# Dynamics of liver GH/IGF axis and selected stress markers in juvenile gilthead sea bream (*Sparus aurata*) exposed to acute confinement. Differential stress response of growth hormone receptors

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**Abstract**

The time courses of liver GH/IGF axis and selected stress-markers were analyzed in juvenile gilthead sea bream (*Sparus aurata*) sampled at zero time and at fixed intervals (1.5, 3, 6, 24, 72 and 120 h) after acute confinement (120 kg/$m^3$). Fish remained unfed throughout the course of the confinement study, and the fasting-induced increases in plasma growth hormone (GH) levels were partially masked by the GH-stress inhibitory tone. Hepatic mRNA levels of growth hormone receptor-I (GHR-I) were not significantly altered by confinement, but a persistent 2-fold decrease in GHR-II transcripts was found at 24 and 120 h. A consistent decrease in circulating levels of insulin-like growth factor-I (IGF-I) was also found through most of the experimental period, and the down-regulated expression of GHR-II was positively correlated with changes in hepatic IGF-I and IGF-II transcripts. This stress-specific response was concurrent with plasma increases in cortisol and glucose levels, reflecting the cortisol peak (60-70 ng/ml) the intensity and duration of the stressor when data found in the literature were compared. Adaptive responses against oxidative damage were also found, and a rapid enhanced expression was reported in the liver tissue for mitochondrial heat-shock proteins (glucose regulated protein 75). At the same time, the down-regulated expression of proinflammatory cytokines (tumour necrosis factor-α) and detoxifying enzymes (cytochrome P450 1A1) might dictate the hepatic depletion of potential sources of reactive oxygen species. These results provide suitable evidence for a functional partitioning of hepatic GHRs under states of reduced IGF production and changing cellular environment resulting from acute confinement.

**Key words:** cortisol, glucose regulated protein 75, tumour necrosis factor-α, cytochrome P450 1A1.

## 1. Introduction

Over the last decade, growth hormone receptors (GHRs) have been cloned and sequenced in more than forty fish species covering almost all fish lineages (Calduch-Giner et al., 2001; Lee et al., 2001; Benedet et al., 2005). Initially, these receptors were clustered in two clades encompassing GHRs of salmonid (GHR-II) and non-salmonid fish (GHR-I), although this observation led to the suggestion that both receptors are retained in the same fish through teleost radiation and evolution. This hypothesis is supported by the coexistence of duplicated GHRs in rainbow trout and several Mediterranean perciform fish, such as gilthead sea bream, common dentex and European sea bass (Saera-Vila et al., 2005; Bermejo-Nogales et al., 2007). Likewise, Jiao *et al*. (2006) demonstrated the occurrence of two GHRs in black sea bream, Southern catfish and Nile tilapia. More recently, duplicated GHRs have been demonstrated in Mozambique tilapia (Pierce et al., 2007), orange-spotted grouper (Li et al., 2007) and Atlantic halibut (Hildahl et al., 2007; Hildahl et al., 2008), which indicates that duplication and divergence of fish GHRs might have taken place in an early fish ancestor.

Nevertheless, receptor specificity remains unclear and GHR-I of masu salmon binds somatolactin (SL) and growth hormone (GH), but the binding affinity is higher for SL than GH and it was then named SL receptor by Fukada el al. (2005). The orthologous medaka gene might also mediate SL signalling (Fukamachi et al., 2005), but the switch and diversification of GH/SL receptors are highly probable among modern and primitive fish lineages. Thus, GHRs of Japanese eel bind specifically to GH (Ozaki et al., 2006), whereas the GHR might be a promiscuous receptor for GH and SL in the archaic sturgeon and lungfish (Fukamachi and Meyer, 2007). This promiscuity is

not unusual in the Class I cytokine receptor superfamily, although each ligand/receptor interaction may result in unique signalling outcomes (Denley et al., 2005) that may differ among fish species. Indirect evidence for this also exists in gilthead sea bream, as transcriptional studies indicate that insulin-like growth factors (IGFs) in growth (liver, skeletal muscle) and immune (head kidney) relevant tissues are positively correlated with GHR-I rather than GHR-II in parasite-challenges (Sitjà-Bobadilla *et al*., 2008) and different growth models (Benedito-Palos et al., 2007; Saera-Vila et al., 2007). Thus, overall data suggests that GHR-I has evolved in perciform fish, and particularly in gilthed sea bream, as a true orthologous GHR.

Computational analyses also evidence a different promoter organization of GHRs in gilthead sea bream (Saera-Vila et al., 2007). It is noteworthy that surrounding the transcription start site of GHR-II, but not in GHR-I, there are several consensus elements for redox and stress regulatory elements (activating proteins,APs, 1 and 4, and cAMP-responsive elements, CRE), which would contribute to delineate a differential and stress-specific regulation of fish GHRs. Confinement exposure is a common stressor in aquaculture practise that impacts negatively on the immune system, reproduction and growth performance (Pickering 1993; Van Weerd and Komen, 1998). Thus, this experimental condition was chosen to analyze in juvenile fish the functional partitioning of hepatic GHRs after acute stress confinement. The study includes data on GHR transcripts, hepatic IGF production and plasma levels of GH. Circulating levels of cortisol and glucose were measured as conventional stress markers to assess the reliability of stress response triggered under our experimental conditions. Since mitochondrial heat-shock proteins (glucose regulated protein 75, GRP75), proinflammatory cytokines (tumour necrosis factor-α, TNF-α) and detoxifying enzymes (cytochrome P450 1A1, CYP1A1) are also stress-sensitive markers in sparid fish

(Bermejo-Nogales et al., 2007; 2008), these genes were chosen to asses how protein misfolding and potential sources of reactive oxygen species (ROS) were regulated in concert with the liver GH/IGF axis.

## 2. Material and methods

### 2.1. Fish rearing and sampling

Juvenile gilthead sea bream (*Sparus aurata*) of 110-130 g final body weight were reared from July to December in a seawater re-circulatory system (500-l tanks) equipped with physical/biological filters, and a heat-unit system that maintained water temperature above 18-19 ºC. Voluntary feed intake was near to maintenance ration at the time of the experiment (December), and it was stopped one day before confinement exposure to avoid result disturbances due to differences in feed intake between control (undisturbed fish) and stressed fish. Batches of 10 fish were transferred from 500-l tanks (9-10 $kg/m^3$) to cylinder net baskets of 10-l volume (117-123 $Kg/m^3$), each one suspended in 90-l tanks with a seawater flow of 10-l/min to avoid water deterioration (oxygen > 5 ppm; unionised ammonia < 0.02 mg/l). These fish served as zero time and stressed fish at specific sampling times (1.5, 3, 6, 24, 72 and 120 h) after confinement exposure. Additional 500-l tanks (one per each sampling time) were used as control fish donors. No mortality was registered in control and stressed fish over the course of the confinement period.

At each sampling time, eight fish from control and confinement tanks were netted into a bucket containing 0.1 g/l of 3-aminobenzoic acid ethyl ester (MS-222;

Sigma, Saint Louis, MO, USA). Blood was taken from caudal vessels (in less than 2 min for all fish), centrifuged at 3000 *g* for 20 min at 4 ºC, and plasma samples were frozen and stored at -30 ºC until hormone and metabolite analyses were performed. Prior to tissue collection, fish were killed by cervical section and the liver was extracted, immediately frozen in liquid nitrogen, and stored at -80 ºC awaiting RNA isolation. All procedures were carried out according to national (Consejo Superior de Investigaciones Científicas, Institute of Aquaculture Torre de la Sal Review Board) and the current EU legislation on the handling of experimental animals.

2.2. Hormone and metabolite assays

Plasma cortisol levels were assayed using an enzyme immunoassay kit (Diagnostic Systems Laboratories, Webster, TX, USA) based on the competition between unlabelled cortisol and cortisol-horseradish peroxidase for a fixed number of antibody-binding sites. Tetramethylbenzidine was used as a chromogen solution with sensitivity (90% of binding) of 1 ng/ml. Plasma glucose levels were measured by the glucose oxidase method (Thermo Electron, Louisville, CO, USA).

Plasma GH levels were determined by a homologous gilthead sea bream radioimmunoassay (RIA) as reported elsewhere (Martínez-Barberá et al., 1995). The sensitivity and midrange ($ED_{50}$) of the assay were 0.15 and 1.8 ng/ml, respectively. Plasma IGFs were extracted by acid-ethanol cryoprecipitation (Shimizu et al., 2000), and the concentration of IGF-I was measured by means of a generic fish IGF-I RIA validated for Mediterranean perciform fish (Vega-Rubín de Celis et al., 2004). The assay is based on the use of red sea bream (*Pagrus major*) IGF-I (GroPep, Adelaide, Australia) as tracer and standard, and anti-barramundi (*Lates calcarifer*) IGF-I serum

144 (GroPep) (1:8000) as a first antibody. The sensitivity and midrange of the assay were
145 0.05 and 0.7–0.8 ng/ml, respectively.
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147 2.3. RNA extraction and RT procedure
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149 Total RNA extraction from target tissues was performed with the ABI PRISM™
150 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA, USA). Briefly,
151 tissue samples were homogenized at a ratio of 25 mg/ml with a guanidine-detergent
152 lysis reagent. The reaction mixture was treated with proteinase K, and RNA purification
153 was achieved by passing the tissue lysate (0.4 - 0.5 ml) through a purification tray
154 containing an application-specific membrane. Wash solutions containing DNase were
155 applied, and total RNA was eluted into a 96-well PCR plate. The RNA yield was 30-50
156 μg with absorbance measures ($A_{260/280}$) of 1.9-2.1. Reverse transcription (RT) with
157 random decamers was performed with the High-Capacity cDNA Archive Kit (Applied
158 Biosystems). For this purpose, 500 ng total RNA were reverse transcribed into a final
159 volume of 100 μl. RT reactions were incubated for 10 min at 25 ºC and 2 h at 37 ºC.
160 Negative control reactions were run without reverse transcriptase.
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162 2.4. Real-time PCR
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164 Measurements of hepatic transcripts (GRP75, TNF-α, CYP1A1, GHR-I, GHR-
165 II, IGF-I and IGF-II) were taken using an iCycler IQ Real-time Detection System (Bio-
166 Rad, Hercules, CA, USA) as described elsewhere (Bermejo-Nogales et al., 2007).
167 Briefly, diluted RT reactions were used for PCR reactions in 25-μl volume. Each PCR-
168 well contained a SYBR Green Master Mix (Bio-Rad), and specific primers at a final

concentration of 0.3 – 0.9 μM were used to obtain amplicons of 75 – 169 bp in length (Table 1).

β-actin was used as the housekeeping gene, and the efficiency of PCR reactions for the target and the reference gene varied between 87% and 99%, respectively. The dynamic range of standard curves (serial dilutions of RT-PCR reactions) spanned five orders of magnitude, and the amount of product in a particular sample was determined by interpolation of the cycle threshold (Ct) value. The specificity of reaction was verified by analysis of melting curves and by electrophoresis and sequencing of PCR amplified products. Reactions were performed in triplicate and the fluorescence data acquired during the extension phase were normalized to β-actin by the delta-delta method (Livak and Schmittgen, 2001). No changes in β-actin expression were found in response to confinement exposure.

### 2.5. Statistical analysis

The time course of circulating levels of hormones and metabolites was analyzed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls. At each sampling time, stress-specific changes in circulating parameters and hepatic transcripts were analyzed by Student t-test. Pearson Product Moment correlations was used for correlation analyses of circulating GH and IGF-I, and hepatic transcripts of IGFs and GHRs. SPSS for Windows Version 14.0.1 (SPSS Inc) was used as statistical software.

## 3. Results

### 3.1. Circulating and hepatic stress markers

Plasma cortisol titres (< 3 ng/ml) in control fish did not change significantly over the course of the study (Fig. 1A). In stressed fish, plasma cortisol levels peaked up to 60-70 ng/ml after 1.5 h of confinement exposure with a recovery of control values at the 6 h sampling time. After 24 h of confinement exposure plasma cortisol levels peaked again, with a recovery of control values at the last sampling time (120 h).

Plasma glucose levels in control fish did not vary over the course of the confinement study (Fig. 1B). In stressed fish, the plasma glucose concentration was 2-fold increased at the 3 h sampling time, with a progressive recovery of control values during the subsequent 6-120 h sampling times (Fig. 1B).

Hepatic transcripts of GRP75 were significantly up-regulated (2 or 3-fold increase) over the course of the 6-72 h sampling period, with a recovery of control values at the last sampling time (120 h) (Fig. 2A). After 6 h of confinement exposure, TNF-α (Fig. 2B) and CYP1A1 (Fig. 2C) transcripts were not significantly altered, but a 2 or 5-fold decrease was found during the subsequent 24-120 h sampling times.

### 3.2. Liver GH/IGF axis

All fish over the course of the confinement period remained unfed, and GH levels in control fish increased significantly with time (Fig. 3A). This fasting-induced GH rise was partially masked by confinement exposure, and plasma GH levels in stressed fish were 2-fold lower than in control fish at the 72 and 120 h sampling times.

Circulating IGF-I concentration was also lowered by confinement exposure, and stressed fish showed a significant reduction in plasma levels of IGF-I over the course of the 6-72 h period (Fig. 3B), although there was not a significant correlation with GH levels. The maximum decrease (3-fold) was found at the 24 h sampling time, with a recovery of control values at the last sampling (120 h).

After 6 h of confinement exposure, hepatic IGF-I (Fig. 4A) and IGF-II (Fig. 4B) expression was not altered by confinement exposure, but a 2-fold decrease in both IGF transcripts was evidenced at the 24 h sampling time. Later on, the trend was towards recovery of the control values, but a significant reduction was still found at the 120 h sampling time. Regarding the hepatic expression of GHRs, we failed to demonstrate any significant change in the expression of GHR-I (Fig. 5A). In contrast, a 2-fold reduction in GHR-II transcripts was found over the course of the 24-120 h sampling times (Fig. 5B). This stress-specific response was positively correlated with changes in transcripts encoding for IGF-I ($r$=0.605, $P < 0.01$) and IGF-II ($r$=0.514, $P < 0.05$).

## 4. Discussion

Gilthead sea bream is an important aquaculture species in the Mediterranean region, and the stress response of cortisol, glucocorticoid receptors and energy metabolites has been examined in fish subjected to different stressors (Tort et al., 1996; Arends et al., 1999; Acerete et al., 2008). There is also now experimental evidence for an inhibitory effect of handling and crowding stress upon circulating GH levels (Rotllant et al., 2000; 2001), but the effects of acute and chronic stressors on hepatic IGF production and expression of duplicated fish GHRs have not been evaluated simultaneously. This study is, therefore, the first report in non salmonid fish considering the time course of selected stress markers and key components of the liver GH/IGF axis in a changing cellular environment resulting from acute confinement.

Cortisol and glucose are currently used as primary/secondary stress biomarkers in a wide variety of fish species (Barton and Iwama, 1991; Wendelaar Bonga, 1997). In the present study, cortisol titres in control fish enter into the ideal fish levels (>5 ng/ml) (Pickering and Pottinger, 1989), and the stress-associated increases in plasma cortisol and glucose levels followed the expected response in this fish species (Ortuno et al., 2001; Sangiao-Alvarellos et al., 2005). Moreover, the cortisol peak (60-70 ng/ml) did not deviate from gilthead sea bream literature data when plasma titres were related to stocking density: (i) 15-20 ng/ml at 26-30 kg/$m^3$ (Rotllant et al., 2000; Barton et al., 2005), (ii) 30 ng/ml at 70 kg/$m^3$ (Sangiao-Alvarellos et al., 2005) and (iii) 178 ng/ml at 200 kg/$m^3$ (Rotllant et al., 2001). This finding supports a close relationship between cortisol response and the intensity/duration of confinement, although we found a bimodal cortisol rise as reported elsewhere by Arends et al. (1999). These authors considered that the first cortisol peak is due to a rapid activation of the hypothalamic-

257 pituitary-interrenal axis (HPI) leading to exhaustion and/or negative feedback regulation
258 of this axis. The second cortisol peak might be ACTH-independent and, in our case, it
259 was associated to a wide variety of tissue repair and remodelling processes that were
260 mostly mediated by the stress response of the endoplasmic reticulum (Calduch-Giner et
261 al., 2008).

262 The transition from normal to stressful conditions is also accompanied by a
263 robust up-regulation of heat shock proteins (Hsp), which damps the cytotoxicity caused
264 by misfolded and denaturated proteins (Anckar and Sistonen, 2007). These proteins thus
265 have housekeeping functions, which makes the mitochondrial GRP75 and its yeast
266 homologue (SSC1p) life-essential (Craig et al., 1989; Kaul et al., 2007). Few studies
267 have examined the role of GRP75 in fish, but it is now recognized that hepatic GRP75
268 is up-regulated in zebrafish toxicogenomic models (Lam et al., 2006). The up-regulated
269 expression of hepatic GRP75 has also been demonstrated in a previous gilthead sea
270 bream study with fish exposed for short (24 h) and long (3 weeks/pair fed study) periods
271 of time to high (120 $kg/m^3$) and mild (50 $kg/m^3$) loading densities, respectively
272 (Bermejo-Nogales et al., 2008). This enhanced expression protects mitochondria against
273 oxidative damage, and not surprisingly the hepatic GRP75 showed in the present study
274 a rapid and robust up-regulation, which stopped after 5 days of acute confinement
275 exposure. This loss of responsiveness can be understood as the reestablishment of a new
276 redox homeostasis that was encompassed by a down-regulated expression of hepatic
277 TNFα and CYP1A1 genes. The former is a pro-inflammatory cytokine that increases
278 leukocyte and mitochondrial ROS production (Yang et al., 2007), and experimental data
279 support a reduced or enhanced expression of TNFα and TNF decoy receptors in stressed
280 rats and fish, respectively (Connor et al., 2005; Momoda et al., 2007). Likewise, hepatic
281 CYP1A1 is a major hepatic metabolizing enzyme that transforms endogenous

282 substrates, procarcinogens and pollutants into less- or non-toxic metabolites (van der
283 Oost et al., 2003). These processes are, however, potential sources of ROS, and
284 CYP1A1 expression is generally repressed by oxidative stress (Barouki and Morel,
285 2001).

286 The modulation of ROS production and antioxidant defences by the endocrine
287 system is also a well-documented phenomenon (Haddad et al., 2002). Thus, in long-
288 lived dwarf mice, the reduced signalling of GH and IGF-I contributes to maintain an
289 appropriate cellular redox state (Holzenberger et al., 2003; Bartke and Brown-Borg,
290 2004). Conversely, animals over expressing GH combat oxidative stress less efficiently
291 than normal and dwarf mice (Brown-Borg et al., 1999; Brown-Borg and Rakoczy,
292 2000). In this way, it is not surprising the stress-induced reduction in plasma GH levels.
293 This stress response after confinement exposure has been reported in a wide variety of
294 fish species, including gilthead sea bream (Rotllant et al., 2000; 2001), tilapia (Auperin
295 et al., 1997), rainbow trout (Pickering et al., 1991) and Atlantic salmon (Wilkinson et
296 al., 2006). However, the link between somatotropic and HPI axes is not clear, since the
297 *in vivo* cortisol and GH negative correlation disagrees with the *in vitro* GH stimulatory
298 action of cortisol (Nishioka et al., 1985; Wendelaar Bonga, 1997; Yada et al., 2005).
299 This apparent inconsistency is also found in mammals, and short-term glucocorticoid
300 treatments stimulate GH secretion whereas long-term treatments exert an inhibitory
301 action (Casanueva el al., 1990; Guiustina et al., 1992; Miell et al., 1991). The present
302 study was conducted under a regimen of natural photoperiod, and the shortened light-
303 dark cycle at the time of the study produced the known winter decreases in cortisol and
304 GH titres (Mingarro et al., 2002). This scenario of seasonal hyposomatotropism and
305 hypocortisolism makes difficult any additional enhancement of the inhibitory GH-tonus,
306 but the achieved results indicate that confinement exposure partially blunted the fasting-

induced increase in plasma GH levels. This elevated plasma GH levels during fasting and malnutrition presumably reflects the insensitivity of liver to GH action, and thereby a reduced negative feedback effect of IGF-I on pituitary GH release (Pérez-Sánchez et al., 1992; 1995). Indeed, more recent studies in gilthead sea bream (Saera-Vila et al., 2005) and hybrid striped bass (Picha et al., 2008) indicate that this fasting state of GH resistance is mediated at long-term by a down-regulated expression of GHR-I and II.

Time series analyses of IGFs evidenced a lag-time among changes in hepatic IGF transcripts and circulating levels of protein. However, the hepatic tissue is the primary source of circulating IGF-I and its depletion by stress and nutritional disorders reflects, in this and previous gilthead sea bream studies (Gómez-Requeni et al., 2004; Benedito-Palos et al., 2007), the down-regulated expression of hepatic IGF-I. In rainbow trout and Atlantic salmon, handling and confinement also reduce circulating levels of IGF-II (Wilkinson et al., 2006). Thus far, there is no information in gilthead sea bream on the effects of aquaculture stressors on circulating levels of IGF-II. Nevertheless, the results presented here evidenced a robust down-regulation of hepatic IGF-II, which might reflect reduced circulating levels of IGF-II. Experimental evidence in catfish (Small et al., 2006), tilapia (Kajimura et al., 2003) and sunshine bass (Davis and Peterson, 2006) also indicate that confinement and cortisol treatment reduce IGF activity and sensitivity to GH. Therefore, the mechanisms by which stressful conditions inhibit growth are apparently conserved throughout evolution, but more complete information on the sequence of events that adjusts growth to each particular condition requires species-specific studies. Thus, in gilthead sea bream, the reduced hepatic IGF-I production can be compensated at the local tissue level (skeletal muscle) by the enhanced expression of IGF-II (Benedito-Palos et al., 2007; Saera-Vila et al., 2007). In

support of this, growth differences between families of channel catfish have been related to differences in muscle IGF-II expression (Peterson et al., 2004).

In gilthead sea bream, local compensatory mechanisms of growth can also be mediated by GHRs, and the muscle expression of GHR-II is increased by the large dietary replacement of fish oil with vegetable oils regardless of that found for IGFs (Benedito-Palos et al., 2007). Both in gilthead sea bream (Saera-Vila et al., 2005) and hybrid striped bass (Picha et al., 2008), the muscle expression of GHR-II is also up-regulated by fasting, which may serve to repair and preserve tissue functions as reported for rat GHRs in atrophied muscle fibers (Casse et al., 2003). In the same way, we found in the present study that hepatic transcripts of GHR-II were down-regulated after acute confinement exposure, whereas no significant changes were reported for GHR-I. Since glucocorticoids inhibit the expression of GHRs in mammals (Beauloye et al., 1999; Gabrielsson et al., 1995) and GHR-I exhibit a higher affinity for SL in salmonids (Fukada et al., 2005), it may be argued that GHR-II is the true ortologous of mammalian GHR whereas GHR-I might be mostly evolved as a SL receptor. However, SL has not a growth-promoting action in gilthead sea bream (Pérez-Sánchez et al., 2002; Vega-Rubín de Celis et al., 2003), and nice positive correlations between GHR-I and IGF transcripts have been reported in different experimental models of this marine fish (Benedito-Palos et al., 2007; Saera-Vila et al., 2007, Sitjà-Bobadilla *et al*., 2008). Moreover, GHRs of Japanese eel bind specifically to GH (Ozaki et al., 2006) and Jiao et al. (2006) did not found in black sea bream any response to SL in GHR transfected cells. If so, there is not an easy pattern of transcription and function of GHRs in non-salmonid fish, although it is reasonable to imagine that GHR-II has a dual function preserving cell survival and limiting at the same time growth and hepatic IGF production under life-stress conditions.

Prior to the present study, there is not a time course study addressing the differential stress regulation of GHRs in fish, although several lines of evidence support a complex cross talk between glucocorticoids and fish GHRs. Thus, cortisol injection in black sea bream enhances the expression of hepatic GHR-I without significant effects on GHR-II (Jiao et al., 2006). Pierce *et al*. (2005) reported an increased expression of GHR-II in primary cultures of salmon hepatocytes exposed to dexamethasone, and Small *et al.* (2006) demonstrated a reduced expression of GHR-II in catfish fed with cortisol for 4 weeks. More recently, Uchida et al. (2009) reported that handling and confinement exposure enhanced in tilapia the hepatic expression of GHR-II. To date there is no an easy explanation for these apparent contradictory findings, although it is suspected that the glucocorticoid response is a dynamic process having both stimulatory and inhibitory effects upon the somatotropic axis, which may reflect physiological and pharmacological responses and/or differences between short- and long-term treatments as found in mammals (Vottero et al., 2003). In particular for gilthead sea bream, we found in the present study that the stress-specific response of GHR-II was encompassed by adaptive changes in ROS production and mitochondrial chaperones. Furthermore, a previous computational study (Saera-Vila et al., 2007) recognized a subset of stress-sequence elements (CRE and APs) surrounding the transcription start site of GHR-II. These sequence elements can activate and/or inhibit the expression of target genes depending on promoter organization and cellular redox context (Abate et al., 1991; Hai and Hartman, 2001; Glahder et al., 2003). Taking all these findings together, there is now increasing evidence for a functional partitioning of GHRs in gilthead sea bream, but some overlapping and redundancy may occur, and the possibility of GHR-I/GHR-II heterodimers cannot be excluded.

In summary, stress-mediated changes in primary and secondary stress markers (cortisol, glucose, GRP75, TNF-α, CYP1A1) evidenced a robust stress response with the establishment of a new cellular homeostasis in fish exposed to acute confinement. In this changing cellular environment, the time course of the liver GH/IGF axis revealed delayed stimulatory effects of fasting on plasma GH titres, a reduced hepatic IGF production and a differential expression of the two types of gilthead sea bream GHRs. This can represent an interesting field of research for the exploration of the different stress-susceptibilities of fish species and strains to aquaculture stressors.

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668

669 Table 1. Gilthead sea bream primer sequences used for real-time PCR.
670

| Gene | accession number | primer sequence | position |
|---|---|---|---|
| GRP-75 | DQ524993 | F TCC GGT GTG GAT CTG ACC AAA GAC | 358-381 |
| | | R TGT TTA GGC CCA GAA GCA TCC ATG | 500-477 |
| TNF α | AJ413189 | F CAG GCG TCG TTC AGA GTC TC | 587-606 |
| | | R CTG TGG CTG AGA GGT GTG TG | 663-644 |
| CYP 1A1 | AF011223 | F GCA TCA ACG ACC GCT TCA ACG C | 903-924 |
| | | R CCT ACA ACC TTC TCA TCC GAC ATC TGG | 1071-1045 |
| GHR-I | AF438176 | F ACC TGT CAG CCA CCA CAT GA | 1275-1294 |
| | | R TCG TGC AGA TCT GGG TCG TA | 1373-1354 |
| GHR-II | AY573601 | F GAG TGA ACC CGG CCT GAC AG | 1690-1709 |
| | | R GCG GTG GTA TCT GAT TCA TGG T | 1764-1743 |
| IGF-I | AY996779 | F TGT CTA GCG CTC TTT CCT TTC A | 112-133 |
| | | R AGA GGG TGT GGC TAC AGG AGA TAC | 195-172 |
| IGF-II | AY996778 | F TGG GAT CGT AGA GGA GTG TTG T | 406-427 |
| | | R CTG TAG AGA GGT GGC CGA CA | 514-495 |
| β-actin | X89920 | F TCC TGC GGA ATC CAT GAG A | 811-829 |
| | | R GAC GTC GCA CTT CAT GAT GCT | 861-841 |

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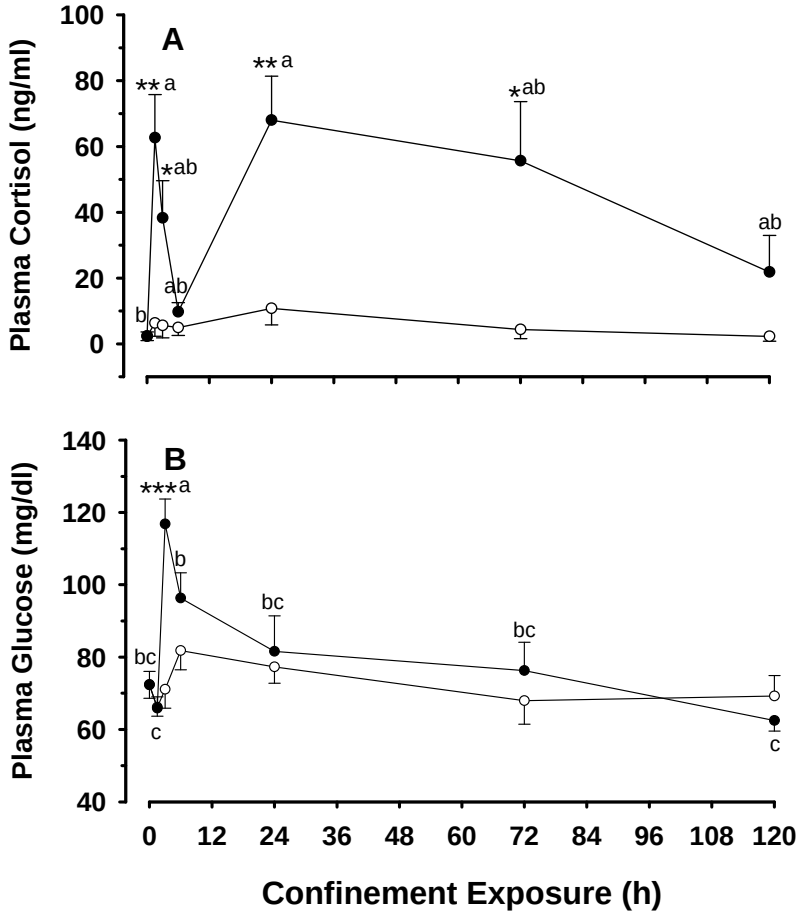
675 Figure 1. Time course of plasma levels of cortisol (A) and glucose (B) in control (○) and
676 stressed (●) fish. Data are the mean ± SEM (n = 6-8). Different letters indicate statistically
677 significant changes over the course of the experiment in stressed fish (ANOVA, $P<0.05$).
678 Statistically significant differences between stressed and control fish were analyzed at each
679 sampling time by means of Student t-test (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).
680

681 Figure 2. Box-Whisker plots representing the time course of the relative gene expression of
682 GRP75 (A), TNF-α (B) and CYP1A1 (C) in stressed fish. Data in control fish were used as
683 arbitrary reference values at each sampling time in the normalization procedure (values > 1 or
684 < 1 indicate increase or decrease respect to reference values). The lower boundary of the box
685 indicates the 25th percentile and the upper boundary of the box indicates the 75th percentile.
686 Whiskers above and below the box indicate the 90th and 10th percentiles. Continuous line
687 inside the box is the median; non-continuous line inside the box is the mean. Statistically
688 significant differences respect to the control group were analysed by means of Student t-test
689 (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).
690

691 Figure 3. Time course of plasma levels of GH (A) and IGF-I (B) of control (white) and
692 stressed (black) gilthead sea bream. Data are the mean±SEM (n = 6-8). Different letters
693 indicate statistically significant changes over the course of the experiment in control (regular
694 font) and stressed fish (italic font, ANOVA, $P<0.05$). Statistically significant differences
695 between stressed and control fish were analyzed at each sampling time by means of Student t-
696 test (* $P<0.05$, *** $P<0.001$).
697

Figure 4. Box-Whisker plots representing the time course of the relative gene expression of IGF-I (A), and IGF-II (B) in stressed fish. Data in control fish were used as arbitrary reference values at each sampling time in the normalization procedure (values > 1 or < 1 indicate increase or decrease with respect to reference values). The lower boundary of the box indicates the 25th percentile and the upper boundary of the box indicates the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles. Continuous line inside the box is the median; non-continuous line inside the box is the mean. Statistically significant differences respect to the control group were analysed by means of Student t-test (* $P<0.05$, ** $P<0.01$).

Figure 5. Box-Whisker plots representing the time course of the relative gene expression of GHR-I (A), and GHR-II (B) in stressed fish. Data in control fish were used as arbitrary reference values at each sampling time in the normalization procedure (values > 1 or < 1 indicate increase or decrease with respect to reference values). The lower boundary of the box indicates the 25th percentile and the upper boundary of the box indicates the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles. Continuous line inside the box is the median; non-continuous line inside the box is the mean. Statistically significant differences respect to the control group were analysed by means of Student t-test (* $P<0.05$).


A
Plasma Cortisol (ng/ml)
100
80
60
40
20
0
**a
*ab
**a
*ab
ab
ab
b
B
Plasma Glucose (mg/dl)
140
120
100
80
60
40
***a
b
bc
bc
bc
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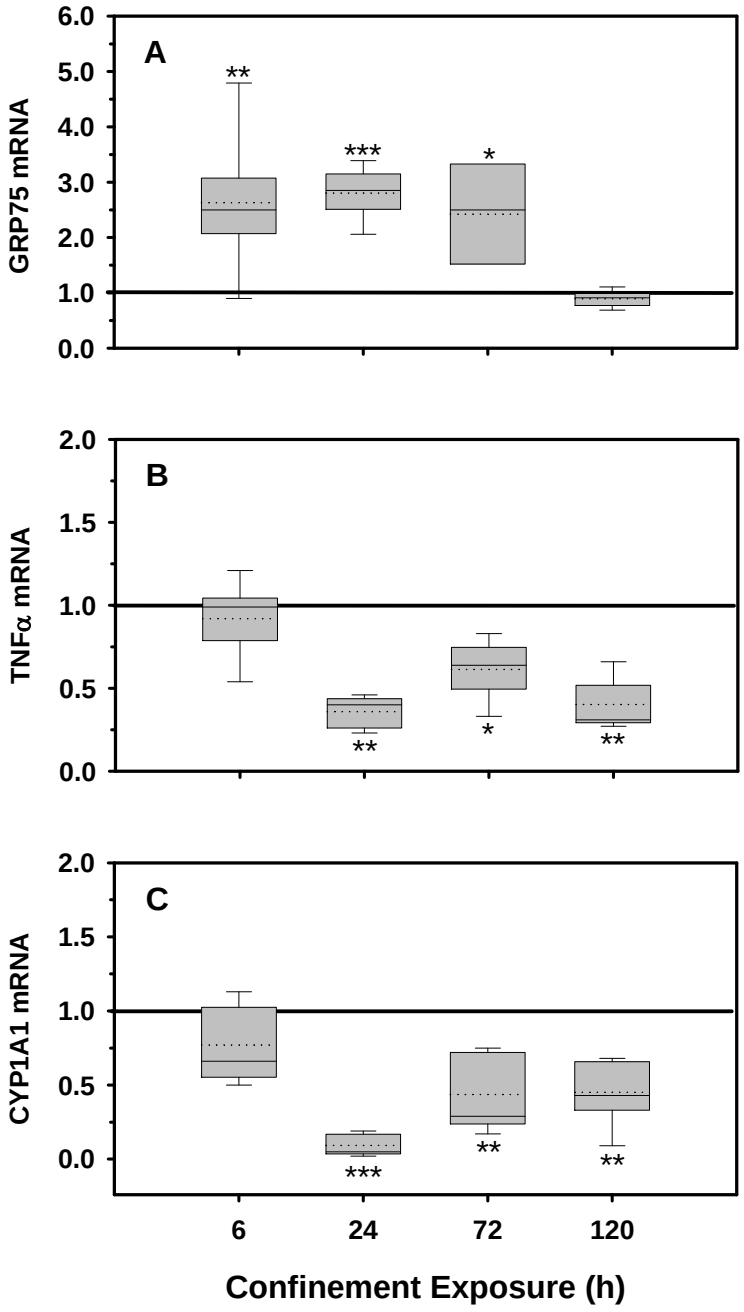
c
0
12
24
36
48
60
72
84
96
108
120
Confinement Exposure (h)

**Figure 1**


A
B
C
GRP75 mRNA
TNFα mRNA
CYP1A1 mRNA
6.0
5.0
4.0
3.0
2.0
1.0
0.0
2.0
1.5
1.0
0.5
0.0
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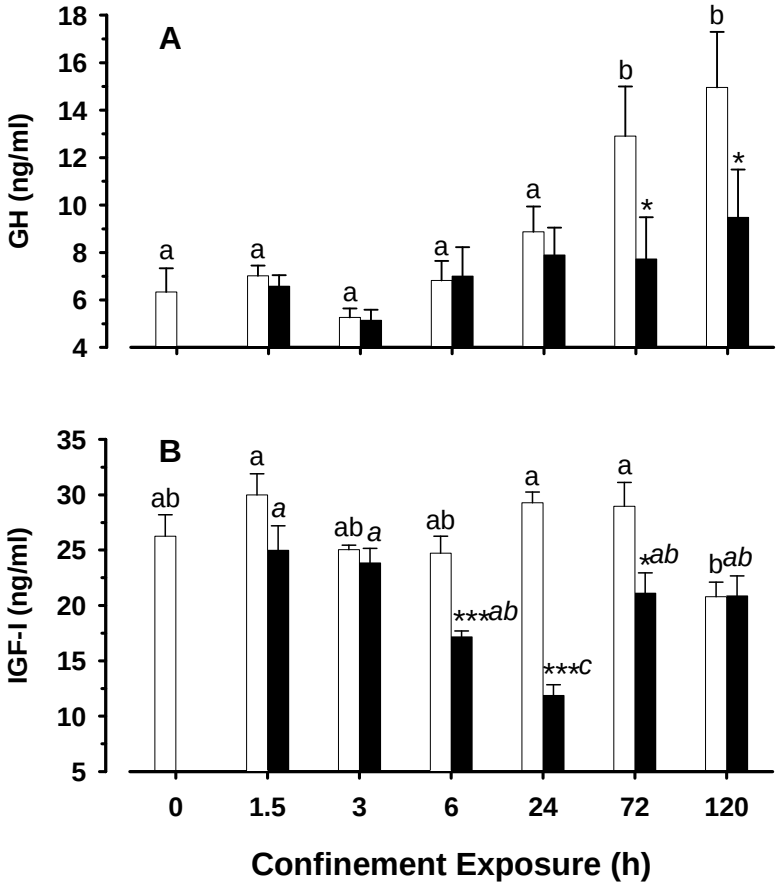
***
*
6
24
72
120
Confinement Exposure (h)

**Figure 2**


A
GH (ng/ml)
18
16
14
12
10
8
6
4
a
a
a
a
a
b
b
*
*
B
IGF-I (ng/ml)
35
30
25
20
15
10
5
ab
a
a
ab
a
ab
a
***ab
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b
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1.5
3
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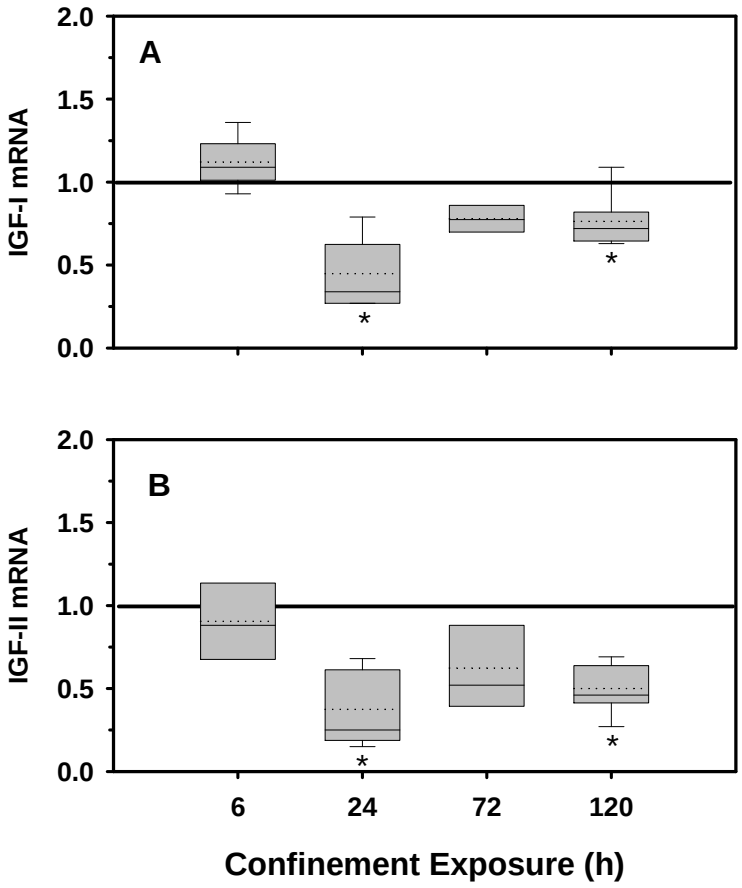
24
72
120
Confinement Exposure (h)

**Figure 3**


A
IGF-I mRNA
2.0
1.5
1.0
0.5
0.0
*
*
B
IGF-II mRNA
2.0
1.5
1.0
0.5
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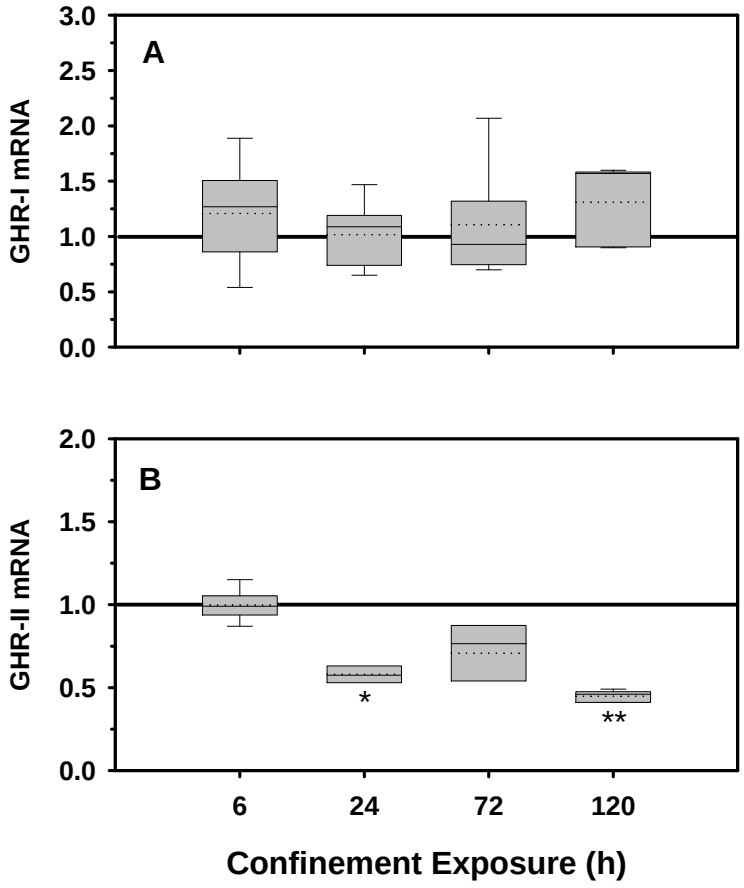
24
72
120
Confinement Exposure (h)

**Figure 4**


A
B
GHR-I mRNA
GHR-II mRNA
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2.0
1.5
1.0
0.5
0.0
*
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6
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72
120
Confinement Exposure (h)

**Figure 5**