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Switching to fast growth: the insulin-like growth factor (IGF) system in skeletal muscle of Atlantic salmon

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SUMMARY

In this study we describe the complete coding sequence for insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II), insulin-like growth factor binding protein (IGFBP) 1, 2, 4, 5 and 6 and IGFBP-related protein 1 (IGFBP-rP1) of Atlantic salmon (*Salmo salar* L.). We also report the characterisation of two gene paralogues of IGFBP-2 and IGFBP-5. Following 22 days restricted feeding (0 d) to achieve zero growth, fish were fed to satiation and sampled at 3, 5, 7, 14, 30 and 60 days. Expression profiles for genes involved in the IGF signalling pathway in fast myotomal muscle were determined using real-time quantitative RT-PCR. The transition from zero to fast growth is characterised by constitutive upregulation of IGF-I and IGFBP-4, a transient increase in IGFBP-5.2, and downregulation of IGFBP-2.1, IGF-II, IGF2R (IGF-II receptor) and IGFR1a (IGF-I receptor a). Expression of IGFBP-2.2, IGFBP-5.1, IGFBP-6, IGFBP-rP1 and IGFR1b showed little or no response to feeding. Expression of the myogenic marker genes myogenin, MHC and MLC2 were higher with feed restriction, and decreased as an early response to feeding, before increasing to a peak at 14 days, corresponding with a peak in IGF-I expression. IGFBP-4, which contains a putative connective tissue localisation signal, was the only IGFBP constitutively upregulated following feeding, and was positively correlated with IGF-I expression. Together, these data show that switching to fast growth in Atlantic salmon skeletal muscle involves the local upregulation of IGF-I, IGFBP-5.2 and IGFBP-4, with downregulation of IGFBP-2.1.

Key words: IGF-I, IGF-I receptor, IGF binding proteins, teleost fish, growth, skeletal muscle, myogenesis, rainbow trout, coho salmon, feeding, nutrition.

INTRODUCTION

The insulin-like growth factor (IGF) system includes the growth hormones IGF-I, IGF-II, their corresponding receptors and the IGF binding proteins (IGFBPs). This system has been extensively studied in mammals (reviewed by Stewart and Rotwein, 1996; LeRoith et al., 2001). IGF-I and IGF-II are conserved polypeptides that are expressed as pre-prohormones consisting of A, B, C, D and E domains, with the E domain removed by a proteolytic processes to yield the mature IGF-I peptide. IGFs have multiple functions and are necessary for normal development and survival controlled *via* endocrine and autocrine/paracrine pathways (Liu et al., 1993; Baker et al., 1993). Circulating levels of IGFs are primarily determined by their secretion from the liver under the control of the pituitary/growth hormone axis (LeRoith et al., 2001). Both IGF-I and IGF-II are synthesised locally in numerous tissues including skeletal muscle where an IGF-I splice variant is induced in response to mechanical stimuli (Yamaguchi et al., 2006). IGF-I expression in skeletal muscle has also been shown to respond to hormones (Bonaventure et al., 2002) while IGF-II mRNA expression is regulated by mTOR and amino acid availability in C2C12 myoblasts (Erbay et al., 2003).

In addition, IGF-I and IGF-II promote growth by stimulating the proliferation and differentiation of skeletal muscle satellite cells as well as stimulating the hypertrophy of mature muscle cells. Stimulation of the cellular responses to IGF-I is mediated through the IGF-I receptor, a member of the tyrosine kinase family. Both IGF-I and IGF-II are able to bind the IGF-I receptor, leading to the activation of multiple signal transduction cascades including the PI3/AKT/mTOR and MAP kinase pathways, and so to cell proliferation (Engert et al., 1996), survival and differentiation (Jones and Clemmons, 1995; Duan et al., 2000; Coolican et al., 1997). The IGF-II receptor is identical to the mannose 6-phosphate receptor. This receptor does not possess any tyrosine kinase activity and its function remains unknown, although recently an IGF2R signalling cascade was identified that results in hypertrophy of cardiac myoblast cells (Chu et al., 2008). Also, binding of IGF-II to the type 2 receptor has been shown to lead to degradation of the ligand, resulting in stabilisation of IGF concentrations (Boker et al., 1997).

The availability of IGFs is regulated by the insulin-like growth factor binding proteins (IGFBPs). The IGFBPs are a family of secreted proteins with high affinity for IGFs, and six distinct IGFBPs have been characterised in several mammalian species (Clemmons, 2001; Duan, 2002; Firth and Baxter, 2002). Different tissues express different IGFBPs at various developmental stages, and the IGFBPs are thought to protect IGFs from proteolytic degradation. Expression of IGFBPs in skeletal muscle has been shown to be regulated by hormones (Awede et al., 2002) and mechanical stimulation (Awede et al., 1999). The IGFBPs have a higher affinity for the ligand than does the receptor (Clemmons, 1998); thus IGFBPs limit the availability of IGF-I to bind with its receptor. Some IGFBPs contain domains that can bind other proteins such as cell surface proteins and proteins present in the extracellular matrix (ECM) (Jones et al., 1993a; Jones et al., 1993b). Binding to these proteins results in a reduction in the affinity of IGFBPs for IGF-I. In this way, IGF-I can be targeted to particular tissues, and to particular cell types within

those tissues. The targeted degradation of IGFBPs by specific proteases can also lead to the release of IGF-I (Jones et al., 1993a), again allowing IGF-I to be targeted to particular tissues (Smith et al., 2001). As well as having a role in regulating IGF-I availability, some IGFBPs have been shown to possess regulatory activity independent of ligand binding. For example, an IGFBP-5 proteolytic fragment with limited capacity to bind IGF-I stimulated osteoblast mitogenesis (Andress and Birnbaum, 1992; Andress et al., 1993), and administration of recombinant human IGFBP-5 osteoblast clones derived from IGF-I knockout mice increased proliferation and increased the activity of the osteoblast differentiation marker alkaline phosphatase (Miyakoshi et al., 2000).

For muscle growth to occur, myogenic signalling pathways need to be induced. Myogenesis is a multi-step process that produces new muscle fibres through the fusion of myoblasts to form short myotubes (hyperplasia), and contributes new cells that are able to fuse to existing muscle fibres (hypertrophy) from myogenic precursor cells (Johnston, 2006). The signalling pathways controlling vertebrate myogenesis have been extensively studied, with genes involved in each of these steps identified. Late markers of myogenesis include the transcription factor myogenin, involved in the initiation and stabilisation of the differentiation programme, and the myofibrillar proteins myosin heavy chain and myosin light chain 2. By monitoring the expression patterns of these marker genes, the effect that nutritional status has on muscle growth can be determined.

In fish, it appears that the IGF signalling pathway is a conserved system with orthologues of mammalian IGFs, IGFBPs and IGF receptors identified and shown to function in growth, reproduction and osmoregulation (reviewed by Wood et al., 2005a). Through the use of morpholino gene knockdown experiments, it has been shown that IGFBP-2 is involved in cardiovascular development in zebrafish embryos (Wood et al., 2005b) and IGFBP-3 is involved in pharyngeal skeleton morphogenesis (Li et al., 2005), while IGFBP-1 restricts IGF-stimulated growth and development processes under hypoxic stress (Kajimura et al., 2005). Due to physiological differences between ectothermic fish and homeothermic mammals, it is likely that the IGF system is differently regulated. For example, the duration of increased oxygen consumption following a single meal is a few hours in mammals, compared with 2–10 days in teleosts depending on temperature (Johnston and Battram, 1993). Furthermore, unlike mammals, many teleost species can undergo long periods of fasting in their natural environment associated with seasonal changes in water temperature and prey availability. In addition, early in teleost evolution, around 320–350 million years ago, a whole genome duplication event occurred (Amores et al., 1998; Jaillon et al., 2004) so the IGF system in teleosts is likely to be further complicated by the presence of gene paralogues. For example, in zebrafish there are two IGF-I receptors, IGFR1a and IGFR1b (Maures et al., 2002), whereas only one receptor is present in mammalian species. This situation is further complicated in salmonids, as the teleost whole genome duplication was followed by an additional duplication event specifically within the salmonid lineage 25–100 million years ago (Allendorf and Thogaard, 1984). These duplication events have resulted in the presence of up to four salmonid paralogues for each mammalian gene. It has been estimated that only 50% of the duplicated genes have subsequently been lost from the genome (Bailey et al., 1978) with retained paralogues able to undergo subfunctionalisation leading to altered expression (e.g. Macqueen and Johnston, 2008).

Only limited information is available on the nutritional regulation of the various components of the IGF signalling pathway in fish. Starvation and refeeding experiments have been used as the model system to study the regulation of muscle growth in fish. We propose a variation to this method whereby fish are maintained at zero growth and are then fed to satiation with a high protein diet. This experimental protocol benefits from the fact that the fish do not undergo such profound changes in metabolism as they do during starvation, and thus an increase in the net flux through anabolic pathways is more likely to be observed, rather than the dramatic switch from the catabolic to the anabolic state. The first aim of the present study was to completely characterise the IGF system including retained paralogues, and examine gene expression during the transition from a state of zero growth to fast growth. The second aim was to test the hypothesis that IGFBPs and their paralogues show differential regulation with feeding.

MATERIALS AND METHODS

Animals and experimental design

One-hundred and ten Atlantic salmon (*Salmo salar* L., 840±152 g, mean ± s.d., N=110) were individually passive induced transponder (PIT) tagged (Fish Eagle, Lechlade, Gloucestershire, UK) and fed a maintenance diet (25% normal ration) to achieve a zero or slightly negative growth rate. Growth was measured as the thermal growth co-efficient, TGC=[($M_2^{0.333}$–$M_1^{0.333}$)(degree days)$^{-1}$×1000], where M_1 and M_2 are start and final body masses, respectively, and degree days values are the sum of the temperature values (in °C) for each day of the experiment. Fish were reared in tanks with an average temperature of 7.8°C, 13.96 mg l^{-1} oxygen and average salinity of 28.9 p.p.m. Zero growth was achieved over a period of 22 days, and then fish were fed to satiation with a commercial feed (EWOS Innovation) for 60 days. Sampling of fish occurred at 0, 3, 5, 7, 14, 30 and 60 days with 10 fish sampled at each time point. Fish were humanely killed following Schedule 1 of the Animals (Scientific Procedures) Act 1986 (Home Office Code of Practice. HMSO: London January 1997) and individual mass and fork lengths measured. Fast muscle was dissected from the dorsal myotome between 0.6 and 0.7 standard length (tip of snout to last vertebrae). Tissues were snap frozen in liquid nitrogen and stored at –80°C until analysed.

RNA extraction and cDNA synthesis

Total RNA was extracted by addition of 100 mg of muscle or liver to Lysing matrix D (Qbiogene, Irvine, CA, USA) with 1 ml TRI Reagent (Sigma, Gillingham, Dorset, UK) and homogenised using a Fast Prep instrument (Qbiogene). Total RNA was quantified based on absorbance at 260 nm. Genomic DNA contamination was removed by treatment with Turbo DNA-free (Ambion, Austin, TX, USA), and the integrity of the purified RNA was confirmed by agarose gel electrophoresis. First strand cDNA was synthesised from 1 µg total RNA using Superscript III (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's guidelines.

PCR amplification and cloning of genes encoding IGF-I, IGF-II and IGFBPs

Primers to amplify the coding sequence for each of the IGFBPs and IGF-I and IGF-II were designed based on EST sequences and previously published sequences (Kamangar et al., 2006) and are listed in Table 1. Rapid amplification of cDNA ends (RACE) was performed to obtain 5′ and 3′ sequences for IGF-I and IGF-II using Smart RACE kit (Clontech, Mountain View, CA, USA) and GeneRacer kit (Invitrogen). PCR was performed in a 50 µl reaction volume using BioTaq PCR kit (Bioline, London, UK) with 35 cycles (95°C 30 s, 60°C 30 s and 72°C 2 min), with muscle

Table 1. Primer sequences used for cloning genes encoding IGFBPs, IGF-I and IGF-II and for RACE for IGF-I and IGF-II

Gene	Forward primer 5′–3′	Reverse primer 5′–3′
IGF-I	ATGTCTAGCGCTCATTTCTT	GAATTCTTACATTCGGTAGTTCCTT
IGF-II	GACATGGAAACCCAGAAAAGACACGAA	GGGCTCAATTGTGGCTGACGTA
IGF-I	AGTCAGGGTTAGGACGCACAG (5′ RACE)	GCATTTATGTGATGTCTTCAAGAGTG (3′ RACE)
IGF-II	GTACTGCTCCAACAGGTTGA (5′ RACE)	GGAAAACACAGAATGAAGGTCAA (3′ RACE)
IGFBP-1	ATGCTTGGATTATATAAGAAGTT	GAATTCAGAGCTCCAGCTGGCACTCTAAG
IGFBP-2.1	ATGATATCGTATTCAGGCT	GAATTCATTTCTGGGCTGAGGCGAG
IGFBP-2.2	ATGGTGCTATATTTTAGCTG	GAATTCATATCTCTGCCATCTGAAGGA
IGFBP-4	ATGGTGCGAGGTGGCGGTGC	CTCCTCACTCTCTCATGGTTGC
IGFBP-5.1	ATGTTTCTCAGTTTTTGTCT	GAATTCACTCGTTGTTGCTGCTGC
IGFBP-5.2	ATTACAAGCGTTCCATGCAGTAC	GTTTTTGTTGCTGTTGCTCTCCG
IGFBP-6	ATGCCTCTCCTTTCTAACTTAA	GAATTCAATCTCCATCGCATGGTA
IGFBP-rP1	ATGTTAGTGTTTTTCGCTGTGGTC	GAATTCACAGCTCATCATCCTTGGCCAC

IGF, insulin-like growth factor; IGFBP, IGF binding protein; IGFBP-rP1, IGFBP-related protein 1.

or liver cDNA as template. The PCR products were cloned into a T/A pCR4-TOPO vector (Invitrogen) and transformed into chemically competent TOP10 *Escherichia coli* cells (Invitrogen). Individual colonies were grown and plasmids purified using the Fastprep plasmid purification method (Eppendorf, Hamburg, Germany). Sequencing was performed using T3 and T7 sequencing primers with Big Dye terminator v3.1 sequencing chemistry (Applied Biosystems, Foster City, CA, USA) at the University of Dundee Sequencing Facility.

Quantitative PCR

Quantitative PCR (qPCR) was performed using a Stratagene MX3005P QPCR system (Stratagene, La Jolla, CA, USA) with SYBR green chemistry (Power SYBR, Applied Biosystems). cDNA used in qPCR assays was first diluted 80-fold. Each qPCR reaction mixture contained 7.5 µl 1×Power SYBR green master mix, 6 µl cDNA (80-fold dilution), 500 nmol l^{-1} each primer and RNAse-free water to a final volume of 15 µl. Amplification was performed in duplicate in 96 well plates (Stratagene) with the following thermal cycling conditions: initial activation 95°C for 10 min, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C. Dissociation analysis of the PCR products was performed by running a gradient from 60 to 95°C to confirm the presence of a single PCR product. Products were also sequenced to confirm their identity. A dilution series made from known concentrations of plasmid containing the PCR inserts was used to calculate absolute copy numbers for each of the genes examined.

Standards for calculating absolute copy number for each gene were prepared by cloning the PCR product from each primer pair into a T/A pCR4-TOPO vector (Invitrogen) and transformation of chemically competent TOP10 *Escherichia coli* cells (Invitrogen). Individual colonies were grown and plasmids purified using the Fastprep plasmid purification method (Eppendorf). The concentration of each plasmid was calculated based on absorbance at 260 nm, and a dilution series produced for calculation of copy number *via* qPCR.

Primers were designed using Net primer (Premier BioSoft) to have a T_m of 60°C and, where possible, were designed to cross an exon–exon junction to avoid amplification of contaminating genomic DNA. The primers used for qPCR are listed in Table 2.

Table 2. Primer sequences used for real-time PCR (qPCR) analysis

Gene	Forward primer 5′–3′	Reverse primer 5′–3′	Product size (bp)	Accession number
IGF-I	CCTGTTCGCTAAATCTCACTTC	TACAGCACATCGCACTCTTGA	226	EF432852
IGF-II	GGAAAACACAAGAATGAAGGTCAA	CCACCAGCTCTCCTCCACATA	127	EF432854
IGFR1a	GGGGCTCTCCTTCTGTCCTA	AGAGATAGACGACGCCTCCTA	175	EU861008
IGFR1b	CCTAAATCTGTAGGAGACCTGGAG	GGTTAGCCACGCCAAATAGATCC	138	EU861006
IGF2R	CTTCATCCACGCTCAGCAG	ACCCTGGGCCGTGTCTAC	168	CX325971
IGFBP-1	AGGACCAGGGACAAGAGGAAG	CTGTTCCACCAGTTTCTTGC	154	EF432856
IGFBP-2.1	CGGTGAGGAAGGCCACTAAGG	ATATCACAGTTGGGGATGT	249	EF432858
IGFBP-2.2	TTCCATGATAACAGGGGACCAG	GACCGTGGGTGGACATGTGG	108	EF432860
IGFBP-4	ACTTCCATGCCAAGCAGTGC	GGTCCCATCCTCACTCTCTC	164	EU861007
IGFBP-5.1	ATCACGGAGGACCAACTGC	TGCTTGTCAATGGGTAGTGG	169	EF432862
IGFBP-5.2	TTCTCCAGAGGAAGCTATGTTAG	TCAAGGCTGCTGACAGAGTG	170	EU861009
IGFBP-6	GCTGCGTGCCTCTTCCTCA	TTACGGCAGGGTGCCTTTTC	159	EF432864
IGFBP-rP1	GAAGTGTGTGGCTCCGATG	GTTTTCCGCTGGTGACCTTCT	249	EF432866
Myogenin	GTGGAGATCCTGAGGAGTGC	CTCACTCGACGACGAGACC	146	DQ294029
MHC	AGAAGCACGCCACTGAAAAC	AACCCTCAAGGTCGTCCACT	209	DN164736
MLC2	CCATCAACTTCACCGTCTTCCTCAC	CAGCCCACAGGTTCTTCATCTCC	194	NM_001123716
EF1-α	GAATCGGCTATGCCTGGTGAC	GGATGATGACCTGAGCGGTG	141	BG933853
β-Actin	TGACCCAGATCATGTTTGAGACC	CTCGTAGATGGGTACTGTGTGGG	146	G933897
RNA pol II	CCAATACATGACCAAATATGAAAGG	ATGATGATGGGGATCTTCCTGC	157	BG936649
HPRT1	CCGCCTCAAGAGCTACTGTAAT	GTCTGGAACCTCAAACCCTATG	255	EG866745
18S	TCGGCGTCCAACTTCTTA	GCAATCCCCAATCCCTATC	189	AJ427629

IGFR1a, IGFR1b and IGF2R, IGF receptors; MHC, myosin heavy chain; MLC, myosin light chain; EF1-α, elongation factor 1-α; RNA pol II, RNA polymerase II; HPRT, hypoxanthine phosphoribosyltransferase 1.

Data analysis

Genorm (Vandesompele et al., 2002) was used to analyse the stability of several housekeeping genes including 18S ribosomal, hypoxanthine phosphoribosyltransferase 1 (HPRT1), β-actin, RNA polymerase II and elongation factor 1-α (EF1-α). Analysis revealed EF1-α, β-actin and RNA polymerase II to be the most stable genes in this experimental system (M=0.27), thus the geometric average of these genes was used for normalisation of qPCR data, and gene expression values are displayed as arbitrary units. Statistical analysis was performed using minitab (Minitab, State College, USA). Significant differences in expression between time points were calculated by ANOVA using Fisher's individual error rate *post hoc* tests. Correlations in gene expression were calculated using linear regression.

Bioinformatics

Predictions for size, isoelectric point (pI) and potential *N*-glycosylation sites were performed from deduced amino acid sequences using the ExPaSy proteomics server of the Swiss Institute of Bioinformatics (http://www.expasy.ch). Multiple amino acid alignments were performed using T-coffee clustal W software (Notredame et al., 2000). Graphical representation and clustering analysis of gene expression data were performed using Permutmatrix (Caraux and Pinloche, 2005).

RESULTS

Characterisation of *Salmo salar* IGF-I and IGF-II

The deduced amino acid sequence of the full-length preproIGF-I cDNA (EF432852) contains 176 amino acids and consists of a 44 amino acid signal peptide, a 70 amino acid mature peptide and a 62 amino acid E peptide. The mature peptide of Atlantic salmon IGF-I has 80% identity with human IGF-I and 94.4% identity with zebrafish orthologues, whereas the preproIGF-I has 64% and 74% identity with human and zebrafish preproIGF-I, respectively. Atlantic salmon IGF-II (EF462854) deduced amino acid sequence contains 214 amino acids and consists of a 47 amino acid signal peptide, a 70 amino acid mature peptide and a 97 amino acid E peptide. PreproIGF-II has 50.6% and 61.1% identity while the mature peptide has 83.3% and 76.8% identity with human and zebrafish orthologues, respectively.

Characterisation of *Salmo salar* IGFBPs

IGFBP-1

The coding sequence of IGFBP-1 (EF432856) is 735 nucleotides in length and encodes a protein of 245 amino acids with a putative molecular mass of 26.5 kDa and pI of 6.58. The amino acid sequence contains 18 cysteines and a signal peptide of 25 amino acids, and has 39.2% and 59.6% identity with human and zebra fish orthologues, respectively. The *Salmo salar* IGFBP-1 sequence does not contain the RGD integrin recognition moitif found in mammalian IGFBP-1. Predicted phosphorylation sites within the amino acid sequence are at six serine, three threonine and one tyrosine residue (Fig. 1).

IGFBP-2 paralogue 1

The coding sequence of IGFBP-2.1 (EF432858) is translated into a protein of 280 amino acids with 19 cysteine residues and a 22 amino acid signal peptide. Paralogue 1 has 50.7% and 76.6% identity with human and zebrafish IGFBP-2, respectively. The amino acid sequence has an estimated mass of 31.1 kDa, a putative *N*-glycosylation site at position 262 and predicted phosphorylation sites at eight serine, five threonine and two tyrosine residues. Similar to IGFBP-2 of mammals, the IGFBP-2.1 protein contains a C-terminal RGD motif (Fig. 1).

IGFBP-2 paralogue 2

The Atlantic salmon IGFBP-2 paralogue 2 nucleotide sequence (EF432860) encodes a protein of 283 amino acids in length, with a molecular mass of 31.6 kDa and a pI of 6.03. IGFBP-2.2 has 59.6% identity with IGFBP-2.1, and differs from IGFBP-2.1 in having three predicted *N*-glycosylation sites at positions 46, 112 and 258, 20 cysteine residues and a signal peptide of 23 amino acids. Comparison with human and zebrafish IGFBP-2 shows identity of 43.3% and 55.4%, respectively, and 60.0% identity with zebrafish IGFBP-2b (NP001119936). The amino acid sequence has several predicted phosphorylation sites, at five serine, six threonine and one tyrosine residue. Like the IGFBP-2.1, this protein also contains a C-terminal RGD motif (Fig. 1).

IGFBP-4

IGFBP-4 (EU861007) encodes a protein of 264 amino acids with an estimated molecular mass of 28.7 kDa and a pI of 6.93 and contains 22 cysteine residues (Fig. 1). The amino acid sequence has 59.8% identity with human IGFBP-4, is not predicted to undergo *N*-glycosylation but has predicted phosphorylation sites at seven serine, two threonine and one tyrosine residue.

IGFBP-5

Blast searches revealed the presence of at least three paralogues of IGFBP-5. We have cloned and obtained full-length coding sequences for two IGFBP-5 paralogues.

IGFBP-5 paralogue 1

The 270 amino acid IGFBP-5 paralogue 1 (IGFBP-5.1, EF432862) has a predicted molecular mass of 29.8 kDa and a pI of 8.55, and is most similar (96.3% identity) to the previously reported rainbow trout IGFBP-5 (Kamangar et al., 2006). This paralogue has 55.1% and 81.3% identity with human and zebrafish orthologues, contains a putative nuclear localisation signal and is predicted to bind to DNA. It also has 19 cysteine residues and a 20 amino acid signal peptide, with predicted phosphorylation sites at six serine, one threonine and three tyrosine residues. Similar to mammalian IGFBP-5, the *Salmo salar* IGFBP-5 contains a heparin binding site (FKRKQCKP) at positions 222–229 (Fig. 1).

IGFBP-5 paralogue 2

IGFBP-5.2 (EU861009) encodes a protein of 268 amino acids with a predicted molecular mass of 29.5 kDa and a pI of 9.1. Unlike IGFBP5.1, this paralogue does not contain a nuclear localisation signal or DNA binding motif. IGFBP-5.2 has 72.4% identity to IGFBP-5.1, and 48.9% and 70.2% identity to human and zebrafish orthologues. Like paralogue 1, the C-terminus of IGFBP-5.2 also contains a heparin binding site (FKHKQCKP) at positions 222–229 (Fig. 1).

IGFBP-6

The coding sequence for IGFBP-6 (EF432864) encodes a protein of 199 amino acids, with an estimated molecular mass of 21.5 kDa, containing 14 cysteines and a 24 amino acid signal peptide. IGFBP-6 contains a putative heparin binding site, YRKKQCRS, at amino acids 158–166 (Fig. 1).

IGFBP-related protein 1

The IGFBP-related protein 1 (IGFBP-rP1) coding sequence (EF432866) encodes a protein of 263 amino acids, with an

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                                                    *  *       *      *
IGFBP-1         MLGLYKKLTLLAAMSLSLLTSLAQSSPVVGPEPIRCAPCTQEKLDECPAISPDCKQVLRE
IGFBP-2.1       -------MISYSGCSL-LLLSVAFVGASFAEMVFRCPSCTAERQAACPKLTETCAEIVRE
IGFBP-2.2       -------MVLYFSCGLLLLMLLGLPGLSLGDLVFHCPKCTAERQTACPELTINCTEIVRE
IGFBP-4         MVRGGGAAIAPPCWGLWAVGALSLATLCLTEEAIRCPVCSEERLASCQLPDGSCEETVRE
IGFBP-5.1       ---------MFLSFCLLLTFVLGLTG-SFGSY-VPCEPCDQKALSMCPPVPVGC-QLVKE
IGFBP-5.2       ---------MLISFSLLATLLLSESG-CLGSF-VPCEPCDQKLMSMCPPVPVGC-QLVKE
IGFBP-6         -------MPLLSNLTTIILLLIA-----------HCG CSTLANRLGPYK--GC-LSCKE
                                    :.           *  *             *    :*
                  *        **  *       *      *     *                    *
IGFBP-1         PGCG-------CCLACALEKGASCGVYTAHCAQGLKCSPRPGDPRPLHSLTRGQAICTED
IGFBP-2.1       PGCG-------CCPVCARQEGELCGVYTPRCSSGLRCYPKPDSDLPLEQLVQGLGLCGHK
IGFBP-2.2       PACG-------CCPVCARLEGEFCGVYTPRCSTGLRCYPTADSKLPLEQLVQGLGRCSQK
IGFBP-4         AGCG-------CCPTCALAKGVHCGVYSPRCGTGLRCYPPRNVERPLHSLMHGQGVCTDE
IGFBP-5.1       PGCG-------CCLTCALSEGQACGVYTGTCTHGLRCLPRNGEEKPLHALLHGRGVCTNE
IGFBP-5.2       PGCG-------CCLTCALPEGQSCGVYTGTCTHGLRCLPKNGEEKPLHALLHGRGVCANE
IGFBP-6         PGRGPRDHIGQAGGTSMLAQGEPCGVYTMNCAKGLRCVPLPQEHSPLQALLQGRGFCTKH
                .. *       .  ..   :*  ****:  *  **:* *      **. * :* . * ..

IGFBP-1         QGQEEVEGVPDHSSLTYF----------------LGL-----N-------TPFDTKNEGA
IGFBP-2.1       VVTEPTGSQEHREQFSGEVVDLLDT----SLTEIPPVRKATKD-------NPWLGPKENA
IGFBP-2.2       VDPVPNR-----------TQEHQGTSEELQGTEGPTQKKLTKD------VRIWILSKDQA
IGFBP-4         RDVEENS-----------ALDRQDEIIP-EHPNNSNIRCSPQD-------KRCI---QKT
IGFBP-5.1       KGYKPLHPPIDHE-----SLEHEDT----LTTEITEDQLQPAKVPLLPKQDLINSKKIQA
IGFBP-5.2       KMYRPLYPGRDGY-----S--PEEA----MLAEVPKSLLPQAKVPLYGGRDHISSRKAQA
IGFBP-6         SRTSPTERP---------------------------------------------------

                                                *
IGFBP-1         QESI--KAKVNTIRKK---------------LVEQGPCHIELHAALDKITSS----QQEL
IGFBP-2.1       MRQH--RQEMKTKMKSNK-PEDPKTP-----RGKQIQCQQELDQVLERISKMPFRDNRGP
IGFBP-2.2       IRQAQN--ELKTKMKNNNCAEEPKT-----QEPMKGPCKQELEKVLEEISKMSFHDNRGP
IGFBP-4         LARH--PAKSTNQRSNNA-REDPKA--------ALAPCRAELQRALDRLVS-----NTRT
IGFBP-5.1       MRKDKDRKRAQAKLRSIG-PMDYSPLPIDKHEPEFGPCRRKLDGIIQGMKDTS----RVM
IGFBP-5.2       MRQAKDRKRQQAKLHSVS-SLDYSTLALDKLQPEFGPCRRKLDGIIQRIKNTS----RVL
IGFBP-6         --------HPTGPH------------PSHSGEIEKAPCRKLLNSVLRGVELTIFLSD---
                        .                          *:  *.  :  :
                          *          *          * *
IGFBP-1         GEKFTNFYLPNCDKHGFYKAKQCESSLVGPPARCWCVFSWNGKKIPGSNYLLGGLECQLE
IGFBP-2.1       LEDLYALHIPNCDKRGQYNLKQCKMSLHGQRGECWCVNPHTGRPIPSAPTVRGDPNCSQY
IGFBP-2.2       VENLYQLKFPNCEKIGQYNLKQCHISTHGQRGECWCVNPFTGVQIAQSTKVRGDPNCSQY
IGFBP-4         HEDLYSIPIPNCDKNGDFHAKQCQPARDGQRGKCWCVDQKTGMKLPGPLELRGELDCHQL
IGFBP-5.1       A---LSLYLPNCDRKGFFKRKQCKPSRGRKRGICWCVDKY-GSQVPGTDYSGGDIQCKDL
IGFBP-5.2       A---LSLYLPNCDKKGFFKHKQCKPSRGRKRGICWCVDRF-GVQLPGTDYSGVDIQCKDP
IGFBP-6         ----RDIYIPNCDTLGFYRKKQCRSSKGIQRGLCWCVDEL-GTALSSRASEDGTLPCDGD
                      : :***:  * :. ***. :     . ****    *  :.            *

IGFBP-1         L
IGFBP-2.1       LRGPEMDT-------LASAQK
IGFBP-2.2       VEEQEMETGTQSTTILQMAEI
IGFBP-4         MTATMRE
IGFBP-5.1       ESSSNNE
IGFBP-5.2       ESNSNKN
IGFBP-6
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Fig. 1. Amino acid alignment for insulin-like growth factor binding proteins (IGFBP) 1–6. Conserved cysteine residues are indicated by a bold asterisk above the alignment. The CWCV and GCGCCXXC, RGD and heparin binding motifs are highlighted in bold text. The predicted nuclear localisation signal for IGFBP-5.1 is underlined. The asterisks below the alignment indicate completely conserved residues; **:**, indicates highly conserved residues; **.**, indicates less conserved residues.

estimated molecular mass of 27.6 kDa and a pI of 7.35, and contains 18 cysteine residues and an 18 amino acid signal peptide. This sequence, which has 56.3% identity with human IGFBP-7, contains a putative *N*-glycosylation site at position 154 and has predicted phosphorylation sites at 11 serine, eight threonine and eight tyrosine residues (Fig. 1).

Growth model

Prior to the first sample being taken, fish were fed a maintenance diet for 21 days such that net growth relative to day 0 was zero or slightly negative as shown in Fig. 2 (TGC=–0.26±0.4, mean ± s.d., *N*=10). After feeding, early TGC calculations are unreliable as the food present in the gut will give a false indication of growth. Comparing growth rates of the fish at later time points provides a more reliable indicator of growth. At 3 and 5 days, there was an increase in mass of 41.8±14.1 g and 66.5±15.4 g, respectively, contributed by food in the gut. At 7 days, there was an increase in mass of 106.5±25.6 g, which was probably due to the presence of food in the gut and the presence of digested food in the bowel, with a small amount due to growth. If we subtract the 106.5 g contributed by food and digested food observed for 7 days from the 14, 30 and

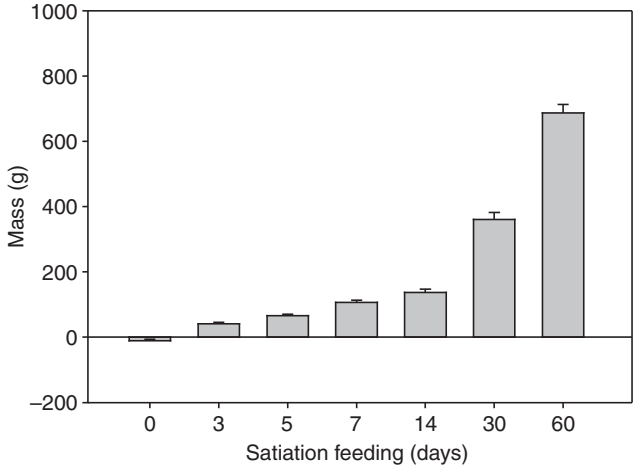

Fig. 2. Change in body mass over the time course of the experiment. Fish were fed a maintenance diet for 22 days to achieve zero or slightly negative growth rate, and then fed to satiation with a commercial fish feed (EWOS Innovation) to stimulate rapid growth. Values represent means ± s.e.m., *N*=10 per sample point.

60 day values, we can obtain a better indication of the growth that has occurred over this period. Therefore at 14, 30 and 60 days, fish had accumulated 43.5±24.5, 245.1±80.2 and 580.4±113.2 g (mean ± s.d.) in mass, respectively, all of which can be attributed to growth. These values are equivalent to average TGC values of 1.4, 3.4 and 3.6 for 14, 30 and 60 days, respectively. It is assumed that fish sampled at day 60 represent steady-state fast growth for this diet. If the mass of food present in the gut is not considered, then the fish would have TGCs for days 14, 30 and 60 of 4.4, 4.9 and 4.2, respectively.

Effect of nutritional status on IGF-system genes

IGF-I and IGF-II expression

Expression of IGF-I was modulated by the nutritional status of the fish, with IGF-I expression increasing in response to feeding. Significant upregulation of IGF-I mRNA occurred at 3, 5, 14 ($P<0.05$), 30 and 60 days ($P<0.01$) following the switch to satiation feeding. There appear to be two peaks in IGF-I expression with the first occurring at 3 and 5 days, and the second occurring at 14 and 30 days (Fig. 3).

Expression of IGF-II was significantly reduced in response to feeding at all time points ($P<0.05$; days 5, 7, 14 and 60, $P<0.01$) with the greatest decrease observed at 7 days followed by a gradual increase with continued feeding (Fig. 3).

IGF receptors

In common with zebrafish, the Atlantic salmon genome contains two IGF-I receptors, designated IGFR1a and IGFR1b. Expression of IGFR1a was downregulated at all time points in response to feeding (Fig. 3), with significant downregulation observed at 3, 14, 30 and 60 days ($P<0.05$). Expression of IGFR1a was negatively correlated with IGF-I expression ($r^2=0.72$, $P=0.016$). In contrast, the expression of IGFR1b was not significantly changed in response to feeding (Fig. 3).

Expression levels of the IGF-II receptor IGF2R were significantly decreased in response to feeding from 3 days ($P<0.05$); the lowest expression was observed at 7 days followed by a gradual increase with continual feeding, although values remained below those at 0 days (Fig. 3). Expression of IGF2R was positively correlated with IGF-II expression ($r^2=0.73$, $P=0.015$).

IGF binding proteins

IGFBP-1

IGFBP-1 expression was not detected in fast skeletal muscle, but was found in liver (data not shown).

IGFBP-2

IGFBP-2 paralogue 1 expression was significantly downregulated at 7, 14 and 30 days ($P<0.05$) with the lowest level of expression

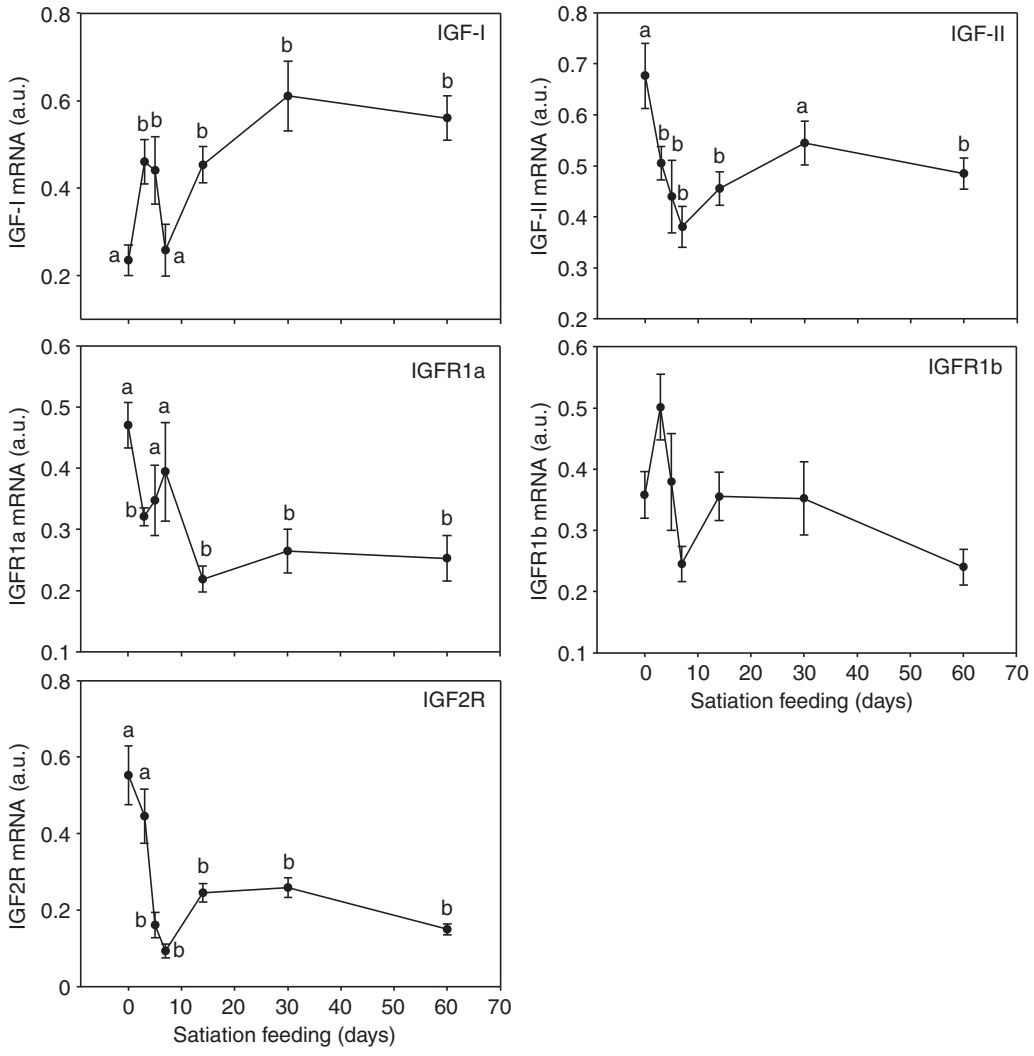

Fig. 3. Expression profiles for IGF-I, IGF-II, and IGF receptors IGFR1a, IGFR1b and IGF2R in fast skeletal muscle of Atlantic salmon from fish with zero growth rate (0 days) that were then fed to satiation and sampled at 3, 5, 7, 14, 30 and 60 days. Gene expression was normalised to the geometric average of three reference genes (Genorm analysis); see text for details. Values represent means ± s.e.m., 10 fish per sample point. Significant differences between means are indicated by different letters.

obtained at 14 days and remaining at that level with continued feeding. Expression of IGFBP-2 paralogue 2 was significantly downregulated at 5 and 60 days in response to feeding, with expression at all other time points close to 0 day values (Fig. 4).

IGFBP-4

Expression of IGFBP-4 was significantly upregulated (P<0.001) at all time points after feeding (Fig. 4) and was positively correlated with IGF-I expression (r^2=0.62, P=0.036).

IGFBP-5

Of the two IGFBP-5 paralogues, only expression of paralogue 2 was significantly modulated. Paralogue 2 was significantly upregulated at 3 days (P<0.001) and downregulated at 7 days (P<0.01; Fig. 4).

IGFBP-6

IGFBP-6 expression was significantly downregulated at 5 days (P<0.01), but then increased with continued feeding to 0 day levels (Fig. 4).

IGFBP-related protein 1

Expression of IGFBP-rP1 was significantly downregulated at 7 days (P<0.05; Fig. 4), and was highly correlated with MLC2 gene expression (r^2=0.93, P=0.0001).

Muscle-specific genes

The expression profiles of myogenin, myosin light chain and myosin heavy chain were measured as markers of myogenesis. Myogenin is one of the four myogenic transcriptional regulatory

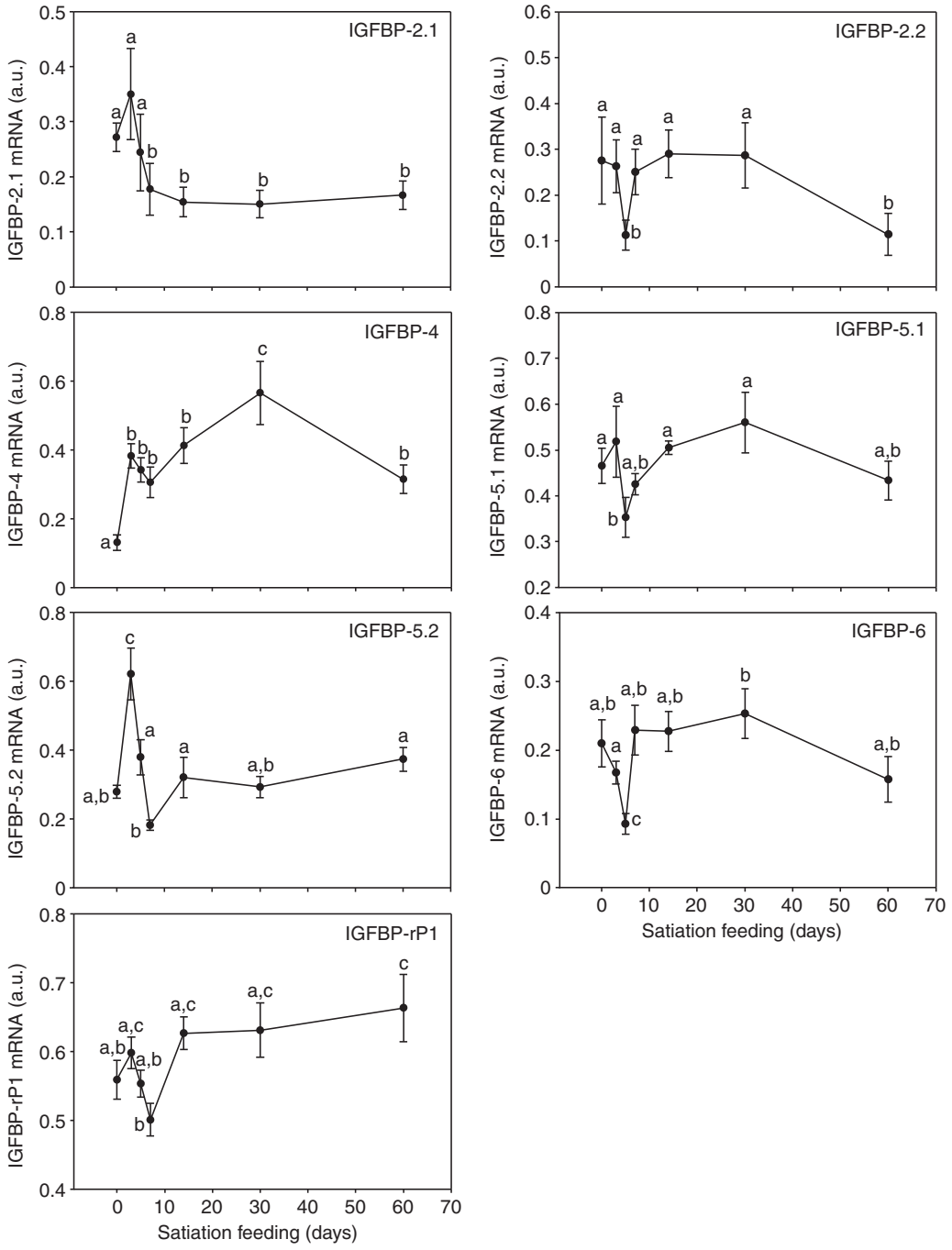

Fig. 4. Expression profiles for IGFBPs 2–6 and IGFBP-related protein 1 (IGFBP-rP1) in fast muscle of Atlantic salmon from fish with zero growth rate (0 days) that were then fed to satiation at days 3, 5, 7, 14, 30 and 60. Gene expression was normalised to the geometric average of three reference genes (Genorm analysis); see text for details. Values represent means ± s.e.m., 10 fish per sample point. Significant differences between means are indicated by different letters.

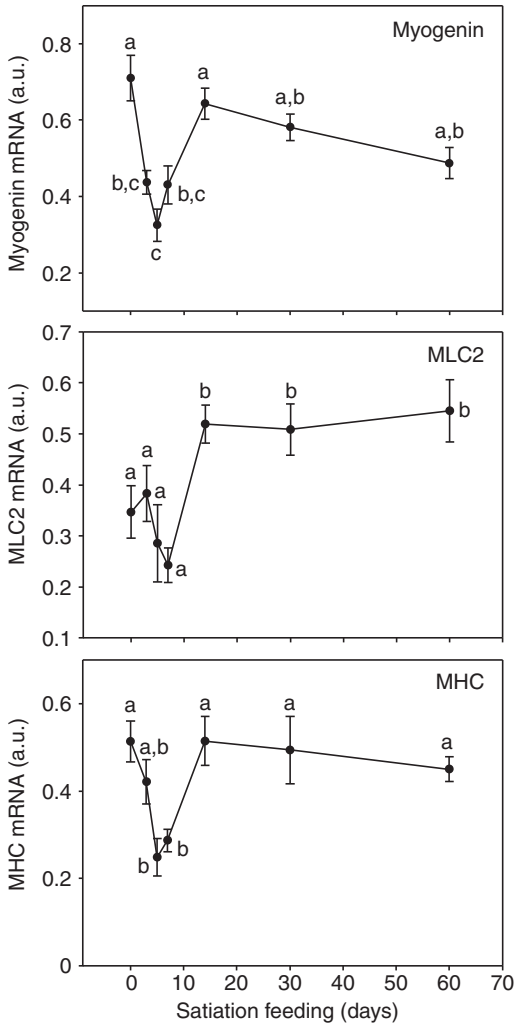

Fig. 5. Expression profiles for myogenin, MHC and MLC2 in fast muscle of Atlantic salmon from fish with zero growth rate (0 days) that were then fed to satiation at 3, 5, 7, 14, 30 and 60 days. Gene expression was normalised to the geometric average of three reference genes (Genorm analysis); see text for details. Values represent means ± s.e.m., 10 fish per sample point. Significant differences between means are indicated by different letters.

factors that function to initiate and stabilise the myogenic differentiation programme. MHC and MLC2 are polypeptide components of myosin, the most abundant contractile protein. Expression of MHC was significantly downregulated at 5 and 7 days (P<0.01), with expression increasing for 14, 30 and 60 days to levels similar to that at 0 days (Fig. 5). Myogenin expression was positively correlated with MHC expression (r^2=0.79, P=0.007; Fig. 6F) with significantly decreased expression observed at 3, 5 and 7 days, and an increased expression at 14, 30 and 60 days (significant relative to 3, 5 and 7 days, P<0.05, but still less than that at 0 days; Fig. 5). Expression of MLC2 was significantly increased at 14, 30 and 60 days (P<0.05) relative to that at the start of feeding (0 days; Fig. 5).

DISCUSSION

IGFBP characterisation

We report here for the first time the nucleotide sequence for the IGFBPs from *Atlantic salmon* including paralogues for IGFBPs 2 and 5. Similar to the IGFBPs from mammals, the Atlantic salmon proteins contain cysteine-rich N- and C-terminal domains, a hinge domain lacking in cysteine residues and a characteristic thyroglobulin type I motif in the C-terminus (lacking in the IGFBP-rP1). The GCGCCXXC 'IGFBP' motif, characteristic of all mammalian IGFBPs apart from IGFBP-6, is present in all of the Atlantic salmon IGFBPs with the exception of IGFBP-6 and IGFBP-rP1. The CVCW motif, which is clearly implicated in IGF-I binding (Baxter, 2000), is present in all the Atlantic salmon sequences, with the exception of IGFBP-rP1. It is likely that the IGFBP-rP1 in Atlantic salmon, in common with that in mammals, has only weak affinity for IGF-I.

IGFBPs have a higher affinity for IGFs than do the IGF receptors responsible for IGF-mediated signalling. For IGFs to bind their receptors, the IGF needs to be released from the binding protein (Clemmons, 1998). Binding of IGFBPs to various proteins has been shown to cause a decrease in their affinity for IGF-I; for example, binding of IGFBP-5 to the ECM results in an 8-fold reduction in its affinity for IGF-I, and may be required to potentiate the actions of IGF-I (Clemmons, 1998). Both Atlantic salmon IGFBP-2 paralogues contain RGD motifs, implicated in binding integrins, while both IGFBP-5 paralogues and IGFBP-6 contain a heparin binding motif implicated in binding to glycosaminoglycan side-chains of proteins of the ECM and cell surfaces. IGFBP-4 does not contain any putative binding motifs, and like its mammalian orthologue is likely to require proteolytic cleavage to potentiate the effects of IGF-I.

IGF signalling during feeding

This study reports the expression profiles for components of the IGF system during a transition from zero growth to fast growth in Atlantic salmon over a period of 60 days. Examination of the expression patterns of genes involved in the IGF signalling pathway can enable identification of the key components regulating skeletal muscle growth in Atlantic salmon.

In order to examine the modulation of the IGF system during muscle growth, fish were fed a restricted diet for a period of 21 days, to achieve zero or slightly negative growth rate prior to feeding, and then fed to satiation. Glencross has discussed the advantages of a restricted feeding model to better detect nutritional effects (Glencross et al., 2003). Increased mass (calculated with food in the gut subtracted) for 14 (43.5 g), 30 (254 g) and 60 days (580 g) can be considered to be due to growth. The TGCs (calculated following adjustment for the mass of food in the gut) for fish at 14, 30 and 60 days were 1.4, 3.6 and 3.4, indicating that maximum growth rates are achieved between 14 and 30 days post-feeding.

Fish switching between zero and fast growth showed increased expression of IGF-I in fast skeletal muscle as previously reported (Gabillard et al., 2006; Chauvigne et al., 2003). The response to feeding in muscle was observed as early as 3 days after feeding, with expression levels exceeding that at day zero with continued feeding. During the transition from zero to fast growth, there were two peaks in IGF-I expression, the first at 3 and 5 days, and the second at 14 and 30 days. This second peak at 14 and 30 days in IGF-I expression occurred at the same time as the peak in MLC2, MHC and myogenin expression, which has also been observed in refeeding experiments using trout (Gabillard et al., 2006; Chauvigne et al., 2003). Musaro and Rosenthal suggest that IGF-I is involved in post-mitotic growth and is required for maturation of the myogenic programme (Musaro and Rosenthal, 1999). The second peak in IGF-I expression may represent a marker for the resumption of myogenesis as indicated by the corresponding peak in MLC2, MHC and myogenin expression.

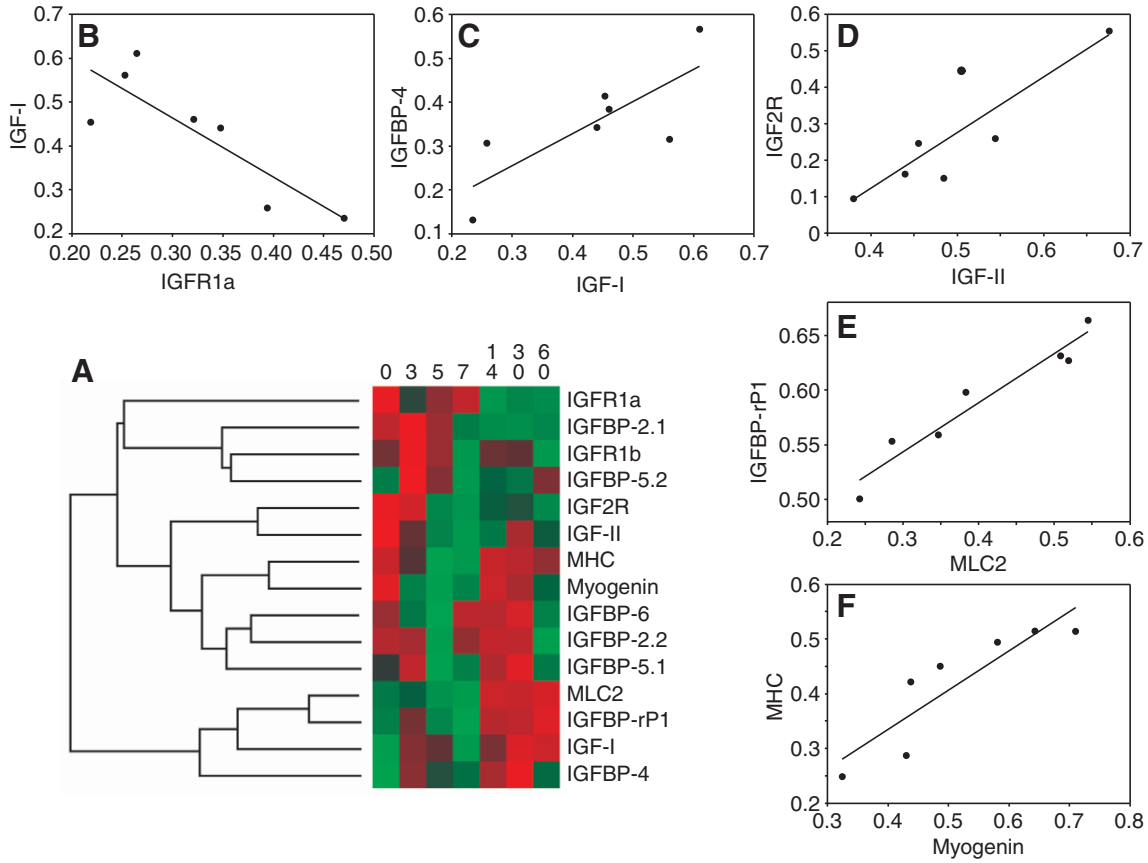

Fig. 6. Heat map summary and hierarchical clustering for components of the IGF signalling pathway during the transition from zero growth (day 0) to fast growth at 3 to 60 days (A). Rows are standardised to have a mean of 0 and s.d. of 1 so that red indicates high and green indicates low values. Regression analysis (10 fish per sample point) showing negative correlation (B) between IGF-I and IGFR1a (r^2=–0.72, P=0.016), and positive correlation (C) between IGF-I and IGFBP-4 (r^2=0.62, P=0.036), (D) IGF-II and IGF2R (r^2=0.72, P=0.015), (E) MLC2 and IGFBP-rP1 (r^2=0.93, P=0.0001) and (F) MHC and myogenin (r^2=0.79, P=0.007).

Expression of the muscle-specific genes myogenin and MHC initially decreased in response to feeding and values were lower at 60 days than at 0 days. If we consider the fish sampled at 60 days to represent steady-state fast growth for this diet, it can be postulated that expression had increased during feed restriction. Montserrat and colleagues found that expression of myogenin increased after 4 weeks of fasting (Montserrat et al., 2007). They suggest that in this situation, myogenin may play a role in muscle maintenance. Similar findings of decreased expression of MHC in response to refeeding have been reported for rainbow trout (*Oncorhynchus mykiss*) (Johansen and Overturf, 2006). Svanberg and colleagues observed that MHC mRNA expression was elevated during starvation and reduced after feeding in human skeletal muscle, suggesting that the increased expression of myosin during periods of starvation facilitates rapid recovery of myofibrillar protein with feeding (Svanberg et al., 2000). It should be noted that, in the current experiment, the primers used to amplify MHC were designed to a conserved region of the gene and could potentially amplify multiple isoforms of MHC. An increase in expression of MHC, MLC2 and myogenin relative to that at 3, 5 and 7 days was observed from day 14 onwards, suggesting that from 14 days, late differentiation of the myogenic programme is occurring. These increases in expression at 14 days onwards correspond to gains in body mass (Fig. 2).

IGF-II levels were decreased in response to feeding as reported in rainbow trout (Montserrat et al., 2007). Chauvigne and colleagues found that expression of IGF-II in trout fast muscle increased during refeeding after a period of starvation (Chauvigne et al., 2003) and Hevrøy and colleagues found increased IGF-II expression in Atlantic salmon fed increased lysine (Hevrøy et al., 2006). Differences in the response observed could be due to differences in developmental stage and/or the different treatments prior to feeding. The downregulation of IGF-II in response to feeding was associated with a downregulation in IGF-II receptor (IGF2R) expression. Since the function of this receptor is yet to be identified, the significance of this finding is unknown. IGF2R has been reported to target ligands for degradation *via* the lysosomal pathway, indicating that a downregulation of this receptor could increase the availability of IGF-II to bind with the IGF-I receptors and could constitute a further level of regulation mediating cross-talk between different signalling cascades. It is interesting to note that the expression of IGF-II and IGF2R was correlated, suggesting that these genes are co-regulated or that some form of feedback regulation occurs for this pathway, which could stabilise IGF-II protein levels. Analysis of IGF-II protein levels by western blotting in future experiments could be used to determine whether the decrease in expression of the receptor leads to an increase in IGF-II levels.

In response to feeding, IGFR1a expression was significantly decreased. Similar observations have been reported in trout for IGFR1a (Chauvigne et al., 2003) and IGFR1b (Montserrat et al., 2007), and have also been reported in mammals, where the IGFR

expression increased during fasting in rat muscle (Lowe et al., 1989). This suggests that during periods of low food intake, increased sensitivity to IGF-I in muscle is achieved through increasing the abundance of IGF-I receptors. Expression of IGFR1a was negatively correlated with IGF-I expression, suggesting that in Atlantic salmon a similar situation occurs to that in mammals, where IGF-I has been shown to decrease IGF-I receptor mRNA levels in a muscle cell line (Hernandez-Sanchez et al., 1997). The change in expression in response to feeding observed for IGFR1a was not seen with IGFR1b. These differences in expression suggest that these two genes have evolved distinct *cis*-regulatory elements with only IGFR1a being responsive to nutrition.

Expression of the IGFBPs was examined in response to nutrition, with the expression of several IGFBPs found to be modulated by feeding. IGFBP-related protein 1 has recently been cloned in rainbow trout (Kamangar et al., 2006), and has been reported to be differentially regulated in response to feeding (Gabillard et al., 2006) with expression correlated with that of IGF-I. In contrast, the Atlantic salmon orthologue did not show any differential expression in response to feeding apart from a transient downregulation at 7 days but, intriguingly, its expression was highly correlated with that of MLC2, suggesting some level of co-regulation. Considering the overall lack of response to feeding, along with the absence of the CWCV motif required for high affinity IGF-I binding, it appears unlikely that IGFBP-related protein 1 participates in IGF-I signalling.

Similar to previous findings (Gabillard et al., 2006), IGFBP-1 expression was not detected in muscle but was expressed in liver (data not shown). IGFBP-2 paralogue 1 levels were significantly downregulated from days 14 to 60. IGFBP-2 has been demonstrated to inhibit IGF-I-stimulated cell proliferation and DNA synthesis, and is considered to be a negative regulator of growth (Duan et al., 1999). In zebrafish, expression increased during starvation and was reduced by growth hormone treatment (Duan et al., 1999). The downregulation of IGFBP-2.1 during the period of rapid growth from 14 days onwards in the present study is likely to be the result of an increased availability of IGF-I to the IGF-I receptor.

IGFBP-2 paralogue 2 is the orthologue of the previously misnamed rainbow trout IGFBP-3 (Kamangar et al., 2006) as also suggested by Rodgers and colleagues (Rodgers et al., 2008). Based on the low amino acid sequence homology between IGFBP-2 paralogues 1 and 2, and preliminary phylogenetic analysis (D. J. Macqueen, personal communication), it is likely that these two sequences diverged early after the teleost whole genome duplication. Consistent with this, the expression patterns of the two genes were quite dissimilar, with paralogue 2 not responding to feeding, suggesting that expression of IGFBP-2 paralogue 2 does not contribute to muscle growth regulation in Atlantic salmon.

It is interesting that of all the IGFBPs examined, only IGFBP-4 expression was constitutively upregulated following feeding. In mammals, IGFBP-4 is thought to inhibit the mitogenic properties of IGF-I, although this has yet to be confirmed in fish. Considering the overall homology of 55% with mammalian IGFBP-4, it is quite possible that the fish protein possesses some other biological activity. Expression of IGFBP-4 was correlated with that of IGF-I, and IGFBP-4 expression was the highest of all the binding proteins measured in fast muscle. IGFBP-4 is also known to be highly expressed in mammalian connective tissue (Boes et al., 1992; Jennische and Hall, 2000). Knudtson and colleagues reported that a C-terminal basic region of 20 amino acids was required for targeting the rat IGFBP-4 to connective tissue (Knudtson et al., 2001). There is 75% identity between the rat and salmon sequences in this 20 amino acid region (CDKNGDFHAKQCQPARDGQR), suggesting that in salmonids IGFBP-4 is also targeted to connective tissue. Although IGFBP-4 may be targeted to the connective tissue, its contribution to muscle growth should not be overlooked. Muscle is a complex tissue made up of several cell types that must be co-ordinately regulated during growth. The ECM (connective tissue) has been found to control the development and cellular metabolism of muscle fibres (Fisher and Rathgaber, 2006). The importance of the ECM in regulating muscle growth and differentiation has been highlighted. For example expression of myogenin alone was not sufficient for the formation of skeletal muscle, with ECM being required to allow complete differentiation of cultured skeletal muscle cells (Osses and Brandan, 2002; Massague et al., 1986). Contained within the ECM is the basement membrane, which appears to play a role as a guide for newly forming myotubes (Fisher and Rathgaber, 2006). Further analysis of IGFBP-4 expression in fish species should elucidate its tissue localisation as well as growth inhibitory/promotion properties. Crucially, the expression of the IGFBP-4 protease has not been examined, and has yet to be identified in salmonids. This protease has been found to be co-regulated with IGFBP-4 expression in rat, thereby allowing the targeted release of IGF-I for binding to the IGF-I receptor (Smith et al., 2001).

IGFBP-5 has been shown to stimulate mitogenesis in other species, although it is worth noting that in this experiment it was the IGFBP-5.2 paralogue that was upregulated in response to feeding and this protein lacks a nuclear localisation signal and DNA binding motif. Interestingly, the ligand-independent actions of IGFBP-5 have been reported to arise from the C-terminal fragment of the peptide. Given that the DNA binding motif is located in the C-terminus of IGFBP-5.1, it seems unlikely that the C-terminus of paralogue 2 possesses mitogenic activity. The salmonid IGFBP-5 paralogues, like their mammalian counterparts, contain a heparin binding motif. Heparin binding motifs are present in proteins that bind to glycosaminoglycan side-chains of many cell surface and ECM proteins. In mammals, to potentiate the actions of IGF-I, it is necessary for IGFBP-5 to bind to the ECM (Clemmons, 1998). Like IGFBP-4, salmonid IGFBP-5 could also target IGF-I to the ECM, suggesting that regulation of the ECM could be a key component in the resumption of muscle growth. The difference in expression patterns between paralogues 1 and 2 suggests that these genes have subfunctionalised, with paralogue 2 retaining *cis*-regulatory elements responsible for regulation by nutrition in muscle.

IGFBP-6 expression was significantly downregulated at day 5. IGFBP-6 in mammals has a low affinity for IGF-I, with a 20- to 100-fold binding preference for IGF-II, leading to the assumption that it is a regulator of IGF-II (Bach, 2005). IGFBP-6 has been found to inhibit the action of IGF-I (Duan and Xu, 2005), and has also been found to inhibit cell proliferation (Bach, 2005). After 5 days, IGFBP-6 returns to levels observed at day zero.

In conclusion, as well as characterising components of the IGF signalling system, namely IGF-I, IGF-II, IGFBPs 1, 2, 4, 5 and 6, and IGFBP-rP1, from Atlantic salmon, this paper reports the characterisation of two IGFBP paralogues. We have demonstrated that the IGFBPs and their paralogues are differentially regulated with nutritional status, highlighting the need to identify and characterise gene paralogues in salmonids. Our results suggest that during times of nutrient restriction, sensitivity to IGF-I in muscle is increased through the increased abundance of IGFR1a. We have shown that the transition from zero to fast growth is marked by a constitutive upregulation of IGF-I and IGFBP-4, with constitutive

downregulation of IGFBP-2.1. It is plausible that upregulation of IGFBP-4 targets IGF-I to the ECM, constituting a necessary step for muscle growth, and this hypothesis is worth further investigation.

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REFERENCES

Allendorf, F. W. and Thogaard, G. H. (1984). Tetraploidy and evolution of salmonids fishes. In *Evolutionary Genetics of Fishes* (ed. B. J. Turner), pp. 1-53. New York: Plenum.

Amores, A., Force, A., Yan, Y. L., Joly, L., Amemiya, C., Fritz, A., Ho, R. K., Langeland, J., Prince, V., Wang, Y. L. et al. (1998). Zebrafish hox clusters and vertebrate genome evolution. *Science* **282**, 1711-1714.

Andress, D. L. and Birnbaum, R. S. (1992). Human osteoblast-derived insulin-like growth-factor (Igf) binding Protein-5 stimulates osteoblast mitogenesis and potentiates Igf action. *J. Biol. Chem.* **267**, 22467-22472.

Andress, D. L., Loop, S. M., Zapf, J. and Kiefer, M. C. (1993). Carboxy-truncated insulin-like growth-factor binding Protein-5 stimulates mitogenesis in osteoblast-like cells. *Biochem. Biophys. Res. Commun.* **195**, 25-30.

Awede, B., Thissen, J. P., Gailly, P. and Lebacq, J. (1999). Regulation of IGF-I, IGFBP-4 and IGFBP-5 gene expression by loading in mouse skeletal muscle. *FEBS Lett.* **461**, 263-267.

Awede, B. L., Thissen, J.-P. and Lebacq, J. (2002). Role of IGF-I and IGFBPs in the changes of mass and phenotype induced in rat soleus muscle by clenbuterol. *Am. J. Physiol. Endocrinol. Metab.* **282**, E31-37.

Bach, L. A. (2005). IGFBP-6 five years on: not so 'forgotten'? *Growth Horm. IGF Res.* **15**, 185-192.

Bailey, G. S., Poulter, R. T. M. and Stockwell, P. A. (1978). Gene duplication in tetraploid fish – model for gene silencing at unlinked duplicated loci. *Proc. Natl. Acad. Sci. USA.* **75**, 5575-5579.

Baker, J., Liu, J. P., Robertson, E. J. and Efstratiadis, A. (1993). Role of insulin-like growth-factors in embryonic and postnatal-growth. *Cell* **75**, 73-82.

Baxter, R. C. (2000). Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. *Am. J. Physiol. Endocrinol. Metab.* **278**, E967-E976.

Boes, M., Booth, B. A., Sandra, A., Dake, B. L., Bergold, A. and Bar, R. S. (1992). Insulin-like growth-factor binding-protein (Igfbp)4 accounts for the connective-tissue distribution of endothelial-cell Igfbps perfused through the isolated heart. *Endocrinology* **131**, 327-330.

Boker, C., vonFigura, K. and HilleRehfeld, A. (1997). The carboxy-terminal peptides of 46 kDa and 300 kDa mannose 6-phosphate receptors share partial sequence homology and contain information for sorting in the early endosomal pathway. *J. Cell Sci.* **110**, 1023-1032.

Caraux, G. and Pinloche, S. (2005). PermutMatrix: a graphical environment to arrange gene expression profiles in optimal linear order. *Bioinformatics* **21**, 1280-1281.

Chauvigne, F., Gabillard, J. C., Weil, C. and Rescan, P. Y. (2003). Effect of refeeding on IGFI, IGFII, IGF receptors, FGF2, FGF6, and myostatin mRNA expression in rainbow trout myotomal muscle. *Gen. Comp. Endocrinol.* **132**, 209-215.

Chu, C. H., Tzang, B. S., Chen, L. M., Kuo, C. H., Cheng, Y. C., Chen, L. Y., Tsai, F. J., Tsai, C. H., Kuo, W. W. and Huang, C. Y. (2008). IGF-II/mannose-6-phosphate receptor signaling induced cell hypertrophy and atrial natriuretic peptide/BNP expression via G alpha q interaction and protein kinase C-alpha/CaMKII activation in H9c2 cardiomyoblast cells. *J. Endocrinol.* **197**, 381-390.

Clemmons, D. R. (1998). Role of insulin-like growth factor binding proteins in controlling IGF actions. *Mol. Cell Endocrinol.* **140**, 19-24.

Clemmons, D. R. (2001). Use of mutagenesis to probe IGF-binding protein structure/function relationships. *Endocr. Rev.* **22**, 800-817.

Coolican, S. A., Samuel, D. S., Ewton, D. Z., McWade, F. J. and Florini, J. R. (1997). The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. *J. Biol. Chem.* **272**, 6653-6662.

Duan, C. (2002). Specifying the cellular responses to IGF signals: roles of IGF-binding proteins. *J. Endocrinol.* **175**, 41-54.

Duan, C. M. and Xu, Q. J. (2005). Roles of insulin-like growth factor (IGF) binding proteins in regulating IGF actions. *Gen. Comp. Endocrinol.* **142**, 44-52.

Duan, C. M., Ding, J., Li, Q., Tsai, W. and Pozios, K. (1999). Insulin-like growth factor binding protein 2 is a growth inhibitory protein conserved in zebrafish. *Proc. Natl. Acad. Sci. USA* **96**, 15274-15279.

Duan, C., Bauchat, J. R. and Hsieh, T. (2000). Phosphatidylinositol 3-kinase is required for insulin-like growth factor-I-induced vascular smooth muscle cell proliferation and migration. *Circ. Res.* **86**, 15-23.

Engert, J. C., Berglund, E. B. and Rosenthal, N. (1996). Proliferation precedes differentiation in IGF-I-stimulated myogenesis. *J. Cell Biol.* **135**, 431-440.

Erbay, E., Park, I. H., Nuzzi, P. D., Schoenherr, C. J. and Chen, J. (2003). IGF-II transcription in skeletal myogenesis is controlled by mTOR and nutrients. *J. Cell. Biol.* **163**, 931-936.

Firth, S. M. and Baxter, R. C. (2002). Cellular actions of the insulin-like growth factor binding proteins. *Endocr. Rev.* **23**, 824-854.

Fisher, B. D. and Rathgaber, M. (2006). An overview of muscle regeneration following acute injury. *J. Phys. Ther. Sci.* **18**, 57-66.

Gabillard, J. C., Kamangar, B. B. and Montserrat, N. (2006). Coordinated regulation of the GH/IGF system genes during refeeding in rainbow trout (Oncorhynchus mykiss). *J Endocrinol.* **191**, 15-24.

Glencross, B., Curnow, J., Hawkins, W., Kissil, G. W. M. and Peterson, D. (2003). Evaluation of the feed value of a transgenic strain of the narrow-leaf lupin (*Lupinus angustifolius*) in the diet of the marine fish, *Pargus auratus*. *Aquac. Nutr.* **9**, 197-206.

Hernandez-Sanchez, C., Werner, H., Roberts, C. T., Woo, E. J., Hum, D. W., Rosenthal, S. M. and LeRoith, D. (1997). Differential regulation of insulin-like growth factor-I (IGF-I) receptor gene expression by IGF-I and basic fibroblastic growth factor. *J. Biol. Chem.* **272**, 4663-4670.

Hevrøy, E. M., El-Mowafi, A., Taylor, R. G., Olsvik, P. A., Norberg, B. and Espe, M. (2006). Lysine intake affects gene expression of anabolic hormones in Atlantic salmon (*Salmo salar*). *Gen. Comp. Endocrinol.* **152**, 39-46.

Jaillon, O., Aury, J. M., Brunet, F., Petit, J. L., Stange-Thomann, N., Mauceli, E., Bouneau, L., Fischer, C., Ozouf-Costaz, C., Bernot, A. et al. (2004). Genome duplication in the teleost fish Tetraodon nigroviridis reveals the early vertebrate proto-karyotype. *Nature* **431**, 946-957.

Jennische, E. and Hall, C. M. (2000). Expression and localisation of IGF-binding protein mRNAs in regenerating rat skeletal muscle. *APMIS* **108**, 747-755.

Johansen, K. A. and Overturf, K. (2006). Alterations in expression of genes associated with muscle metabolism and growth during nutritional restriction and refeeding in rainbow trout. *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.* **144**, 119-127.

Johnston, I. A. (2006). Environment and plasticity of myogenesis in teleost fish. *J. Exp. Biol.* **209**, 2249-2264.

Johnston, I. A. and Battram, J. (1993). Feeding energetics and metabolism in demersal fish species from Antartctica, temperate and tropical environments. *Mar. Biol.* **115**, 7-14.

Jones, J. I. and Clemmons, D. R. (1995). Insulin-like growth-factors and their binding-proteins: biological actions. *Endocr. Rev.* **16**, 3-34.

Jones, J. I., Gockerman, A., Busby, W. H., Camachohubner, C. and Clemmons, D. R. (1993a). Extracellular-matrix contains insulin-like growth-factor binding protein-5: potentiation of the effects of Igf-I. *J. Cell Biol.* **121**, 679-687.

Jones, J. I., Gockerman, A., Busby, W. H., Wright, G. and Clemmons, D. R. (1993b). Insulin-like growth-factor binding Protein-1 stimulates cell-migration and binds to the Alpha-5-Beta-1 integrin by means of its Arg-Gly-Asp sequence. *Proc. Natl. Acad. Sci. USA* **90**, 10553-10557.

Kajimura, S., Aida, K. and Duan, C. M. (2005). Insulin-like growth factor-binding protein-1 (IGFBP-1) mediates hypoxia-induced embryonic growth and developmental retardation. *Proc. Natl. Acad. Sci. USA* **102**, 1240-1245.

Kamangar, B. B., Gabillard, J. C. and Bobe, J. (2006). Insulin-like growth factor-binding protein (IGFBP)-1,-2,-3,-4,-5, and -6 and IGFBP-related protein 1 during rainbow trout postvitellogenesis and oocyte maturation: molecular characterization, expression profiles, and hormonal regulation. *Endocrinology* **147**, 2399-2410.

Knudtson, K. L., Boes, M., Sandra, A., Dake, B. L., Booth, B. A. and Bar, R. S. (2001). Distribution of chimeric IGF binding protein (IGFBP)-3 and IGFBP-4 in the rat heart: importance of C-terminal basic region. *Endocrinology* **142**, 3749-3755.

LeRoith, D., Bondy, C., Yakar, S., Liu, J. and Butler, A. (2001). The somatemedin hypothesis: 2001. *Endocr. Rev.* **22**, 53-74.

Li, Y., Xiang, J. and Duan, C. (2005). Insulin-like growth factor binding protein-3 (IGFBP-3) plays an important role in regulating pharyngeal skeleton inner ear formation and differentiation. *J. Biol. Chem.* **280**, 3613-3620.

Liu, J. P., Baker, J., Perkins, A. S., Robertson, E. J. and Efstratiadis, A. (1993). Mice carrying null mutations of the genes encoding Insulin-Like Growth Factor-I (Igf-1) and Type-1 Igf Receptor (Igf1r). *Cell* **75**, 59-72.

Lowe, W. L., Adamo, M., Werner, H., Roberts, C. T. and Leroith, D. (1989). Regulation by fasting of rat Insulin-Like Growth Factor-I and its receptor-effects on gene-expression and binding. *J. Clin. Invest.* **84**, 619-626.

Macqueen, D. J. and Johnston, I. A. (2008). Evolution of follistatin in teleosts revealed through phylogenetic, genomic and expression analyses. *Dev. Genes Evol.* **218**, 1-14.

Massague, J., Cheifetz, S., Endo, T. and Nadal Ginard, B. (1986). Type β transforming growth factor is an inhibitor of myogenic differentiation. *Proc. Natl. Acad. Sci. USA* **83**, 8206-8210.

Maures, T., Chan, S. J., Xu, B., Sun, H., Ding, J. and Duan, C. M. (2002). Structural, biochemical, and expression analysis of two distinct insulin-like growth factor I receptors and their ligands in zebrafish. *Endocrinology* **143**, 1858-1871.

Miyakoshi, N., Richman, C., Linkhart, T. A., Baylink, D. J. and Mohan, S. (2000). Evidence that IGF binding protein (IGFBP)-5 functions as a growth factor: studies using IGF-I knockout mice. *J. Bone Miner. Res.* **15**, S190-S190.

Montserrat, N., Gabillard, J. C., Capilla, E., Navarro, M. I. and Gutierrez, J. (2007). Role of insulin, insulin-like growth factors, and muscle regulatory factors in the compensatory growth of the trout (Oncorhynchus mykiss). *Gen. Comp. Endocrinol.* **150**, 462-472.

Musaro, A. and Rosenthal, N. (1999). Maturation of the myogenic program is induced by postmitotic expression of insulin-like growth factor I. *Mol. Cell. Biol.* **19**, 3115-3124.

Notredame, C., Higgins, D. G. and Heringa, J. (2000). T-Coffee: a novel method for fast and accurate multiple sequence alignment. *J. Mol. Biol.* **302**, 205-217.

Osses, N. and Brandan, E. (2002). ECM is required for skeletal muscle differentiation independently of muscle regulatory factor expression. *Am. J. Physiol. Cell Physiol.* **282**, C383-C394.

Rodgers, B. D., Roalson, E. H. and Thompson, C. (2008). Phylogenetic analysis of the insulin-like growth factor binding protein (IGFBP) and IGFBP-related protein gene families. *Gen. Comp. Endocrinol.* **155**, 201-207.

Smith, E. P., Kamyar, A., Niu, W., Wang, J., Cercek, B., Chernausek, S. D. and Fagin, J. A. (2001). IGF-binding protein-4 expression and IGF-binding protein-4 protease activity are regulated coordinately in smooth muscle during postnatal development and after vascular injury. *Endocrinology* **142**, 4420-4427.

Stewart, C. E. H. and Rotwein, P. (1996). Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors. *Physiol. Rev.* **76**, 1005-1026.
Svanberg, E., Ennion, S., Isgaard, J. and Goldspink, G. (2000). Postprandial resynthesis of myofibrillar proteins is translationally rather than transcriptionally regulated in human skeletal muscle. *Nutrition* **16**, 42-46.
Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3:RESEARCH0034.
Wood, A. W., Duan, C. M. and Bern, H. A. (2005a). Insulin-like growth factor signaling in fish. *Int. Rev. Cytol.* **243**, 215-285.
Wood, A. W., Schlueter, P. J. and Duan, C. (2005b). Targeted knockdown of insulin-like growth factor binding protein-2 disrupts cardiovascular development in zebrafish embryos. *Mol. Endocrinol* **19**, 1024-1034.
Yamaguchi, A., Fujikawa, T., Shimada, S., Kanbayashi, I., Tateoka, M., Soya, H., Takeda, H., Morita, I., Matsubara, K. and Hirai, T. (2006). Muscle IGF-I Ea, MGF, and myostatin mRNA expressions after compensatory overload in hypophysectomized rats. *Pflugers Arch.* **453**, 203-210.