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# Metabolic molecular indicators of chronic stress in gilthead seabream (*Sparus aurata*) using comparative proteomics

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ABSTRACT

The aim of this study was to identify possible metabolic molecular indicators of chronic stress in gilthead sea bream *Sparus aurata*. Two potential stressful conditions were tested: repeated handling and crowding at high stocking density. Gilthead seabream kept under optimized rearing conditions were used as control fish. Cortisol was measured as primary stress indicator and the liver proteome of stressed fish was compared to that of control fish using comparative proteomics. Plasma cortisol levels in sea bream repeatedly handled and crowded at high stocking density were significantly higher than in undisturbed control fish. A total of 560 *spots* were detected and the statistical analysis revealed a differential expression in about 50% of all detected proteins. Spots with greater than 2-fold or lower than 0.5-fold changes were identified by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Proteins like fatty acid binding protein (lipid transport and antioxidant role), heat shock cognate protein (chaperoning), calmodulin ($Ca^{2+}$ signaling), mitochondrial porine — voltage-dependent anion channel (lipid oxidation), glutamine synthetase (ammonia metabolism), cofilin and beta-tubulin (cytoskeleton), hemoglobin and several other proteins involved in carbohydrate metabolism (triose-phosphate isomerase, pyruvate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, alfa-enolase) were differentially expressed in fish under chronic stress. Some of these proteins may be used in the future as chronic stress and/or part of a panel of welfare biomarkers, after validation studies using RT-PCR and ELISA assays.



## 1. Introduction

Farmed animals welfare is increasingly becoming a relevant and significant societal topic (Veissier et al., 2008). Both consumer awareness and ethical reasons make this issue a top priority on animal farming conditions. On the other hand, animal stress and welfare are also an important issue for producers, since growth and reproductive performances, the health status and diseases susceptibility are strictly related to stress conditions (Conte, 2004). Welfare studies on terrestrial farmed animals are nowadays common in scientific research (e.g., Cockram, 2007; Smulders et al., 2006; Tovar and Giraldo, 2006). However, only recently, welfare of farmed fish has emerged (Conte, 2004; Huntingford et al., 2006; Branson, 2008). Defining the welfare status of fish is difficult and the identification of reliable welfare indicators represents the main challenge. A wide range of physical, physiological and behavioral measures are used to assess fish welfare (Huntingford and Kadri, 2008), and an accurate evaluation can only be made through the integration and interpretation of several indicators (Ashley, 2007; Turnbull and Kadri, 2007).

Avoidance of adverse consequences of exposure to chronic stressors is a central welfare goal in aquaculture (Conte, 2004; Ashley, 2007). It is well reported that some aquaculture practices such as repetitive handling, confinement and crowding of fish represent potential chronic stressors affecting fish physiology and the welfare status (Pagés et al., 1995; Mugnier et al., 1998; Arends et al., 1999; Barton et al., 2005). Therefore, improvement of aquaculture practices in order to minimize chronic stress conditions on farm and preserve fish welfare is one of the main challenges in aquaculture research. In line with this, recently the European Food Safety Authority identified some hazards and risk factors potentially affecting welfare of several farmed species, included gilthead seabream (EFSA, 2008).

In addition, there is an objective difficulty for designing an appropriate experimental approach to test welfare, which currently would be absence of stress, i.e., normality or control condition. An objective approach for welfare evaluation is the study of the stress response (Pottinger, 2008). The stress response in fish occurs at three

*Abbreviations:* 2-DE, Two-dimensional gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl) dimethylamonio]-1-propanesulphonate; CID, collision-induced dissociation; Da, dalton (molecular mass); DTT, Dithiothreitol; IPG, immobilized pH gradient; LC–MS/MS, liquid chromatography–tandem mass spectrometry; *m/z*, mass-to-charge ratio; MOPS, 3-(N-morpholino) ethanesulphonic acid; PFF, peptide fragment fingerprinting.

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levels. The primary response involves the rapid activation of the brain–sympathetic–chromaffin cell axis (BSC-axis) and the hypothalamic–pituitary–interrenal axis (HPI-axis) with the release of catecholamines and cortisol into the bloodstream. The secondary response is defined by the hormonal effects on blood and tissues, including changes in metabolism, hydromineral balance, cardio-respiratory and immune functions. Tertiary response includes physiological effects related to whole-animal performance such as growth, disease resistance, behavior and survival (Barton, 2002; Wendelaar Bonga, 1997; Iwama, 2007).

Usually stress response in fish is evaluated by measuring levels of hormonal, metabolic, haematological and hydromineral parameters in the blood; growth and feeding parameters, and organosomatic indexes are generally used as tertiary stress indicators (Barton, 2002). Beside the reliability of clinical biochemical parameters, including plasma cortisol, glucose, lactate, electrolytes as primary and secondary stress indicators, their use in some circumstances, poses some difficulties in the interpretation of results because stress response is subjected to modulation by several factors, both intrinsic and extrinsic to the fish (Barton, 1997, 2002; Mommsen et al., 1999; Pottinger, 2008). For example, traditional parameters such as plasma cortisol, may be not sufficient to assess physiological and welfare conditions when chronic stress occurred (Procarione et al., 1999; Montero et al., 1999; Haukenes and Barton, 2004; Barton et al., 2005) due to HPI-axis acclimation.

Nevertheless, recent development in cutting edge technologies such as proteomics, genomics and metabonomics could give rise to a better insight of the mechanisms involved in stress-related processes in fish, thus facilitating the identification of stress and/or welfare indicators. Indeed these technologies are probably the best approaches as they offer a comprehensive method to study biochemical systems by expanding the level of investigation from single biomolecules to a wide range of molecules present in a cell or a tissue at once, in terms of their presence and relative abundance, without "a priori" knowledge. Gene expression analysis has recently begun to emerge as an alternative approach to assess stress and welfare conditions (Cairs et al., 2008; Gornati et al., 2004a,b; Krasnov et al., 2005; Momoda et al., 2007; Ribas et al., 2004). Proteomic techniques, such as bidimensional electrophoresis (Cowan and Vera, 2008; Han and Wang, 2007; Lopez, 2007; Westermeier et al., 2008), are also promising and potentially new approaches as alternatives to conventional methods.

The main objective of the present study was the identification of potential chronic stress metabolic biomarkers in gilthead seabream. Liver proteome was compared in fish stressed by repetitive handling and by crowding at high stocking density and in control fish using comparative proteomics. Gilthead seabream was used as a model due to its economic importance and to the available knowledge on its stress physiology (Arends et al., 1999; Barton et al., 2005; Montero et al., 1999, 2001; Ortunõ et al., 2001; Roncarati et al., 2006; Rotllant et al., 2000, 2001; Sangiao-Alvarellos et al., 2005; Tort et al., 2001). The liver was chosen as the target organ for comparative proteomic analysis, due to its major role in several key metabolic processes.

## 2. Materials and methods

### 2.1. Animals, experimental conditions and sampling procedure

The experiment was carried out at the Ramalhete Research Station (CCMAR, University of Algarve, Faro, Portugal). The trial lasted from September 18 until October 16 of 2007, after a 4 month adaptation period in experimental tanks. Fish were supplied by a commercial fish farm.

In this study, two potential chronic stressful conditions were tested: repeated handling (HND) and crowding produced by high stocking density (HSD). A control group (CTRL) was also reared in an initial density of 2.52 kg/m$^3$, that according to previous studies (Arends et al., 1999; Montero et al., 1999) should not induce chronic stress to gilthead seabream. The experimental treatments were tested in duplicate. To induce handling stress, fish were netted, and air exposed for 1 min, twice a week. It has been reported that such a short air exposure is an acute stressor, from which seabream recovers easily (Arends et al., 1999).

Forty-four specimens of 376.37 ± 43.37 g (mean ± standard deviation) were distributed among six polyethylene tanks of 1000 L. Tanks were supplied with in flow-through Ria Formosa Lagoon seawater. Temperature (22.45 ± 1.55 °C), salinity (36.92 ± 0.90), and oxygen saturation level (>80% saturation) were daily monitored.

Six fish (initial density — 2.52 kg/m$^3$) were used for each CTRL and HND tanks and, eleven fish (initial density — 46.15 kg/m$^3$) for each HSD tank. A cage system was mounted inside the HSD tanks in order to reduce the rearing volume at approximately 100 L with a water flow system allowing a good water quality.

At initial sampling, fish were anesthetized with 250 ppm of 2-phenoxyethanol (Sigma) for weight and length measurement.

During the trial, fish were fed twice a day (10:00 am and 02:30 pm) *ad libitum* with a diet based on 94.63% of dry matter (DM), 50.73% DM crude protein, 20.26% crude fat and crude energy 24.36 kJ/g DM. HND fish were fed once a day when subjected to handling treatment.

At the end of the experiment, fish were anesthetized and euthanized with an overdose of 2-phenoxyethanol (1500 ppm) for blood sampling and biometric measurement. The blood was collected from the caudal vein with a heparinized syringe, placed on ice, incubated at 4 °C and centrifuged at 3000 rpm for 10 min as reported by Montero et al. (1999). Plasma samples were collected, quickly frozen in liquid nitrogen and then stored at −80 °C.

Plasma cortisol concentration was measured using a commercial solid-phase competitive chemiluminescent enzyme immunoassay Immulite on 10 µl of plasma sample (Siemens Medical Solution Diagnostic, Los Angeles USA). Chemiluminescence was measured by the Immulite One analyzer. The immunoassay was previously compared to $^{125}$Iodine radioimmunoassay Coat-A-Count Cortisol (D.P.C. Los Angeles, CA) on 22 plasma samples, obtaining a significant linear correlation ($R^2 = 0.98$). Cortisol analyses were performed at Fish Physiology and Health Laboratory of Institute for Environmental Protection and Research (ISPRA, Italy).

For proteome analysis, the liver was quickly removed, washed with MilliQ water, frozen immediately in liquid nitrogen, and kept at −80 °C. To avoid contaminations, extra care was taken during liver handling, to prevent protein degradation by liver proteases.

### 2.2. Liver proteome analysis

Four technical replicates were done for each experimental condition, using a pooling strategy to reduce the influence of inter-individual variation (and, thus, of biological outliers) enabling to focus on consistent global changes in protein expression (Westermeier et al., 2008).

#### 2.2.1. Protein extraction and purification

Prior to protein extraction, liver samples were always handled in liquid nitrogen (to avoid protease activity). For protein extraction, 100 mg of tissue (pooled from all livers for each condition) was dissolved in 1 mL of extraction buffer containing 7 M urea (Sigma-Aldrich), 2 M thiourea (Sigma-Aldrich), 4% w/v CHAPS (Sigma-Aldrich), 0.3% w/v DTT (Sigma-Aldrich), 1% v/v ampholytes IPG Buffer pH 3–10 (GE Healthcare) and 1% v/v protease inhibitor cocktail (Sigma-Aldrich) and then incubated on ice for 30 min. After incubation period, the lysate was homogenized with an Ultra-Turrax IKA T8 (IKA-WERG), sonicated four times for 4 s, and centrifuged at 19,000 *g* for 5 min at 4 °C. Considering the high fat content of the samples, two extra centrifugation steps were performed, at the same speed and temperature, during 10 and 5 min, respectively.