salinity on plasma glucose levels was also found, as detected by one-way ANOVA ($P = 0.023$). However, the post hoc tests could not identify the significantly different groups, which are probably related to the high variability found for this parameter. Nevertheless, the mean plasma glucose level tended to be lower in specimens acclimated to extreme environmental salinities (5, 55 g L^{-1}), with respect to the other salinities tested.

Plasma total free amino acid concentrations were affected by environmental salinities, showing a "U-shaped" relationship with respect to salinity, well described by a polynomial equation (Fig. 1). The lowest plasma total free amino acid concentration was found in fish acclimated to 15 g L^{-1}, this being significantly lower than in fish acclimated to 5 g L^{-1} or maintained at 38 g L^{-1}. Plasma total indispensable amino acid concentrations followed the same

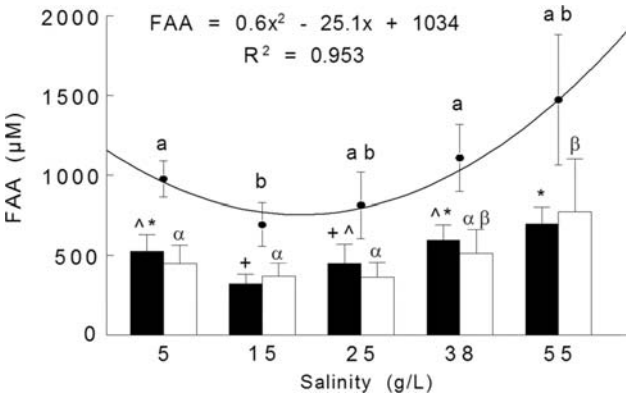

Fig. 1 Plasma concentrations of total free amino acids in *S. senegalensis* juveniles acclimated to different salinities for 2 weeks. Total (*filled circles*), dispensable (*black bars*), and indispensable (*white bars*) amino acids. Results are expressed as mean values + SD ($n = 6$). *Different superscripts* indicate significant differences among the treatments for total (*a* or *b*), dispensable (*plus*, *hat*, or *asterisk*), or indispensable amino acids (α or β). A *polynomial line* has been fitted to total free amino acid data, where *x* represents the salinity

Table 1 Plasma cortisol, glucose, and osmolality levels in *S. senegalensis* juveniles acclimated to different salinities for 2 weeks

Salinity (g L^{-1})	Cortisol (ng mL^{-1})	Glucose (mM)	Osmolality (mOsm kg^{-1})
5	15 ± 16§	2.0 ± 0.9	357 ± 8§
15	18 ± 8§	4.0 ± 0.8	345 ± 10§
25	20 ± 18§	3.7 ± 1.5	370 ± 6§
38	38 ± 66§	3.2 ± 6.0	357 ± 26§
55	220 ± 92*	2.0 ± 1.5	398 ± 18*

Data are shown as mean values ± SD ($n = 6$). Different symbols within the same column indicate significant differences among the treatments for the same parameter. Significant differences among the treatments were identified for glucose ($P = 0.023$, one-way ANOVA), but these could not be identified through post hoc tests

pattern as total free amino acid concentrations. Plasma concentration of total dispensable amino acids tended to increase as salinity increases, being significantly higher at 55 g L^{-1} than at 5, 15, and 25 g L^{-1}.

Regarding the individual plasma amino acid concentrations (Fig. 2), it was observed that among the indispensable amino acids, the branched-chain amino acids (isoleucine, leucine, and valine) tended to increase with salinity. These concentrations were significantly higher in specimens acclimated to 55 g L^{-1} than in fish acclimated to 5 or 15 g L^{-1} (Fig. 2a). Plasma concentrations of lysine showed a "U-shaped" relationship with respect to environmental salinity. Lysine concentrations were significantly lower at 15 and 25 g L^{-1} and significantly higher

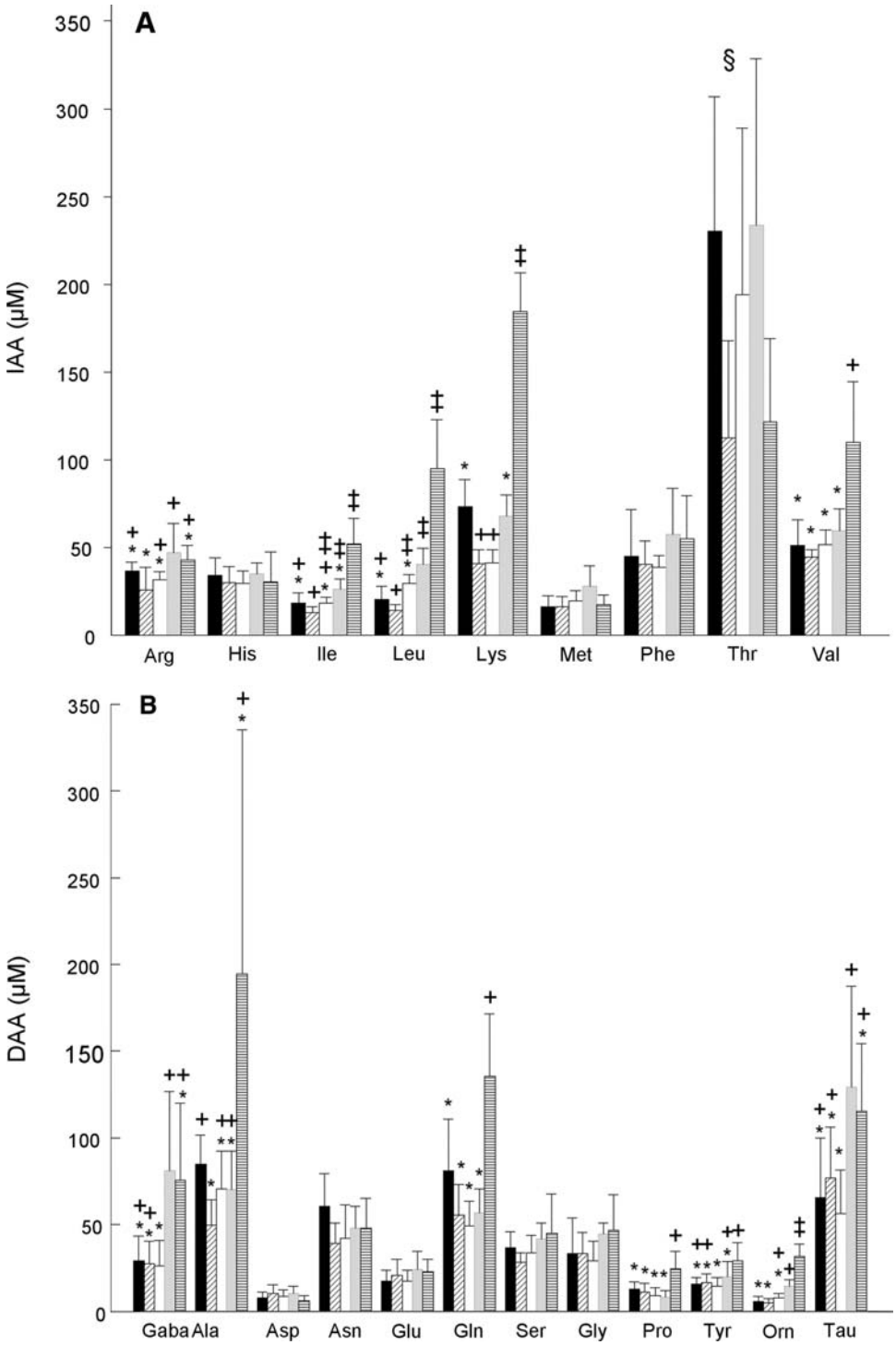

Fig. 2 Plasma concentrations for the different indispensable (**a**) and dispensable (**b**) amino acids in *S. senegalensis* juveniles acclimated to different salinities for 2 weeks (*black bars*, 5; ▨, 15; *white bars*, 25; ▩, 38; ▤, 55 g L^{-1}). Results are expressed as mean values + SD ($n = 6$). *Different symbols* indicate significant differences among salinities for the same amino acid

at 55 g L^{-1} than at the other salinities tested (Fig. 2a). No clear tendency in plasma arginine and threonine levels with respect to environmental salinities were observed, while the levels of the other indispensable amino acids assessed (histidine, methionine, and phenylalanine) did not change significantly with environmental salinity (Fig. 2a). Regarding the individual dispensable amino acids, it was observed that plasma concentrations of glutamine, proline, and ornithine were significantly higher in fish acclimated to 55 g L^{-1} than at the other salinities tested (Fig. 2b). Plasma concentrations of alanine, tyrosine, γ-amino-*n*-butyric acid (GABA), and taurine tended to increase as salinity increases.

Discussion

This experiment tested the effect of acclimation to different environmental salinities on selected plasma stress-related parameters and amino acid levels in Senegalese sole juveniles. Results suggested a high euryhalinity capacity of this species with modifications in plasma osmolality, glucose, and cortisol levels. These results agree with that reported previously for juvenile specimens of Senegalese sole submitted to a similar range of experimental salinities (Arjona et al. 2007, 2008). In addition, a clear effect of salinity on plasma levels of free amino acids has been observed.

The results from the present study show a tendency for a lower plasma glucose concentration in fish acclimated to the lowest (5 g L^{-1}) and highest (55 g L^{-1}) salinities with respect to specimens acclimated to other salinities (15, 25, and 38 g L^{-1}). The fish reared at the extreme salinities could be using higher amounts of glucose to face the higher energy requirements of different tissues involved directly or indirectly on osmoregulatory work, as observed in other euryhaline fish species (Laiz-Carrión et al. 2005; Sangiao-Alvarellos et al. 2003, 2005) and also suggested in this species (Arjona et al. 2007, 2008).

Salinity acclimation did result in several changes in the plasma amino acid levels of Senegalese sole. Changes in free amino acid levels in relation to salinity acclimation have been observed in different animals such as mussels (Deaton 2001), crabs (Fujimori and Abe 2002), insect larvae (Edwards 1982), or fish (Bystriansky et al. 2007). Most of the observed changes in plasma amino acid levels appear to be directly or indirectly involved in osmoregulatory processes. The results of the current experiment indicated that the lowest plasma amino acids levels were found in fish acclimated to 15 g L^{-1}, an environmental salinity where Senegalese sole is isosmotic with its medium and net ion and water flux is almost zero (Arjona et al. 2007). However, at extreme salinities (5, 55 g L^{-1}) where fish osmolality is clearly different from that of the surrounding water, several changes in the plasma levels of free amino acids were found.

In euryhaline fish species, both taurine and GABA have been described as important osmolytes (Huxtable 1992; Schaarschmidt et al. 1999). An increase in the concentration of taurine and GABA in the plasma of Senegalese sole was concomitant with an increase in water osmolality. This ultimately resulted in a higher plasma osmolality in fish maintained at 55 g L^{-1} than at the other environmental salinities tested. A rise in free amino acid levels in response to elevated salinity has also been observed for other teleost fish (Ballantyne 2001).

However, not all changes in free amino acid levels are related to changes in plasma osmolality. Some of these changes may be due to energetic costs of osmoregulation. As already mentioned, fish can use different metabolites (glucose, lactate, and even amino acids) to get energy for facing the energetic costs of osmoregulatory work (Soengas et al. 2008). Among other functions, cortisol may increase proteolytic activity (Milligan 1997; Mommsen et al. 1999). A general tendency for an increase in plasma levels of some amino acids at the highest salinity tested (55 g L^{-1}) was observed and this may result from an increased proteolysis due to cortisol action in fish acclimated to this salinity. Several of these amino acids can be easily used as energetic substrates: (i) the branched-chain amino acids are used in skeletal muscle, which is the major site of branched-chain amino acid oxidation (Van den Thillart 1986; Van Waarde 1988); (ii) glutamine, proline, and ornithine are members of the "glutamate family" (Brosnan 2000), which means that they are easily transaminated into glutamate and glutamate transdeamination is the main pathway of amino acid oxidation in fish liver (Ballantyne 2001); (iii) alanine is a preferential substrate for liver gluconeogenesis (Ballantyne 2001). It has been shown that cortisol enhanced hepatic gluconeogenic activity (Laiz-Carrión et al. 2003; Vijayan et al. 1997). Since Senegalese sole specimens reared at 55 g L^{-1} presented higher plasma cortisol levels than fish at the other salinities tested, it is possible that gluconeogenesis from amino acids assume a special importance in these fish. In order to use the carbon skeletons of amino acids as energy sources or in gluconeogenesis, deamination must occur and this probably leads to an increase in ammonia levels. Glutamine synthesis is an important pathway for ammonia detoxification in several fish species (Ip et al. 2001) and an increase in plasma glutamine levels in Senegalese sole juveniles chronically exposed to exogenous ammonia has been previously observed (Pinto et al. 2007). Therefore, this pathway may assume a special importance in fish reared at the highest environmental salinity tested, which could explain the twofold increase of the plasma glutamine concentration in these fish.

Results from the current experiment show that more metabolic changes occur when Senegalese sole juveniles are transferred from seawater (38 g L^{-1}) to hypersaline (55 g L^{-1}) than to hyposaline (5 g L^{-1}) water. The same was observed when gilthead seabream (*Sparus aurata*) was transferred from seawater to hypersaline water (55 g L^{-1}), then to low salinity water (6 g L^{-1}, Sangiao-Alvarellos et al. 2005). According to these authors, in euryhaline marine fish species acclimation to low-salinity water is less energy costly than acclimation to hypersaline water.

This study confirms that Senegalese sole juveniles are able to adapt to a wide range of environmental salinities, but during this process several metabolic adjustments are observed. Changes in plasma levels of cortisol, glucose, and amino acids indicate that fish reared at the extreme salinities tested (5, 55 g L^{-1}) were facing extra energy costs, probably related to osmoregulatory processes. Amino acids seem to play an important role so fish can adjust to the different environmental salinities, either as energy sources or as important osmolytes for cell volume regulation. Further studies analysing the effects of salinity on some key metabolic enzymes, for instance, enzymes involved in amino acid catabolism, gluconeogenesis, or in ammonia detoxification, could provide a clear picture on the role of amino acids during salinity acclimation in fish.

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