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Quantification of a glucocorticoid receptor in sea bass (*Dicentrarchus labrax*, L.) reared at high stocking density

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Abstract

To assess the effects of environmental stress conditions on fish, the examination of a suite of biomarkers, including endocrine parameters, has been suggested. In teleosts, glucocorticoids, including cortisol and corticosterone, are known to mediate stress response. Inside the cell, they bind to a high-affinity cytosolic glucocorticoid receptor (GR), which acts as ligand-dependent transcription factor to control and regulate gene expression. Receptor number or affinity may directly influence the degree of reactivity of target cells.

GR transcripts have been cloned and characterized in different fish species, but no studies, to date, are available on the quantification of GR in sea bass (*Dicentrarchus labrax*, L.), although this is one of the most important species in Mediterranean aquaculture. These considerations prompted our interest in cloning the GR in this species and investigating the impact of long-term exposure to crowding stress on GR expression in the liver of sea bass. Our data clearly demonstrate that a high rearing density stress affects GR mRNA, whose abundance in the liver decreased inversely with blood cortisol levels.

Keywords: Biomarker; Animal biotechnology; Fish welfare; Aquaculture

1. Introduction

There is increasing public, governmental, and commercial interest in the welfare of intensively farmed fish, and stocking density has been highlighted as an area of particular concern (Ellis et al., 2002). Protecting the welfare of farmed animals is a central requirement of any animal-rearing system. Stress resulting from husbandry practices represents a major influence on both the health and welfare of farmed animals. The development of appropriate environmentally related husbandry practices can minimize the stress on farmed fish, reduce the incidence of diseases, and maintain high standards of animal welfare. Knowledge of these stress levels, therefore, represents a key parameter in achieving and maintaining standards of good fish husbandry and welfare.

In addition to several indicators that have been proposed for assessing fish welfare, molecular biomarkers directly indicating gene activity may have the characteristics for being useful early indicators. A biomarker is defined as any biological response (ranging from molecular through cellular and physiological responses to behavioural changes) to a stress factor measured inside an organism indicating a deviation from the normal state that cannot be detected in the intact organism (Van Gastel and van Brummelen, 1996). In order to assess the exposure of fish to environmental stress conditions, it was suggested that a suite of biomarkers be examined, including evaluation of endocrine parameters.

In teleosts, stress activates the hypothalamus–pituitary–interrenal (HPI) axis, leading to a rapid release of adrenocorticotropic hormone (ACTH) into the blood stream and a subsequent secretion of the glucocorticoid hormone

Abbreviations: mRNA, messenger RNA; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; DTT, dithiotreitol; dNTPs, deoxynucleotide triphosphate; cDNA, DNA complementary to RNA; T_m, melting temperature; bp, base pairs.

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cortisol by the interrenal tissue, the tissue analogous to the adrenal cortex in mammals (Bern and Madsen, 1992; Wendelaar Bonga, 1997). The effects of cortisol in fish consist in mediating stress-induced hyperglycemia, which is crucial for supporting the increased energy demand associated with stress, through activation of phosphoenolpyruvate carboxykinase (PEPCK) and gluconeogenesis (Mommsen et al., 1999; Saplosky et al., 2000). Cortisol released into the circulatory system enters cells by passive diffusion or is facilitated by a carrier-mediated process (Vijayan et al., 1997). Inside the cell, it binds to a high-affinity cytosolic glucocorticoid receptor (GR), which acts as a ligand-dependent transcription factor to control and regulate gene expression (Evans, 1998). Receptor number or affinity may directly influence the degree of reactivity of target cells (Vanderbilt et al., 1987).

The significance of cortisol, which is frequently used to describe the condition of fish together with other direct parameters such as haematocrit, blood glucose or total haemoglobin, may be limited when the chronic stress of rearing fish at high densities is concerned (Van Weerd and Komen, 1998). The results of previous studies investigating whether increased stocking densities activates the stress response are conflicting, depending upon the species used and the experimental design (Tort et al., 1996; Procarione et al., 1999). These findings suggest that the use of plasma cortisol as an indicator of chronic stress may not be very informative, in particular, due to the acclimation of the interrenal gland during chronic stress and the influence of negative feedback mechanisms on the HPI axis (Rotllant et al., 2000). Thus, besides cortisol, other biomarkers can serve as indicators of stress levels in commercial farming situations (Gornati et al., 2004b). In this context, we examined glucocorticoid receptor expression in response to high rearing density stress conditions by real-time PCR.

2. Materials and methods

2.1. Animals

Fingerling sea bass (*Dicentrarchus labrax*, L.) obtained from Nuova Azzurro commercial hatchery (Civitavecchia, RM Italy) were reared, with inconsistent mortality, at a low biomass density (<10 kg/m^3) in a 4-m^3 fiberglass tank connected to a water recycling system supplied with about 24 water refillings per day. The salinity (obtained by adding salt Oceanfish 600 LT from Prodac Int® to dechlorinated tap water) was 10 g/L. Sea bass is a strongly euryhaline marine fish that may be found under natural conditions at salinities ranging from freshwater to 90 g/L (Barnabe, 1990). Recent studies show good growth performances at salinities ranging from 10 to 37 g/L. A low salinity preference was shown for juveniles of these species (Saillant et al., 2003), while Dalla Via et al. (1998) reported good metabolic performances at salinities as low as 2 g/L.

Other water parameters were strictly controlled: temperature 20±1 °C, PO_2 95–105% of the saturation value (obtained with pure O_2 insufflation), free CO_2<15 mg/L, N-NH_4^+<0.03 mg/L, total gas pressure 98–100% of the saturation value, N-NO_3^-<20 mg/L, pH 7.2, and alkalinity 140–200 mg/L $CaCO_3$.

At an average size of 50 g, three populations were randomly selected and transferred to 200-L fiberglass tanks at different nominal densities, corresponding to 10, 80, and 100 kg/m^3, with the same environmental conditions and water refilling rate. Food was automatically distributed daily to ensure a 1.5% body mass ratio, with extruded pellet for marine fish (TROUVIT Power HQII®). Temperature, PO_2 and pH were continuously monitored, while other parameters were assessed weekly. Cortisolemia was checked on a monthly basis in blood samples with the aim of monitoring the stress status of the animals. For this, fish were anaesthetized with 3-aminobenzoic acid ethyl (MS222, 100 ppm) and the blood was drawn from the caudal vein with a heparinized syringe, in less than 1 min (usually 30–40 s). Catching an individual fish did not cause stress to remaining ones, as the fish from each experimental group were taken out of tank and punctured at the same moment by different operators. The blood was centrifuged (10,000×g for 5 min) and plasma was stored at −70 °C for later hormonal analyses. Plasma cortisol was measured using an ELISA kit from IBL-Hamburg on a 96-well microplate reader (λ=450 nm). To maintain the nominal densities, calibrated sampling was performed weekly in each of the experimental tanks. After 3 months, during which no mortality was observed, three animals from each group were randomly sampled, immediately stunned, and sacrificed. Liver and brain were removed in toto, frozen in liquid N_2, and stored at −80 °C until molecular biology analysis.

Samples of sea bass farmed at 50 kg/m^3, a density considered relatively high for commercial farming, were obtained from Agroittica Toscana (Piombino, Italy), a land-based farm that utilizes marine water and liquid O_2.

2.2. Glucocorticoid receptor sequencing

2.2.1. RNA purification

Total RNA was extracted from 1 g of liver tissue, using 10 ml TRIzol RNA isolation reagent (Invitrogen). Briefly, extraction solution was combined with the sample at a 1:1 (v/w) ratio and homogenized. The liquid phase was incubated with chloroform for phase separation. Total RNA was finally extracted using one step of isopropanol washing. The RNA pellet was resuspended in DEPC-treated water (Invitrogen). The quantity of the RNA was calculated using the absorbance at 260 nm. The integrity and relative quantity of RNA was checked by electrophoresis. After two DNAse treatments (DNA-free, Ambion), 3 μg of total RNA was retrotranscribed into cDNA in a volume of 12 μl containing 1 μl of oligo dT16 primer (50 pmol) and 1 μl