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Dietary arginine and repeated handling increase disease resistance and modulate innate immune mechanisms of Senegalese sole (*Solea senegalensis* Kaup, 1858)

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ABSTRACT

Stress is known to impair immune function and disease resistance in fish. In the present study, repeated handling was employed as a chronic stressor in order to verify whether its attributed immunosuppressive effects could be minimized by dietary arginine supplementation. Therefore, Senegalese sole (*Solea senegalensis*) were air exposed daily for 3 min during 14 days (handling) or left undisturbed (control). In addition, both control and handled specimens were fed 3 diets with graded levels of arginine (Arg 4.4, Arg 5.7 and Arg 6.9 g 16 g^{-1} N). Following the 14 days stress challenge and feeding on those diets, fish were infected with *Photobacterium damselae* subsp. *piscicida* (strain PC566.1; LD_{50} 5 × 10^3 cfu mL^{-1}) and fed the same experimental diets. Respiratory burst activity and nitric oxide production of head–kidney leucocytes increased parallel to dietary arginine supplementation. HIF-1, HAMP-1, MIP1-alpha and gLYS expression values and some humoral parameters augmented in control specimens fed the Arg 5.7 and Arg 6.9 diets. Interestingly, repeated acute stress increased both disease resistance and some innate immune mechanisms in handled fish. The role of dietary arginine and repeated handling on Senegalese sole innate immunity and disease resistance are discussed.

1. Introduction

Senegalese sole (*Solea senegalensis*) is a high-value flatfish that presents a great 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 [25]. Among the different factors that may induce high mortality during the juvenile stage, stress might be one of the key issues. Osmoregulatory and metabolic changes associated to stress responses have been assessed previously in this species [2–4,12,13]. However, little attention has been paid to study alterations in innate immune mechanisms after a stress challenge [14,44].

Confinement, high density, handling and transport are stress inducers which are highly relevant to aquaculture and have received considerable attention [6,12,14,22,42,43]. The effect of stress on the immune system has been widely investigated and, although acute stress can have beneficial effects, chronic stress was found to inhibit an optimal immune response in both mammals and teleost fish [22,45,49,54], leading to increased susceptibility to pathogens [53]. Corticosteroids can influence multiple aspects of the innate immune defense mechanisms in fish [51]. Cortisol usually down-regulates the production of pro-inflammatory cytokines and nitric oxide (NO) to prevent damage due to an excessive inflammatory response [42]. Moreover, phagocytosis of peripheral blood leucocytes from common carp (*Cyprinus carpio*) and hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) was significantly depressed after *in vitro* cortisol administration [28]. Additionally, mRNA levels of g-type lysozyme and hepcidin antimicrobial peptide-1 genes decreased in liver and kidney of Senegalese sole submitted to high stocking density [44]. Furthermore, high plasma cortisol levels resulted in reduction of leucocyte proliferation, numbers of antibody

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producing cells, and levels of virus-neutralising antibodies in fish [51].

Stress conditions that induced high plasma cortisol levels also modified amino acid (AA) metabolism in Senegalese sole [2,3,12–14,59]. Moreover, changes in plasma free AA levels may be indicative of AA requirements in fish [56]. 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 [2,3,12]. 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 in fish have been recently reviewed [29]. Thus, AA not only serve as constituents of proteins and energy sources, but are also converted into important biochemically active substances *in vivo*. Particularly, arginine is the precursor for the synthesis of nitric oxide (NO) and polyamines [46]. NO causes vasodilatation and stimulates microcirculation, improving thereby cardiac, pulmonary and cerebral functions in humans [40]. In fish, macrophage NO production plays an important role in the cellular defense mechanisms and has been demonstrated in stimulated macrophages in several fish species [7,35,50]. Moreover, different studies revealed that L-arginine administration improved wound and bone healing in mammals [40]. In fish, a positive effect of an arginine-enriched diet on the resistance of channel catfish (*Ictalurus punctatus*) to infection with *Edwardsiella ictaluri* has been also demonstrated [8].

Therefore, the present study aimed to assess whether dietary arginine supplementation can minimize the immunosuppressive effects attributed to chronic stress in fish. Repeated handling stress will be used in the current study since it has been already established as a chronic stressor for this species. Similarly, it is also intended to verify to what extent dietary treatment can influence disease resistance and some aspects of the innate immune system in Senegalese sole.

2. Materials and methods

2.1. Formulation and analytical procedures with experimental diets

Three practical diets were formulated to be isonitrogenous, isolipidic and isoenergetic (54% protein, 8% lipids, 21 kJ g^{-1} energy on a dry-matter (DM) basis). Marine-derived ingredients represented only 15% of the formula. The rest of the protein fraction was achieved by means of a variable blend of soybean meal, soy protein concentrate, wheat meal, corn and wheat gluten, and wheat DDGS (dried distillers grains with solubles), whereas fish oil was the main fat source. Thus, the plant-protein fraction in these experimental diets was around 75%. Moreover, L-arginine (0.8 and 1.5%) was added to two of the diets to obtain graded levels of arginine (4.5, 6 and 7.5% of crude protein) at the expenses of wheat gluten. Following analytical procedures final values were 4.4, 5.7 and 6.9 g 16 g^{-1} N, respectively. Therefore, experimental diets are referred from now on as Arg 4.4, Arg 5.7 and Arg 6.9. The Arg 4.4 diet was formulated according to known nutritional requirements of Senegalese sole and served as control [18]. These plant protein-rich diets were further supplemented with lysine and mono-calcium phosphate to avoid imbalances. In the absence of specific data on vitamin, mineral and trace element requirements of Senegalese sole, requirement data for other species were applied [26,36]. Experimental diets were manufactured by SPAROS Lda. (Faro, Portugal). Main ingredients were grinded (below 250 micron) in a Micropulverizer hammer mill (Hosokawa, model #1). Powder ingredients were then mixed accordingly to the target formulation in a double-helix mixer (TGC, model 500L). All diets were manufactured by extrusion (pellet size 3 mm), by means of a pilot-scale twin-screw extruder (CLEXTRAL BC45) with a screw diameter of 55.5 mm and temperature ranging 105–110 °C. Upon extrusion, extruded feeds were dried in a convection oven (LTE OP 750-UF) for 2 h at 60 °C. Following drying, pellets were allowed to cool at room temperature, and subsequently the supplemental L-arginine and fish oil were added under vacuum coating conditions in a DINNISEN Pegasus vacuum mixer (PG-10VCLAB). Formulation and proximate composition of experimental diets are presented in Table 1.

Diets were analysed for total amino acids content. Diet samples were hydrolyzed in 6°M HCl at 106 °C over 24 h in nitrogen-flushed glass vials. Afterwards, samples were pre-column derivatized with phenylisothiocyanate (PITC; Pierce), using the PicoTag method (Waters, USA) described by [11]. 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 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 [11]. Resulting peaks were analysed with the Breeze software (Waters). During analytical procedures, asparagine is converted to aspartate and glutamine to glutamate during acid hydrolysis, so the reported values for these amino acids (Asx and Glx) represent the sum of the respective amine and acid. Moreover, tryptophan was not determined since it is destroyed by acid hydrolysis. Amino acid profile of experimental diets is presented in Table 2.

Table 1
Ingredients and proximal composition of experimental diets.

	Experimental diets		
	Arg 4.4	Arg 5.7	Arg 6.9
Ingredients (%)			
Fishmeal 70 LT	5.0	5.0	5.0
CPSP	5.0	5.0	5.0
Squid meal	5.0	5.0	5.0
Soybean meal	17.0	17.0	17.0
Soycomil (PC)	3.2	3.2	3.2
Wheat gluten	13.9	13.0	12.1
Wheat meal	17.2	17.3	17.5
Wheat DDGS	10.0	10.0	10.0
Corn gluten	15.0	15.0	15.0
Fish oil	4.0	4.0	4.0
Vit & Min Premix[a]	1.0	1.0	1.0
DCP	2.7	2.7	2.7
L-Lysine	1.0	1.0	1.0
L-Arginine	–	0.8	1.5
Proximate composition			
Dry matter (% DM)	90.7	92.3	92.5
Crude protein (% DM)	53.9	54.1	56.4
Crude fat (% DM)	7.4	8.1	8.1
Ash (% DM)	6.6	6.6	6.8
Gross Energy (kJ g^{-1} DM)	21.7	21.3	21.1
NFE	31.1	31.2	28.7

CPSP, fish soluble protein concentrate (hydrolysed white fish meal); DCP, dibasic calcium phosphate; DDGS, dried distillers grains with solubles; DM, dry matter; NFE, nitrogen free extracts = 100 − (CP + CL + CA).

[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), 300 mg; Zn (zinc oxide), 30 mg; Se (sodium selenite), 0.3 mg; Fe (iron sulphate), 56 mg; Ca (calcium carbonate), 80 mg; KCl (potassium chloride), 750 mg; NaCl (sodium chloride), 0.4 g. Vitamins (mg kg^{-1} diet): vitamin A (retinyl acetate), 2.75 mg; vitamin D3 (DL-cholecalciferol), 0.04 mg; vitamin K3 (menadione sodium bisulfite), 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.