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# Expression of pituitary prolactin, growth hormone and somatolactin is modified in response to different stressors (salinity, crowding and food-deprivation) in gilthead sea bream *Sparus auratus*

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ABSTRACT

Prolactin (PRL), growth hormone (GH) and somatolactin (SL) expression was studied in gilthead sea bream (*Sparus auratus*) in response to several different stressors (salinity, food deprivation or stocking density). In the first experiment, specimens were acclimated during 100 days at three different environmental salinities: low salinity water (LSW, 6 ppt), brackish water (BW, 12 ppt) and seawater (SW, 38 ppt). Osmoregulatory parameters corresponded to those previously reported for this species under similar osmotic conditions. Pituitary PRL expression increased with decreasing environmental salinity, and was significantly different between SW- and LSW-acclimated fish. Pituitary GH expression was similar between SW- and BW-acclimated fish but decreased in LSW-acclimated specimens. Pituitary SL expression had a "U-shaped" relationship to environmental salinity with the lowest expression in BW-acclimated fish. In a second experiment SW-acclimated specimens were randomly assigned to one of four treatments and maintained for 14 days: (1) fed fish under low density (LD, 4 kg $m^{-3}$); (2) fed fish under high density (HD, 70 kg $m^{-3}$); (3) food deprived fish under LD; and (4) food deprived fish under HD. Plasma glucose and cortisol levels corresponded to those previously reported in *S. auratus* under similar experimental conditions. Pituitary PRL and SL expression increased in fish maintained under HD and decreased in food deprived fish. In conclusion, an effect of environmental salinity on pituitary PRL and GH expression has been demonstrated. In addition, crowding stress seems to interact with food deprivation in *S. auratus* and this is reflected by changes in pituitary PRL, GH and SL expression levels.


## 1. Introduction

The endocrine system plays a key role in the maintenance of homeostasis in the face of short or long term changes in the environment. Frequently environmental challenges are complex and multi-parameter making experimental simulation of such situations complex but pertinent. Numerous studies have demonstrated that members of the GH/PRL family which includes prolactin (PRL), growth hormone (GH) and somatolactin (SL) (Rand-Weaver and Kawauchi, 1993; Kaneko, 1996) play a key role in regulating homeostasis of a number of physiological processes in response to environmental challenges.

SL has no single regulatory action but appears to play a role in osmoregulation (Kaneko, 1996), stress (Rand-Weaver et al., 1993), acid–base regulation (Kakizawa et al., 1996) and metabolism (Vega-Rubin de Celis et al., 2004). PRL in contrast, is essential for acclimation to hyposmotic environments (Hirano, 1986; McCormick, 1995, 2001; Manzon, 2002) and morphological studies have demonstrated stimulation of PRL cells in several teleost species (Nishioka et al., 1988) including sea bream (*Sparus auratus* L.) (Mancera et al., 1993b). In addition to its osmoregulatory action, PRL is implicated in processes such as stress and metabolism. Chronic stressors such as confinement or food deprivation increase plasma PRL levels in *Oncorhynchus kisutch, Oreochromis niloticus* and *O. mosambicus* (Avella et al., 1991; Auperin et al., 1995; Weber and Grau, 1999).

GH regulates growth (Björnsson, 1997), but also has an osmoregulatory action in both salmonids and non-salmonids (Sakamoto et al., 1993; Mancera and McCormick, 1998). Moreover, an increase in opercular chloride cell number, gill $Na^+$,$K^+$-ATPase activity and salinity tolerance has been reported in some species when GH is administered (Flik et al., 1993; McCormick, 1995, 2001; Sakamoto et al., 1997). A negative relationship exists between stress and growth and in several teleosts, including *S. auratus*, stress causes a rise in plasma cortisol which is accompanied by a decrease in

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GH levels (Pérez-Sanchez and Le Bail, 1999; Pickering et al., 1991; Leatherland, 1993; Rotllant et al., 2000). In contrast, stress associated with food-deprivation in *S. auratus*, enhances plasma GH levels (Pérez-Sanchez and Le Bail, 1999; Mingarro et al., 2002).

In the present study the effect on GH/PRL family members of exposure simultaneously to several different stressors (salinity, food deprivation or stocking density) is evaluated and related to physiological parameters associated with stress. The biological model utilised is the gilthead sea bream in which the physiology of osmoregulation (Mancera et al., 1993a,b, 2002; Sangiao-Alvarellos et al., 2003, 2005a,b; Laiz-Carrión et al., 2005a,b) and stress (Arends et al., 1999; Rotllant et al., 2000, 2001; Montero et al., 1999; Mancera et al., 2008) are well characterised.

## 2. Materials and methods

### 2.1. Fish

Immature sea bream (*S. auratus*) were used in all experiments (see below for details), and were fed daily with 1% body weight using commercial dry pellets containing 48% crude protein, 6% carbohydrates, 25% crude fat, and 11.5% ash (20.2 mega J/kg of feed) (Dibaq-Diproteg SA, Segovia, Spain). In all experiments fish were fasted for 24 h before sampling. The experiments described comply with the Guidelines of the European Union Council (86/609/EU), the Spanish Government (RD 1201/2005) and of the University of Cádiz (Spain) for the use of laboratory animals.

### 2.2. Experiment 1: Effect of environmental salinity

The experimental conditions were chosen to modify the osmoregulatory system in *S. auratus* (see, Laiz-Carrión et al., 2005b). Immature *S. auratus* (17–23 g body weight, $n = 480$) were provided by ACUINOVA S.L. (San Fernando, Cádiz, Spain) and were acclimated for at least 2 weeks at the Faculty of Marine Science (Puerto Real, Cádiz) in 2500 l tanks (six tanks, 80 fish per tank) in an open circuit sea water (SW, 38 ppt salinity) system. Fish were maintained under natural photoperiod (July–October 2002) and temperature (18–27 °C) and constant water quality (hardness, pH, and concentration of oxygen, carbon dioxide, hydrogen sulphide, nitrite, nitrate, ammonia, calcium, chlorine and suspended solids) during the experiment. After acclimation experiments were initiated and replicate tanks of fish were either maintained at full salinity (SW group, control) or salinity progressively changed (2 ppt per hour by mixing dechlorinated tap water) to generate brackish water (BW, 12 ppt salinity) or low salinity water (LSW, 6 ppt salinity). Water salinity was checked daily and corrected when necessary. No mortality was observed during the experiments.

Fish were maintained for 100 days in experimental condition and at the end of the trial (120–150 g body weight, $n = 480$) fish were sacrificed in excess anaesthetic (2-phenoxyethanol, 1 ml $l^{-1}$ water, Sigma, Madrid, Spain), weighed and sampled. Blood was collected into ammonium-heparinized syringes by caudal puncture, centrifuged (1 min at 10,000 g) and plasma immediately frozen on dry ice and stored at −80 °C. In order to assess gill $Na^+,K^+$-ATPase activity, 3–5 filaments of the second branchial arch were cut just above the septum with fine scissors and placed in 100 µl of ice-cold SEI buffer (150 mmol $l^{-1}$ sucrose, 10 mmol $l^{-1}$ EDTA, 50 mmol $l^{-1}$ imidazole, pH 7.3) and frozen at −80 °C. Pituitaries were removed, frozen on dry ice, and stored at −80 °C.

### 2.3. Experiment 2: Effect of high stocking density and food deprivation

Immature *S. auratus* (100–150 g body weight, $n = 48$) provided by Planta de Cultivos Marinos (CASEM, Universidad de Cádiz, Puerto Real, Cádiz, Spain) were transferred to the Faculty of Marine Science (Puerto Real, Cádiz). The experimental conditions were selected to activate the stress response in *S. auratus* (Sangiao-Alvarellos et al., 2005b; Mancera et al., 2008). Fish were acclimated to 300 l aquaria containing a round plasticised wire-net cage with a final volume of 250 l (inner diameter 60 cm) in an open circuit SW (38 ppt salinity) system under natural photoperiod (April) and constant temperature (18 °C) for at least 2 weeks prior to experiments.

At the start of experiments the wire-net cages in selected tanks were lifted to increase the stocking density from 4 to 70 kg $m^{-3}$ to give the following treatments (two tanks/treatment): (1) fed fish, low density (LD, 4 kg.$m^{-3}$); (2) fed fish, high density (HD, 70 kg.$m^{-3}$); (3) food deprived fish, LD; (4) food deprived fish, HD. In the food deprived group food was withheld from the onset of the change in stocking density. After 14 days, 12 fish from each treatment (six from each replicate tank) were removed using a dip-net and blood and tissue samples were taken as described in Section 2.2. No mortality was observed during the experiments.

### 2.4. Analytical techniques

Plasma osmolality was measured with a vapour pressure osmometer (Fiske One-Ten Osmometer, Fiske, VT, USA). Plasma $Na^+$levels were measured using a flame atomic absorption spectrophotometer (UNICAM 939, UNICAM, Cambridge, UK) in diluted samples 1:400 (v/v). Plasma $Cl^-$ and glucose levels were measured in duplicate using colorimetric commercial kits from Spinreact (Barcelona, Spain) adapted to microplates. Gill $Na^+,K^+$-ATPase activity was determined using a micro assay method (McCormick, 1993) adapted for *S. auratus* (as described in Mancera et al., 2002). All colorimetric assays were performed with a Bio-Kinetics EL-340i Automated Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA) using DeltaSoft3 software for Macintosh (BioMetallics, Inc. NJ). Plasma cortisol levels were measured in 50 µl samples using an indirect enzyme immunoassay (ELISA) validated for *S. auratus* (Tintos et al., 2005).

### 2.5. Pituitary expression of GH, PRL and SL mRNA

Total RNA was extracted from individual pituitary glands using TRI Reagent according to the manufacturer's protocol (Sigma). The quality and quantity of the isolated RNA was assessed by spectrophotometry using a GeneQuant Spectrophotometer (Amersham Biosciences, UK) and agarose gel electrophoresis. First-strand synthesis of pituitary cDNA was carried out by denaturing 1 µg total RNA at 70 °C for 10 min, placing on ice and adding synthesis mix to a final volume of 30 µl: 0.2 µl M-MLV Reverse Transcriptase (200 U/µl GIBCO BRL, USA), 2 µl oligo (dT) primer (1 µg/µl, Amersham Biosciences, UK), 3 µl DTT (0.1 M), 3 µl dNTPs (10 mM, Amersham Biosciences), 0.2 µl RNase inhibitor (40 U/µl, Amersham Biosciences) and 6 µl 5× RT buffer (250 mM Tris–HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$; GIBCO BRL) and incubating for 2 h at 37°.

Extensive validation of the RT- PCR method was carried out and involved construction of standard curves with the cloned target template to ensure linearity of amplification, determination of the optimal number of PCR cycles for amplification of target cDNA (all reactions were terminated in the logarithmic phase of the amplification). RT-PCR amplification of each of the pituitary hormones was performed using a thermo-cycler (RoboCycler, Stratagene). Reactions (25 µl) contained 1.5 µl of 25 mM $MgCl_2$, 2.5 µl of 10× $Mg^{2+}$free buffer (100 mM Tris–HCl, pH 9.0, 500 mM KCl, 1% Triton X-100), 0.5 µl of dNTPs (10 mM), 50 pmol of appropriate forward and reverse primers (Table 1) and 0.75 U of *Taq* polymerase (Promega, Madison, WI, USA). The same thermocycling protocol was used for all primer pairs and consisted of an initial step