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Regulation of the somatotropic axis by dietary factors in rainbow trout (*Oncorhynchus mykiss*)

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The activity of the somatotropic axis was analysed in juvenile rainbow trout (*Oncorhynchus mykiss*) fed either a fishmeal-based diet (FM) or graded levels of plant proteins to replace 50 % (PP50 diet), 75 % (PP75 diet) or 100 % (PP100 diet) of the fishmeal protein. For this purpose, partial cloning and sequencing of the gene encoding rainbow trout growth hormone receptor (GHR) was first accomplished by RT-PCR, using degenerate primers based on the sequences of non-salmonid fish *GHR*. Growth rates and energy retention were lowered by the PP75 and PP100 diets and a concurrent and progressive increase in plasma levels of growth hormone (GH) was found. However, no changes in hepatic GH binding and total plasma insulin-like growth factor (IGF)-I levels were observed among the four experimental groups. This fact agrees with the lack of changes in hepatic measurements of GHR and IGF-I transcripts. No consistent changes in IGF transcripts were found in peri-visceral adipose tissue and skeletal muscle, but GHR mRNA was up-regulated in the peri-visceral adipose tissue of fish fed the PP75 and PP100 diets, which would favour the lipolytic action of GH. Two specific bands (47 and 33 kDa) of IGF-binding proteins were found in the plasma of all analysed fish, but the sum of the two integrated areas increased progressively with plant protein supply, which might reflect a reduced free IGF availability. Therefore, in our experimental model, the growth impairment could be due, at least in part, to a lowered availability of biologically active IGF (free IGF fraction) rather than to liver GH desensitization or defect in IGF synthesis and release at the systemic and/or paracrine–autocrine level.

Rainbow trout: Plant proteins: Growth hormone: Growth hormone receptor: Insulin-like growth factor

Growth hormone (GH) plays a central role as a pluripotent endocrine regulator of physiological functions in fish and higher vertebrates (Björnsson, 1997; Pérez-Sánchez, 2000). In mammals, it is now recognized that this pituitary hormone exerts its action by direct interaction with a GH receptor (GHR) on the cell membrane of target tissues, triggering a phosphorylation cascade for signalling and gene expression (Behncken & Waters, 1999; Zhu *et al.* 2001). Transmission of the intracellular GHR signal remains unexplored in fish, although this receptor has been cloned and sequenced from several fish species including the goldfish (*Carassius auratus*; Lee *et al.* 2001), turbot (*Scophthalmus maximus*; Calduch-Giner *et al.* 2001), black sea bream (*Acanthopagrus schlegeli*; Tse *et al.* 2003), gilthead sea bream (*Sparus aurata*; Calduch-Giner *et al.* 2003), catfish (*Silurus meridionalis*; GenBank accession no. AY336104), Japanese flounder (*Paralichthys olivaceus*; GenBank accession no. AB058418), grass carp (*Ctenopharyngodon idella*; GenBank accession no. AY283778), masu salmon (*Oncorhynchus masou*; Fukada *et al.* 2004) and coho salmon (*Oncorhynchus kisutch*; GenBank accession no. AF403539 and AF403540). Furthermore, the organization of the *GHR* gene has been elucidated in turbot and gilthead sea bream (Pérez-Sánchez *et al.* 2002), and a high conservation of exon–intron boundaries through the evolution of vertebrate species has been reported despite the occurrence of an exclusive fish intron (10/10A) at the C-terminus.

The first observation in fish that shifts in GHR mRNA levels are associated with changes in growth rates has been made in gilthead sea bream, in which the hepatic expression of GHR is up-regulated during the summer growth spurt (Calduch-Giner *et al.* 2003). In this fish species, we have also found that hepatic GHR are down-regulated by the replacement of fishmeal by plant proteins, which in turn results in decreased growth and expression of hepatic insulin-like growth factor (IGF)-I (Gómez-Requeni *et al.* 2004). Currently, there are no data in rainbow trout (*Oncorhynchus mykiss*) about the effect of dietary N source on the activity and regulation of the somatotropic axis. To address this issue, in the present work trout *GHR* was partially cloned and sequenced in order to establish the tissue-specific regulation of GHR transcripts in fish fed diets containing fishmeal or plant proteins. Further, hepatic GH binding, tissue-specific expression of IGF, and plasma levels of GH, IGF-I and IGF carriers (IGF-binding proteins, IGFBP) were analysed.

Abbreviations: GH, growth hormone; GHR, growth hormone receptor; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; TBS, Tris-buffered saline.

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Materials and methods

Experimental diets and feeding trial

As shown in Table 1, four diets were formulated to contain either fishmeal as the sole protein source (FM diet) or graded levels of plant proteins to replace 50 % (PP50 diet), 75 % (PP75 diet) or 100 % (PP100 diet) of the fishmeal protein. Crystalline amino acids were added to plant protein-based diets to meet the indispensable amino acid profile. Fish oil was added to maintain the dietary crude fat content constant in all diets. To measure digestibility, 1 % chromic oxide was added as an inert marker and it was determined after perchloric acid digestion (Bolin *et al*. 1952). Faeces were collected twice daily using a faecal collection apparatus (Choubert *et al*. 1982), and apparent digestibility coefficients were calculated as described elsewhere (Maynard & Loosli, 1969).

The feeding trial was performed in the experimental fish farm of INRA (Donzacq, Landes, France) under natural photoperiod. Rainbow trout of 19 g initial body weight were reared in sixteen circular 1000-litre glass-fibre tanks in groups of seventy-five fish each. Water was supplied from natural springs and its temperature ranged from 16 to 18°C over the course of the trial (21 March to 7 June).

Each experimental diet was randomly distributed by hand to quadruplicate groups of fish for 11 weeks. Feed was offered twice daily near to visual satiety and feed consumption was recorded daily. Every 3 weeks fish were counted and group-weighed.

Table 1. Ingredients, chemical composition and apparent digestibility coefficients (ADC) of the four experimental diets

	FM	PP50	PP75	PP100
Ingredient (g/kg)				
Fishmeal	637·9	319·0	159·5	0
Corn gluten meal	0	116·0	177·1	232·4
Wheat gluten	0	100	150	200
Extruded peas (Aquatex)	0	80·0	120·0	163·3
Rapeseed meal (Primor 00)	0	46·9	75·0	100·0
Extruded whole wheat	203·4	110·2	42·5	0
Fish oil	128·6	143·6	151·1	158·7
Binder (Na alginate)	10	10	10	10
Mineral premix*	10	10	10	10
Vitamin premix*	10	10	10	10
$CaHPO_4 \cdot 2H_2O$ (18 % P)	0	16·1	37·8	40
L-Arg	0	6·2	9·5	12·5
L-His	0	1·9	2·9	3·9
L-Lys	0	13·8	20·8	27·6
Dl-Met	0	2·0	3·1	4·1
L-Trp	0	1·3	2·1	2·7
L-Thr	0	4·1	6·2	8·3
L-Ile	0	3·5	5·3	7·1
L-Val	0	4·7	7·0	9·4
Analytical composition				
DM (%)	94·4	92·2	91·5	91·6
Crude protein (% DM)	51·5	50·3	49·1	48·6
Crude fat (% DM)	19·7	19·6	19·6	19·2
Gross energy (kJ/g DM)	22·7	23·5	23·9	23·6
IAA (% DM)	22·2	22·9	22·1	23·9
DAA (% DM)	21·8	23·3	22·1	23·7
IAA:DAA	1·0	0·9	1·0	1·0
ADC values (%)				
DM	83·4	79·2	76·8	72·0
Crude protein	93·4	93·7	93·9	93·6
Crude fat	95·6	92·8	91·6	90·3
Starch	99·2	80·7	75·5	56·2
Energy	92·0	87·8	86·4	82·7

IAA, indispensable amino acids; DAA, dispensable amino acids.
* As per National Research Council (1993).

Whole-body composition for the calculation of retention efficiencies was determined in an initial pooled sample of fifteen fish and in pools of six fish per tank (twenty-four fish per diet) at the end of the growth trial. Specimens for body analysis were ground, and small aliquots were dried (110°C) to estimate water content. The remaining samples were freeze-dried and chemical analyses for protein, fat and ash were made following published methods (Association of Official Analytical Chemists, 1990).

Blood and tissue sampling

At the end of the growth trial and following overnight fasting, blood samples were taken from caudal vessels with heparinized syringes (twenty animals per diet). Plasma was drawn after centrifugation at 3000***g*** for 20 min at 4°C and stored at −30°C until hormone analysis. Liver, white muscle and peri-visceral adipose tissue of twelve animals per diet were rapidly excised, frozen in liquid N_2, and stored at −80°C until use for GH-binding and real-time PCR assays.

Plasma hormone assays

Plasma GH levels were determined by a double antibody RIA using recombinant trout GH (Eurogentec, Liège, Belgium) as tracer and standard. Anti-salmon GH serum (GroPep, Adelaide, Australia) was used as a first antibody (1:25 000). A goat anti-rabbit IgG (1:20) (Biogenesis, Poole, UK) was used as precipitating antibody, and the sensitivity and mid range of the assay were 0·05 and 0·6 μg/l, respectively.

After acid–ethanol precipitation to avoid IGFBP interference, total plasma levels of IGF-I were measured by a homologous RIA based on the use of recombinant trout IGF-I (GroPep) as tracer and standard, and anti-trout IGF-I serum (GroPep; 1:16 000) as a first antibody. A goat anti-rabbit IgG (1:20; Biogenesis) was used as a precipitating antibody. The sensitivity and mid range of the assay were 0·05 and 0·8 μg/l, respectively.

Insulin-like growth factor-binding protein activity

Plasma samples were diluted 1:4 in sample buffer (10 % glycerol, 12·5 % Tris–OH, 2 % SDS and 0·05 % bromophenol blue), heated at 60°C for 15 min and immediately put in ice for 1 min. Then, 15 μl samples were electrophoresed under non-reducing conditions in a 12 % separating polyacrylamide gel (SDS-PAGE) for 1 h at 200 W using a Mini-Protean II (Bio-Rad, Hercules, CA, USA). Gels were placed in transfer buffer (25 mM-Tris–OH, 192 mM-glycine, 20 % CH_3OH; pH 8·1–8·5) for 15 min and electroblotted to Immun-Blot PVDF membranes (Bio-Rad) at 100 W for 90 min at room temperature. Membranes were then placed in a blocking solution (5 % dry non-fat milk in Tris-buffered saline (TBS): 20 mM-Tris–OH, 500 mM-NaCl, 0·05 % NaN_3; pH 7·5) for 1 h at room temperature, and washed for 10 min with 0·1 % Tween-20 in TBS. After this, membranes were incubated for 15 h at 4°C in the probe buffer (1 % bovine serum albumin, 0·1 % Tween-20 in TBS) containing [^{125}I]-labelled recombinant trout IGF-I (200 000 cpm/ml). The binding specificity was established by adding an excess of human IGF-I (2 μg/ml). Membranes were exposed for 6 days at −80°C to Biomax MS films (Kodak, Rochester, NY, USA). Autoradiographs were scanned with a GS-710 Cali-

brated Imaging Densitometer (Bio-Rad), and bands quantified using Quantity-One 4.2.3 software (Bio-Rad).

Growth hormone binding

Hepatic GH binding was assayed by a radioreceptor assay (Yao *et al.* 1991), based on the use of recombinant trout GH (Eurogentec) as iodinated tracer and cold hormone to measure total and non-specific GH binding.

Partial cloning and sequencing of trout growth hormone receptor

Total liver RNA was extracted by the acid guanidium thiocyanate–phenol–chloroform method (Chomczynski & Sacchi, 1987). The purified RNA was treated with DNase I, and 2 μg were reverse-transcribed with 200 U Superscript II (Life Technologies, Gaithesburg, MD, USA) using oligo$(dT)_{17}$ as anchor primer. Degenerate forward (GHR1: 5′-GGA GAC NTT YCG NTG YTG GTG GA) and reverse (GHR2: 5′-TGT CGG ACA CCT GGG CRT ARA ART C) primers for *GHR* cloning and sequencing were designed on the basis of their previous success in turbot and gilthead sea bream (Calduch-Giner *et al.* 2001, 2003). For PCR, 2 μl RT reaction were amplified with 0·2 mM each dNTP, 1 μM forward and reverse primers and 2·5 U Platinum *Taq* DNA Polymerase (Life Technologies) in a final volume of 50 μl of reaction buffer. After 35 cycles (94°C for 1 min, 54°C for 2 min, 72°C for 3 min), amplified fragments were run in agarose gels, and bands of interest were purified (Wizard PCR Preps; Promega, Madison, WI, USA) and sequenced by the dideoxy chain termination method (ABI PRISM dRhodamine terminator cycle sequencing kit; Perkin-Elmer, Wellesley, MA, USA). The resulting sequence has been submitted to the GenBank database under accession no. AF438178.

Structure and phylogenetic analysis of growth hormone receptors

Putative transmembrane regions were predicted with HMMTOP version 2.0 (http://www.enzim.hu/hmmtop; Tusnády & Simon, 2001). Potential N-linked glycosylation sites were determined with Gene Runner version 3.02 software (Hastings Software, Hastings, NY, USA). Protein sequence alignments were made by means of ClustalX software (Thompson *et al.* 1994). Phylogenetic comparison of protein sequences was carried out with MEGA2 version 2.1 software (Kumar *et al.* 2001).

Northern Blot

A cDNA probe comprising 552 nt of the extracellular domain of trout *GHR* was PCR-amplified with 5′-GAA TTC ACG GAA CCC GGA GCA CTG and 5′-GAA TTC CGT TGA CTC TTT ATT GGG A as forward and reverse primers, respectively. Amplified PCR fragments were ligated into pGEM-T vector according to the manufacturer's instructions (Promega) and used to transform competent *Escherichia coli* (JM109 strain). Recombinant plasmids were purified (SV Minipreps; Promega), and the probe was excised with appropriate restriction enzymes, gel-purified and labelled with 0·74 Mbq [^{32}P]dCTP by decanucleotide random priming (Decalabel DNA Labeling Kit; Fermentas, Hanover, MD, USA). Liver total RNA (30 μg) from fish fed FM and PP100 diets was separated in a 1 % denaturing agarose/formaldehyde gel, transferred into positively charged nylon membranes by vacuum transfer (Bio-Rad) in standard saline citrate (10 ×), and UV cross-linked. Prehybridization (3 h) and hybridization (overnight) were performed at 65°C in 0·5 mM-Na_2HPO_4 (pH 7·2), 1 mM-EDTA, 7 % SDS and 1 % bovine serum albumin. After washing at room temperature, dried membranes were exposed to Biomax MS films (Kodak) with intensifying screens for 7 days at −80°C.

Real-time PCR assays

Tissue expression of *GHR* and *IGF* genes was assessed in liver, skeletal muscle and adipose tissue by means of SYBR Green I real-time PCR assays as described elsewhere in gilthead sea bream (Calduch-Giner *et al.* 2003). *β-Actin* was chosen as a housekeeping gene, and specific primers for each gene of interest were designed with the Primer Express software of Applied Biosystems (Foster City, CA, USA). Primers for *GHR* (forward: 5′-CGA TAC CTT GTG CTT CGA ATT G; reverse: 5′-TGA TGT TCA GCA GCG TCC AA) amplified a 80 bp amplicon between 203 and 282 nt positions of trout GHR. *IGF-I* primers (forward: 5′-GCG ATG TGC TGT GTC TCC TG; reverse: 5′-AGC CTC TCT CTC CAC ACA CAA AC) amplified a 148 bp amplicon between the signal peptide and the B domain, with no alternative splicing in this region (Chen *et al.* 1994). Primers for trout *IGF-II* (forward: 5′-TAC CAC TCA GTT TGT CAC ACC T; reverse: 5′-CCG AAG CCA CTT CAA CAA TGT A) amplified a 130 bp amplicon between the signal peptide and the B domain. Primers for *β-actin* (forward: 5′-GAT GGG CCA GAA AGA CAG CTA; reverse: 5′-TCG TCC CAG TTG GTG ACG AT) amplified a 105 bp amplicon.

Total RNA (1 μg) from tissues was extracted, treated with DNase I and reverse-transcribed with oligo-dT as described earlier (cloning and sequencing). PCR amplification and analysis was performed on an ABI PRISM 5700 Sequence Detection System (Applied Biosystems). The final volume of PCR reactions was 25 μl, using SYBR Green PCR Master Mix (Applied Biosystems) and specific primers (0·9 μM). The real-time PCR protocol was 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Standard curves were generated by amplifying serial dilutions of known quantities of recombinant plasmids for each gene of interest. The dynamic range of standard curves spanned at least five orders of magnitude, and the amount of product in a particular sample was determined by interpolation of the cycle threshold value (Ct). For each particular gene, the efficiency of the PCR reaction (91–98 %) was the same for serial dilutions of standards and RT reactions. Specificity of amplified PCR products was determined by DNA sequencing and analysis of melting curves on real-time PCR assays. Data were normalized by the delta–delta method (Livak & Schmittgen, 2001), after the verification that Ct values for *β-actin* amplification did not change significantly among tissues and experimental conditions (Ct = 19·9–20·3).

Statistical analysis

Data were analysed by one-way ANOVA, followed by the Student–Newman–Keuls test at a significance level of $P<0·05$. Tank average values were used as experimental units for statistical analysis of growth performance.

Results

Molecular characterization of trout growth hormone receptor

The partial nucleotide sequence of trout *GHR* was determined by RT-PCR, using degenerated primers based on available sequences

of *GHR* of non-salmonid fish. PCR amplification yielded a single cDNA fragment with six potential N-linked glycosylation sites, four of which are conserved in all the available sequences of non-salmonid *GHR* (Fig. 1(A)). A strict conservation of extracellular cysteine residues, the characteristic GHR ligand-binding motif (Y/F)GEFS, box 1 and box 2 cytoplasmatic domains and intracellular tyrosine residues were also found (Fig. 1(B)).

Northern blot of liver RNA with a specific cDNA probe for the trout *GHR* outlined the expression of a single transcript around 8 kb (Fig. 2). The overall identity of the deduced amino acid sequence of trout GHR remained high compared with GHR of non-salmonid fish (turbot: 58 %; black sea bream: 57 %; gilthead sea bream and Japanese flounder: 56 %; grass carp and goldfish: 55 %; catfish: 47 %), but decreased by up to 45 % when comparisons

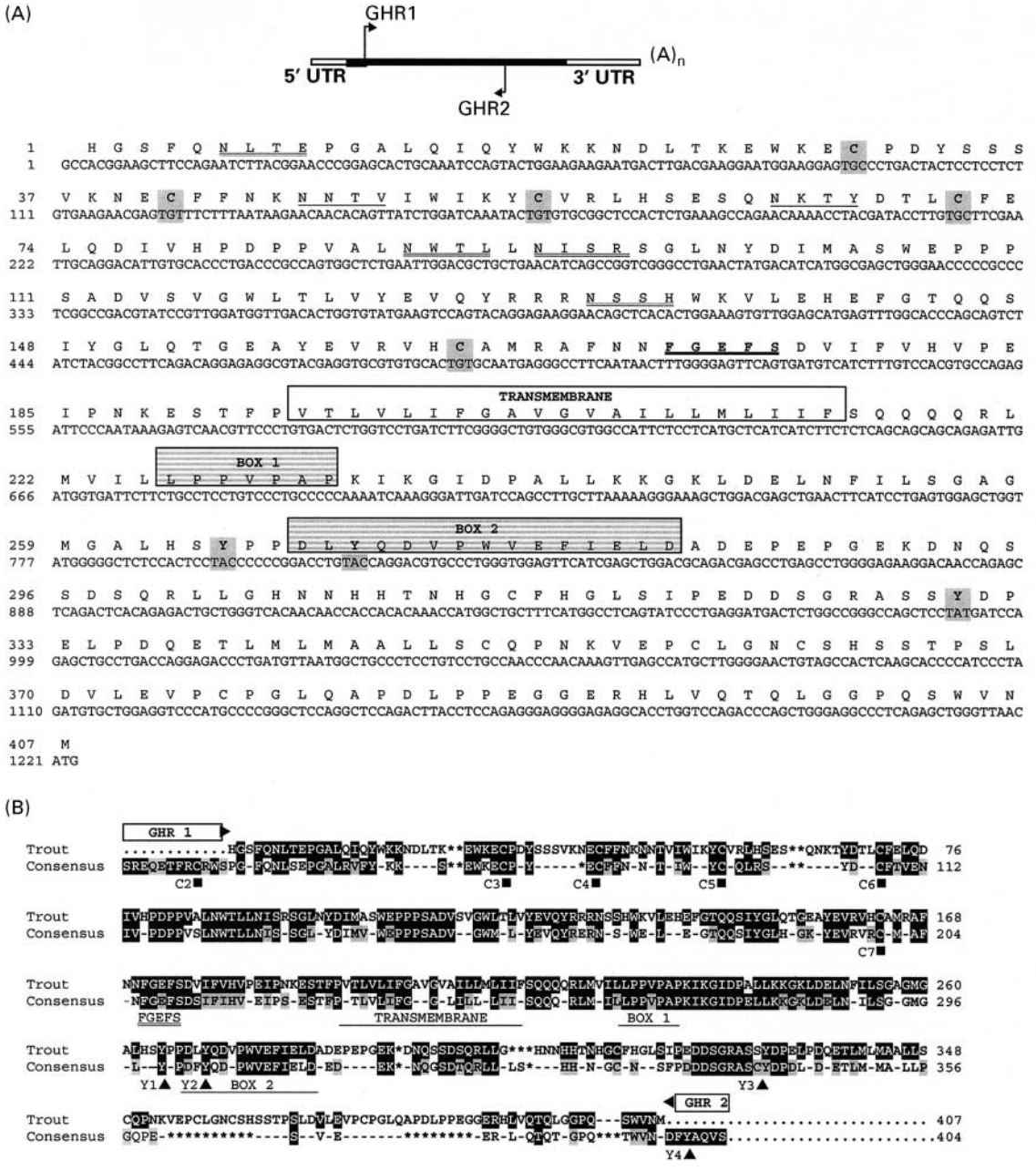

Fig. 1. (A) Schematic representation of PCR strategy for cloning and sequencing of the trout growth hormone receptor (GHR) using degenerate forward and reverse primers (GHR1 and GHR2, respectively; UTR, untranslated region. Potential N-linked glycosylation sites are single or double underlined. Double underline indicates glycosylation sites strictly conserved in all the available GHR of non-salmonid fish. FGFES motif is in bold and underlined. Extracellular cysteines and cytoplasmic tyrosines are in bold and boxed in grey. Transmembrane, box 1 and box 2 domains are boxed. (B) Amino acid alignment of rainbow trout GHR and consensus sequence of non-salmonid GHR (catfish (AY336104), goldfish (AF293417), grass carp (AY283778), gilthead sea bream (AF438176), black sea bream (AF502071), turbot (AF352396), Japanese flounder (AB058418) and masu salmon (AB071216)). Identical and homologous amino acids are in black in trout and consensus sequences. Hyphens in the consensus sequence indicate a lack of conservation. Letters with a white background in the consensus sequence indicate amino acid conservation higher than 50 %, grey background indicates conservation higher than 65 %, and black background indicates conservation from 85 % to 100 %. Asterisks indicate positions where gaps are introduced for a better alignment. Transmembrane domain, FGEFS motif, box 1 and box 2 domains are indicated. Extracellular cysteine and intracellular tyrosine residues are labelled by squares and triangles.

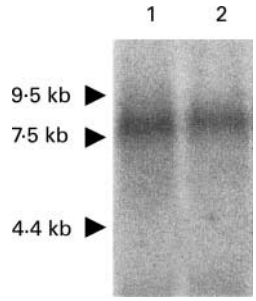

Fig. 2. Northern blot analysis of total liver RNA with an extracellular cDNA probe encoding for the trout growth hormone receptor: lane 1, fish fed diet FM; lane 2, fish fed diet PP100 (for details of the experimental diets, see Table 1, p. 354). Migration of RNA size markers is indicated.

were made with coho and masu salmon GHR. Thus, in the phylogenetic analysis, trout GHR and GHR of non-salmonid fish were put in the same node according to the present hierarchy of fish. A second node was found for the available sequences of coho salmon and masu salmon, although all fish GHR were put in the same cluster when GHR of tetrapods were included as an outgroup (Fig. 3).

Growth performance and nutritional regulation of the somatotropic axis

Juvenile rainbow trout grew from 19 g to 111–147 g over the course of trial. Feed intake in fish fed PP75 and PP100 diets was significantly higher than in the other two experimental groups. However, final body weight, specific growth rates, feed efficiency, N retention and energy retention were progressively and significantly reduced with the 75 and 100 % replacement diets (Table 2).

Plasma levels of GH increased progressively with poorer growth, and significant differences between FM and PP100 groups were found at the end of the trial in overnight fasted fish (Fig. 4(A)). No significant changes in hepatic GH binding and total plasma IGF-I levels were found among groups (Fig. 4(B) and (C)).

As shown in Table 3, measurements of GHR and IGF transcripts of the liver tissue of the two extreme groups (FM and PP100 fish) did not reveal any significant effect of dietary treatment. In muscle and peri-visceral adipose tissue, no consistent changes in IGF-I and IGF-II expression were found among all the experimental groups. However, in the peri-visceral adipose tissue, GHR transcripts were up-regulated by the 75 and 100 % replacement diets. The same was found in the muscle of fish fed diet PP100, although this increase was not statistically significant.

Western ligand blotting showed the existence of two specific IGFBP bands (47 and 33 kDa) regardless of dietary treatment. The sum of the integrated area for these two bands increased with poorer growth, and this increase was parallel to the increase of the ratio 47:33 kDa IGFBP (Fig. 5).

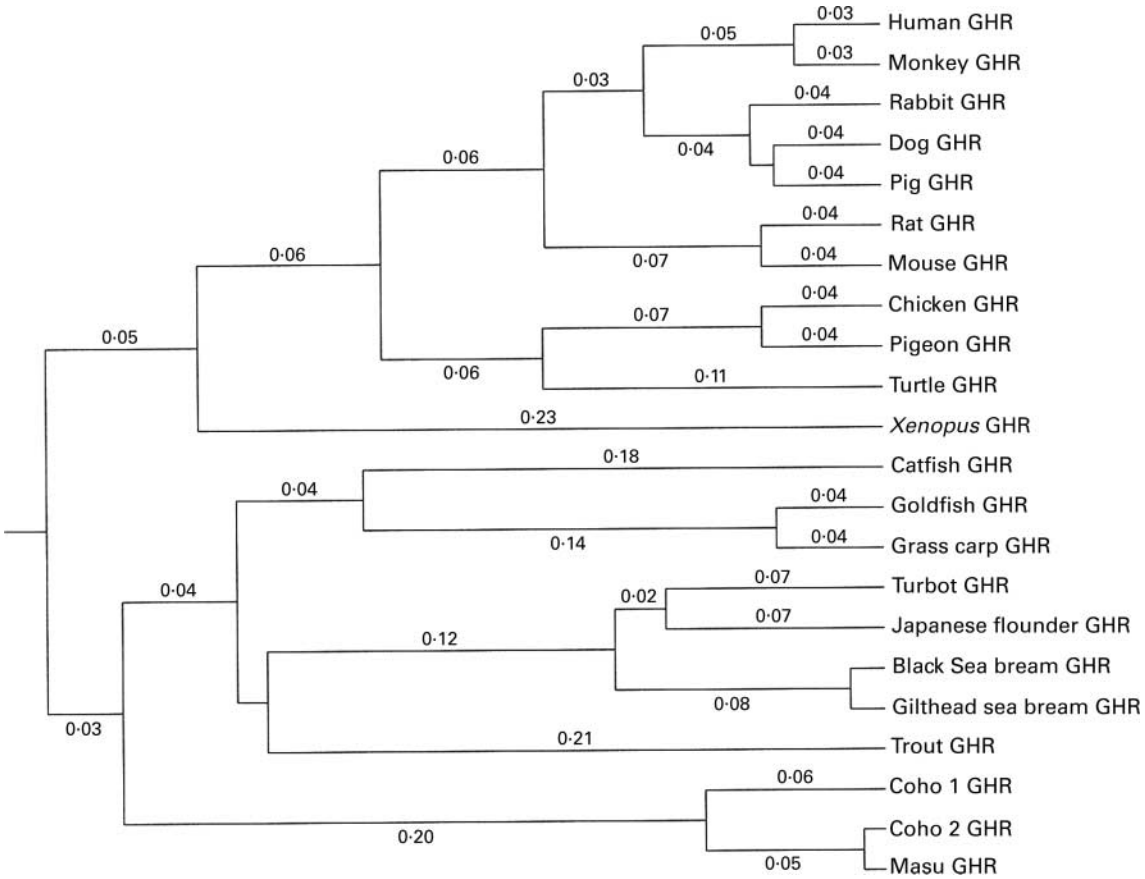

Fig. 3. Phylogenetic tree of growth hormone receptors (GHR) made with MEGA2 version 2.1 software (Kumar *et al.* 2001). GenBank accession no.: man, NM_000163; monkey, U84589; dog, AF133835; pig, X54429; rabbit, AF015252; mouse, NM_010284; rat, NM_017094; turtle, AF211173; chicken, M74057; pigeon, D84308; *Xenopus*, AF193799; turbot, AF352396; Japanese flounder, AB058418; black sea bream, AF502071; gilthead sea bream, AF438176; rainbow trout, AF438178; catfish, AY336104; goldfish, AF293417; grass carp, AY283778; coho 1, AF403539; coho 2, AF403540; masu, AB071216.

Table 2. Data on growth performance and nutrient retention of fish fed the four experimental diets*

(Mean values with their standard error of four groups)

	FM		PP50		PP75		PP100	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Initial body weight (g)	19·2	0·1	19·2	0·1	19·2	0·1	19·2	0·05
Final body weight (g)	147·1[c]	0·9	142·4[c]	1·3	135·6[b]	2·4	111·1[a]	1·2
Feed intake (g DM/kg ABW per d)	14·9[a]	0·1	14·8[a]	0·1	15·4[b]	0·1	15·4[b]	0·05
SGR† (%)	2·61[c]	0·01	2·57[c]	0·005	2·51[b]	0·02	2·25[a]	0·01
FE‡	1·33[c]	0·01	1·29[c]	0·01	1·25[b]	0·005	1·17[a]	0·005
PER§	2·58[c]	0·02	2·63[c]	0·02	2·55[b]	0·01	2·42[a]	0·005
Retention (% digestible intake)								
N	48·2[c]	0·6	46·9[c]	0·2	44·3[b]	0·5	40·3[a]	0·9
Lipid	93·9	2·0	94·9	2·8	94·2	0·3	90·9	1·8
Energy	61·0[b]	1·1	59·9[b]	0·7	57·1[a]	0·3	56·6[a]	0·1

ABW, average body weight; SGR, specific growth rate; FE, feed efficiency; PER, protein efficiency ratio.

[a,b,c] Mean values within a row with unlike superscript letters were significantly different among dietary treatments (Student–Newman–Keuls test; $P<0·05$).

* For details of the experimental diets, see Table 1 (p. 354).

† SGR = [100 × (ln final fish weight (ln initial fish weight)]/days.

‡ FE = wet weight gain/dry feed intake.

§ PER = wet weight gain/protein intake.

Discussion

Knowledge on the mode of action and regulation of the GH–IGF axis in fish is increasing considerably. One major advance is the cloning and sequencing of GHR in salmonid and non-salmonid fish. However, it is noticeable that available sequences of coho salmon and masu salmon lack one pair of extracellular cysteine residues (C5, C6) and three intracellular tyrosine ones (Y1, Y3, Y6), which are already conserved in the GHR of tetrapods and non-salmonid fish (Zhu *et al.* 2001; Pérez-Sánchez *et al.* 2002). Several authors have speculated about a divergent evolution of GHR in salmonids (Björnsson *et al.* 2002). Nevertheless, the trout sequence reported herein retains all cysteine and tyrosine residues, and in consequence this salmonid cytokine receptor is related in phylogenetic trees to GHR of non-salmonid fish rather than to previously reported GHR of salmonid fish. The tissue expression of trout GHR has been confirmed by Northern blot, and interestingly the molecular weight of the detected band was two-fold higher than that found for gilthead sea bream GHR mRNA (Calduch-Giner *et al.* 2003), which suggests the presence of long untranslated regions in trout GHR. In this scenario, it must be noted that ongoing studies support the duplication of GHR in the genome of most fish species, including salmonid and non-salmonid fish (Saera-Vila *et al.* 2005). In the present study we have analysed the same GHR as that first described in non-salmonid fish, which acts as a functional and specific GHR in transfection assays (Lee *et al.* 2001; Tse *et al.* 2003), and is perhaps the retained *GHR* gene in all vertebrate species.

Plasma GH levels are increased by fasting and nutritional changes arising from decreased dietary protein:energy in a wide variety of fish species, including salmonids (Varnavsky *et al.* 1995; Johnsson *et al.* 1996; Pottinger *et al.* 2003), striped bass (*Morone saxatilis*; Small *et al.* 2002), tilapia (*Oreochromis mossambicus*; Weber & Grau, 1999) and gilthead sea bream (Pérez-Sánchez *et al.* 1995; Company *et al.* 2001). Recent gilthead sea bream studies also indicate that plasma GH levels are up-regulated in fish fed diets with reduced nutritive value as a result of

Table 3. Data on liver, muscle and adipose tissue expression of growth hormone receptor (GHR), insulin-like growth factor (IGF)-I and IGF-II (real-time PCR assays) in fish fed the four experimental diets*

(Mean values with their standard error of five to seven fish per groups)

	FM		PP50		PP75		PP100	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Liver								
GHR	0·90	0·06					1·00	0·12
IGF-I	0·84	0·02					1·00	0·06
IGF-II	0·99	0·16					1·00	0·04
White muscle								
GHR	0·77	0·11	0·81	0·17	0·77	0·13	1·00	0·18
IGF-I	0·86	0·11	0·87	0·10	0·89	0·14	0·90	0·09
IGF-II	0·96	0·15	1·00	0·17	0·61	0·06	0·72	0·07
Adipose tissue								
GHR	0·56[a]	0·11	0·58[ab]	0·09	0·70[b]	0·10	1·00[b]	0·11
IGF-I	0·70	0·13	0·94	0·05	0·98	0·25	1·03	0·18
IGF-II	0·96	0·08	0·93	0·03	0·94	0·05	1·00	0·20

Values presented are in arbitrary units.

[a,b] Mean values within a row with unlike superscript letters were significantly different among dietary treatments (Student–Newman–Keuls test; $P<0·05$).

* For details of the experimental diets, see Table 1 (p. 354).

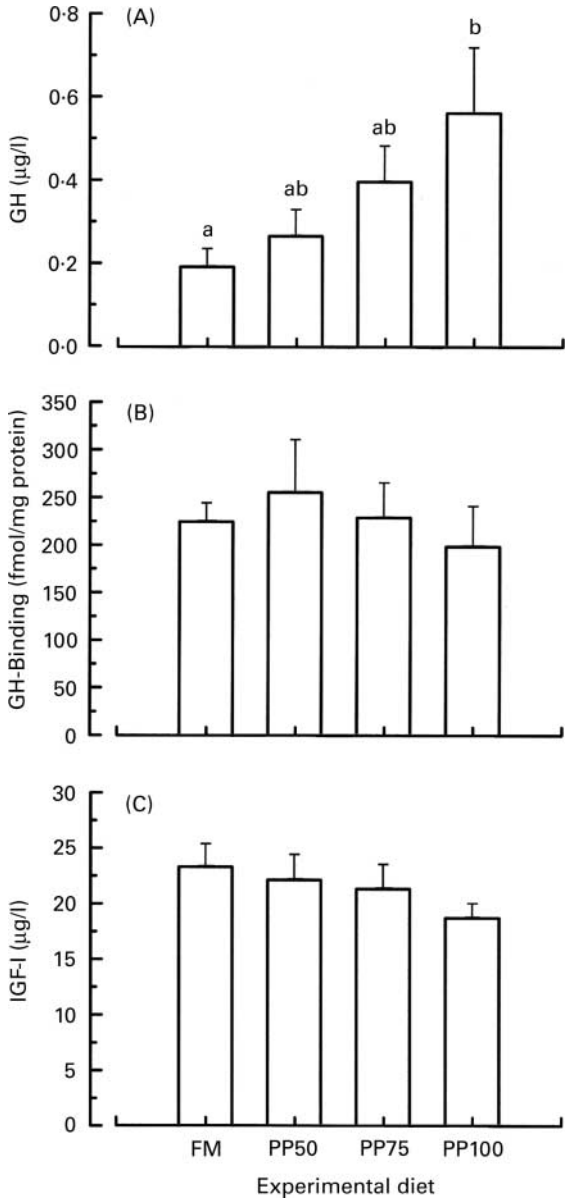

Fig. 4. (A) Plasma levels of growth hormone (GH), (B) hepatic GH binding and (C) plasma levels of insulin-like growth factor (IGF)-I in fish fed the four experimental diets. For details of the experimental diets, see Table 1 (p. 354). Values are means with their standard errors shown by vertical bars for ten to fifteen animals. [a,b]Mean values with unlike superscript letters were significantly different ($P<0·05$).

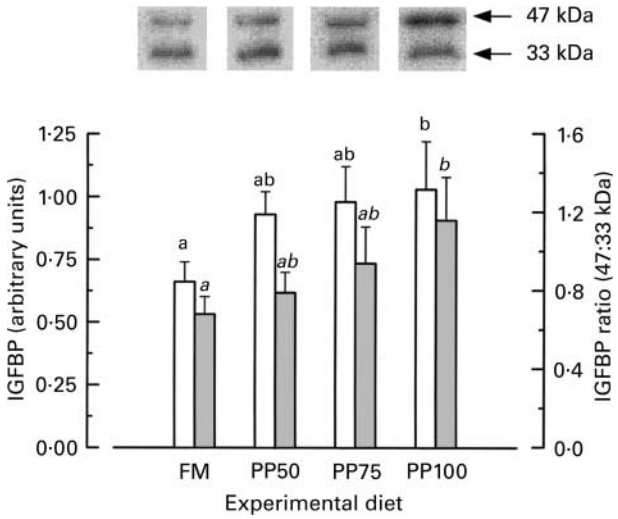

Fig. 5. A representative Western ligand blot of insulin-like growth factor-binding proteins (IGFBP) in fish fed the four experimental diets is shown at the top of the figure. The plot below indicates the integrated area (arbitrary units) for the two IGFBP bands (□) and the calculated ratio of 47 kDa IGFBP to 33 kDa IGFBP (■). For details of the experimental diets, see Table 1 (p. 354). Values are means with their standard error shown by vertical bars for eight animals. [a,b]Mean values with unlike superscript letters (roman and italic) were significantly different ($P<0·05$).

changes in dietary amino acid profile (Gómez-Requeni *et al.* 2003) and protein source (Gómez-Requeni *et al.* 2004). In this context, the GH rise is probably due to a reduced negative feedback inhibition of hepatic IGF-I. This is consistent with a decreased hepatic expression of *GHR* and *IGF-I* genes, which in turn results in a lowered plasma IGF-I concentration in conjunction with a reduction of feed intake and weight gain in juvenile gilthead sea bream fed with full plant protein diets (Gómez-Requeni *et al.* 2004). In trout, the same kind of experimental diets induced herein a progressive decrease of weight gain with the 75 % and 100 % replacement of fishmeal, but this condition was related to the increase of feed intake, which suggests some state of enhanced energy expenditure. This was evidenced by the reduction of feed efficiency and the retention of digestible N and lipid nutrients. However, it remains to be established the extent to which this growth impairment is due to the presence of anti-nutritional factors and/or changes in the amino acid profile and other specific nutrients.

In the present study, we also found that plasma GH levels increased in fish fed high plant protein diets, but we did not detect changes in hepatic GH binding (a measure of GHR protein expression). Similarly, hepatic GHR transcripts remained unaltered, but in peri-visceral adipose tissue and in some extent in white muscle, GHR mRNA were up-regulated in a progressive manner with the 75 % and 100 % replacement of fishmeal. Since GH has a lipolytic effect not mediated by IGF in fish (Vega-Rubín de Celis *et al.* 2003), it can be argued that the up-regulation of plasma GH levels and adipose tissue GHR mRNA represents an effective manner to mobilize and metabolize energy substrates from peripheral lipid depots. In mammals, it is now recognized that catabolic states characterized by the increase of plasma GH titres are usually accompanied by the increased expression of GHR in skeletal muscle (Combes *et al.* 1997), but this is the first report of the up-regulation of GHR in a peripheral GH target tissue of a lower vertebrate species. This can be of special relevance to modulate GH action in a fish species with a clear hyposomatotropism (GH <2 ng/ml) compared with other salmonid and non-salmonid fish (Pérez-Sánchez & Le Bail, 1999).

The precise mechanism involved in the plasma GH rise in the absence of liver GH desensitization (no changes in hepatic GHR and IGF transcripts) remains to be elucidated. However, there is now increasing evidence for a regulatory role of IGFBP in IGF function and activity, and it appears likely that the increase in plasma IGFBP activity reported herein represents an effective way to limit the amount of biologically active IGF (free IGF fraction) for GH feedback inhibition in fish fed diets with a high plant

protein content. In fish, there is now evidence of at least three IGFBP, including a high-molecular-weight form (40–50 kDa) and two IGFBP in the 31–24 kDa size range (Kelley *et al.* 1992; Niu & Le Bail, 1993; Park *et al.* 2000; Cheng *et al.* 2002). As in mammals, the higher-molecular-weight IGFBP is the most abundant circulating IGF carrier under normal physiological conditions. In contrast, lower-molecular weight IGFBP are often at or below the limit of detection in fed fish, and up-regulated several fold under catabolic conditions (Kelley *et al.* 2001, 2002; Peterson & Small, 2004). Since the cloning and sequencing of gilthead sea bream IGFBP-2 (Funkenstein *et al.* 2002) and zebrafish IGFBP-1 and -2 (Duan *et al.*, 1999; Maures & Duan, 2002), it becomes likely that the ≤31 kDa IGFBP, originally identified by Western ligand blotting, might indeed be IGFBP-1 and -2. Additionally, doublets of higher-molecular-weight proteins might represent different glycosylated forms of IGFBP-3.

Two glycosylation sites are always utilized in mammalian IGFBP-3, whereas the alternative glycosylation site incorporates 5 kDa of carbohydrates, accounting for the characteristic doublet (4–45 kDa) with a core protein size of 29 kDa (Firth & Baxter, 2002). Fish IGFBP-3 also exists as glycosylated protein (Shimizu *et al.* 2003), and a doublet of 45–34 kDa has been found in coho salmon serum (Shimizu *et al.* 1999). In trout, we have also detected an IGFBP doublet of higher molecular weight, increasing the total IGFBP activity and the 47:33 kDa IGFBP with plant protein supply. It is now accepted that proteolytic cleavage is the predominant mechanism to release IGF from IGFBP (Bunn & Fowlkes, 2003). However, post-translational modifications such as glycosylation regulate the rate of IGFBP proteolysis, protecting them against the action of metalloproteases. In trout, the precise mechanism responsible for the increased 47:33 kDa IGFBP remains unknown, but this condition can protect IGFP-3 against degradation, avoiding the release of free IGF (biologically active form).

In summary, partial cloning and sequencing of a trout GHR was accomplished by RT-PCR, and a tissue-specific regulation of GHR mRNA was evidenced as a result of a different nutritional condition. In fish fed diets high in plant protein, the combined increase of plasma GH titres and GHR mRNA in adipose tissue would enhance the lipolytic action of GH. Further, we suggest that the decrease in growth rates with plant protein supply is primarily due to a lower availability of free plasma IGF rather than to liver GH desensitization or defect in IGF synthesis and release at the systemic and autocrine–paracrine level.

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