Co-expression of IGFs and GH receptors (GHRs) in gilthead sea bream (*Sparus aurata* L.): sequence analysis of the GHR-flanking region

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Abstract

The tissue-specific expression of IGFs and GH receptors (GHRs) was analyzed in gilthead sea bream (*Sparus aurata* L.) as an attempt to understand the functional partitioning of duplicated GHRs on the regulation of fish growth by season and aging. Gene transcripts were measured in liver, muscle, and adipose tissue by means of quantitative real-time PCR assays. In juvenile fish, concurrent increases in circulating levels of GH and IGF-I and hepatic mRNA levels of IGF-I and GHR-I were evidenced with the summer growth spurt. Conversely, muscle and adipose tissue expression of GHR-I and IGF-II were significantly upregulated by overwintering. The aging decrease of growth rates was accompanied by a reduced activity of the liver GH/IGF axis, and parallel increases in muscle IGF expression would be dictated at the local tissue level by the enhanced expression of GHR-I. Extra-hepatic expression of

IGFs and GHR-II did not correlate seasonally in juvenile fish, and nonsignificant effects of aging were found on the summer expression of GHR-II in any analyzed tissue. One transcription start site was identified by RLM-RACE in GHR-I and GHR-II. Sequence analyses indicated that both genes have TATA-less promoters containing consensus initiator sequences and downstream promoter elements surrounding the transcription start site. Conserved CCAAT-boxes and GC-rich regions were retrieved in the GHR-I promoter, whereas stress- and redox-sequence elements (cAMP-responsive element-binding protein, activator proteins; AP-1, and AP-4) were characteristic features of GHR-II. All this supports the functional partitioning of fish GHRs regardless of fish species differences.

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Introduction

Biological actions of growth hormone (GH) are initiated by binding to specific receptors (GH receptors; GHRs) localized on the cell surface membrane of central and peripheral target tissues. These GHRs belong to the hematopoietic receptor superfamily, which includes among others receptors for prolactin (PRL), leptin, erythropoietin, granulocyte-stimulating factor, and interleukins (Kelly *et al.* 1991, Kopchick & Andry 2000). Common characteristics are a single transmembrane domain, one or two pairs of positionally conserved cysteines, two regions of homology to the type III module of fibronectin, a WSXWS motif that is conserved as YXXFS in the mammalian GHR, and proline-rich Box 1 and Box 2 that are important for signal transduction (Kopchick & Andry 2000). As a characteristic feature, expression of GHRs takes place at varying tissue levels, and the use of alternate promoters plays a major role in orchestrating a tissue-specific pattern. Heterogeneity in the 5'-untranslated region is in fact a well-documented phenomenon, and at least eight GHR mRNA variants splicing just upstream of the translation start site in exon 2 are reported for the human GHRs

(Goodyer *et al.* 2001, Orlovskii *et al.* 2004, Wei *et al.* 2006).

Fish GHRs were first cloned and sequenced in turbot (*Scophthalmus maximus*; Calduch-Giner *et al.* 2001) and goldfish (*Carassius auratus*; Lee *et al.* 2001). Later on the GHR cDNA sequences of many species including gilthead sea bream (*Sparus aurata*; Calduch-Giner *et al.* 2003), black sea bream (*Acanthopagrus schlegelii*; Tse *et al.* 2003), Japanese flounder (*Paralichthys olivaceus*; Nakao *et al.* 2004), common carp (*Cyprinus carpio*; GenBank accession number AY691176), grass carp (*Ctenopharyngodon idella*; AY283778), channel catfish (*Ictalurus punctatus*; DQ103502), Mozambique tilapia (*Oreochromis mossambicus*; Kajimura *et al.* 2004), coho salmon (*Oncorhynchus kisutch*; AF403539, AF403540), masu salmon (*Oncorhynchus masou*; Fukada *et al.* 2004), rainbow trout (*Oncorhynchus mykiss*; Very *et al.* 2005), Atlantic salmon (*Salmo salar*; Benedet *et al.* 2005), and Japanese eel (*Anguilla japonica*; Ozaki *et al.* 2006) have been reported. Most of these GHRs share several common features, and amino acid alignments reveal a relative high degree of identity (35–40%) among GHRs of tetrapods and non-salmonid fish. Nevertheless, amino acid identity among GHRs of tetrapods and salmonids decreases up to 27–34% with a lack of three conserved cytoplasmatic tyrosine residues and two

extracellular cysteines involved in a short disulphide link. Initially, fish GHRs were clustered in two clades: the first one corresponded to GHRs thus far described in non-salmonid fish (GHR type I) and the other clade encompassed most GHR sequences described in salmonid fish (GHR type II). This observation led to the suggestion that two different lineages of GHRs are present in teleost evolution. This assumption has now changed by the finding of two genomic contigs with a strict conservation of intron–exon junctions in fugu (*Fugu rubripes*), zebrafish (*Danio rerio*), and the Mediterranean gilthead sea bream (Saera-Vila *et al.* 2005). In the same study, the coexistence of duplicated GHR genes was experimentally supported in rainbow trout and European sea bass (*Dicentrarchus labrax*). Likewise, Jiao *et al.* (2006) cloned and sequenced the GHR-II in black sea bream, Southern catfish (*Silurus meridionalis*), and Nile tilapia (*Oreochromis niloticus*), providing further evidence for the coexistence of two GHR genes in a single fish species.

It is believed that duplication of genes and entire genomes are important mechanisms for morphological and functional innovation in evolution. At this standpoint, polyploidy has long been recognized in fish (Zhou *et al.* 2001, Volf 2005), and duplication and divergence of fish GHRs would take place on an early ancestor of fish lineage. Salmonids are, however, considered recent tetraploids, and two isoforms of GHR-II are differentially expressed in rainbow trout (Very *et al.* 2005, Gabillard *et al.* 2006). Truncated variants of GHR-I have been characterized in turbot (Calduch-Giner *et al.* 2001) and Japanese flounder (Nakao *et al.* 2004), and this fact might allow the silencing and/or apparent genomic loss of GHR-II in the flatfish lineage (Saera-Vila *et al.* 2005). These truncated variants of fish GHRs comprise extracellular and transmembrane domains, the first 28 amino acid residues of the intracellular domain and a divergence sequence of 21–26 amino acid residues, which is the result of the lack of the alternative splicing of intron 9/10 (see Pérez-Sánchez *et al.* 2002).

Cloned GHR-I and GHR-II have shown to be functional in goldfish (Lee *et al.* 2001), black sea bream (Tse *et al.* 2003, Jiao *et al.* 2006), and Atlantic salmon (Benedet *et al.* 2005). In these studies, mammalian CHO-K1 were transfected with GHR cDNA and GH exposure triggers a strong proliferation response, which can also be induced by PRL at supraphysiological doses. Binding studies in masu salmon evidenced that GH and somatotactin (SL) may functionally interact through GHR-I (Fukada *et al.* 2005). Experimental evidence also indicates that the expression of GHR-II is modulated by water temperature in rainbow trout (Gabillard *et al.* 2006), whereas GHR-I and GHR-II are differentially regulated by cortisol and testosterone in black sea bream (Jiao *et al.* 2006). Previous studies in gilthead sea bream also revealed that duplicated GHRs are differentially regulated by fasting (Saera-Vila *et al.* 2005), although the relative contribution of each gene in the regulation of fish growth still remains unclear. Thus, the major goal of the present study was to analyze the tissue-specific expression of gilthead sea bream GHRs and insulin-like growth factors (IGFs) in relation with changes in growth (season and age models).

Additionally, transcription start sites and GHR-flanking regions were mapped as a first attempt to characterize GHR promoters in fish.

Materials and Methods

Fish rearing and sampling

Juvenile fish of 20–25 g initial body weight (8 months old) were reared until marketable size in triplicate 2500 l tanks at the indoor experimental facilities of Instituto de Acuicultura de Torre de la Sal. Photoperiod and water temperature followed natural changes, and fish were fed with a commercial fish meal-based diet (Proaqua, Palencia, Spain) containing 47% protein and 21% lipid. Feed was adjusted to maximize growth rates and feed conversion efficiency through the entire productive cycle as described elsewhere (Mingarro *et al.* 2002). Specific growth rates (SGRs) were calculated monthly ($SGR = (\ln \text{ final wt} - \ln \text{ initial wt}) \times 100 / \text{days}$). At critical step windows over the course of year (October, January, May, and July), overnight fasted fish were randomly selected (five fish per tank) and killed by a blow on the head under anesthesia (3-aminobenzoic acid ethyl ester, 100 mg/l). Blood was taken from caudal vessels with heparinized syringes, and the resulting plasma samples (3000 g for 20 min at 4 °C) were stored at –30 °C until hormone assays. Liver, mesenteric adipose tissue, and dorsal skeletal muscle (white muscle) were rapidly excised, frozen in liquid nitrogen, and stored at –80 °C until RNA extraction and analysis.

Gilthead sea bream is a protandrous fish and 1-year-old (immature males), 2-year-old (mature males), and 3-year-old (mature females) fish were reared in triplicate groups as a second experimental set in 2500 l tanks. Fish were fed with fish meal-based diets under standardized conditions, and randomly selected fish (5 fish per tank and 15 fish per group of age) were taken for blood and tissue collection during the summer growth spurt (July).

Hormone assays

Plasma GH levels were determined by a homologous gilthead sea bream RIA as reported elsewhere (Martínez-Barberá *et al.* 1995). The sensitivity and midrange (ED50) of the assay were 0.15 and 1.8 ng/ml respectively.

Circulating levels of IGF-I were measured by means of a generic fish IGF-I RIA validated for Mediterranean perciform fish (Vega-Rubín de Celis *et al.* 2004a). 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 (GroPep; 1:8000) as a first antibody. The sensitivity and midrange of the assay were 0.05 and 0.7–0.8 ng/ml respectively.

RNA/DNA extraction

Total RNA was extracted by the acid guanidium thiocyanate–phenol–chloroform method (Chomczynski & Sacchi 1987). Genomic DNA was isolated from blood with the High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's instructions. Quantity and purity of RNA and DNA samples were determined by absorbance measures at 260 and 280 nm respectively. The integrity of isolated nucleic acids was tested by electrophoresis in agarose gels.

Gene expression

Transcripts of GHRs, IGFs, and β -actin were quantified in liver, adipose tissue, and muscle by means of real-time quantitative PCR assays. Briefly, after DNase I treatment, 2 μ g total RNA were reverse transcribed with 200 U Superscript II (Invitrogen: Life Technologies) using oligo (dT)₁₇ as anchor primer. Specific primers for GHR-I, GHR-II, IGF-I, and β -actin amplification were made as described elsewhere (Calduch-Giner *et al.* 2003, Saera-Vila *et al.* 2005). Primers for IGF-II (forward: TGGGATCGTAGAGGAGTGTGT; reverse: CTGTAGAGAGGTGGCCGACA) were designed to amplify a 109 bp amplicon, comprised between 392 and 500 nt positions (Duguay *et al.* 1996).

The iCycler iQ Real Time PCR Detection System (Bio-Rad Laboratories Inc.) was used for sample cDNA quantification. Each reaction contained a SYBR Green Master Mix (Bio-Rad) and specific primers at a final concentration of 0.9 μ M. The PCR protocol was 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Standard curves were generated by amplification of serial dilutions of known quantities of recombinant plasmids. For target and reference genes, the efficiency of PCR amplification was 94–96% for serial dilutions of standards and RT reactions. Specificity of reaction was verified by the analysis of melting curves and by electrophoresis and sequencing of PCR amplified products. Reactions were performed in triplicate and fluorescence data were analyzed by interpolation of the cycle threshold (Ct) value. Each transcript level was normalized to β -actin using the delta-delta method (Livak & Schmittgen 2001). Tissue-specific levels of β -actin mRNA did not vary with experimental variables.

Transcription start site

Mapping of the transcription start site was made with the RLM-RACE kit (Ambion, Austin, TX, USA) with minor modifications. Briefly, 1 μ g total RNA was dephosphorylated with calf intestinal phosphatase at 37 °C for 1 h. The mRNA cap structure was removed, and the purified mRNA was ligated with T4 ligase to an adapter oligonucleotide sequence. The ligation product was reverse transcribed with random decamers and Moloney murine leukemia virus (M-MLV) reverse transcriptase. PCR amplification was performed with an external primer and specific reverse primers for GHR-I

(CCTGGACTCCACCAACATCGGAATG; 362–338 nt position, GeneBank AF438176) and GHR-II (CGAGCG-GAGCTGGACTTTGTAAAG; 526–504 nt position, GeneBank AY573601). The nested PCR was conducted with a 5'RACE inner primer and specific reverse primers for GHR-I (GGAAACCAGGAGAAGGAGCAGGAGATTG; 228–201 nt position) and GHR-II (GGCTTGGAGAGGTTCTGGA-GAGTG, 366–343 nt position). Conditions for PCR amplification were 2 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C with a final extension of 15 min at 72 °C. The amplified PCR products were gel extracted (QIAquick gel extraction, Qiagen) and sequenced by the deoxy chain termination method (ABI PRISM dRhodamine terminator cycle sequencing kit, Perkin-Elmer, Wellesley, MA, USA).

Analysis of GHR-flanking region

Genomic DNA was used for the construction of Genome Walker libraries by means of the Universal GenomeWalker kit (BD Biosciences, Bedford, UK). Briefly, 2.5 μ g aliquots of genomic DNA were digested with DraI, EcoRV, and PvuII respectively. Digested DNAs were extracted, precipitated, and synthetic adapters were ligated to genomic DNA fragments using T4 DNA ligase. Two reverse primers surrounding the transcription start site of GHR-I (TGGTACGAAGTCTC-GAGGTGGTG and TTTCACAACCTTGGTCATCTGATGGC) and GHR-II (TGAGAACCACACAG AAAC TGTTC AAC and CTCCATGTAAGCTGGTGACGCTG) were designed to be used with adapter primers in primary and secondary PCRs (35 cycles for 30 s at 94 °C, 30 s at 57 °C, and 300 s at 72 °C). PCR-amplified fragments were purified and sequenced as described earlier.

Bioinformatics analyses of regulatory elements were performed with the MatInspector software (<http://www.genomatix.de>). A preliminary analysis was made with a core similarity of 0.75 and an optimized matrix similarity value to allow the recovery of a comprehensive list of candidate factors. To discard false positives, MatInspector was rerun with a core similarity threshold and a matrix similarity value of 1 and 0.90 respectively.

Statistical analysis

Data were analyzed by Student's *t*-test and one-way ANOVA followed by Student–Newman–Keuls test ($P < 0.05$). Correlation analyses were made by Pearson product moment correlations.

Results

Seasonal trial

In juvenile fish, circulating levels of GH and IGF-I varied together ($r = 0.68$, $P < 0.001$), following seasonal changes in

growth rates and environmental cues. Thus, the growth spurt of summer occurred in coincidence with the highest circulating concentration of GH and IGF-I (Fig. 1).

Hepatic transcripts of GHR-I increased with the rise of growth rates and the maximum gene expression was attained in July (Fig. 2A). By contrast, in adipose tissue (Fig. 2B) and muscle (Fig. 2C), the highest amount of GHR-I transcripts was found in January. The liver transcriptional profile of GHR-II paralleled that reported for GHR-I ($r=0.711$, $P<0.001$; Fig. 2D). However, a different tissue regulation was found in peripheral tissues, and adipose tissue transcripts of GHR-II peaked in July (Fig. 2E), whereas muscle expression remained high from January to July (Fig. 2F).

In liver, the highest amount of IGF-I mRNA levels was attained in July (Fig. 3A), and a close positive correlation was found with circulating levels of IGF-I over season ($r=0.77$, $P<0.001$). Hepatic transcripts of IGF-I were also positively correlated with hepatic mRNA levels of GHR-I ($r=0.703$, $P<0.05$) and GHR-II ($r=0.804$, $P<0.001$). Muscle and adipose tissue expression of IGF-I was 20- to 100-fold lower than in liver, and nonsignificant changes over the course of season were detected in these extra-hepatic tissues (Fig. 3B and C).

Seasonal changes in hepatic *IGF-II* gene expression were not found in juvenile fish (Fig. 3D). Extra-hepatic expression of IGF-II remained relatively high, and a maximum for IGF-II transcripts was found in January (Fig. 3E and F). Thus, a positive correlation between IGF-II and GHR-I transcripts was reported in adipose tissue ($r=0.528$, $P<0.05$) and muscle ($r=0.479$, $P<0.05$).

Age trial

In summer, growth rates and circulating levels of GH and IGF-I decreased progressively and significantly with advancing age in 1-, 2- and 3-year-old fish (Fig. 4). Older fish also showed a decreased hepatic expression of GHR-I (Fig. 5A), whereas the amount of these transcripts did not vary significantly in adipose tissue (Fig. 5B). By contrast, muscle expression of GHR-I was significantly increased in older fish (Fig. 5C). Significant age-related changes in the expression of GHR-II were not found in any examined tissue (Fig. 5D–F).

The age-related changes in IGF-I and IGF-II transcripts followed similar trends (Fig. 6). However, the range of individual variation was higher for IGF-II, and nonsignificant changes in IGF-II expression were detected in any analyzed tissue. By contrast, hepatic expression of IGF-I decreased significantly in older fish (Fig. 6A). This trend was opposite to that found in skeletal muscle (Fig. 6C), and a positive correlation between GHR-I and IGF-I transcripts was reported in liver ($r=0.682$, $P<0.05$) and muscle ($r=0.626$, $P<0.05$).

Alternative splicing and sequence analysis

Two bands of 500 and 400 bp were obtained by RLM-RACE of GHR-I and GHR-II respectively. The reliability of this

Figure 1 Seasonal changes in water temperature (continuous line), daylength (dotted line), and daily feed intake of growing juvenile fish (bars); arrows indicate sampling times for blood and tissue collection (A). Body weight (white bars) and specific growth rates (gray bars) at sampling times (B). Plasma levels of GH (C) and IGF-I (D). Growth parameters are the average values (mean $\pm$ S.E.M.) of triplicates tanks. Hormone data are the mean of ten fish. Different letters above each bar indicate statistically significant differences between sampling times ($P<0.05$, Student–Newman–Keuls).

finding was checked by PCR, and the results allow us to determinate two transcription start sites (one for each gene) delimiting the 5'-flanking region of exon 1. This exon codes entirely for the 5'-UTR sequence, which was verified by the screening of Genome Walker libraries of GHR-I (Fig. 7A) and GHR-II (Fig. 7B). Exon 1 of GHR-I was located 9 kb upstream of exon 2 as further demonstrated by long PCRs with intact genomic DNA and specific primers surrounding flanking regions of exon 1 (forward primer, GCTCTACGCTGGCCATCAGATGAC) and exon 2 (reverse primer, GGCTGTCAGAATTCCTACACAGG-TAG). A similar strategy was used for GHR-II but no positive results were obtained, which suggests a long intron 1 that may be difficult to amplify by PCR.

Figure 2 Seasonal changes in the tissue-specific expression (relative units) of GHR-I and GHR-II. For each gene, the highest expression among tissues was used as reference value in the normalization procedure. Data are the mean $\pm$ S.E.M. ($n=6$). For each tissue, different letters above each bar indicate statistically significant differences between sampling times ($P<0.05$, Student–Newman–Keuls).

Sequence analysis of the 5′-flanking region of GHR-I (1589 pb; GeneBank accession number AH014067) and GHR-II (1262 pb; AH014068) did not reveal a consensus TATA-box surrounding the transcription start site. However, regulatory elements similar to the consensus for the initiator element (Inr) were retrieved in the promoter region of both genes. Also, sequences similar to downstream promoter element (DPE) were found at positions +38 (GHR-I) and +29 (GHR-II). CCAAT-boxes and GC-rich sequences were only retrieved in the proximal-flanking region of GHR-I (see Fig. 7A). Binding sites for cAMP-responsive element-binding protein (CREB) and activator proteins (AP-1 and AP-4) were exclusively found in the GHR-II-flanking region (see Fig. 7B).

Discussion

The use of alternate promoters orchestrates in mammals a tissue-specific pattern of GHR expression (Goodyer *et al.* 2001, Orlovskii *et al.* 2004). This schema is apparently simplified in fish, and only one transcription start site was evidenced herein in gilthead sea bream GHRs. However, genome duplication offers a second level of regulation, and two functional *GHR* genes (with additional isoforms in salmonids) have been conserved in several lineages of modern bony fish. These two GHRs span more than 20 kb in gilthead sea bream, and share a strict conservation of exon–intron junctions (see Pérez-Sánchez *et al.* 2002, Saera-Vila *et al.* 2005). In the present study, the gene organization was completed at the 5′-flanking region, and 1.5–1.2 kb just

Figure 3 Seasonal changes in the tissue-specific expression (relative units) of IGF-I and IGF-II. For each gene, the highest expression among tissues was used as reference value in the normalization procedure. Data are the mean $\pm$ S.E.M ($n=6$). For each tissue, different letters above each bar indicate statistically significant differences between sampling times ($P<0.05$, Student–Newman–Keuls).

upstream of the transcription start site were sequenced in GHR-I and GHR-II respectively. This represents a first attempt to characterize the promoter region of fish GHRs, which may serve to better understand the complex regulatory processes driving the tissue-specific expression of IGFs in different growth and living conditions.

Both in mammals and fish, the liver is the most important target tissue of GH and the primary source of systemic IGF-I (endocrine form). Thus, plasma levels of IGF-I are a good indicator of growth rates in European sea bass (Vega-Rubín de Celis *et al.* 2004a) and channel catfish (Silverstein *et al.* 2000, Li *et al.* 2006). Circulating levels of IGF-I are positively correlated with growth rates and dietary protein levels in barramundi and Atlantic salmon (Dyer *et al.* 2004). In salmonids, the regulation of circulating IGF-I is well documented on the basis of seasonal and nutritional cues

(Dickhoff *et al.* 1997, Larsen *et al.* 2001, Nordgarden *et al.* 2005). In gilthead sea bream, circulating levels of IGF-I are higher than those reported in salmonids and also correlate with growth shifts, derived from changes in season (Mingarro *et al.* 2002) and nutritional condition (Gómez-Requeni *et al.* 2003, 2004, Benedito-Palos *et al.* 2007). In the present study, this notion was further supported by concurrent changes in growth rates, circulating levels of GH and IGF-I, and hepatic mRNA levels of IGF-I and GHR-I. Hence, systemic increases of IGF-I would be mostly mediated in season and aging by the hepatic transcriptional activation of GHR-I. Moreover, hepatic mRNA levels of GHR-II did not vary with aging, and GHR-II emerges in older fish as a ubiquitous gene that apparently did not mediate the age-related decrease in growth rates and hepatic IGF expression. Alternatively, GHR-II may be involved in tissue repair and survival, which

Figure 4 Summer age-related changes in body weight (white bars) and specific growth rates (gray bars) (A). Plasma levels of GH (B) and IGF-I (C). Growth parameters are the average values (mean $\pm$ S.E.M.) of triplicates tanks. Hormone data are the mean of ten fish. Different letters above each bar indicate statistically significant differences between sampling times ($P < 0.05$, Student–Newman–Keuls).

would explain the reported upregulated expression of muscle GHR-II by fasting (Saera-Vila *et al.* 2005). Muscle disuse atrophy was also associated in rats to the enhanced expression of GHRs, although this attempt of muscle repair requires intact insulin and IGF-I receptor signaling (Casse *et al.* 2003, Kim *et al.* 2005).

Hepatic IGF-I is not crucial for postnatal growth in mice, and liver IGF-I knockouts show normal growth due to the compensatory action of autocrine/paracrine IGF-I (see Le Roith *et al.* 2001a,b). Postnatally elevated levels of IGF-II fail to rescue the dwarfism of IGF-I-deficient mice (Moerth *et al.* 2007), although species-specific differences appear to be important in IGF-II expression and function. Thus, transcripts of IGF-II decrease quickly during the postnatal development of mice and rats (Rotwein 1991), but substantial amounts are found later in life in humans and in a wide range of fish species, including common carp (Vong *et al.* 2003), rainbow trout (Chauvigné *et al.* 2003), Nile tilapia (Caelers *et al.* 2004), channel catfish (Peterson *et al.* 2004), and gilthead sea bream (Duguay *et al.* 1996). Of note, a relative high expression of IGF-II is retained in extra-hepatic tissues of

most fish species, and compensatory increases of muscle IGF-II have been documented in fast-growing juveniles of gilthead sea bream when they were fed practical diets with increased amounts of feed-borne contaminants (Benedito-Palos *et al.* 2007). The muscle expression of GHR-I was not significantly altered by this dietary intervention, which agrees with the common notion that autocrine/paracrine IGF effects are mostly GH independent (Wood *et al.* 2005). However, in the present study, correlation analyses suggested that the increased muscle expression of IGF-II by overwintering would be mediated by the upregulated expression of GHR-I. Parallel increases of IGFs and GHR-I mRNA levels also occurred in the muscle tissue of older fish during the summer growth spurt. Hence, this locally enhanced expression of GHR-I may be considered adaptive to face up a reduced activity of the liver GH/IGF-I axis. However, the relative contribution of fish GHRs on growth and IGF regulation probably depends not only on fish lineage, but also on each particular age, nutritional, and environmental condition. Jiao *et al.* (2006) showed that duplicated *GHR* genes are differentially regulated by cortisol in black sea bream, a closely related sparid fish. In this regard, the evolutionary scenario for most paralog genes is consistent with the partitioning of ancestral functions after degenerative mutations in different regulatory and/or structural sequences (sub-functionalization model; see Volf *et al.* 2005). The microphthalmia-associated transcription factor (MITF) is perhaps one of the most illustrative examples, mammals and birds having a unique *MITF* gene which generates different isoforms through the use of alternate promoters. By contrast, fish have two different *MITF* genes that are present in species as divergent as zebrafish, pufferfish, and platyfish.

The sub-functionalization model might also be applied to GHRs, which evolved in gilthead sea bream as duplicated genes with a single transcription start site. By contrast, GHRs of higher vertebrates have multiple untranslated exons that are alternatively spliced to a common acceptor site. These spliced transcripts are modulated by different regulatory elements, having liver-specific GHRs, a TATA-box surrounding the transcription start site (Goodyer *et al.* 2001). Other conserved and ubiquitous mammalian GHR variants have TATA-less promoters, and the transcription initiation is determined in the bovine P1 promoter by Inr substitutes (Jiang *et al.* 2000). Sequences similar to consensus Inr were also found herein, which indicate that transcription initiation of GHRs can be dictated in fish and higher vertebrates by Inr-like sequences that were initially sub-estimated in metazoan genomes (Gross & Oelgeschläger 2006). Moreover, many core promoters contain downstream elements, and consensus sequences for the DPE appear to be essential for the activity of most Inr promoters (Smale 1997, Smale & Kadonaga 2003). This may be the case of the two gilthead sea bream GHRs, which have retained Inr and DPEs surrounding the transcription start site.

Typically, TATA-less promoters have CCAAT-boxes in the forward or reverse orientation between -60 and -100 of the

Figure 5 Summer age-related changes in the tissue-specific expression (relative units) of GHR-I and GHR-II. For each gene, the highest expression among tissues was used as reference value in the normalization procedure. Data are the mean $\pm$ S.E.M. ($n=6-7$). For each graph, different letters above each bar indicate statistically significant differences between sampling times ($P<0.05$, Student–Newman–Keuls).

major start site (Mantovani 1999). Consensus GC-boxes are also common elements in TATA-less promoters, and functional CCAAT-boxes and Sp1-binding sites have been identified in human V2, bovine 1B, ovine 1B, mouse L2, and rat GHR2 promoters (see Goodyer *et al.* 2001). Functional binding sites for the ubiquitous ZBP-89 have also been reported in the bovine GHR1A promoter (Xu *et al.* 2006). In this study, a conserved CCAAT-box and a GC-rich region (identified as a ZBP-89/Sp1-binding site) were retrieved in the GHR-I-flanking region of gilthead sea bream. Hence, structural, transcriptional, and regulatory features suggest that GHR-I of sparid fish evolved as a true orthologous gene of mammalian GHRs. Nevertheless, the regulation of duplicated fish GHRs is a complicated and intriguing process, and we cannot exclude some overlapping and functional

redundancy of fish GHRs. Thus, ongoing studies indicate that the reduction of hepatic IGF expression during crowding stress might be dictated by GHR-II rather than GHR-I (unpublished results). Supporting this, CREB and AP-1 recognition sites were found in the GHR-II-flanking region of gilthead sea bream. The regulation of CREB (Hai & Hartman 2001) and AP-1 (Prabhakar 2001, Kim *et al.* 2002) have been extensively studied in mammals, and serve as models of stress- and redox-sensitive transcription factors. Of note, AP-4 is a closely related transcription factor that is rapidly downregulated by glucocorticoids (Tsujimoto *et al.* 2005), and various AP-4 sequence elements were retrieved in the proximal 5'-flanking region of gilthead sea bream GHR-II, although its functional relevance remains still to be demonstrated.

Figure 6 Summer age-related changes in the tissue-specific expression (relative units) of IGF-I and IGF-II. For each gene, the highest expression among tissues was used as reference value in the normalization procedure. Data are the mean $\pm$ S.E.M. ($n=6-7$). For each tissue, different letters above each bar indicate statistically significant differences between sampling times ($P<0.05$, Student–Newman–Keuls).

Experimental evidence shows that SL rather than GH binds to GHR-I of masu salmon (Fukada *et al.* 2005). Orthologous medaka genes could also mediate SL signaling (Fukamachi *et al.* 2005), but the switch and diversification of GHR functions are highly probable among fish lineages. Thus, the binding capabilities of GHRs have been characterized in Japanese eel, and the recombinant eel GHR-I binds specifically to GH and does not cross-react with eel SL (Ozaki *et al.* 2006). In the same context, GHR-II is apparently lost or silenced in turbot, and GHR-I and truncated isoforms emerge as the unique functional GHR in the flatfish lineage (see Saera-Vila *et al.* 2005). Regardless of this, each ligand/receptor interaction can result in unique signaling outcomes as was reported in mammals for insulin receptor and insulin/IGF ligands (Denley *et al.* 2005). Thus, recombinant SL does not exert a growth-promoting action in gilthead sea bream, but has a lipolytic action similar to that found with GH preparations, which was evidenced by

changes in the respiratory quotient and activities of lipolytic enzymes (Vega-Rubín de Celis *et al.* 2003). Additionally, differences in binding affinities and ligand abilities may contribute to make a hypothetical scenario in which GH and SL work through a same receptor. Combined data on plasma GH and SL levels have not been included in this article, although previous studies clearly proved in gilthead sea bream a specific seasonal pattern for each GH/PRL family member (Mingarro *et al.* 2002, Pérez-Sánchez *et al.* 2002). Furthermore, opposite trends for circulating GH and SL have been reported with advancing age and changes in nutritional condition, arising from shifts in ration size, dietary composition, and secretagog effects of arginine (Company *et al.* 2001, Vega-Rubín de Celis *et al.* 2004b).

In summary, coexpression analyses suggest a key role of GHR-I in the tissue-specific regulation of IGFs in a non-salmonid fish of economical relevance for the Mediterranean aquaculture. Some functional redundancy of GHR-I and

Figure 7 Exon-intron organization and sequence analysis of the 5'-flanking region of GHR-I (AH014067) and GHR-II (AH014068). In both genes, exon 1 codes for 5'-UTR; exon 2 for signal peptide; exons 4–7 for extracellular domains; exon 8 for trans-membrane domain; exons 9 and 10 for intracellular domains; exon 10A for intracellular and 3'-UTR domains. Exon 1 is in capital letters and splicing consensus is printed in bold. Transcription start sites of GHR-I and GHR-II are indicated by arrows. Consensus sequences (CCAAT, GC rich, AP-1, AP-4, CREB, Inr, and DPE) surrounding transcription start sites are underlined.

GHR-II cannot be excluded, emerging GHR-II as a stress- and redox-sensitive genes. This notion is supported by sequence analysis of the 5'-flanking region, although functional studies remain to be implemented to document the physiological relevance of consensus-binding sites in the core promoter of fish GHRs. Additionally, detailed studies merit to be conducted to explore and better understand the growth plasticity and functional diversification of IGFs and GHRs among fish lineages.

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References

- Benedet S, Johansson V, Sweeney G, Galay-Burgos M & Björnsson B 2005 Cloning of two Atlantic salmon growth hormone receptor isoforms and *in vitro* ligand-binding response. *Fish Physiology and Biochemistry* **31** 315–329.
- Benedito-Palos L, Saera-Vila A, Caldach-Giner JA, Kaushik S & Pérez-Sánchez J 2007 Combined replacement of fish meal and oil in practical diets for fast growing juveniles of gilthead sea bream (*Sparus aurata* L.): networking of systemic and local components of GH/IGF axis. *Aquaculture* In Press.
- Caelers A, Berishvili G, Meli ML, Eppler E & Reinecke M 2004 Establishment of a real-time RT-PCR for the determination of absolute amounts of *IGF-I* and *IGF-II* gene expression in liver and extrahepatic sites of the tilapia. *General and Comparative Endocrinology* **137** 196–204.
- Caldach-Giner JA, Duval H, Chesnel F, Boeuf G, Pérez-Sánchez J & Boujard D 2001 Fish growth hormone receptor: molecular characterization of two membrane-anchored forms. *Endocrinology* **142** 3269–3273.
- Caldach-Giner JA, Mingarro M, Vega-Rubín de Celis S, Boujard D & Pérez-Sánchez J 2007 Molecular cloning and characterization of gilthead sea bream (*Sparus aurata*) growth hormone receptor (GHR). Assessment of alternative splicing. *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology* **136** 1–13.
- Casse AH, Desplanches D, Mayet-Sornay MH, Raccourt M, Jegou S & Morel G 2003 Growth hormone receptor expression in atrophying muscle fibers of rats. *Endocrinology* **144** 3692–3697.
- Chauvigné F, Gabillard JC, Weil C & Rescan PY 2003 Effect of refeeding on IGF-I, IGF-II, IGF receptors, FGF2, FGF6, and myostatin mRNA expression in rainbow trout myotomal muscle. *General and Comparative Endocrinology* **132** 209–215.
- Chomczynski P & Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162** 156–159.
- Company R, Astola A, Pendón C, Valdivia MM & Pérez-Sánchez J 2001 Somatotrophic regulation of fish growth and adiposity: growth hormone (GH) and somatolactin (SL) relationship. *Comparative Biochemistry and Physiology. Part C, Pharmacology and Toxicology* **132** 435–445.
- Denley A, Cosgrove LJ, Booker GW, Wallace JC & Forbes BE 2005 Molecular interactions of the IGF system. *Cytokine and Growth Factor Reviews* **16** 421–439.
- Dickhoff WW, Beckman BR, Larsen DA, Duan C & Moriyama S 1997 The role of growth in endocrine regulation of salmon smoltification. *Fish Physiology and Biochemistry* **17** 231–236.
- Duguay SJ, Lai-Zhang J, Steiner DF, Funkenstein B & Chan SJ 1996 Developmental and tissue-regulated expression of IGF-I and IGF-II mRNAs in *Sparus aurata*. *Journal of Molecular Endocrinology* **16** 123–132.
- Dyer AR, Barlow CG, Bransden MP, Carter CG, Glencross BD, Richardson N, Thomas PM, Williams KC & Carragher JF 2004 Correlation of plasma IGF-I concentrations and growth rate in aquacultured finfish: a tool for assessing the potential of new diets. *Aquaculture* **236** 583–592.
- Fukada H, Ozaki Y, Pierce AL, Adachi S, Yamauchi K, Hara A, Swanson P & Dickhoff WW 2004 Salmon growth hormone receptor: molecular cloning, ligand specificity, and response to fasting. *General and Comparative Endocrinology* **139** 61–71.
- Fukada H, Ozaki Y, Pierce AL, Adachi S, Yamauchi K, Hara A, Swanson P & Dickhoff WW 2005 Identification of the salmon somatolactin receptor, a new member of the cytokine receptor family. *Endocrinology* **146** 2354–2361.
- Fukamachi S, Yada T & Mitani H 2005 Medaka receptors for somatolactin and growth hormone: phylogenetic paradox among fish growth hormone receptors. *Genetics* **171** 1875–1883.
- Gabillard J-C, Yao K, Vanputte M, Gutiérrez J & Le Bail P-Y 2006 Differential expression of two GH receptor mRNAs following temperature change in rainbow trout (*Oncorhynchus mykiss*). *Journal of Endocrinology* **190** 29–37.
- Gómez-Requeni P, Mingarro M, Kirchner S, Caldach-Giner JA, Médale F, Corraze G, Panserat S, Martin SAM, Houlihan DF, Kaushik SJ *et al.* 2003 Effects of dietary amino acid profile on growth performance, key metabolic enzymes and somatotrophic axis responsiveness of gilthead sea bream (*Sparus aurata*). *Aquaculture* **220** 749–767.
- Gómez-Requeni P, Mingarro M, Caldach-Giner JA, Médale F, Martin SAM, Houlihan DF, Kaushik S & Pérez-Sánchez J 2004 Protein growth performance, amino acid utilisation and somatotrophic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*). *Aquaculture* **232** 493–510.
- Goodyer CG, Zogopoulos G, Schwartzbauer G, Zheng H, Hendy GN & Menon RK 2001 Organization and evolution of the human growth hormone receptor gene 5'-flanking region. *Endocrinology* **142** 1923–1934.
- Gross P & Oelgeschläger T 2006 Core promoter-selective RNA polymerase II transcription. *Biochemical Society Symposium* 225–236.
- Hai T & Hartman MG 2001 The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. *Gene* **273** 1–11.
- Jiang H, Okamura CS, Boyd CK & Lucy MC 2000 Identification of Sp1 as the transcription factor for the alternative promoter P2 of the bovine growth hormone receptor gene. *Journal of Molecular Endocrinology* **24** 203–214.
- Jiao B, Huang X, Chan CB, Zhang L, Wang D & Cheng CHK 2006 The co-existence of two growth hormone receptors in teleost fish and their differential signal transduction, tissue distribution and hormonal regulation of expression in seabream. *Journal of Molecular Endocrinology* **36** 23–40.
- Kajimura S, Kawaguchi N, Kaneko T, Kawazoe I, Hirano T, Visitation N, Grau EG & Aida K 2004 Identification of the growth hormone receptor in an advanced teleost, the tilapia (*Oreochromis mossambicus*) with special reference to its distinct expression pattern in the ovary. *Journal of Endocrinology* **181** 65–76.
- Kelly PA, Djiane J, Postel-Vinay MC & Edery M 1991 The prolactin/growth hormone receptor family. *Endocrine Reviews* **12** 235–251.
- Kim HJ, Jung KJ, Yu BP, Cho CG, Choi JS & Chung HY 2002 Modulation of redox-sensitive transcription factors by calorie restriction during aging. *Mechanisms of Ageing and Development* **123** 1589–1595.
- Kim H, Barton E, Muja N, Yakar S, Pennisi P & LeRoith D 2005 Intact insulin and insulin-like growth factor-I receptor signaling is required for growth hormone effects on skeletal muscle growth and function *in vivo*. *Endocrinology* **146** 1772–1779.
- Kopchick JJ & Andry JM 2000 Growth hormone (GH), GH receptor, and signal transduction. *Molecular Genetics and Metabolism* **71** 293–314.
- Larsen DA, Beckman BR & Dickhoff WW 2001 The effect of low temperature and fasting during the winter on metabolic stores and

- endocrine physiology (insulin, insulin-like growth factor-I, and thyroxine) of coho salmon, *Oncorhynchus kisutch*. *General and Comparative Endocrinology* **123** 308–323.
- Lee LTO, Nong G, Chan YH, Tse DLY & Cheng CHK 2001 Molecular cloning of a teleost growth hormone receptor and its functional interaction with human growth hormone. *Gene* **270** 121–129.
- Li MH, Peterson BC, Janes CL & Robinson EH 2006 Comparison of diets containing various fish meal levels on growth performance, body composition, and insulin-like growth factor-I of juvenile channel catfish *Ictalurus punctatus* of different strains. *Aquaculture* **253** 628–635.
- Livak KJ & Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* **25** 402–408.
- Mantovani R 1999 The molecular biology of the CCAAT-binding factor NF-Y. *Gene* **239** 15–27.
- Martínez-Barberá JP, Pendón C, Martí-Palanca H, Calduch-Giner JA, Rodríguez RB, Valdivia MM & Pérez-Sánchez J 1995 The use of recombinant gilthead sea bream (*Sparus aurata*) growth hormone for radioiodination and standard preparation in radioimmunoassay. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology* **110** 335–340.
- Mingarro M, Vega-Rubín de Celis S, Astola A, Pendón C, Martínez Valdivia M & Pérez-Sánchez J 2002 Endocrine mediators of seasonal growth in gilthead sea bream (*Sparus aurata*): the growth hormone and somatolactin paradigm. *General and Comparative Endocrinology* **128** 102–111.
- Moerth C, Schneider MR, Renner-Mueller I, Blutke A, Elmlinger MW, Erben RG, Camacho-Hübner C, Hoefflich A & Wolf E 2007 Postnatally elevated levels of insulin-like growth factor (IGF)-II Fail to rescue the dwarfism of IGF-I-deficient mice except kidney weight. *Endocrinology* **148** 441–451.
- Nakao N, Higashimoto Y, Ohkubo T, Yoshizato H, Nakai N, Nakashima K & Tanaka M 2004 Characterization of structure and expression of the growth hormone receptor gene of the Japanese flounder (*Paralichthys olivaceus*). *Journal of Endocrinology* **182** 157–164.
- Nordgarden U, Hansen T, Henre GI, Sundby A & Björnsson BT 2005 Endocrine growth regulation of adult Atlantic salmon in seawater: the effects of light regime on plasma growth hormone, insulin-like growth factor-I, and insulin levels. *Aquaculture* **250** 862–871.
- Orlovskii IV, Sverdlova PS & Rubtsov PM 2004 Fine structure, expression, and polymorphism of the human growth hormone receptor gene. *Molecular Biology* **38** 24–33.
- Ozaki Y, Fukada H, Kazeto Y, Adachi S, Hara A & Yamauchi K 2006 Molecular cloning and characterization of growth hormone receptor and its homologue in the Japanese eel (*Anguilla japonica*). *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology* **143** 422–431.
- Pérez-Sánchez J, Calduch-Giner JA, Mingarro M, Vega-Rubín de Celis S, Gómez-Requeni P, Saera-Vila A, Astola A & Valdivia MM 2002 Overview of fish growth hormone family. New insights in genomic organization and heterogeneity of growth hormone receptors. *Fish Physiology and Biochemistry* **27** 243–258.
- Peterson BC, Waldbieser GC & Bilodeau L 2004 IGF-I and IGF-II mRNA expression in slow and fast growing families of USDA103 channel catfish (*Ictalurus punctatus*). *Comparative Biochemistry and Physiology. Part A. Molecular and Integrative Physiology* **139** 317–323.
- Prabhakar NR 2001 Physiological and genomic consequences of intermittent hypoxia: invited review: oxygen sensing during intermittent hypoxia: cellular and molecular mechanisms. *Journal of Applied Physiology* **90** 1986–1994.
- Le Roith D, Bondy C, Yakar S, Liu JL & Butler A 2001a The somatomedin hypothesis: 2001. *Endocrine Reviews* **22** 53–74.
- Le Roith D, Scavo L & Butler A 2001b What is the role of circulating IGF-I? *Trends in Endocrinology and Metabolism* **12** 48–52.
- Rotwein P 1991 Structure, evolution, expression and regulation of insulin-like growth factors I and II. *Growth Factors* **5** 3–18.
- Saera-Vila A, Calduch-Giner JA & Pérez-Sánchez J 2005 Duplication of growth hormone receptor (GHR) in fish genome: gene organization and transcriptional regulation of GHR type I and II in gilthead sea bream (*Sparus aurata*). *General and Comparative Endocrinology* **142** 193–203.
- Silverstein JT, Wolters WR, Shimizu M & Dickhoff WW 2000 Bovine growth hormone treatment of channel catfish: strain and temperature effects on growth, plasma IGF-I levels, feed intake and efficiency and body composition. *Aquaculture* **190** 77–88.
- Smale ST 1997 Transcription initiation from TATA-less promoters within eukaryotic protein-coding genes. *Biochimica et Biophysica Acta* **1351** 73–88.
- Smale ST & Kadonaga JT 2003 The RNA polymerase II core promoter. *Annual Review of Biochemistry* **72** 449–479.
- Tse DLY, Tse MCL, Chan CB, Deng L, Zhang WM, Lin HR & Cheng CHK 2003 Seabream growth hormone receptor: molecular cloning and functional studies of the full-length cDNA, and tissue expression of two alternatively spliced forms. *Biochimica et Biophysica Acta* **1625** 64–76.
- Tsujimoto K, Ono T, Sato M, Nishida T, Oguma T & Tadakuma T 2005 Regulation of the expression of caspase-9 by the transcription factor activator protein-4 in glucocorticoid-induced apoptosis. *Journal of Biological Chemistry* **280** 27638–27644.
- Vega-Rubín de Celis S, Gómez-P, Calduch-Giner JA, Médale F & Pérez-Sánchez J 2003 Expression and characterization of european sea bass (*Dicentrarchus labrax*) somatolactin: assessment of *in vivo* metabolic effects. *Marine Biotechnology* **5** 92–101.
- Vega-Rubín de Celis S, Gómez-Requeni P & Pérez-Sánchez J 2004a Production and characterization of recombinantly derived peptides and antibodies for accurate determinations of somatolactin, growth hormone and insulin-like growth factor-I in European sea bass (*Dicentrarchus labrax*). *General and Comparative Endocrinology* **139** 266–277.
- Vega-Rubín de Celis S, Rojas P, Gómez-Requeni P, Albalat A, Gutiérrez J, Médale F, Kaushik SJ, Navarro I & Pérez-Sánchez J 2004b Nutritional assessment of somatolactin function in gilthead sea bream (*Sparus aurata*): concurrent changes in somatotrophic axis and pancreatic hormones. *Comparative Biochemistry and Physiology. Part A, Molecular and Integrative Physiology* **138** 533–542.
- Very NM, Kittilson JD, Norbeck LA & Sheridan MA 2005 Isolation, characterization, and distribution of two cDNAs encoding for growth hormone receptor in rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology* **140** 615–628.
- Vollf JN 2005 Genome evolution and biodiversity in teleost fish. *Heredity* **94** 280–294.
- Vong QR, Chan KM & Cheng CH 2003 Quantification of common carp (*Cyprinus carpio*) IGF-I and IGF-II mRNA by real-time PCR: differential regulation of expression by GH. *Journal of Endocrinology* **178** 513–521.
- Wei Y, Rhani Z & Goodyer CG 2006 Characterization of growth hormone receptor messenger ribonucleic acid variants in human adipocytes. *Journal of Clinical Endocrinology and Metabolism* **91** 1901–1908.
- Wood AW, Duan C & Bern HA 2005 Insulin-like growth factor signaling in fish. In *International Review of Cytology*, Ed K W Joan vol 243. New York: Academic Press, pp 215–285.
- Xu Q, Springer L, Merchant JL & Jiang H 2006 Identification of zinc finger binding protein 89 (ZBP-89) as a transcriptional activator for a major bovine growth hormone receptor promoter. *Molecular and Cellular Endocrinology* **251** 88–95.
- Zhou R, Cheng H & Tiersch TR 2001 Differential genome duplication and fish diversity. *Reviews in Fish Biology and Fisheries* **11** 331–337.

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