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. Journal of Endocrinology (2007) 194, 361–372

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 schilgehi; 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 (Oncorhyncus kisutch; AF403539, AF403540), masu salmon (Oncorhynus masou; Fukada et al. 2004), rainbow trout (Oncorhynus 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), zebrasfish (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 bass, 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 2001; Vo lff 2005), and duplication and divergence offish 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 somatolactin (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 (Proaquia, 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 final wt – ln initial wt) × 100/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.
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 μg 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: TGGGATCGTAGAGGAGTGTT; reverse: CTGTAGAGACAGGAGTGGCG) were designed to amplify a 109 bp amplicon, comprised between 392 and 500 nt positions (Duguy 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 μM. 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 μg 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 (CCTGACTCCAAAACATCGGAATG; 362–338 nt position, GeneBank AF438176) and GHR–II (CGAGCGGAGCTGGACTTTGTAAG; 526–504 nt position, GeneBank AF573601). The nested PCR was conducted with a 5’RACE inner primer and specific reverse primers for GHR–I (GGAAGCAGGAGAAGAGGAGGAGGATTG; 228–201 nt position) and GHR–II (GCTTGGAGGTTCTGGA-GAGGT; 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 μg aliquots of genomic DNA were digested with DraI, EcoRV, and PvuII respectively. Digest DNA samples 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 (TGGTACGAAGTGCTGTTGAT; reverse: CTGTAGAGACAGGAGTGGCG) and GHR–II (TGAGAACCACACAGAAACGGTGACCG; reverse: CTGTAGAGACAGGAGTGGCG) 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 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, GCTCTCACGCTGGCCATCAGATGAC) and exon 2 (reverse primer, GCCTGTCAGAATTCCTACACAGGTAG). 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 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).

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 C₃₈ (GHR-I) and C₂₉ (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...
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-Rubínd et al. 2004) 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 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±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).
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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 ± 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 Wolf 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 underestimated 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
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 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 ± 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).
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 nonsalmonid fish of economical relevance for the Mediterranean aquaculture. Some functional redundancy of GHR-I and

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**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 ± 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).
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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