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Stress response and changes in amino acid requirements in Senegalese sole (*Solea senegalensis* Kaup 1858)

C. Aragão, J. Corte-Real, B. Costas, M. T. Dinis, and **L. E. C. Conceição**

CCMAR, Universidade do Algarve, Campus de Gambelas, Faro, Portugal

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Summary. Fish in aquaculture are often exposed to various stressors that may change their ability to survive or limit growth. Amino acids are used for processes other than growth, including stress response. This study intended to analyse how repeated acute handling stress can affect growth and amino acid requirements in fish. Senegalese sole juveniles were weekly held in the air during 3 min (*Handling*) for 9 weeks; *Control* groups were left undisturbed. Growth and plasma levels of stress indicators and of free amino acids were assessed at the end of the experiment. Plasma cortisol and osmolality levels showed that fish in the *Handling* treatment were stressed, but growth was unaffected. Plasma amino acid concentrations indicate that their requirements in stressed fish were altered, which probably reflects the synthesis of proteins or other specific compounds related to stress response.

Keywords: Handling stress – Cortisol – Osmolality – Amino acids – Requirements – *Solea senegalensis*

Introduction

Fish in aquaculture are often exposed to various stressors, such as grading, transport, crowding, and vaccination. Stressors are important factors that limit fish performance under aquaculture conditions. Fish will respond to a stressor in an attempt to compensate for the challenge imposed upon it and thereby cope with the stress. This response diverts energy from normal metabolic processes including growth, to deliver energy to the physiological systems activated to adapt to the stressor. The several endocrine and physiological alterations often result in changes in the ability of the fish to survive, increase the incidence of diseases, or limit growth (Pickering, 1998).

Amino acids are used for processes other than growth, including stress response. In Humans, injury, infection, and stress increase the demand for some amino acids, either due to synthesis of specific proteins, to selective catabolism, or to use in the synthesis of specific molecules (Obled et al., 2002; Reeds and Jahoor, 2001). In fish, during stress conditions, some studies show that the total amino acid content in the plasma increases and the concentration of some free amino acids is greatly affected (Milligan, 1997; Vijayan et al., 1997). Changes in plasma free amino acid levels may be indicative of amino acid requirements in fish (Wilson, 2002). Moreover, plasma free amino acid levels have also been used as indicators of amino acid requirements in other animals, such as kitten (Strieker et al., 2006; Taylor et al., 1997).

Much work has been done in the requirements of individual amino acids and particular aspects of their metabolism, in fish (e.g. Lall et al., 1994; Simmons et al., 1999; Tibaldi and Tulli, 1999; Wilson, 1994) and especially in Humans and farmed animals (e.g. Farrell et al., 1999; Fürst and Stehle, 2004; Imura and Okada, 1998; Roush and Cravener, 2002). However, work is still needed to establish the relation between stressful husbandry conditions and amino acid metabolism and to understand how stress can affect animal growth and amino acid requirements.

The objective of this study is to analyse to what extent repeated acute handling stress can affect animal growth and amino acid requirements. Juvenile Senegalese sole (*Solea senegalensis*) will be used as model species, since it is resistant to stress in terms of survival and also due to its importance for the Southern European marine aquaculture industry. Changes in the plasma levels of individual free amino acids will be used as an indicator of additional amino acid requirements due to stressful husbandry conditions.

Materials and methods

Experimental procedures

The experiment was carried out at the Ramalhete Research Station (CCMAR, Faro, Portugal), using a partial-recirculated seawater system (temperature: 20 ± 1 °C; salinity: 36 g L^{-1}; dissolved oxygen: above 80% saturation level), comprised by flat-bottom sand coloured fibreglass tanks (70 cm length × 30 cm width × 20 cm depth, volume 20 L, water flow rate 54 L h^{-1}). Senegalese sole (*Solea senegalensis*) juveniles (78 ± 19 g wet weight) were used as a model. Initial density inside the tanks was 3 kg m^{-2}. Tanks were daily cleaned and temperature, salinity, and dissolved oxygen were daily measured. Ammonia and nitrite levels in water were measured twice a week using test kits and never exceeded 0.025 and 0.3 mg L^{-1}, respectively, during the whole experiment. Fish were kept at a photoperiod of 9 h light:15 h dark and the light intensity at water surface was 200 lux. At the beginning of the experiment, fish were anaesthetised with 2-phenoxyethanol (300 ppm; Sigma-Aldrich, Germany) and individually measured, weighed, and marked with water ink (Acualux Titan, Spain) on the ventral side. Fish were distributed by six tanks, each containing eight fish. Two treatments were tested in triplicate: *Control* and *Handling*. *Handling* groups were weekly chased with a net in order to capture all fish inside each tank and thereafter the net was held in the air during three min. Immediately after handling the fish were returned to the experimental tanks. Fish in *Control* groups were reared without any disturbance, except from daily tank cleaning procedures.

Fish were fed every day by hand four times a day (9:30, 12:30, 15:00, and 17:00) with a commercial diet ("Dourasoja^{extra}", Sorgal, Portugal; 43% crude protein). Fish were initially fed with 2% of their biomass and this amount was subsequently daily adjusted (±0.5%) based on visual inspection of the feed remaining, so that the fish were fed close to satiation.

The experiment lasted for nine weeks and final sampling occurred six days after the last handling stress. Fish were fasted for 24 h prior to final sampling in order to avoid any influence of feeding on cortisol and glucose levels (Arends et al., 1999). Each tank was individually sampled. Firstly, anaesthetic 2-phenoxyethanol (Sigma; 500 ppm) was added into each target tank in order to avoid stress due to handling. After 1 min, five fish were quickly taken out from each tank at a time and anaesthetised with 2-phenoxyethanol (Sigma; 1500 ppm). Blood was withdrawn individually from the caudal vein of these five fish using heparinised syringes. The blood collection lasted less than 3 min to avoid a cortisol increase due to manipulation during sampling. After each tank sampling, stored blood was centrifuged at 1500 × *g* for 2 min. The collected plasma was stored at −25 °C for further analysis of cortisol, glucose, osmolality, and free amino acid levels. In addition, all fish were weighed and measured. In fish used to withdrawn blood, liver was dissected on a glass placed over ice bed, weighed, frozen in liquid nitrogen, and kept at −80 °C for further enzymatic analysis.

Analytical procedures

Plasma cortisol was determined by radioimmunoassay (RIA) as described by Rotllant et al. (2006). Briefly, 50 µL of plasma samples were 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.

Glucose analysis was performed on plasma samples using a commercially available kit (Boehringer Mannheim, R-Biopharm AG., Darmstadt, Germany), after deproteinisation with Carrez reagents. Plasma osmolality was determined using a cryo-osmometer (Osmomat 030).

Plasma free amino acids levels were analysed by high pressure liquid chromatography (HPLC) in a Pico-Tag Amino Acid Analysis System (Waters, USA), using norleucine as internal standard and according to the procedures described by Cohen et al. (1989). Resulting peaks were analysed with the Breeze software (Waters, USA). Due to technical constraints, aspartic acid and phenylalanine in the samples were not quantified.

Livers were individually assayed for selected amino acid catabolic enzyme activities. Crude extracts were obtained by homogenisation of frozen tissue in ice–cold buffer (30 mM Hepes, 0.25 mM saccharose, 0.5 mM EDTA, 5 mM K_2HPO_4, 1 mM dithiothreitol; pH 7.4) using an UltraTurrax, followed by centrifugation at 1000 × *g* at 4 °C for 10 min. The supernatants were then treated by ultrasound and centrifuged again at 15000 × *g* at 4 °C for 20 min. Activities of alanine aminotransferase (ALAT, EC 2.6.1.2), aspartate aminotransferase (ASAT, EC 2.6.1.1), and glutamate dehydrogenase (GDH, EC 1.4.1.2) were measured on supernatants at 37 °C using spectrophotometric procedures, according to Aragão et al. (2003). Enzyme activity units (IU), defined as micromoles of substrate converted to product per minute at assay temperature, are expressed per gram of liver (total activity). Due to technical constraints, not all analytical procedures were performed for all samples.

Data analysis

Relative growth rate (RGR), condition factor (K), and hepatosomatic index (HSI) were calculated for each fish as follows:

- RGR (% body weight day^{-1}) = $(e^g - 1) \times 100$, with $g = [\ln(WW_2) - \ln(WW_1)]$ $days^{-1}$, where WW_1 and WW_2 are the initial and final wet weights, respectively;
- K (g cm^{-3}) = (wet weight) × (total length)$^{-3}$ × 100;
- HSI (%) = (liver weight) × (fish wet weight)$^{-1}$ × 100.

All results are expressed as means ± standard deviation (SD). Data among triplicate tanks was previously tested by one-way ANOVA. In the absence of significance differences between tanks, data from the same treatment was pooled together and analysed by *t*-test. $P \leq 0.05$ was considered as statistically significant.

Results

Growth and survival

Survival was 100% for both treatments at the end of the experimental period. No significant effect of weekly han-

Table 1. Growth and condition parameters of *S. senegalensis* juveniles in *Control* and *Handling* treatments

Treatment	Wet weight (g)		RGR (% day^{-1})	K (g cm^{-3})	HSI ‡ (%)
	Initial	Final			
Control	76.7 ± 18.1	113.1 ± 24.6	0.62 ± 0.15	1.25 ± 0.10	1.88 ± 0.38
Handling	80.1 ± 20.1	116.2 ± 29.7	0.59 ± 0.17	1.25 ± 0.18	1.91 ± 0.57

Values are means ± SD of 24 fish (‡ 15 fish). Differences were not significant between treatments (*t*-test; $P > 0.5$).
RGR Relative growth rate, *K* condition factor, *HSI* hepatosomatic index

dling was found for final wet weight, RGR, K, or HSI in Senegalese sole juveniles (Table 1).

Stress indicators

Plasma cortisol levels were affected by weekly handling, being significantly higher at the end of the experiment in *Handling* than in *Control* (Fig. 1). However, plasma glucose levels were not significantly different between treatments (Fig. 2). On the other hand, plasma osmolality followed the same pattern observed for cortisol (Fig. 3).

Free amino acid concentrations and enzymatic activities

Total plasma free amino acid concentrations were not significantly affected ($P > 0.1$) by the handling stress

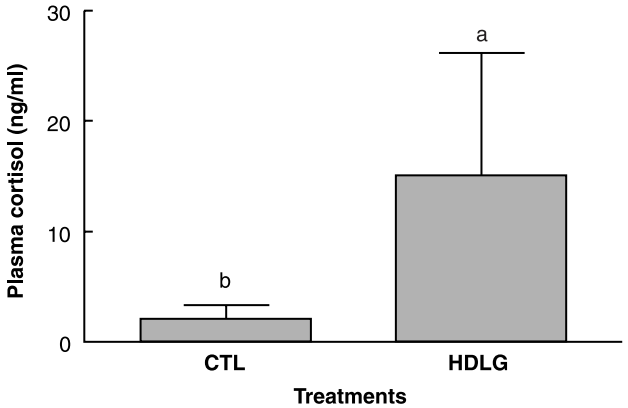

Fig. 1. Plasma cortisol levels in *S. senegalensis* juveniles in *Control* (CTL) and *Handling* (HDLG) treatments. Values are means ± SD ($n = 5$ for CTL; $n = 10$ for HDLG). Different letters above bars indicate significant differences between treatments (*t*-test; $P < 0.01$)

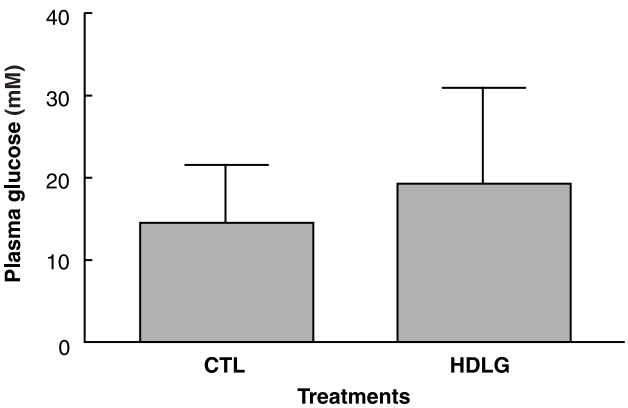

Fig. 2. Plasma glucose concentrations in *S. Senegalensis* juveniles in *Control* (CTL) and *Handling* (HDLG) treatments. Values are means ± SD ($n = 7$ for CTL; $n = 13$ for HDLG). Differences were not significant between treatments (*t*-test; $P > 0.3$)

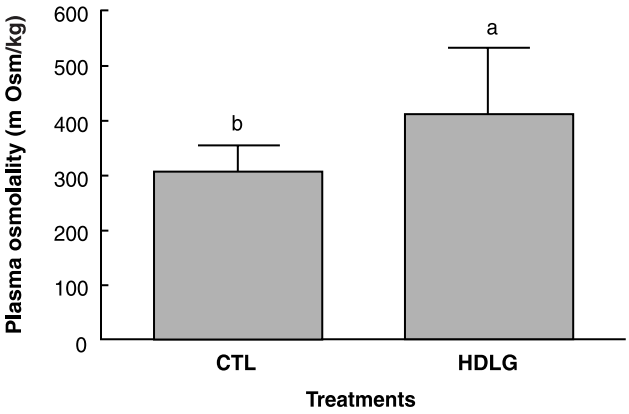

Fig. 3. Plasma osmolality in *S. senegalensis* juveniles in *Control* (CTL) and *Handling* (HDLG) treatments. Values are means ± SD ($n = 7$ for CTL; $n = 14$ for HDLG). Different letters above bars indicate significant differences between treatments (*t*-test; $P < 0.02$)

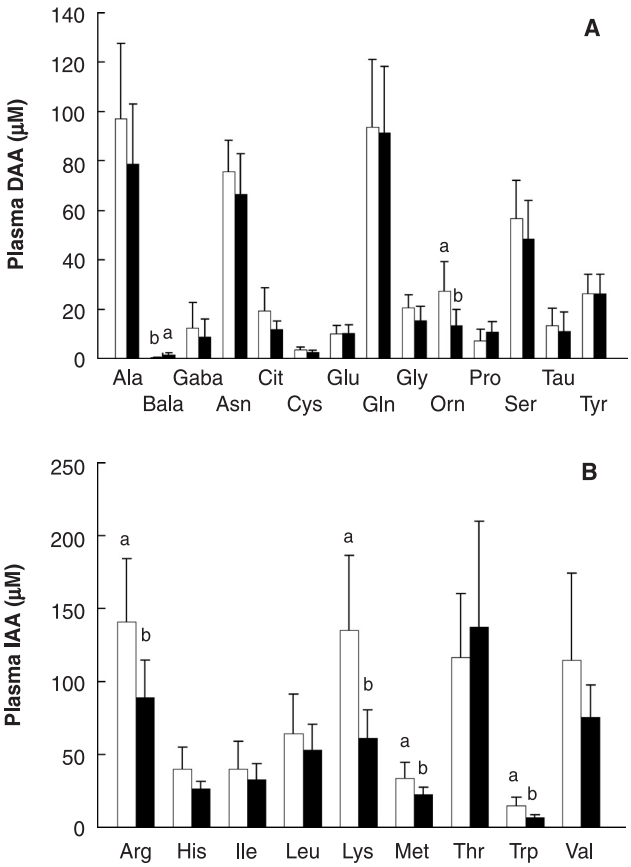

Fig. 4. Dispensable (**A**) and indispensable (**B**) amino acid concentrations in plasma of *S. senegalensis* juveniles in *Control* (□) and *Handling* (■) treatments. Values are means ± SD ($n = 6$ for *Control*; $n = 9$ for *Handling*). Different letters above bars indicate significant differences between treatments (*t*-test; $P < 0.01$, except for MET, $P = 0.05$). Alanine (Ala), β-alanine (Bala), γ-aminobutyric acid (Gaba), asparagine (Asn), citrulline (Cit), cystine (Cys), glutamic acid (Glu), glutamine (Gln), glycine (Gly), ornithine (Orn), proline (Pro), serine (Ser), taurine (Tau), tyrosine (Tyr), arginine (Arg), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), threonine (Thr), tryptophan (Trp), valine (Val)

Table 2. Activities of amino acid catabolic enzymes in liver of *S. senegalensis* juveniles in *Control* and *Handling* treatments

Treatment/ enzymes	ALAT (IU g^{-1} liver)	ASAT (IU g^{-1} liver)	GDH (IU g^{-1} liver)
Control	31.2 ± 4.7	111.5 ± 31.4	4.6 ± 1.8
Handling	31.1 ± 5.4	116.2 ± 44.5	4.7 ± 1.2

Values are means $\pm$ SD of 14 livers. Differences were not significant between treatments (*t*-test; $P > 0.7$). *ALAT* Alanine aminotransferase, *ASAT* aspartate aminotransferase, *GDH* glutamate dehydrogenase

(910 ± 240 and 1174 ± 317 μM, respectively for *Control* and *Handling* treatments). The sum of either total dispensable ($P = 0.07$) and total indispensable ($P = 0.27$) amino acids in the plasma was also unaffected by the treatments, but the concentration of some specific amino acids was significantly different. Regarding dispensable amino acids, only β-alanine and ornithine were significantly different between *Control* and *Handling* treatments (Fig. 4A). However, several indispensable amino acids were affected by the treatments (arginine, lysine, methionine and tryptophan), being significantly lower in *Handling* than in *Control* groups (Fig. 4B).

No significant differences were found between treatments for any of the three main enzymes involved in amino acid catabolism (ALAT, ASAT, GDH). Total activities determined in fish livers are shown in Table 2. The differences between treatments were also not significant when the results are expressed as specific activity (IU g^{-1} soluble protein; results not shown).

Discussion

Although it is widely accepted that stress can reduce growth in fish (Pickering, 1998), several studies provide conflicting results, in part since different responses may arise from different types of stressors, their intensity, and duration. In Atlantic salmon parr, repeated acute handling stress applied once or twice daily decreased growth and food consumption in a 40 day experiment (McCormick et al., 1998). Handling procedures twice weekly also decreased growth in Eurasian perch and rainbow trout during an 8-week experiment (Jentoft et al., 2005). However, another study with rainbow trout showed that growth was unaffected by daily handling stress during 10 weeks (Barton et al., 1987). In the current experiment, it seems clear that the handling procedure stressed the fish, based on the results of plasma cortisol. However, no differences in growth were observed. Reduced feeding has been suggested to be one of the causes by which stress decreases growth rate (McCormick et al., 1998). In this study, the same amount of food has been given to both groups along the experiment and since fish were being fed to apparent satiety, this suggests that both *Control* and *Handling* groups were eating the same. This may indicate that the applied stressor did not compromise feeding in Senegalese sole juveniles, which may be one of the reasons for the similar growth between stressed and unstressed fish groups.

Plasma glucose concentration was not significantly affected due to the handling stress imposed on the fish after nine weeks of experiment. This may be due to a possible habituation to the stressful conditions. When the acute stressor is repeated over a period of time, some habituation may occur, which may result in reductions in post-stress cortisol and glucose levels (Jentoft et al., 2005). However, cortisol enhances the rate of gluconeogenesis, by increasing the activity of all key gluconeogenic enzymes. This may not lead necessarily to an increase in plasma glucose concentrations, since this is related to the rate of glucose turnover in the fish (Mommsen et al., 1999). Therefore, the fact that the plasma glucose concentration in this study did not increase, contrary to what was observed for cortisol, is not surprising and it does not mean that the fish did not present an increased gluconeogenic activity. Besides the possible stress habituation, this lack of increase in plasma glucose concentration in stressed fish as been observed for turbot (Waring et al., 1996) and in flounder only a mild increase was observed (Waring et al., 1992). This may suggest a possible different stress physiology response in flatfish submitted to handling procedures than, for instance, in pelagic fish species such as gilthead seabream (Arends et al., 1999; Papoutsoglou et al., 1999) or Atlantic salmon (Waring et al., 1992). This possible difference in carbohydrate metabolism in flatfish species may partly explain why in the current experiment growth was unaffected by stress, since a reduction in growth due to glucose mobilization under stressful conditions could be expected.

One of the physiological changes that may occur in fish physically handled is an osmotic and ionic disturbance. The cardiovascular adjustments in stressed fish and the increased permeability of the surface epithelia, due to an increase in cathecolamine concentrations, may result in difficulties in controlling the osmotic water efflux and the ions influx in saltwater species (Pickering, 1998). This effect was observed in the present study, where the fish in the *Handling* treatment presented an increase in plasma osmolality when compared to *Control* treatment. Handling experiments in other species such as seabass

(Varsamos et al., 2006), gilthead seabream (Arends et al., 1999; Papoutsoglou et al., 1999), flounder or Atlantic salmon (Waring et al., 1992) also induced an increase in plasma osmolality, although air exposure did not affect plasma osmolality in turbot (Waring et al., 1996).

In this study, no differences in plasma total amino acids were found between stressed and unstressed fish. In the same way, acute handling stress did not increase plasma total amino acid levels in common dentex up to 12 h after the applied stressor (Morales et al., 2005). However, confinement and exhaustive exercise have been shown to increase plasma total amino acids in tilapia and rainbow trout, respectively, and a relation between increased cortisol levels and tissue amino acid mobilization has been proposed (Milligan, 1997; Vijayan et al., 1997). Therefore, this may suggest that in this study, the type of stress imposed to the fish did not increase protein degradation as a way to increase the availability of amino acids for energy production. This hypothesis is further reinforced by the fact that the activities of the catabolic amino acid enzymes analysed were not significantly affected in the stressed (*Handling*) compared with control fish. However, evidences from the present study suggest a strong influence of stress on amino acid metabolism.

The changes detected in the plasma free amino acid concentrations within the current study, suggest changes in amino acid requirements. It is not always possible to use plasma levels of amino acids as indicators of its requirements (see Wilson, 2002), but this does not seem to be the case for the current experiment. First, since fish in both treatments were growing at the same rate, this excludes the possibility of the variations in plasma free amino acid concentrations were being affected by differences in growth. Second, since samples were collected in fasted fish, no influence of feeding is expected. Moreover, several studies with fish have confirmed the possible use of plasma free amino acid responses as a indicator of their requirements assessed by growth trials (e.g. Alam et al., 2002; Lall et al., 1994; Tibaldi and Tulli, 1999). Therefore, plasma free amino acid concentrations indicate that amino acid requirements changed in Senegalese sole juveniles exposed to handling stress, which appears to be related with the synthesis of proteins or other specific compounds related to stress response.

The major alterations in plasma free amino acid concentrations were found for indispensable amino acids. Several of those are directly or indirectly involved in the synthesis of compounds important in the stress response. For instance, arginine is involved in several important metabolic functions, including nitric oxide synthesis, which plays important roles in many diverse processes, as vasodilation, immune response, and neurotransmission (Wu and Morris, 1998). Arginine is also involved in urea synthesis (Walsh and Mommsen, 2001). Urea seems to be an important nitrogen-end product in flatfish species (Dosdat et al., 1996; Verbeteen et al., 1999) and this may be also the case in Senegalese sole. A possible increase in urea production in stressed fish as a consequence of increased nitrogen excretion or for osmoregulation purposes seems a matter for further studies. On the other hand, tryptophan is a precursor of serotonin and changes in their availability may modulate melatonin synthesis, which may suffer alterations during stress conditions in fish (Kulczykowska, 2001; Larson et al., 2004). Regarding methionine, it is the precursor of cysteine and glutathionine, a compound with a critical role in cellular peroxidative protection (Obled et al., 2002; Reeds and Jahoor, 2001).

In summary, physical handling once a week induced stress in Senegalese sole juveniles, resulting in an increased plasma cortisol and osmolality levels and in changes in the free amino acid pool. However, fish seem to be able to cope with this stress situation, resulting in similar growth rates in handled and control groups. Moreover, it seems that the amino acid requirements in fish under this stress situation are altered, and may result in an increase in the requirement of some indispensable amino acids involved in the synthesis of proteins and other compounds related with the stress response.

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Authors' address: C. Aragão, CCMAR, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal,
Fax: +351 289 800 069, E-mail: caragao@ualg.pt