Dynamic transcriptional regulation of autocrine/paracrine *igfbp1, 2, 3, 4, 5, and 6* in the skeletal muscle of the fine flounder during different nutritional statuses

Diego Safian*, Eduardo N Fuentes*, Juan Antonio Valdés and Alfredo Molina

Laboratorio de Biotecnología Molecular, Departamento de Ciencias Biológicas, Facultad de Biología, Universidad Andres Bello, Avenida Republica 217, 8370146 Santiago, Chile

(Correspondence should be addressed to A Molina; Email: amolina@unab.cl)

*(D Safian and E N Fuentes contributed equally to this work)

Abstract

The IGF-binding proteins (IGFBPs) play a dual role in the regulation of the activity and bioavailability of IGFs in different tissues. Diverse evidence has shown that IGFBPs can inhibit and/or potentiate IGF actions. In this study, *igfbp1, 2, 3, 4, 5, and 6* were isolated in the fine flounder, a flat fish species that shows slow growth and inherent Gh resistance in muscle. Subsequently, the expression of all *igfbps* was assessed in the skeletal muscle of flounder that underwent different nutritional statuses. *igfbp1* was not expressed in muscle during any of the nutritional conditions, whereas *igfbp3* and *igfbp5* were the lowest and the highest *igfbps* expressed respectively. A dynamic expression pattern was found in all the *igfbps* expressed in skeletal muscle, which depended on the nutritional status and sampling period. During the fasting

period, *igfbp2, 4, and 5* were downregulated, whereas *igfbp3* was upregulated during part of the fasting period. The restoration of food modulated the expression of the *igfbps* dynamically, showing significant changes during both the long- and short-term refeeding. *igfbp3* and *igfbp6* were downregulated during short-term refeeding, whereas *igfbp5* was upregulated, and *igfbp2* and *igfbp4* remained stable. During long-term refeeding, the expression of *igfbp2, 4, 5, and 6* increased, while *igfbp3* remained unchanged. In conclusion, this study shows for the first time the isolation of all *igfbps* in a single fish species, in addition to describing a dynamic nutritional and time-dependent response in the expression of *igfbps* in the skeletal muscle of a nonmammalian species.

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Introduction

The insulin-like growth factor (IGF) system regulates somatic growth in vertebrates and is integrated by the IGFs (IGF1 and IGF2), the IGF receptors (IGF1R and IGF2R), and the IGF-binding proteins (IGFBPs; Duan *et al.* 2010). IGFs are mainly synthesized by the liver; however, they can be locally produced in numerous tissues, including skeletal muscle acting in an autocrine/paracrine fashion (Velloso 2008). In particular, muscle-derived IGF1 has been proposed as the main regulator of growth in mammals (Sjögren *et al.* 1999, Yakar *et al.* 1999). As in mammals, some recent evidence has shown that in fish, local Igf1 is of importance in muscle growth (Eppler *et al.* 2007, Fox *et al.* 2010, Fuentes *et al.* 2012a). The dynamic of the biological actions of IGFs is modulated by the binding of these growth factors with either specific membrane receptors and/or the IGFBPs. The IGFBPs are a family of proteins that show high affinity for IGFs and are composed of six members, denominated IGFBP1–6 (Jones & Clemmons 1995). In circulation, the IGFBPs bind to IGFs, transporting and increasing their

half-life by preventing their proteolytic degradation (Hwa *et al.* 1999). The IGFBPs have a higher affinity for IGF than IGF receptors; thus, they are implicated in the modulation of the activity and bioavailability of IGFs in different tissues (Clemmons 1998). Numerous evidences indicate that IGFBPs can inhibit and/or potentiate IGF actions depending on the physiological context (Duan & Xu 2005, Duan *et al.* 2010). IGFBPs can also be synthesized in different cell types and tissues, exerting an important role in the local regulation of IGFs (Jones & Clemmons 1995, Firth & Baxter 2002, Duan *et al.* 2010). Particularly, in skeletal muscle, the *igfbps* are expressed differentially during myogenesis and are implicated in the modulation of muscle growth, showing ligand-dependent and -independent actions (James *et al.* 1993, Ewton & Florini 1995, Bayol *et al.* 2000, Awede *et al.* 2002, Foulstone *et al.* 2003, Ren *et al.* 2008).

In fish, several studies have described the isolation of individual *igfbps* (Duan *et al.* 1999, Maures & Duan 2002, Chen *et al.* 2004, Kamangar *et al.* 2006, Bower *et al.* 2008, Li *et al.* 2009, Pedroso *et al.* 2009a, Peterson & Waldbieser 2009, Wang *et al.* 2009, Dai *et al.* 2010, Rahman & Thomas 2011).

However, until now, the isolation and assessment of the expression patterns of all six *igfbps* in the same species have not been achieved. Previous studies on the biology of Igfbps in fish have shown that Igfbps regulate embryonic development (Duan *et al.* 1999, Kajimura *et al.* 2005, Li *et al.* 2009, Wang *et al.* 2009) and exert ligand-dependent and -independent mechanisms (Dai *et al.* 2010, Zhong *et al.* 2011), as well as showing that Igfbps are modulated by Gh treatment (Duan *et al.* 1999, Cheng *et al.* 2002, Chen *et al.* 2004, 2010, Pedrosa *et al.* 2009b), oxygen availability (Kajimura *et al.* 2005, Kamei *et al.* 2008, Rahman & Thomas 2011, Sun *et al.* 2011), reproduction (Kamangar *et al.* 2006, Chen *et al.* 2010), and nutrition (Gabillard *et al.* 2006, Bower *et al.* 2008, Li *et al.* 2009, Pedrosa *et al.* 2009a, Peterson & Waldbieser 2009, Amaral & Johnston 2011, Macqueen *et al.* 2011). Nevertheless, studies on local muscle-derived *igfbps* (autocrine/paracrine) have been scarce and have recently shown contrasting results, reflecting a complex physiology of Igfbps and suggesting that intricate patterns of regulation have evolved between and within teleost lineages (Gabillard *et al.* 2006, Bower *et al.* 2008, Amaral & Johnston 2011, Macqueen *et al.* 2011).

The fine flounder (*Paralichthys adspersus* (*P. adspersus*)) is an endemic flatfish species of the southern East Pacific that shows a slow growth rate and poor food intake even under favorable nutritional conditions (Fuentes *et al.* 2012a,b). Previously, we have described part of the molecular and endocrine basis accounting for the slow growth of this teleost species, showing that fine flounder present an inherent Gh resistance in skeletal muscle, which results in low basal levels of muscle-derived Igf1 (Fuentes *et al.* 2012a,b). Thus, the low production of autocrine/paracrine Igf1 was the responsible mechanism for growth deficit in this species; however, the regulation of the main modulators of Igf1 availability and actions, such as the Igfbps, remained unknown as did their possible consequences on growth in this species. Thus, use of this intriguing fish model will allow us to go further in the understanding of *igfbps* physiology in fish, particularly in the understanding of local *igfbps* produced in the muscle. In this context, the aim of this work was to assess a detailed time course changes in the expression pattern of all six *igfbps* in skeletal muscle in the fine flounder, evaluating two contrasting nutritional periods, fasting and refeeding (short and long term). In order to achieve this goal, six *igfbps* were previously isolated.

Materials and Methods

Animals, sampling, and experimental design

Three-year-old juvenile fine flounder (*P. adspersus*) with an average weight of 300 ± 10 g and length of 27 ± 2 cm were maintained under natural temperature and photoperiod conditions corresponding to the geographic location of CIMARQ ($33^{\circ}13'S$; $71^{\circ}38'W$) during the spring season

of 2009 in the southern hemisphere. Fish were randomly divided into two tanks and acclimatized for 2 weeks under satiety feeding conditions. At the beginning of the experiment (week 0), food was withheld from one group for 3 weeks (experimental (EXP)), inducing a nutritionally catabolic state of fasting. Then, fish were subjected to a 4-week satiety refeeding period, returning the fish to an anabolic state. The other group (control (CTRL)) was fed until satiety throughout the 7-week study. Samples were obtained weekly over the trial to study long-term regulation and dynamics of the *igfbps* expression in skeletal muscle. At the beginning of the refeeding period, fish were sampled at 2, 4, and 24 h in order to assess short-term changes in the expression of *igfbps*. In order to study the expression of the *igfbps* in different tissues (e.g. tissues from gills, intestine, stomach, spleen, heart, liver, and red and white muscle) of the fine flounder, tissues were collected from nonmanipulated flounder maintained in the conditions mentioned earlier. For each sampling point, three individuals were sampled ($n=3$). Sampling was performed under anesthesia (3-aminobenzoic acid ethyl ester, 100 mg/l) and tissue was subsequently collected. Tissue was frozen immediately in liquid nitrogen and stored at $-80^{\circ}C$ until processing. The study adhered to animal welfare procedures and was approved by the Bioethical Committees of the Universidad Andres Bello and the National Commission for Scientific and Technological Research of the Chilean government.

RNA extraction and cDNA synthesis

Total RNA was extracted from tissue using the RNeasy Mini Kit (Qiagen) following the manufacturer's recommendations. RNA was quantified using NanoDrop technology with the Epoch Multi-Volume Spectrophotometer System (BioTek, Winooski, VT, USA). Assessment of RNA quality was performed by electrophoresis on a 1.2% formaldehyde agarose gel containing ethidium bromide. Only RNAs with an A260:A280 ratio between 1.9 and 2.1 were used for cDNA synthesis. Residual genomic DNA was removed using the genomic DNA wipeout buffer included in the Quantitect RT kit (Qiagen). Subsequently, 800 ng RNA was reverse transcribed into cDNA for 30 min at $42^{\circ}C$ using the manufacturer's recommendations.

Isolation and identification of igfbps in fine flounder

Once high-quality cDNA was obtained from the liver as described previously, *igfbp* 1, 2, 3, 4, 5, and 6 were isolated and deposited in the GenBank (Table 1). Primers used for obtaining the sequences of *igfbps* were designed by multiple alignments of sequences from fish species using ClustalW, identifying evolutionary conservative regions (Table 1). PCR was performed using 1 μ l cDNA template, 10 μ l PCR buffer $10\times$, 200 μ M each dNTP, 500 nM each primers, 0.3 μ l Taq DNA polymerase (12 U/ μ l) (Promega), and RNase-free water to a final volume of 50 μ l. Primers used for isolating the

Table 1 Primer sequences for cloning and quantitative PCR (qPCR) assay of the *igfbps* of the fine flounder (**M**=A/C, **R**=A/G, **Y**=C/T, **S**=G/C, and **K**=G/T). Amplicon size (pb), qPCR efficiencies (*E* (%)), coefficient of standard curves (*R*²), and GenBank accession number are also shown

Gene	Primer	Sequence (5'–3')	Size (bp)	<i>E</i> (%)	<i>R</i> ²	Accession number
<i>igfbp1</i>	Forward cloning	GAKCKKGGMTGYGGCTGCT	226	99.5	0.986	JN635274
	Reverse cloning	TGTCACAGTTGGGGAGGTAGA				
<i>igfbp2</i>	Forward qPCR	GCCAAGAGGACACTGAAGGT	172	102.6	0.998	JN126038
	Reverse qPCR	GGGAGGTAGAAGGTGGTGAA				
<i>igfbp3</i>	Forward cloning	TTCCG MT GTCC R AGYTGAC	80	99.3	0.953	JN126039
	Reverse cloning	GACATST TR CACTGTTTSAG				
<i>igfbp4</i>	Forward qPCR	TTGGCATTGGCAAGAGTCC	189	95	0.993	JN126040
	Reverse qPCR	TCTGTTATCCTCAGCGGTCA				
<i>igfbp5</i>	Forward cloning	AAACCKCTGCAGGCKCTGCT	164	99.3	0.997	JN126041
	Reverse cloning	AGGCTGTCC R TACTTGTCCA				
<i>igfbp6</i>	Forward qPCR	CCTGAGTATGGTCCTGTCCG	124	100.2	0.997	JN635275
	Reverse qPCR	CCTCTGGGGTTGAGAATGTC				
<i>igfbp7</i>	Forward cloning	GAGAGGGAG K GTGGARGAGAA	124	100.2	0.997	JN635275
	Reverse cloning	TCACAGTTGGGKATGGGGAT				
<i>igfbp8</i>	Forward qPCR	CAGCGATGGACAGGCAGGATG	124	100.2	0.997	JN635275
	Reverse qPCR	GTAGCTCAGCACGACATGGA				
<i>igfbp9</i>	Forward cloning	KCACGCSCTTCTCCAYGG	124	100.2	0.997	JN635275
	Reverse cloning	AG K GGYTTACACTGCTTGC				
<i>igfbp10</i>	Forward qPCR	GCAAGGAGCAGAAGAGGAAG	124	100.2	0.997	JN635275
	Reverse qPCR	CAGAGCCATTACACGAGAAGT				
<i>igfbp11</i>	Forward cloning	GAGAGCCCTGTGGGGTGTA	124	100.2	0.997	JN635275
	Reverse cloning	CGGCACTGCTTTTCTGTGA				
<i>igfbp12</i>	Forward qPCR	GCCAAGCACAGCAGGACTA	124	100.2	0.997	JN635275
	Reverse qPCR	CCAGACCCCTCAGGACACT				

six *igfbps* in the fine flounder are listed in Table 1. Thermal cycling conditions were as follows: initial denaturation of 10 min at 95 °C, followed by 40 cycles of 30 s at 95 °C; 30 s at 53 °C (*igfbp3*), 56 °C (*igfbp1* and *igfbp2*), or 58 °C (*igfbp4–6*); 30 s at 72 °C; and a final extension of 10 min at 72 °C. PCR products were loaded on a 1.5% agarose gel and then isolated and purified using the Wizard SV Gel and PCR Clean-Up System (Promega).

Specific PCR products were cloned into a TOPO TA Cloning system (Invitrogen) using the manufacturer's recommendations. In short, PCR products were ligated into the T/A pCR4-TOPO vector (Invitrogen) and subsequently One Shot TOP10 competent *Escherichia coli* (Invitrogen) was transformed with the vector. Individual colonies were cultured and plasmids were isolated and purified using the Qiagen Plasmid Purification (Qiagen) and subsequently sequenced.

Phylogenetic analyses and logos alignment of Igfbps in fish

In order to further prove that the partial sequences isolated corresponded to six different Igfbps in the fine flounder sequence, alignment and construction of the phylogenetic tree using ClustalW and MEGA 5 were performed (Tamura *et al.* 2011). Owing to the lack of full-length sequences, phylogenetic or evolutionary relationships were not evaluated in this phylogenetic tree. For distance analysis, the neighbor-joining approach with the p-distance method and

bootstrapping (1000 replicates) as support for subgroup on the phylogenetic tree was used.

Logos alignment was performed using the variable central linker (L) domain. The alignment was carried out using LogoBar, a program that allows graph visualization of protein Logos alignment with gaps (Pérez-Bercoff *et al.* 2006).

Assessment of igfbps expression

Primer design Primer designs for quantitative PCR (qPCR) of *igfbps* were based on partial sequences previously obtained and are listed in Table 1. In order to get high-quality primers and avoid secondary structure (hairpins, homo-, and cross-dimers), Amplifx 1.5.4 (<http://ifjr.nord.univ-mrs.fr/AmplifX-Home-page>) and Primer 3 (<http://frodo.wi.mit.edu/primer3/>) programs were used. Subsequently, primer pairs were validated using the software NetPrimer (<http://www.premierbiosoft.com/netprimer/>).

Quantitative real-time PCR All the procedures were carried out according to the protocol outlined by Bower & Johnston (2010), with minor modification. All qPCR assays were carried out to comply with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.* 2009).

Total RNA extraction and cDNA synthesis from different tissues (i.e. *igfbps* expression in different tissue analysis) and skeletal muscle of all sampling points (i.e. *igfbps* during fasting

and refeeding analysis) were performed as described previously (See RNA extraction and cDNA synthesis). qPCR was performed using the Stratagene MX3005P QPCR system (Stratagene, La Jolla, CA, USA). Each qPCR reaction mixture contained 7.5 µl Brilliant II SYBR green master mix (Agilent Technologies, Stratagene), 6 µl cDNA (40-fold dilution), 250 mM each primers, 5 µM ROX, and RNase-free water to a final volume of 15 µl. Amplifications were performed in triplicate with the following thermal cycling conditions: initial activation at 95 °C for 10 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C. CTRL reactions included a no template control (NTC) and a CTRL without reverse transcriptase (–RT). SYBR green fluorescence was always recorded during the linear phase of cycling. In order to confirm the presence of a single PCR product, dissociation curve analysis of the PCR products was performed. Products were also evaluated by electrophoresis on a 2% agarose gel to confirm that a single product was amplified. With the purpose of estimating the assay’s efficiency, twofold dilution series were created from a cDNA pool. Efficiency values were estimated from the slope of the curve following the equation efficiency: $E = 10(-1/\text{slope})^{-1}$ (Table 1). qPCR data were analyzed using C_t values, which were then exported and processed into a Microsoft Excel-based Software application (Q-Gen; Muller *et al.* 2002). For the analysis of *igfbps* expression in different tissue, graphs were expressed as arbitrary units (A.U.) using as a reference gene *18S* rRNA (*18S*). For the analysis of *igfbps* expression during fasting and refeeding, graphs are expressed as a fold change over basal levels found at the beginning of the trial (week 0) for long-term changes. Short-term changes are expressed as fold changes over fasting levels at the end of this period (third week). 40S ribosomal protein S30 (FAU) was used as the most stable reference gene (Fuentes EN, Safian D, Valdes JA & Molina A 2012, unpublished results).

Heat map summary and hierarchical clustering In order to establish relationships among relative expression profiles of the *igfbps* with the rest of the components of the Igf system, a heat map summary and hierarchical clustering analysis of gene expression were performed using

Permutmatrix (Caraux & Pinloche 2005). In order to place the expression pattern of *igfbps* into a global context into the Igf system in muscle, we used the expression of previously published *igf1*, *igf2*, and *igf1r* using the same sampling points (Fuentes *et al.* 2012a,b). Clustering and seriation were based on Pearson’s correlation coefficient of *z*-score-normalized relative transcript abundance values (scaled from 0 to 1). McQuitty’s method of hierarchical clustering was used.

Statistical analysis Statistical analysis used to study differences in gene expression was based on an advanced linear model. This model was the general linear model (GLM) followed by Tukey’s analyses as post-test. All statistical analyses were performed using the STATISTICA 7 Software (Tulsa, OK, USA).

Results

Isolation of *igfbps*

Logos alignment showed a low identity when the L-domain of all *Igfbps* of the fine flounder (*ffIGFBPs*) was compared among them (Tables 2 and 3). The Logos alignment showed that *Igfbp1*, 2, and 3 are very variable among teleost fish whereas *Igfbp4*, 5, and 6 are highly conserved among teleost fish (Fig. 1). Using the partial sequence of all *Igfbps*, including part of the N- and C-domains, equal results were found (data not shown). In order to further corroborate the identity of all six *ffIGFBPs*, a phylogenetic tree using the L-domain from all *Igfbps* was constructed, showing that all *Igfbps* are clustered in different branches with a respective *Igfbp* (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Expression of *igfbps* in different tissues of the fine flounder

igfbp1 and *igfbp2* were predominantly expressed in the liver, showing low expression levels in the other tissues (Fig. 2A and B). Particularly, *igfbp1* was not expressed in red and white muscle, whereas *igfbp2* was not detected in the stomach (Fig. 2A and B). *igfbp3* and *igfbp4* were detected in all analyzed tissues, but *igfbp3* was predominantly expressed in the

Table 2 Amino acid sequence identity between the L-domain of the *Igfbps* of rainbow trout (*rtigfbp*). Accession number for the *rtigfbps*: *igfbp1* (NM_001124561), *igfbp2* (NM_001124649), *igfbp3* (NM_001124557), *igfbp4* (DQ146967), *igfbp5* (NM_001124652), and *igfbp6* (NM_001124560)

	<i>rtigfbp1</i>	<i>rtigfbp2</i>	<i>rtigfbp3</i>	<i>rtigfbp4</i>	<i>rtigfbp5</i>	<i>rtigfbp6</i>
<i>ffigfbp1</i>	58.3	16.7	6.3	12.5	20.8	4.2
<i>ffigfbp2</i>	7.6	37.7	37.9	9.1	15.2	6.1
<i>ffigfbp3</i>	13.6	19.8	18.3	14.8	19.8	12.3
<i>ffigfbp4</i>	7.1	16.1	16.1	82.3	14.4	16.1
<i>ffigfbp5</i>	12.9	21.2	15.3	9.4	80.2	5.6
<i>ffigfbp6</i>	13.8	12.5	4.1	21.0	8.0	79.3

Numbers in bold indicate comparison between respective orthologs sequences.

Table 3 Amino acid sequence identity between the L-domain of the Igfbps of the fine flounder (fflgfbp)

	<i>fflgfbp1</i>	<i>fflgfbp2</i>	<i>fflgfbp3</i>	<i>fflgfbp4</i>	<i>fflgfbp5</i>	<i>fflgfbp6</i>
<i>fflgfbp1</i>	100	11.1	16.6	14.6	14.6	10.4
<i>fflgfbp2</i>		100	18.2	16.7	13.6	5.0
<i>fflgfbp3</i>			100	14.8	11.1	16.7
<i>fflgfbp4</i>				100	19.6	16.1
<i>fflgfbp5</i>					100	9.4
<i>fflgfbp6</i>						100

liver, whereas *igfbp4* was highly expressed in the intestine, stomach, and spleen (Fig. 2C and D). *igfbp5* expression was also ubiquitous, but particularly highly expressed in the stomach and heart (Fig. 2E). Likewise, *igfbp6* was detected in all tissues assessed, showing high expression in gill, intestine, stomach, and heart and low expression in spleen, red muscle, white muscle, and liver (Fig. 2F).

Expression of igfbps in skeletal muscle during fasting and refeeding

Different results in the gene expression of fine flounder *igfbps* in skeletal muscle were found, which depended on the

feeding status. *igfbp1* was not detected in the skeletal muscle after 40 cycles.

igfbp2 mRNA levels decreased during fasting in the EXP group, showing almost three and 12-fold lower expression than basal levels (0 week) after 2–3 weeks of food withdrawal respectively (Fig. 3A, Supplementary Figure 2A, see section on supplementary data given at the end of this article). After 6 weeks of refeeding, *igfbp2* mRNA returned to basal levels in the EXP group and passed these levels at the seventh week (Fig. 3A, Supplementary Figure 2A, see section on supplementary data given at the end of this article). During short-term refeeding in the EXP group, *igfbp2* expression decreased even further during 2 h of refeeding; however,

Figure 1 Sequence logo alignment for the L-domain of amino acid sequence of fine flounder Igfbps and other teleost fish. Accession number for Igfbp1: turbot (AEB96249), yellowtail (ACD10797), Atlantic salmon (NP_001117096), rainbow trout (NP_001118033), and zebra fish (CAC44453.1). Accession number for Igfbp2: turbot (ADW77214), yellowtail (ACD11355), carp (ACM47497), Atlantic salmon (NP_001117097), European sea bass (ACB15195), and zebra fish (AAF23123). Accession number for Igfbp3: zebra fish (NP_991314), rainbow trout (NP_001118029), and yellowtail (ACD11356). Accession number for Igfbp4: fugu (NP_001139534), Atlantic salmon (NP_001133058), and Arctic charr (ADM93279.1). Accession number for Igfbp5: yellowtail (ACD11357), Atlantic salmon (NP_001117121), rainbow trout (NP_001118124), zebra fish (AAM51549), and rainbow smelt (ACO09073). Accession number for Igfbp6: Atlantic salmon (NP_001117122), rainbow trout (NP_001118032), zebra fish (NP_001154874), and Arctic charr (ADM93278). Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-12-0057>.

Figure 2 mRNA contents of *igfbp1* (A), *igfbp2* (B), *igfbp3* (C), *igfbp4* (D), *igfbp5* (E), and *igfbp6* (F) in different tissues of the fine flounder. Values are expressed as mean (S.E.M.; $n=3$ per each tissue). A GLM followed by Tukey's analysis as a post-test indicates significant differences. Different letters indicate significant differences. Lowercase letters show a significance of $P < 0.05$ and upper case letters show a significance of $P < 0.01$ (A, B, C, D, E and F).

after 4 h, *igfbp2* expression returned to fasting levels (Fig. 4A, Supplementary Figure 2A, see section on supplementary data given at the end of this article). *igfbp2* mRNA contents did not vary significantly in the CTRL fish during the trial (Figs 3A and 4A insert).

igfbp3 mRNA levels in the EXP group increased significantly during 1 and 2 weeks of fasting (four- and three fold respectively), but, interestingly, the expression of *igfbp3* declined and then returned to basal levels at the end of fasting (Fig. 3B, Supplementary Figure 2B, see section on supplementary data given at the end of this article). During long-term refeeding in the EXP group, *igfbp3* expression returned to basal levels. During short-term refeeding in the EXP group, *igfbp3* expression decreased even further (fivefold; Fig. 4B, Supplementary Figure 2B, see section on supplementary data given at the end of this article). *igfbp3* mRNA contents did not vary significantly in the CTRL fish during the trial (Figs 3B and 4B insert).

igfbp4 mRNA levels in the EXP group were significantly downregulated during the third week of fasting (sevenfold lower mRNA levels; Fig. 3C, Supplementary Figure 2C, see section on supplementary data given at the end of this article). During long-term refeeding in the EXP group, *igfbp4* expression was resumed after the fourth week and subsequently showed an increase over basal levels, which was

significant at the seventh week (3.5-fold higher than basal level; Fig. 3C, Supplementary Figure 2C, see section on supplementary data given at the end of this article). No significant changes were observed after 24 h of refeeding in the EXP group (Fig. 4C, Supplementary Figure 2C, see section on supplementary data given at the end of this article). *igfbp4* mRNA contents did not vary significantly in the CTRL fish during the trial (Figs 3C and 4C insert).

igfbp5 mRNA levels decreased after 3 weeks of fasting in the EXP group (fourfold lower expression than 0 week; Fig. 3D, Supplementary Figure 2D, see section on supplementary data given at the end of this article). During refeeding in the EXP group, *igfbp5* mRNA levels increased, finding almost 2.5-fold higher mRNA levels at 6 weeks than at the beginning of the trial (0 week). Rapid changes in gene expression of *igfbp5* in the EXP group were observed during the first 24 h of refeeding, with almost 4.5-fold higher mRNA levels than the fasting period (Fig. 4D, Supplementary Figure 2D, see section on supplementary data given at the end of this article). *igfbp5* mRNA contents did not vary significantly in the CTRL fish during the trial (Figs 3D and 4D insert).

igfbp6 mRNA levels in the EXP group did not change significantly during fasting (Fig. 3E, Supplementary Figure 2E, see section on supplementary data given at the end of this article). During refeeding, particularly during short-term refeeding in the EXP group, *igfbp6* mRNA levels declined rapidly, remaining low during the first 24 h (Fig. 4E, Supplementary Figure 2E, see section on supplementary data given at the end of this article). *igfbp6* mRNA contents did not vary significantly in the CTRL fish during the trial (Figs 3E and 4E insert).

High differences were observed when analyzing the differences in the mRNA contents among all *igfbps* in skeletal muscle of the fine flounder during basal, fasting, and refeeding conditions. *igfbp1* was not detected in skeletal muscle in any of the nutritional conditions assessed. *igfbp2* and *igfbp6* mRNA contents were similar in basal, fasting, and refeeding conditions (Fig. 3F). *igfbp3* expression was the lowest of all *igfbps* (except *igfbp1*; Fig. 3F). *igfbp4* contents were not significantly different in basal and fasting conditions but were significantly different during refeeding. *igfbp5* was always the most abundant of all *igfbps*, independent of nutritional status and sampling period (Fig. 3F). At the beginning of the short-term refeeding (0 h, fasting), *igfbp2*, 3, 4, and 6 contents were not significantly different, with the exception of *igfbp5*, which was the most abundant (Fig. 4F). After 24 h of refeeding, *igfbp2*, 4, and 6 expression was similar, whereas *igfbp3* and *igfbp5* expression was the lowest and the highest respectively (Fig. 4F).

Heat map summary and hierarchical clustering

Hierarchical clustering of Igf system expression during long-term fasting and refeeding showed different variations among different components of the Igf system. During long-term

Figure 3 Transcriptional regulation of the *igfbps* in the skeletal muscle of the fine flounder during long-term fasting and refeeding. Relative expression of *igfbp2* (A), *igfbp3* (B), *igfbp4* (C), *igfbp5* (D), and *igfbp6* (E) in muscle. White, black, and gray bars represent periods of feeding, fasting, and refeeding respectively. Data are presented as changes from initial conditions (0 week) and expressed as mean $\pm$ S.E.M. ($n=3$ per each time). A GLM followed by Tukey's analysis as a post-test indicates significant differences. Different letters indicate significant differences. Lowercase letters show a significance of $P<0.05$ and upper case letters show a significance of $P<0.01$ (A, B, C, D and E). Different numbers of relative mRNA contents of the *igfbps* represent significant differences ($P<0.05$; F). Asterisks indicate a significant difference between the CTRL and EXP groups at a given sampling point. CTRL group of each *igfbp* was added as an insert.

refeeding, two clades were found; the first clade showed a close relation and covariation in the expression of *igf1* and *igfbp2*, 4, 5, and 6 (Fig. 5A), whereas *igf1* showed more similitude with *igfbp5* and *igfbp6* than with *igfbp2* and *igfbp4*. The second clade clustered *igf2*, *igf1r*, and *igfbp3*. During short-term refeeding, two clades were found. The first clade showed two subgroups; the first subgroup clustered *igf2*, *igf1r*, *igfbp2*, and *igfbp6* while the second subgroup clustered *igfbp3* and *igfbp4*. The second clade clustered *igf1* and *igfbp5* (Fig. 5B).

Discussion

Isolation of *igfbp1*, 2, 3, 4, 5, and 6 in the fine flounder

In fish, the *igfbps* have been isolated from different models (Bower *et al.* 2008, Li *et al.* 2009, Pedroso *et al.* 2009a, Peterson & Waldbieser 2009, Rahman & Thomas 2011); however, the isolation and assessment of gene expression of the six *igfbps* in a single teleost had not been achieved until now. Kamangar *et al.* (2006) claimed the isolation of the six *igfbps* in rainbow trout. Nevertheless, in this publication,

Figure 4 Transcriptional regulation of the *igfbps* in the skeletal muscle of the fine flounder during short-term refeeding. Relative expression of *igfbp2* (A), *igfbp3* (B), *igfbp4* (C), *igfbp5* (D), and *igfbp6* (E) in muscle. White, black, and gray bars represent periods of feeding, fasting, and refeeding respectively. Data are presented as changes from fed initial conditions (0 week) and expressed as mean $\pm$ S.E.M. ($n=3$ per each time). A GLM followed by Tukey's analysis as a post-test indicates significant differences. Different letters indicate significant differences. Lowercase letters show a significance of $P<0.05$ and upper case letters show a significance of $P<0.01$ (A, B, C, D and E). Different numbers in relative mRNA contents of the *igfbps* represent significant differences ($P<0.05$) (F). Asterisks indicate a significant difference between the CTRL and EXP groups at a given sampling point. CTRL group of each *igfbp* was added as an insert.

Figure 5 Summary of *igfbps* gene expression in the skeletal muscle of the fine flounder during fasting and refeeding. Heat map summary and hierarchical clustering of the *Igf* system expression in the skeletal muscle of the fine flounder during long-term fasting and refeeding (A) and short-term refeeding (B). In the heat map, red and green colors indicate upregulation and downregulation of gene expression during long-term fasting and refeeding and short-term refeeding. Schematic diagram summarizing gene expression pattern of the *igfbps* during fasting, short-term (St), and long-term refeeding (Lt; C). Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-12-0057>.

igfbp3 was misnamed, with the *igfbp2* paralog 2 being the real *igfbp* isolated (Bower *et al.* 2008, Rodgers *et al.* 2008, Ocampo Dasa *et al.* 2011). The similarity among different Igfbps and/or other related proteins has been a common problem in the correct identification of Igfbps (Rodgers *et al.* 2008). All Igfbps have a common structure with a highly conserved N-terminal (also known as IGFBP domain), a conserved C-terminal domain (also known as thyroglobulin type-1 domain), and an internal variable L-domain (Duan & Xu 2005). Moreover, whole genome duplication of teleost has triggered that teleosts often have two copies of each *igfbps*, which is shown to have different expression pattern (Kamei *et al.* 2008, Zhou *et al.* 2008, Wang *et al.* 2009, Bower & Johnston 2010, Dai *et al.* 2010). In this study, we were unable

to isolate paralogs for the *igfbps* in the fine flounder. Genomic resources would be a helpful methodology to try to isolate paralogs in this species, considering that until now all paralogs for *igfbps* have been identified in model fish models either with genome-sequenced (e.g. zebra fish and takifugu) or with EST sequence database (e.g. Atlantic salmon or rainbow trout), whereas in nonmodel fish, just one copy of the *igfbps* have been found (Atlantic croaker, carp, yellowtail, etc.; Cheng *et al.* 2002, Pedrosa *et al.* 2009a, Peterson & Waldbieser 2009, Rahman & Thomas 2011, Sun *et al.* 2011). Comparing the *igfbps*, a very low identity was found, corroborating that they correspond to different Igfbps. In general, the six individual *igfbps* have a high identity with their orthologs. However, the *igfbp3* showed a very low

identity with the rtIgfbp3, highlighting that the flIgfbp3 is not the Igfbp2 paralog (Kamangar *et al.* 2006). In fact, when flIgfbp3 is compared with other orthologs, a higher identity is found. Also by phylogenetic analysis, the flIgfbps are clustered separately in different branches, grouping each of the flIgfbps with their respective orthologs.

Expression of Igfbp in different tissues of the fine flounder

IGFBPs are synthesized mainly in the liver; however, they can also be synthesized in different tissues, regulating the IGF actions in an autocrine/paracrine fashion (Jones & Clemmons 1995, Firth & Baxter 2002, Duan *et al.* 2010). Fine flounder *igfbp1* and *igfbp2* are predominantly expressed in the liver with lower expression in other tissue, which is consistent with the previous findings in other fish species (Maures & Duan 2002, Kamangar *et al.* 2006, Kamei *et al.* 2008, Pedroso *et al.* 2009a). *Igfbp1* is not detected in red and white muscles, similar to the previous reports in other fish species (Maures & Duan 2002, Gabillard *et al.* 2006, Bower *et al.* 2008, Kamei *et al.* 2008, Pedroso *et al.* 2009a). On the other hand, *igfbp2* is highly expressed in the liver of fine flounder. Recently, in Chinook salmon, Igfbp2 was shown to be the most abundant Igfbp into circulation system (Shimizu *et al.* 2011); this coincides with our results pointing out that liver is the major organ for *igfbp2* synthesis. Likewise, *igfbp3* is highly expressed in the liver, but it is also detected in all the tissues assessed, according to the previous reports on tilapia (Cheng *et al.* 2002) and zebra fish (Chen *et al.* 2004). Similar to other fish species, *igfbp4*, 5, and 6 are ubiquitously expressed in different tissues of fine flounder; interestingly, these Igfbps are expressed low in liver, despite this tissue being considered the main source of all Igfbps in fish (Kamangar *et al.* 2006, Li *et al.* 2009, Pedroso *et al.* 2009a, Wang *et al.* 2009). Altogether, these results suggest that Igfbps could be regulating the actions of local Igfs in the fine flounder.

Transcriptional regulation of igfbps in the skeletal muscle during fasting and refeeding

Igfbps regulate the half-life of circulating Igfs as well as modulating their availability and biological activity, either inhibiting or potentiating Igfs' actions depending on the EXP conditions, cell type, tissue, species, and physiological context (Wood *et al.* 2000, Zhou *et al.* 2004, Ning *et al.* 2006, 2008, Ren *et al.* 2008, Dai *et al.* 2010, Amaral & Johnston 2011). Therefore, in the present work, the results were analyzed according to the physiological context of using two contrasting nutritional conditions: a catabolic period of fasting that leads to muscle atrophy followed by an anabolic period of refeeding that leads to hypertrophy of muscle. The fine flounder was used as a model, which is a fish that presents an inherent low production of muscle-derived Igfl in muscle, which is responsible for growth deficiency in this species (Fuentes *et al.* 2012a,b).

The *igfbp1* is not detected in skeletal muscle, although it is highly expressed in the liver, which is concordant with the previous studies on fish (Maures & Duan 2002, Gabillard *et al.* 2006, Bower *et al.* 2008, Kamei *et al.* 2008, Pedroso *et al.* 2009a). Nevertheless, a recent report by Amaral & Johnston (2011) described that *igfbp1* is expressed in skeletal muscle of zebra fish and is regulated by nutrition, increasing during fasting and rapidly returning to basal levels during refeeding. The fact that *igfbp1* is upregulated during fasting has been associated with a negative role in growth in both fish and mammals (Maures & Duan 2002, Kajimura *et al.* 2005, Kajimura & Duan 2007). However, the effects caused by the absence of *igfbp1* expression in muscle of the fine flounder and other teleost fish are unknown and may be indicating a mechanism of fine regulation of local Igfs, independent of Igfbp1.

Mammalian IGFBP2 has been shown to both inhibit and potentiate IGF actions depending on cellular context (Duan & Xu 2005). Studies on Igfbp2 in fish have pointed out that this binding protein is a growth inhibitory protein, regulating Igfl bioavailability (Duan *et al.* 1999, Wood *et al.* 2005). Few reports have described the dynamic of muscle-derived *igfbp2*. In C2C12 cell cultures, *igfbp2* mRNA levels decrease with serum reduction (Bayol *et al.* 2000). In the muscle of fine flounder, *igfbp2* mRNA levels decrease during fasting and return to basal levels during long-term refeeding, in agreement with a previous report on the muscle of rainbow trout (Gabillard *et al.* 2006). In contrast, other recent reports have shown that *igfbp2* expression increases during fasting, supporting the negative role of this binding protein (Bower *et al.* 2008, Macqueen *et al.* 2011).

In mammals, IGFBP3 is the major circulating IGFBP in plasma, transporting and extending the half-life of IGF1 (Firth & Baxter 2002, Yamada & Lee 2009, Domené *et al.* 2011). Locally produced IGFBP3 shows a different dynamic in muscle. This binding protein is not the most abundant IGFBP in muscle and shows both IGF-dependent actions by stimulating myoblast differentiation (Foulstone *et al.* 2003), IGF-independent actions, and suppressing cell proliferation (Pampusch *et al.* 2003). In fish, the role of Igfbp3 in skeletal muscle has not been defined, and no studies have investigated its expression during fasting and refeeding. The *fligfbp3* was the least expressed of all the six *igfbps*. This coincides with the inherent Gh resistance and low basal production of local *igfl* (Fuentes *et al.* 2011). *igfbp3* in fish is regulated by Gh and has been shown to decrease in hypophysectomized striped bass (Siharath *et al.* 1995) and increase with Gh injections in tilapia and yellowtail (Cheng *et al.* 2002, Pedroso *et al.* 2009b); therefore, low levels of *igfbp3* in skeletal muscle of the fine flounder may be associated with an impairment in Gh actions, preventing its expression in this tissue. Interestingly, *igfbp3* was the only binding protein upregulated during fasting, suggesting a negative role of this binding protein in muscle.

In mammals, IGFBP4 is considered a strong inhibitor of the anabolic action of IGFs (Ning *et al.* 2008); however, this binding protein also shows positive effects (Ning *et al.* 2008).

Autocrine/paracrine IGFBP4 in muscle has also been associated with both negative actions, by inhibiting IGF1- and IGF2-stimulated proliferation and differentiation (Damon *et al.* 1998, Ewton *et al.* 1998), and positive actions, by being upregulated concomitant with *igf1* during muscle hypertrophy (Awede *et al.* 1999, 2002) and myoblast proliferation (Ewton & Florini 1995). In teleost fish, an elevated correlation between *igfbp4*, which is highly expressed during proliferation, and *myog*, *myoD*, and *myf5* in myogenic cell has been described (Bower & Johnston 2010). Also in this study, both Igf1 and amino acids synergistically stimulate the expression of *igfbp4* (Bower & Johnston 2010). *In vivo* studies on fish skeletal muscle have shown that *igfbp4* is constitutively upregulated during nutritionally favorable anabolic periods (Gabillard *et al.* 2006, Bower *et al.* 2008, Macqueen *et al.* 2011), coinciding with the present results. Interestingly, when different populations of the Arctic charr were assessed, dwarf fish expresses less *igfbp4* in muscle in basal conditions than normal-sized fish, and during refeeding, the expression of this binding protein was twofold lower in dwarfs than in normal-sized Arctic charr (Macqueen *et al.* 2011). In the fine flounder, *igfbp4* expression is also similar to that of *igf1* long-term fasting and refeeding but not during short-term refeeding, suggesting that Igfbp4 may be a pro-myogenic molecule, as has been suggested previously in fish (Bower *et al.* 2008, Macqueen *et al.* 2011).

Igfbp5 is the most conserved gene of the IGFBP family and is predominantly synthesized in skeletal muscle, similar to the present results (James *et al.* 1993, Kamangar *et al.* 2006, Dai *et al.* 2010). Despite some reports that have shown that IGFBP5 inhibits cell proliferation and differentiation in muscle (Ewton *et al.* 1998), this negative role has been questioned (Duan *et al.* 2010), showing abundant evidence that points out a positive role in muscle growth (Awede *et al.* 2002, Gabillard *et al.* 2006, Lang *et al.* 2006, Bower *et al.* 2008, Ren *et al.* 2008, Bower & Johnston 2010, Macqueen *et al.* 2011). Particularly, IGFBP5 promotes muscle differentiation; its absence impairs myogenesis (Ren *et al.* 2008), and its expression increases with muscle hypertrophy and decreases during muscle atrophy (Awede *et al.* 2002, Lang *et al.* 2006). In fish muscle, only positive effects of *igfbp5* have been described, showing that amino acids alone, as well as the combination of amino acids and Igf1 or Igf2, promote *igfbp5* expression in differentiated myogenic cells (Bower & Johnston 2010), supporting the fact that pro-anabolic molecules (i.e. Gh and Igf1) increase *igfbp5* expression, as was previously shown in mammals (Lang *et al.* 2006). In the fine flounder, *igfbp5* expression decreases under nutritional catabolic periods of fasting and increases during favorable nutritional anabolic periods of refeeding, similar to previous reports in other fish species, supporting the idea that Igfbp5 has a positive role in muscle (Gabillard *et al.* 2006, Bower *et al.* 2008, Macqueen *et al.* 2011).

IGFBP6 is unique among IGFBPs for its IGF2-binding specificity, showing 50- to 100-fold higher affinity for IGF2 than IGF1 (Bach 2005). This binding protein inhibits the

actions of both IGF2 and IGF1, with no evidence of potentiating IGF actions (Bach 2005). In fish, similar findings have been reported, showing that the overexpression of *igfbp6* generates different growth disorders, associated with a delay in corporal size and normal development of somites in zebra fish embryos (Wang *et al.* 2009). Studies on muscle-derived *igfbp6* have been very limited and show that *igfbp6* is present in catabolic periods and in quiescent cells (Ewton & Florini 1995). In fish, similar results have been observed, showing that *igfbp6* is highly expressed in quiescent satellite cells and its expression decreases throughout myogenesis (Bower & Johnston 2010). Interestingly, in the fine flounder, *igfbp6* expression remains stable during fasting and decreases during short-term refeeding but not during long-term refeeding. Previous reports on skeletal muscle of fish show that *igfbp6* expression also decreases during refeeding, but this change was observed during long-term refeeding (Gabillard *et al.* 2006, Bower *et al.* 2008). Thus, although *igfbp6* expression in fish muscle decreases during refeeding, this occurs in different periods, showing that *igfbp6* shows a different temporal expression in different fish species and that this Igfbp is acting as a negative regulator of Igf1 actions in muscle.

Integration of the Igf system in fish skeletal muscle during fasting and refeeding

The components of the Igf system are in a dynamic and organized balance in skeletal muscle. Considering that Igfbps present a complex physiology, an integrative approach of the results, rather than individual interpretation of them, is more appropriate. In this context, the present results of *igfbps* were placed into an endocrine framework of growth in muscle, compiling the present information with our previous reports in order to have an integrative perspective of the endocrine system of growth in the skeletal muscle of a nonmammalian species. We suggest that the absence of Igfbp1 in the muscle of the fine flounder may be compensated by the rest of the Igfbps, as has been previously observed when some of the Igfbps are absent (Wood *et al.* 2000, Ning *et al.* 2006). During fasting, the low basal production of Igf1 in the fine flounder decreases even further (Fuentes *et al.* 2012a,b). During this period, the *igfbp4* and *igfbp5* decreased concomitantly with an increase in Igfbp3 (Fig. 5C). Interestingly, different studies on fish have previously suggested that both Igfbp4 and Igfbp5 may be positive regulators of Igf1, being diminished in catabolic conditions (Gabillard *et al.* 2006, Bower *et al.* 2008, Bower & Johnston 2010, Macqueen *et al.* 2011). On the other hand, Igfbp3 in fish skeletal muscle has not been evaluated until now. We suggest that Igfbp3 could be acting as a negative modulator of Igf1 in skeletal muscle. Owing to low levels of available Igf1, the PI3K/Akt and MAPK/ERK signaling pathways become inactivated, reducing growth in the fine flounder (Fuentes *et al.* 2011). During short-term refeeding, muscle-derived Igf1 increases drastically in this species (Fuentes *et al.* 2012a,b). During this period, *igfbp2*, *3*, and *6* decrease, suggesting inhibitory actions of these Igfbps, as has

been previously pointed out in fish (Duan *et al.* 1999, Gabillard *et al.* 2006, Bower *et al.* 2008, Wang *et al.* 2009, Macqueen *et al.* 2011). Interestingly, during this period, *igfbp5* increases significantly. Therefore, we suggest that Igf1 would be more available to Igf1r, and PI3K/Akt and MAPK/ERK become strongly reactivated, restoring myogenic activity as we previously reported (Fuentes *et al.* 2011). Thus, in this stage, positive signals may be exceeding negative signals, promoting a strong catchup growth, as is observed during the first stages of refeeding in the fine flounder (Fuentes *et al.* 2011, 2012a,b). During long-term refeeding, low production of local *igfbp1* is restored (Fuentes *et al.* 2012a,b). *igfbp4* and *igfbp5* increase even further than basal levels during this period, concomitant with an increase in *igfbp2* and *igfbp6* (Fig. 5C). Interestingly, contents of *igfbp4* and *igfbp5* are higher than *igfbp2* and *igfbp6* at the end of long-term refeeding. These results suggest that different Igfbps might be competing by Igf1, promoting growth, but more attenuated, as has been previously observed in this species full-compensating growth at the end of refeeding (Fuentes *et al.* 2011, 2012a,b).

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-12-0057>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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