Fish Physiol Biochem (2013) 39:1141–1151
DOI 10.1007/s10695-013-9770-1

Interactive effects of a high-quality protein diet and high stocking density on the stress response and some innate immune parameters of Senegalese sole *Solea senegalensis*

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Received: 26 October 2012 / Accepted: 12 January 2013 / Published online: 23 January 2013

Abstract Amino acids (AA) regulate key metabolic pathways, including some immune responses. Therefore, this study aimed to assess whether an increased availability of dietary AA can mitigate the expected increase in plasma cortisol and metabolites levels due to high stocking density and its subsequent immunosuppression. Senegalese sole (*Solea senegalensis*) were maintained at low stocking density (LSD; 3.5 kg m^{-2}) or high stocking density (HSD; 12 kg m^{-2}) for 18 days. Additionally, both treatments were fed a control or a high protein (HP) diet (*LSD*, *LSD HP*, *HSD* and *HSD HP*). The HP diet slightly increased the levels of digestible indispensable AA, together with tyrosine and cysteine. *HSD* was effective in inducing a chronic stress response after 18 days of treatment since fish held at *HSD* presented higher plasma cortisol, glucose and lactate levels. Moreover, this increase in stress indicators translated in a decrease in plasma lysozyme, alternative complement pathway (ACP) and peroxidase activities, suggesting some degree of immunosuppression. Interestingly, while plasma glucose and lactate levels in *HSD HP* specimens decreased to similar values than *LSD* fish, plasma lysozyme, ACP and peroxidase activities increased, with even higher values than *LSD* groups for ACP activity. It is suggested that the HP diet may be used as functional feed since it may represent a metabolic advantage during stressful events and may counteract immunosuppression in sole.

Keywords Arginine · Complement activity · Cortisol · High density · Lysozyme activity · Nitric oxide

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Introduction

Fish welfare is recognized as an important issue in aquaculture, due both to ethical issues and production advantages. For instance, good welfare may lead to lower susceptibility to stress and diseases (e.g. Conceição et al. 2012; Prunet et al. 2012; Segner et al. 2012). The effect of stress on the immune system has been widely investigated, and chronic stress was found to inhibit an optimal immune response in mammals and fish, leading to increased susceptibility to pathogens (Dhabhar 2009; Tort 2011). In particular, cortisol alone

can influence multiple aspects of the innate immune defence mechanisms in fish (Verburg-van Kemenade et al. 2009). Several in vitro studies demonstrated that cortisol affects the cytokine production of immune cells. For instance, cortisol inhibits LPS-induced expression of acute-phase protein serum amyloid S and pro-inflammatory cytokines interleukin (IL)-1β, tumour necrosis factor-α, IL-11 and inducible nitric oxide synthase (Saeij et al. 2003; Fast et al. 2008; Stolte et al. 2008). Cortisol also affects apoptosis and proliferation of immune cells (Verburg-van Kemenade et al. 2009). Macrophages isolated from Atlantic salmon (*Salmo salar*) submitted to repeated handling for 3 and 4 weeks showed decreased survival when exposed to *Aeromonas salmonicida* (Fast et al. 2008). Interestingly, cortisol can induce apoptosis in activated B lymphocytes but not in neutrophils (Weyts et al. 1999). Still, the effects of stress on the immune system are difficult to interpret in vivo, since a number of different hormones are involved via the hypothalamic-pituitary-interrenal axis (Wendelaar Bonga 1997).

Stress conditions that induced high plasma cortisol levels also modified fish amino acid (AA) metabolism in several teleost species (Milligan 1997; Pinto et al. 2007; Aragão et al. 2008, 2010; Costas et al. 2008, 2011a, 2012a). Changes in plasma-free AA levels may be indicative of AA requirements in fish (Wilson 2002). In fact, it has been suggested that fish under stressful conditions present additional AA requirements, due to either increased energetic demands or for the synthesis of stress-related proteins and other compounds related with the stress response (Aragão et al. 2008, 2010; Costas et al. 2008). The role of specific AA and their metabolites on key metabolic pathways that are necessary for growth, immunity or resistance to environmental stressors and pathogens has been recently reviewed in mammals and fish (Li et al. 2007, 2009). Thus, AA not only serves as constituents of proteins and energy sources, but also can be converted into important biochemically active substances in vivo. In particular, arginine is the precursor for the synthesis of nitric oxide (NO) and polyamines in higher vertebrates. In fish, NO production plays an important role in cellular defence mechanisms and has been demonstrated in stimulated macrophages in several fish species (Neumann et al. 1995; Tafalla and Novoa 2000), including Senegalese sole (*Solea senegalensis*) (Costas et al. 2011b). Moreover, dietary tryptophan supplementation can inhibit aggression or reduce cannibalism and stress-induced anorexia and cortisol augmentation in several teleosts (Hseu et al. 2003; Lepage et al. 2003; Höglund et al. 2007). Furthermore, an increase in all dietary indispensable AA appears to minimize some negative effects attributed to cortisol release in chronically handled Senegalese sole (Costas et al. 2012a).

Senegalese sole is a very attractive candidate for marine aquaculture and has a very big potential for future farming at commercial scale. However, growth and survival from juvenile to market-size fish is not fully controlled with regard to rearing technology and husbandry conditions, feeding behaviour and nutritional requirements (Imsland et al. 2003). Among the different factors that may induce high mortality during the juvenile stage, stress might be one of the key issues. In particular, this species appears to be highly susceptible to opportunistic pathogens when stocked at high density (Costas et al. 2008). Therefore, the main objective of this study is to assess whether an increased availability of dietary AA can mitigate the expected increase in plasma cortisol and metabolites levels due to high stocking density. Moreover, it is also intended to verify whether the expected decrease in plasma non-specific immune parameters due to cortisol action can also be mitigated through dietary treatment.

Materials and methods

Experimental diets

Two diets were formulated, a moderate protein reference diet (Control) containing 44.1 % fish meal as the main protein source and a high protein diet (HP) where fish meal was increased up to 46.5 %. Therefore, HP diet had a slightly increase in the levels of digestible indispensable AA, together with tyrosine and cysteine, when compared to the control diet. In addition, L-tryptophan [0.5 % on a dry matter (DM) basis] was added to the HP diet since this indispensable AA is known to reduce cortisol augmentation in fish (see introduction). Wheat gluten and corn gluten were chosen as complementary protein sources due to their high protein content and potential high digestibility in fish, while soybean meal is known to have both high crude protein content (44 % DM) and a reasonably balanced AA profile (Gatlin et al. 2007). In

the absence of specific data on vitamin, mineral and trace element requirements for Senegalese sole, requirement data for other species were applied (NRC 1993; Kaushik 1998). All dietary ingredients were supplied by Sorgal S.A. (Ovar, Portugal) and were finely ground, mixed and dry pelleted through a 3.2 mm die at 50 °C (CPM, C-300 model, San Francisco, CA, USA). The diets were dried at 37 °C for 24-h and stored in a refrigerator (4 ± 1 °C) until use. Formulation and proximate composition of the experimental diets are presented in Table 1 and the corresponding AA profile in Table 2.

Fish

Senegalese sole juveniles (109.6 ± 16.9 g wet weight) originated from the natural spawning of wild broodstock and were reared according to standard larval and juvenile rearing protocols (Dinis et al. 1999). Before the experiment, fish were acclimated for 15 days using a flow-through seawater system (temperature: 20 ± 1 °C; salinity: 36 g l^{-1}; dissolved oxygen: above 90 % saturation level), comprised by a flat-bottomed fibreglass tank.

This study was directed by trained scientists (following FELASA category C recommendations) and conducted according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE.

Experimental design

At the beginning of the experiment, fish were fasted for 24 h, anaesthetized with 2-phenoxyethanol (500 ml l^{-1}, Sigma-Aldrich, Germany) and individually measured and weighed. Fish were distributed in eight flat-bottomed tanks (70 cm length × 30 cm width × 20 cm depth, volume 20 l), using a partial-recirculated seawater system (temperature: 20 ± 1 °C; salinity: 36 g l^{-1}; dissolved oxygen: 90 % above saturation level), to achieve two different initial densities in quadruplicate tanks: 3.5 and 12 kg m^{-2} with 8 and 20 specimens per tank, respectively. Fish maintained at low stocking density (3.5 kg m^{-2}) were regarded as control while fish held at high stocking density (12 kg m^{-2}) were considered chronically stressed. Moreover, fish held at both densities were fed either the Control or the HP diets. Therefore, this experimental set-up comprised four treatments randomly assigned to duplicate tanks: fish held at low stocking density fed the control diet (*LSD*) or the HP diet (*LSD HP*), and fish held at high stocking density fed the control diet (*HSD*) or the HP diet (*HSD HP*). The experimental period lasted for 18 days and fish were fed daily to apparent satiety by automatic feeders, over a 24-h period.

Table 1 Ingredients and proximal composition of experimental diets

	Experimental diets	
	Control	HP
Ingredients (%)		
Fish meal herring	43.4	47.7
Soybean meal	15.0	15.0
Corn gluten	8.0	9.0
Wheat meal	23.0	10.0
Starch	0.0	7.5
Fish oil	9.3	9.0
Agar	1.0	1.0
L-Tryptophan	–	0.5
Choline chloride	0.1	0.1
Mineral mix[a]	0.1	0.1
Vitamin mix[b]	0.1	0.1
Proximate composition		
Dry matter (% DM)	89.7	89.7
Crude protein (% DM)	44.1	46.5
Crude fat (% DM)	14.4	14.4
Ash (% DM)	8.2	9.3
Gross energy (kJ g^{-1} DM)	20.5	20.5

HP high protein; fish oil extracted from sardine, *DM* dry matter

[a] Minerals (g or mg kg^{-1} diet): Mn (manganese oxyde), 20 mg; I (potassium iodide), 1.5 mg; Cu (copper sulphate), 5 mg; Co (cobalt sulphate), 0.1 mg; Mg (magnesium sulphate), 500 mg; Zn (zinc oxide), 30 mg; Se (sodium selenite), 0.3 mg; Fe (iron sulphate), 60 mg; Ca (calcium carbonate), 2.15 g; dibasic calcium phosphate, 5 g; KCl (potassium chloride), 1 g; NaCl (sodium chloride), 0.4 g

[b] Vitamins (mg or IU kg^{-1} diet): vitamin A (retinyl acetate), 8,000 IU; vitamin D3 (DL-cholecalciferol), 1,700 IU; vitamin K3 (menadione sodium bisulphite), 10 mg; vitamin B12 (cyanocobalamin), 0.02 mg; vitamin B1 (thiamine hydrochloride), 8 mg; vitamin B2 (riboflavin), 20 mg; vitamin B6 (pyridoxine hydrochloride), 10 mg; folic acid, 6 mg; biotin, 0.7 mg; inositol, 300 mg; nicotinic acid, 70 mg; pantothenic acid, 30 mg; vitamin E (Lutavit E50), 300 mg; vitamin C (Lutavit C35), 500 mg; betaine (Betafin S1), 500 mg

Table 2 Amino acid composition (g 100 g^{-1} AA) of experimental diets

	Experimental diets	
	Control	HP
IAA		
His	3.4 ± 0.1	3.2 ± 0.1
Arg	7.1 ± 0.1	8.3 ± 0.1*
Ile	4.8 ± 0.1	4.7 ± 0.2
Leu	9.0 ± 0.2	8.9 ± 0.3
Val	4.7 ± 0.1	4.5 ± 0.1
Thr	4.5 ± 0.1	5.1 ± 0.2*
Lys	7.5 ± 0.1	8.1 ± 0.0*
Phe	4.9 ± 0.0	5.0 ± 0.1
DAA		
Asx	9.0 ± 0.2	9.0 ± 0.3
Glx	16.0 ± 0.1	16.5 ± 0.4
Ser	4.6 ± 0.0	4.7 ± 0.1
Gly	5.6 ± 0.0	5.4 ± 0.1
Ala	6.5 ± 0.1	6.3 ± 0.1
Pro	5.5 ± 0.2	5.7 ± 0.2
Tyr	4.2 ± 0.1	4.3 ± 0.1

Trp, Met and Cys were not analysed. Values are mean ± SEM ($n = 3$). Row means followed by asterisk indicate significant differences between experimental diets (Student's t test; $P < 0.05$)

HP high protein

Sampling

Fish were fasted 24-h prior to sampling in order to avoid any influence of feeding on cortisol and glucose levels (Arends et al. 1999). Five fish were quickly taken out from each tank at a time and anaesthetized with lethal doses of 2-phenoxyethanol (1,000 ml l^{-1}, Sigma-Aldrich). Blood was withdrawn from the caudal vein of each fish using heparinized syringes. After each sampling, blood was centrifuged at 2,000×g during 5 min at room temperature. The collected plasma was stored at −25 °C for further analysis.

Analytical procedures

Diets were analysed for total AA contents. Diet samples were hydrolysed in 6 M HCl at 106 °C over 24-h in nitrogen-flushed glass vials. After deproteinization, samples were pre-column derivatized with phenylisothiocyanate (PITC; Pierce), using the PicoTag method (Waters, USA) according to Cohen et al. (1989). External standards were prepared along with the samples, using physiological AA 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 performance liquid chromatography (HPLC) in a Waters Reversed-Phase Amino Acid Analysis System equipped with a PicoTag column (3.9 × 300 mm), using the conditions described by Cohen et al. (1989). Resulting peaks were analysed with the Breeze software (Waters). During acid hydrolysis, asparagine is converted to aspartate and glutamine to glutamate, so the reported values for these AA (Asx and Glx) represent the sum of the respective amine and acid. Moreover, tryptophan was not determined since it is destroyed by acid hydrolysis. Due to technical constraints, cysteine and methionine in the samples were not quantified.

Plasma cortisol levels were 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 and lactate were assessed using commercially available Spinreact kits (Glucose HK Ref. 1001200; Lactate Ref. 1001330), adapted for 96-well microplates. Plasma total proteins were determined in 1:50 (v/v) diluted plasma samples using the bicinchoninic acid (BCA) Protein Assay Kit (Pierce #23225, Rockford, USA) for microplates. Bovine serum albumin served as a standard. All analyses were conducted in triplicates.

Total nitrite plus nitrate in plasma was analysed using a nitrite/nitrate colorimetric method (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, nitrate was reduced to nitrite with nitrate reductase and nitrite was determined colorimetrically after addition of sulfanilamide and *N*-naphthyl-ethylenediamine. Nitrite concentration was calculated by comparison with a sodium nitrite standard curve. Since nitrite and nitrate are endogenously produced as oxidative metabolites of the messenger molecule NO, these compounds are considered as indicative of NO production (Saeij et al. 2003). All analyses were conducted in duplicates.

Alternative complement pathway (ACP) activity was estimated as described by Sunyer and Tort (1995). The following buffers were used: GVB (Isotonic

veronal buffered saline), pH 7.3, containing 0.1 % gelatin; EDTA-GVB, as previous one but containing 20 mM EDTA; and Mg-EGTA-GVB, which is GVB with 10 mM Mg^{+2} and 10 mM EGTA. Rabbit red blood cells (RaRBC; Probiologica Lda, Portugal) were used for ACP determination. RaRBC were washed four times in GVB and resuspended in GVB to a concentration of 2.5 $\times$ 10^8 cells ml^{-1}. Ten microlitre of RaRBC suspension was then added to 40 µl of serially diluted plasma in Mg-EGTA-GVB buffer. Samples were incubated at room temperature for 100 min with regular shaking. The reaction was stopped by adding 150 µl of cold EDTA-GVB. Samples were then centrifuged, and the extent of haemolysis was estimated by measuring the optical density of the supernatant at 414 nm. The ACH50 units were defined as the concentration of serum giving 50 % haemolysis of RaRBC. All analysis was conducted by triplicates.

Lysozyme activity was measured using a turbidimetric assay as described by Costas et al. (2011a). Briefly, a solution of *Micrococcus lysodeikticus* (0.5 mg ml^{-1} 0.05 M sodium phosphate buffer; pH 6.2) was prepared. To a microplate, 15 µl of plasma and 250 µl of the above suspension were added to give a final volume of 265 µl. The reaction was carried out at 25 °C, and the absorbance (450 nm) is measured after 0.5 and 4.5 min. Lyophilized hen egg white lysozyme (Sigma) was serially diluted in sodium phosphate buffer (0.05 M; pH 6.2) and used to develop a standard curve. The amount of lysozyme in the sample was calculated using the formula of the standard curve. All analyses were conducted in triplicate.

Total peroxidase activity in plasma was measured following the procedure described by Quade and Roth (1997). Briefly, 15 µl of plasma (triplicates per fish) was diluted with 135 µl of HBSS without Ca^{+2} and Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µl of 20 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride (Sigma) and 50 µl of 5 mM H_2O_2 were added. The colour-change reaction was stopped after 2 min by adding 50 µl of 2 M sulphuric acid, and the optical density was read at 450 nm in a Powerwave™ microplate spectrophotometer (BioTek, Winooski, USA). The wells without plasma were used as blanks. The peroxidase activity (units ml^{-1} plasma) was determined defining one unit of peroxidase as that which produces an absorbance change of 1 optic density.

Statistics

All results are expressed as mean ± standard error of the mean (SEM). Data were analysed for normality (Kolmogorov–Smirnov test) and homoscedasticity of variance (Levene's test) and, when necessary, they were log-transformed before being treated statistically. Data from experimental diets were analysed by *t* test, while fish data were analysed by two-way analysis of variance (ANOVA) with stress and dietary treatment as dependent variables. When significant differences were obtained from the ANOVA, Student's *t* tests were carried out for paired-comparisons to analyse the effect of stress and dietary treatment. All statistical analyses were performed using the computer package SPSS 15.0 for WINDOWS. The level of significance used was $P \leq 0.05$ for all statistical tests.

Results

Diets

Feed ingredients and feed composition are given in Table 1. HP diet presented higher protein level, while lipids, DM, ash and energy values were similar in both diets. In addition, digestible protein from Control and HP diets was calculated according to Dias et al. (2010), being 41.1 and 43.2 %, respectively. The AA patterns (g 100 g^{-1} AA) presented significant differences between experimental diets, with arginine, threonine and lysine being significantly higher in the HP diet (Table 2).

Experimental diets were well accepted, and no significant differences were found in growth and feed intake among the experimental groups (results not shown). Moreover, survival was 99.1 % for fish held at *HSD* and 100 % for fish in treatments *LSD*, *LSD HP* and *HSD HP* at the end of the experimental period.

Stress indicators

Plasma cortisol levels were significantly higher in specimens from *HSD* when compared to both *LSD* groups. Although cortisol levels also increased in fish held at *HSD HP* treatment, those values were not significantly different than the observed in specimens held at *LSD* treatment (Fig. 1). Plasma glucose and lactate levels showed a similar pattern, being

significantly higher in fish held at *HSD* when compared to specimens held at *LSD*, *LSD HP* and *HSD HP* treatments. In addition, plasma protein levels were not significantly different among treatments (Table 3).

Innate humoral parameters

ACP activity decreased significantly in fish held at *HSD* when compared to all other treatments, while specimens from *HSD HP* treatment showed higher values than those in fish from *LSD* and *HSD* treatments (Fig. 2). Moreover, plasma NO levels showed a trend to decrease in fish held at *HSD* treatment, though no significant changes were observed (Fig. 3). Similar to that observed for ACP values, plasma lysozyme activity decreased significantly in fish held at *HSD* when compared to all other treatments (Fig. 4). Furthermore, plasma peroxidase activity also showed a similar tendency with lower values in fish held at *HSD* when compared to both *LSD* and *LSD HP* treatments. In addition, specimens from *LSD HP* presented higher peroxidase activity than fish from all other treatments (Fig. 5).

Discussion

High stocking density clearly increased plasma cortisol, glucose and lactate levels in fish held at *HSD* treatment from the current study. Moreover, those changes are in agreement with those previously described for Senegalese sole and other fish species under similar stressful conditions (Montero et al. 1999; Costas et al. 2008; Herrera et al. 2009; Conde-Sieira et al. 2010; Salas-Leiton et al. 2010). Measurement of plasma cortisol, glucose and lactate levels provides an effective method to monitor primary and secondary stress responses in fish (Mommsen et al. 1999; Pottinger 2008). Therefore, results from the current study validate the experimental design since *HSD* elicited a chronic stress response, including primary (plasma cortisol) and secondary (plasma glucose and lactate) responses, after 18 days of treatment.

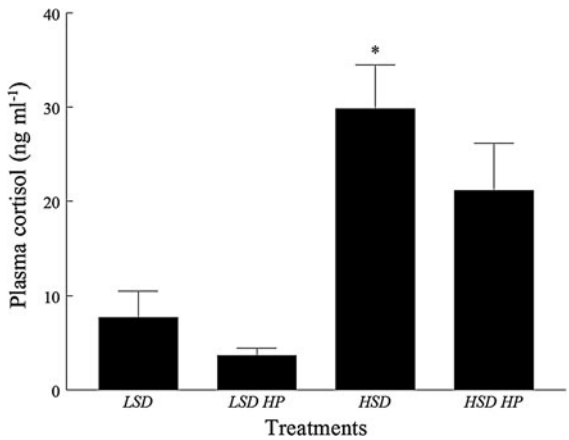

Fig. 1 Plasma cortisol levels in *S. senegalensis* after 18 days held at different treatments. Values are mean ± SEM ($n = 10$). *Significant differences attributed to stress (Student's t test; $P < 0.05$)

Interestingly, plasma glucose and lactate levels decreased significantly in fish held at *HSD HP* up to similar values than *LSD* and *LSD HP* specimens, while plasma cortisol levels were not significantly different than those from controls. Therefore, dietary treatment seems to influence the Senegalese sole primary and secondary stress responses by minimizing cortisol release and the subsequent mobilization of energy substrates, at least after 18 days of feeding and under this particular stressful condition. Similarly, Senegalese sole submitted to repeated handling stress and fed a diet with a slightly increase in arginine, methionine, lysine and phenylalanine for 14 days also decreased plasma glucose and lactate levels (Costas et al. 2012a). Moreover, rainbow trout (*Oncorhynchus mykiss*) fed diets supplemented with tryptophan (4 and 8 times the tryptophan content of the control diets) for 7 days, and thereafter subjected to an acute stress, showed significantly lower cortisol levels than stressed fish fed the control diets (Lepage et al. 2002, 2003). Although HP diet from the current study was supplemented with L-tryptophan, this indispensable AA was not analysed. However, the HP diet presented higher levels of arginine, threonine and lysine than the control diet. Dietary arginine supplementation decreased the level of cortisol in serum of finishing pigs and weaned piglets (Ma et al. 2010; Yao et al. 2011). Additionally, Costas et al. (unpublished results) observed that dietary arginine supplementation beyond minimum requirement for optimal growth can decrease cortisol levels in chronically stressed turbot (*Scophthalmus maximus*). In the present study, it is possible that arginine metabolites or arginine itself may have minimized the release of the adrenocorticotropic hormone from pituitary and/or interfered in the brain monoaminergic system. For instance, an intracerebroventricular injection of arginine induced sedative or

Table 3 Plasma glucose, lactate and protein levels in *S. senegalensis* after 18 days held at different treatments

Parameter	Treatments			
	LSD	*LSD HP*	*HSD*	*HSD HP*
Glucose (mM)	5.0 ± 0.6	6.2 ± 0.5	8.1 ± 0.3*	5.4 ± 0.5
Lactate (mM)	5.3 ± 0.8	4.9 ± 0.5	9.6 ± 0.6*	7.0 ± 0.9
Proteins (mg ml^{-1})	40.1 ± 3.8	37.5 ± 3.6	41.9 ± 9.1	39.8 ± 4.1

Values are mean ± SEM ($n = 10$)

*Significant differences attributed to stress (Student's t test; $P < 0.05$)

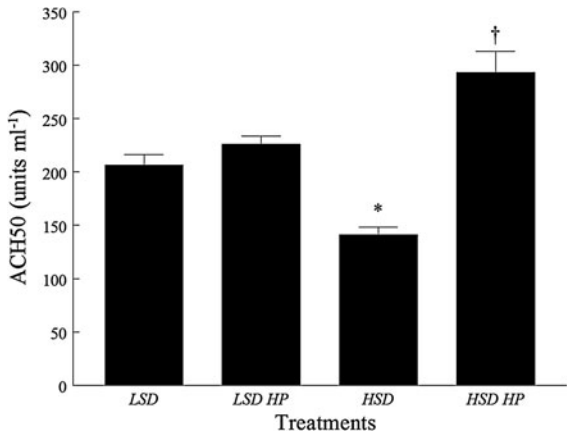

Fig. 2 Alternative complement pathway activity in *S. senegalensis* after 18 days held at different treatments. Data are presented as ACH50 values in plasma, expressed as mean ± SEM ($n = 10$). *Significant differences attributed to stress, and †significant differences attributed to HP diet (Student's t test; $P < 0.05$)

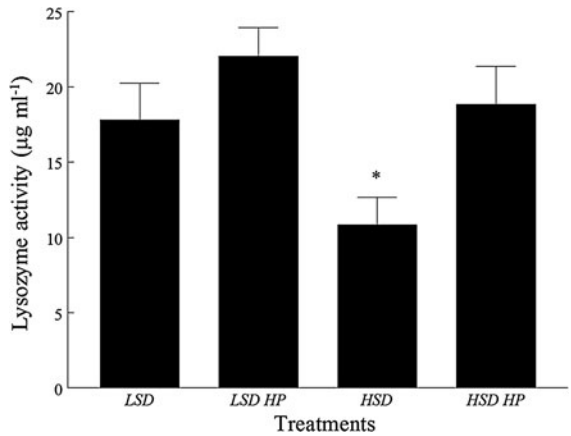

Fig. 4 Plasma lysozyme activity in *S. senegalensis* after 18 days held at different treatments. Values are mean ± SEM ($n = 10$). *Significant differences attributed to stress (Student's t test; $P < 0.05$)

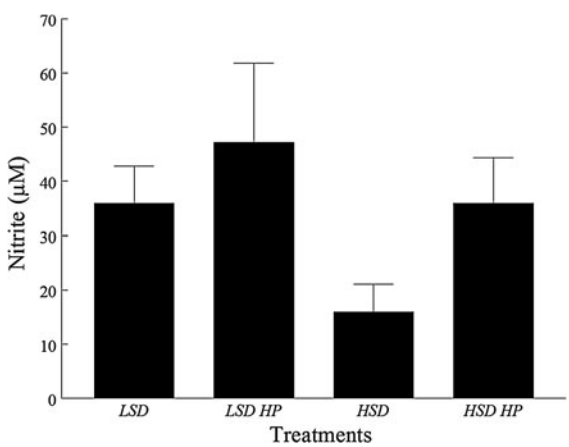

Fig. 3 Nitric oxide levels in *S. senegalensis* after 18 days held at different treatments. Data are presented as nitrite concentration in plasma, expressed as mean ± SEM ($n = 10$). *Significant differences attributed to stress, and †significant differences attributed to HP diet (Student's t test; $P < 0.05$)

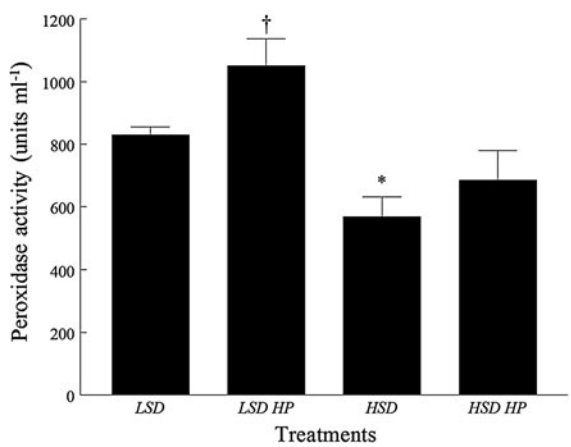

Fig. 5 Plasma peroxidase activity in *S. senegalensis* after 18 days held at different treatments. Values are mean ± SEM ($n = 10$). *Significant differences attributed to stress, and †significant differences attributed to HP diet (Student's t test; $P < 0.05$)

hypnotic effects in chicks exposed to a social isolation stress (Suenaga et al. 2008). However, the underlying mechanisms are unknown and the function of endogenous arginine under stressful husbandry conditions in fish deserves further attention.

The innate humoral immune responses evaluated in this study generally depend on the species and type and duration of the stress imposed. In some cases, plasma lysozyme and ACP activities decrease or no changes are observed (Cuesta et al. 2005; Costas et al. 2011a; Mauri et al. 2011), while in other studies, plasma lysozyme and peroxidase activities significantly increased in stressed specimens (Demers and Bayne 1997; Rotllant et al. 1997; Cuesta et al. 2005; Caipang et al. 2009; Costas et al. 2011b, 2012a). These differential effects may be achieved by differences in overall glucocorticoid sensitivity or receptivity of the immune response being affected (Dhabhar 2009). In the current study, the decrease in plasma lysozyme, ACP and peroxidase activities observed in fish held at *HSD* suggests an impairment of the immune system. Moreover, this drop in humoral responses is consistent with that generally described for chronically stressed fish (Mauri et al. 2011; Tort 2011), including Senegalese sole held at high stocking density (Salas-Leiton et al. 2010). This lower plasma lysozyme, ACP and peroxidase values in fish held at *HSD* may be further associated with decreases in circulating phagocytes. Neutrophils are thought to be the source of plasma lysozyme and peroxidase (Murray and Fletcher 1976; Ellis 1999), and increases in lysozyme and peroxidase levels have been associated with increases in neutrophil numbers (Cerezuela et al. 2009; Costas et al. 2012b). In addition, complement proteins can stimulate phagocytosis by opsonizing pathogens, a process that is mediated by complement receptors on the surface of phagocytic cells (Holland and Lambris 2002). Considering the role of both macrophages and neutrophils on phagocytosing bacteria, it is likely that *HSD* specimens had a lower level of protection than specimens from the other treatments to resist a bacterial infection. In fact, this species appear to be highly susceptible to opportunistic pathogens under similar stressful conditions (Costas et al. 2008).

In the current study, the HP diet increased plasma lysozyme, ACP and peroxidase activities in fish held at *HSD HP* when compared to *HSD* specimens. ACP activity with HP diet was even higher than in *LSD* groups. Moreover, fish held at *LSD HP* also presented higher peroxidase levels than specimens from all other treatments. Therefore, arginine, threonine and/or lysine (and possibly tryptophan) present important roles in non-specific immune mechanisms. Particularly in this study, one or more of these indispensable AA appear to mitigate the immunosuppressive effects attributed to chronic stress action. Similarly, Costas et al. (unpublished results) have recently observed that Senegalese sole fed a diet with higher arginine, isoleucine, leucine, threonine, valine and methionine levels than the control diet increased plasma lysozyme, ACP and peroxidase activities after 12 weeks of feeding. As already specified above (see introduction), many AA regulates key metabolic pathways that are crucial for immune responses. In particular, leucine is an activator of the mTOR signalling pathway that regulates protein synthesis and degradation in cells and appears to exert a greater effect on immune function than isoleucine and valine, which may be explained in part by their differential actions on the mTOR signalling (Li et al. 2007). Furthermore, serotonin, melatonin and *N*-acetylserotonin, products of tryptophan catabolism, can enhance host immunity by inhibiting the production of superoxide, scavenging free radicals and attenuating the production of tumour necrosis factor-alpha (Perianayagam et al. 2005). Since there is a progressive decline in tryptophan concentrations in plasma of animals with inflammation, its catabolism plays a critical role in the functions of both macrophages and lymphocytes (Melchior et al. 2004). At present, a potential use of tryptophan supplementation for animal health management is not fully developed. However, it has been reported that dietary supplementation with tryptophan can inhibit aggression, reduce cannibalism and prevent cortisol-mediated immune suppression in fish (Li et al. 2009). Moreover, a large body of evidence from animal studies indicates that adequate provision of arginine is required for lymphocyte development and that dietary arginine supplementation enhances immune function in various models of immunological challenges (Li et al. 2007). In particular, a positive effect of arginine-enriched diets on disease resistance has even been observed in Senegalese sole (Costas et al. 2011b). Therefore, high protein diets may be used as functional feeds for chronically stressed sole, as an alternative to chemotherapeutic and antibiotic treatments. The term *functional feeds* is used to describe fish feeds that have added benefits beyond the fish

essential nutritional requirements, being both health status and growth expected to improve (Li et al. 2009).

In conclusion, feeding sole a diet with a slightly increase in all indispensable AA, together with tyrosine and cysteine, with respect to a reference diet results in a decrease in post-stress plasma cortisol, glucose and lactate levels after 18 days of treatment, thus minimizing the negative effects attributed to cortisol release after hypothalamic-pituitary-interrenal axis activation. In addition, the decrease in plasma lysozyme, ACP and peroxidase activities due to high stocking density was minimized through HP diet after 18 days. Therefore, it is suggested that a high-quality protein diet may represent a functional feed for chronically stressed sole. A large number of additives or feed ingredients (e.g. prebiotics, probiotics, glucans or nucleotides) are available for inclusion in functional feeds. However, little attention has been paid to the role of individual AA as potential immunostimulants. According to the results from this study and to that already reported by the same authors, supplementing key AA in the diet may represent a metabolic advantage during predictable stressful events (e.g. crowding associated with grading procedures), which may have a significant effect on growth, immunity and welfare in the longer term.

Acknowledgments This study was supported by project STRESSAA-POCTI/CVT/49324/2002 (FCT, Portugal and FEDER). Benjamín Costas and Cláudia Aragão were supported by Fundação para a Ciência e a Tecnologia, Portugal (SFRH/BD/38697/2007 and SFRH/BPD/37197/2007, respectively).

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