Physiological responses of Senegalese sole (*Solea senegalensis* Kaup, 1858) after stress challenge: Effects on non-specific immune parameters, plasma free amino acids and energy metabolism

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ABSTRACT

Physiological responses after an acute handling stress and their subsequent effects on innate immune parameters, plasma free amino acids (AA) and liver energy substrates were assessed in Senegalese sole (*Solea senegalensis*). Eight groups of six specimens (136.1 ± 58.4 g wet weight) were maintained undisturbed, while other eight groups of six specimens were used for acute stress challenge (air exposed during 3 min). A group of six specimens was sampled for blood and head-kidney collection immediately after air exposure (time 0), while the remaining groups were sampled at 5 and 30 min, 1, 2, 4, 6 and 24 h. Undisturbed fish were sampled at the same times and used as control. Fish were fasted for 24 h prior to air exposure and sampling. Plasma cortisol, glucose, lactate and osmolality levels increased immediately after stress peaking at 1 h in air exposed fish. Changes in plasma free AA were also observed at 1 and 24 h after stress. In liver, glycogen levels significantly decreased at 30 min and 1 h, while triglycerides values significantly increased at 1, 2 and 4 h in air exposed fish. In addition, total AA levels in liver augmented significantly at 2 h holding high until 24 h in air exposed specimens. The respiratory burst of head-kidney leucocytes from air exposed fish was significantly higher than that from control groups at 2 and 6 h after air exposure. On the other hand, plasma lysozyme activity significantly decreased at 4 h after acute stress in air exposed fish, while plasma alternative complement pathway followed an inverse linear relationship with respect to cortisol showing the lowest value at 1 h after air exposure. The present study suggests that Senegalese sole presents a stress response comparable to that observed in other teleosts. While some indispensable AA may be used for the synthesis of compounds related to the stress response or fatty acid transport, dispensable AA were probably mainly employed either as energy sources or in gluconeogenesis. Moreover, results from non-specific immune parameters assessed suggest that cortisol may act as regulator of the innate immune system.

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1. Introduction

Fish in aquaculture are often exposed to husbandry-related acute (e.g. handling and temperature changes) and chronic (e.g. rearing density and water quality) stressors, which induce physiological alterations in response to the stress imposed. Plasma cortisol level is widely used as a general indicator of stressful situations in fish (Wendelaar Bonga, 1997; Mommsen et al., 1999). Several stressors, such as air exposure or net confinement, induce a significant increase in plasma cortisol levels in teleosts (e.g., Arends et al., 1999; Acerete et al., 2004). Most studies relating cortisol effect on carbohydrate metabolism in fish rely on plasma glucose and liver glycogen content as indicators of metabolism (Mommsen et al., 1999). In fact, hepatic metabolic changes associated to stressful conditions (e.g. hypoxia, high densities and osmotic challenge) have been reported in fish (Vijayan et al., 1990; Dalla Via et al., 1994; Sangiao-Alvarellos et al., 2005, 2006; Arjona et al., 2009). Furthermore, plasma glucose levels usually augment following stressful situations such as handling, crowding, salinity transfer or acute stress (Waring et al., 1996; Arends et al., 1999; Arjona et al., 2007; Costas et al., 2008). Similarly, plasma lactate concentrations increase significantly in several fish species following severe exercise (Milligan, 1996) or as a result of hypoxia (Arends et al., 1999). In addition, stress conditions that induced high plasma cortisol levels also modified fish amino acid (AA) metabolism

in several teleost species (Milligan, 1997; Vijayan et al., 1997; Pinto et al., 2007; Aragão et al., 2008, 2010; Costas et al., 2008). In fact, it has been suggested that fish under stressful conditions present additional AA requirements, due to higher energy 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). Therefore, the expected increase in cortisol due to an acute stress challenge and the increased energy requirements during this process will probably have a great impact on AA metabolism in fish.

Stress-related physiological changes affect metabolism and cell processes (including the immune cells), compromising the innate defence mechanisms and thereby increasing the outcome of diseases (Espelid et al., 1996; Ellis, 2001). It is now recognized that the neuroendocrine and immune systems interact in a bi-directional way (Verburg-van Kemenade et al., 2009). In fish, the well-established negative effects of stress on immune competence are thought to be maladaptive responses (tertiary stress responses) to chronic or severe stressors. For instance, cortisol may decrease the number of lymphocytes, selectively suppress phagocytic and complement activities in head-kidney and blood and increase susceptibility to infection in teleosts (Pickering and Duston, 1983; Pickering, 1984; Law et al., 2001; Ortuño et al., 2001). However, an acute increase of cortisol levels may signal the immune system to prepare for possible consequences of a stressor and thus serve as an adaptive function (Verburg-van Kemenade et al., 2009).

Senegalese sole (Solea senegalensis) constitutes a new option in aquaculture. The few existing studies focusing on stress response of this species relate to chronic stressors, pointing to an elevation of plasma cortisol values in fish submitted to chronic handling (Aragão et al., 2008), high stocking densities (Costas et al., 2008; Salas-Leiton et al., 2010), osmotic challenge (Arjona et al., 2007, 2009; Aragão et al., 2010) or temperature (Arjona et al., 2010), and lower values in specimens exposed to chronic ammonia (Pinto et al., 2007), when compared to control fish. Furthermore, there is no data available regarding either changes on plasma AA levels or innate immune parameters after an acute stress challenge for this species. Therefore, this study aimed to evaluate the primary and secondary stress responses of Senegalese sole after an acute stress challenge, and to assess to what extent the subsequent stress response may influence plasma AA levels, liver energy substrates, and some innate cellular and humoral immune parameters.

2. Material and methods

2.1. Experimental procedures

The experiment was carried out at the CIIMAR facilities (Porto, Portugal), where 96 Senegalese sole (136.1 ± 58.4 g wet weight) were randomly distributed in two separate recirculated seawater systems (temperature: 18–20 °C; salinity: 34‰; photoperiod: 14 h light/10 h dark; dissolved oxygen: above 90% saturation level). In one of the systems, 48 fish were maintained in eight flat-bottomed rectangular tanks (60 cm length × 35 cm width × 40 cm depth; bottom surface = 0.21 m^2, volume 84 L, water flow rate 114 L/h, n = 6 fish/tank, density = 3.8 kg/m^2) and remained undisturbed except for daily tank cleaning procedures. The remaining 48 fish were maintained in three flat-bottomed round tanks (r = 45 cm; bottom surface = 0.64 m^2, volume 300 L, water flow rate 114 L/h, n = 16 fish/tank, density = 3.4 kg/m^2) and used for acute stress challenge. Fish were acclimated for 14 days (April 2008) and fed twice a day by hand to apparent satiety (based on the assessment of feed remaining in the tanks) with a 3 mm commercial diet (Alpis, A. Coelho e Castro Lda., Póvoa de Varzim, Portugal). After this period, specimens from round tanks were air exposed for 3 min at a time and redistributed in groups of six individuals into seven new tanks (60 cm length × 35 cm width × 40 cm depth; bottom surface = 0.21 m^2, volume 40 L, n = 6 fish/tank, density = 3.8 kg/m^2) independently set up from each other. A group

of six specimens was sampled for blood and tissues collection immediately after air exposure (time 3 min), while the other groups were sampled after 5 and 30 min, 1, 2, 4, 6 and 24 h. Undisturbed fish were sampled at the same times and used as control. Fish were fasted for 24 h prior to air exposure and during the subsequent 24 h sampling period in order to avoid any influence of feeding on stress plasmatic parameters (Arends et al., 1999).

For sampling procedures, all individuals were quickly removed from each tank at a time and anesthetized with ethyl 3-aminobenzoate methanesulfonate (MS-222, 200 mg/L; Sigma-Aldrich, Germany). Blood was withdrawn from the caudal vein of every sampled fish using heparinized syringes. Blood collection lasted less than 3 min in order to avoid a cortisol increase due to manipulation during sampling. Plasma was obtained by centrifugation (10,000 × g for 10 min at 4 °C) and stored at −80 °C for further analysis. After blood collection, fish were individually weighed and head-kidney and liver were subsequently dissected over an ice bed. Liver was weighed and kept at −80 °C for further analysis.

Leucocytes from head-kidney were collected from control and air exposed fish at 0, 2, 4, 6 and 24 h, isolated and maintained essentially as described by Secombes (1990). Briefly, the head-kidney was removed under aseptic conditions, pushed through a 100 μm nylon mesh and suspended in Leibovitz L-15 medium (L-15: Gibco, Scotland, UK) supplemented with 2% foetal calf serum (FCS; Gibco), penicillin (100 IU/mL; P, Gibco), streptomycin (100 μg/mL; S, Gibco) and heparin (20 U/mL; Sigma). The suspensions were then loaded onto a 34:51% Percoll (Sigma) density gradient and centrifuged at 400 × g and 4 °C for 40 min. The band of cells laying at the interface of the Percoll gradient was collected and washed three times at 400 × g and 4 °C for 5 min in L-15, 0.1% FCS, P/S and heparin. The viable cell concentration was determined by the Trypan blue exclusion test. Cells were counted in a hemocytometer and adjusted to 1 × 10^7 cells/mL in L-15, 0.1% FCS, P/S and heparin. Afterwards, cells were plated in 96 well plates at 100 μL per well. After overnight incubation at 18 °C, the non-adherent cells were washed off and the monolayers were maintained with L-15 supplemented with 5% FCS, until the respiratory burst assays were conducted after 24 h of incubation at 18 °C.

2.2. Analytical procedures

Plasma cortisol was measured by radioimmunoassay as described by Metz et al. (2005), which was already performed in Senegalese sole (Arjona et al., 2007, 2009). Plasma osmolality was measured with a vapor pressure osmometer (Fiske One-Ten Osmometer, Fiske, VT, USA) and expressed as mOsm/kg. Plasma glucose, lactate and triglycerides were assessed using commercially available Spinreact kits (Glucose HK Ref. 1001200; Lactate Ref. 1001330; Triglycerides Ref. 1001311), 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. These assays were run on a Bio Kinetics EL-340i Automated Microplate Reader (BioTek Instruments, Winooski, VT, USA) using DeltaSoft3 software for Macintosh (BioMetallics Inc., NJ, USA).

Plasma samples from 0, 1 or 24 h were pooled to one sample per sampling time and used for free AA analysis. All pools were run in triplicates. Due to technical constrains, pool from control samples at 1 h was analyzed only once. All samples were deproteinized by centrifugal ultrafiltration (10 kDa cut-off, 2500 × g, 20 min, 4 °C). After deproteinization, 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 AA standard solutions (acid/neutral and basics from Sigma) and a glutamine solution. Norleucine was used as an internal standard.