UNIVERSITAT DE BARCELONA
FACULTAT DE BIOLOGIA
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PAPER DE LA INSULINA I FACTORS DE CREIXEMENT TIPUS INSULINA EN LA REGULACIÓ DEL CREIXEMENT I METABOLISME EN TRUITA I ORADA

Tesi Doctoral

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Paper de la insulina, els factors de creixement tipus insulina i factors reguladors de la musculatura, en el creixement compensatori de la truita irisada (*Oncorhynchus mykiss*)

Role of insulin, insulin like growth factors, and muscle regulatory factors in the compensatory growth of the trout (*Oncorhynchus mykiss*)

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Resum

En ordre d'analitzar els diferents mecanismes involucrats en el creixement compensatori de la truita irisada (*Onchorynchus mykiss*), es va dissenyar un protocol experimental on després de 1, 2 i 4 setmanes de dejuni, els animals es van alimentar durant un mes amb ració saciant (*ad libitum*).

Mitjançant la tècnica de la RT-PCR a temps real es va analitzar el patró d'expressió de diferents factors músculars així com dels factors de creixement tipus insulina (IGFs) en el múscul blanc i en el fetge.

Els diferents períodes de dejuni van provocar disminucions en el pes final, el factor de condició i les tases específiques de creixement dels animals. La realimentació va provocar una acceleració en el creixement en els grups que havien dejunat prèviament, indicant que en els diferents grups experimentals s'havien donat diferents graus de compensació en el creixement.

Els nivells d'insulina i de glucosa en plasma van decaure durant els diferents períodes de dejuni i van ser recuperats a nivells comparables als del grup control (contínuament alimentat) després de la realimentació.

Els nivells d'expressió del RNAm d'IGF-I en múscul blanc i fetge van disminuir durant el dejuni i després de la realimentació es va observar una recuperació diferent en els nivells d'expressió del RNAm d'IGF-I en els dos òrgans. L'expressió del RNAm d'IGF-II en fetge i en múscul no va variar en cap dels períodes experimentals estudiats.

Es van realitzar estudis de binding en semipurificacions parcials de proteïnes solubles de la musculatura blanca d'aquests animals. El binding d'insulina no es va veure afectat en cap dels períodes experimentals analitzats. Per contra, es van observar increments en el número de receptors d'IGF-I durant el dejuni. L'expressió del RNAm de la isoforma b del receptor d'IGF-I també va incrementar durant el dejuni. L'expressió del RNAm de la isoforma a del receptor va incrementar durant la realimentació. Aquests resultats indiquen una regulació diferencial en l'expressió d'ambdues isoformes en funció de l'estat nutricional de l'animal.

L'expressió del RNAm dels factors musculars i dels FGFs no es va veure afectada en el transcurs de l'experiment. L'expressió muscular de l'ARNm de miogenina, va disminuir durant les primeres setmanes de dejuni i va experimentar un efecte rebot després d'un mes. L'expressió del RNAm de miostatina no va variar durant el dejuni tot i que la realimentació va provocar una disminució significativa.

En el present treball s'ha observat que la realimentació en truita irisada ha provocat diferents graus de creixement compensatori. De la mateixa manera, la regulació diferencial de l'expressió del RNAm d'IGF-I, dels seus receptors i de la miostatina, indica que aquests factors intervenen en aquest procés.

Role of insulin, insulin like growth factors, and muscle regulatory factors in the compensatory growth of the trout (*Oncorhynchus mykiss*)

Submited to General and Comparative Endocrinology

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Abstract

In order to analyze the various mechanisms involved in compensatory growth, an experimental protocol involving 1, 2, or 4 weeks of fasting followed by a single *ad libitum* re-feeding period of 4 weeks was designed for alevins of *Oncorhynchus mykiss*. The expression profile of insulin-like growth factors (IGFs) in trout liver and of the main muscle-growth regulators in trout white muscle was examined using real-time quantitative RT-PCR. Morphological parameters including body weight, specific growth rates (SGR) or coefficient factor, decreased significantly during fasting. Re-feeding accelerated growth and restored final body weight in those groups previously fasted. Plasma insulin and glucose decreased in fasting, while normal levels were restored in all re-fed groups. Fasting decreased the expression of IGF-I mRNA in both liver and white muscle, while re-feeding restored expression to control values. In contrast, IGF-II expression in liver and muscle was not affected by any treatment. Insulin and IGF-I-binding assays in partial semi-purifications (of soluble proteins) in white skeletal muscle showed that insulin binding was not affected by either fasting or re-feeding, whereas fasting up-regulated IGF-I binding. The expression of IGFRIb mRNA in white skeletal muscle also increased with fasting, while IGFRIa increased with re-feeding, indicating that the two receptor isoforms are differentially regulated. The mRNA expression of MRFs and FGFs was not affected throughout the experiment, except for myogenin, which first decreased and the showed a rebound effect after 4 weeks of fasting. Myostatin mRNA expression did not change in fasting, although re-feeding caused a significant decrease.

In conclusion, re-feeding in trout induced compensatory growth. The differential regulation in muscle expression of IGF-I, IGF-I receptors, and myostatin, indicates their contribution to this process.

1. **Introduction**

Compensatory growth has been described as a phase of accelerated growth in which favourable conditions are restored following a period of growth depression (Nicieza et al, 2003) that had been provoked by either restricted food availability or unfavourable environmental conditions.

From a practical point of view, this event is of interest to the aquaculture industry since feeding programs can thence be designed to improve growth rates while minimizing costs (Hayward et *al*., 1997; Nicieza et *al*., 2003). Thus, the effects of fasting and subsequent re-feeding on growth have been widely studied in salmonids such as *Oncorhynchus mykiss* (Tskeredzic et *al*.1995; Jobling and Koskela, 1996; Speare and Arsenault, 1997; Boujard et *al.*2000; Chauvigné et *al.*, 2003), *Oncorhynchus kisutch* (Damsgard and Dill, 1998), and *Oncorhynchus nerka* (Bilton and Robins, 1998). Nevertheless, the endocrine mechanisms of this compensatory process have not been analyzed in detail.

It is well known that in fish, contrary to mammals, muscle growth is characterized by both hyperplasia (cell proliferation resulting in an increase of new myotubes) and hypertrophy (activation/proliferation of myogenic precursor cells that are absorbed by pre-existing fibers) (Rowerlson and Vegetti, 2001; Johansen et *al.*, 2005). Hypertrophy requires the activation of satellite cells (myogenic precursor cells), proliferation, differentiation, and fusion into the myotubes of myoblasts. In mammals, adult skeletal muscle fibres are terminally differentiated such that muscle growth and regeneration are accomplished by satellite cells (Hawke et *al*., 2001), although the majority of postnatal growth occurs mainly via the hypertrophy of existing myotubes, with very little new myoblast differentiation (Stickland et *al*., 1983; Johansen et *al.*, 2005). In mammals and birds, the regulation of muscle development by different myogenic factors has been well characterized. In a variety of fish species, the expression of some myogenic regulator factors (MRFs), myocyte transcription factors (MEFs), fibroblast growth factors (FGFs) and myostatin (a member of the transforming growth factor-β) remains continuous throughout the entire lifespan (Rescan 1998; Chauvigné et. *al.*, 2003; Acosta et *al*., 2005; Terova et *al.,* 2005).

Myostatin (MSTN) acts as a key negative regulator during skeletal muscle growth in mice (McPherron, 1997) and cattle (Kambadur et *al*., 1997; Grobet et *al*., 1997). It has been recently demonstrated, via morpholino knock-down in *Danio rerio,* that myostatin inhibition enhances the expression of MyoD and Myogenin as well as IGF2 and other myo-related genes (Amali et *al*., 2005). On the other hand, FGFs are *in vivo* moderators of critical phases of

muscle development (Rescan 1998; Terova et *al.,* 2005). In mammals, Myo D and myogenin have been described as critical proteins for inducing the successful hypertrophy of skeletal muscle (Ishido et *al*., 2004).

In vertebrates, the IGF system plays an essential role in the formation and maintenance of skeletal muscle (Le Roith, 2001; Stewart and Rotwein, 1996; Benito et *al*., 1996), acting as a potent positive key regulator of muscle growth (Florini et *al.*, 1996, Le Roith, 2001). Few *in vivo* studies have been undertaken in fish that showing how IGF-I gene expression is regulated by nutritional status (Duan and Pliestskaya, 1998; Chauvigné et *al*., 2003). Furthermore, treatment of fish with IGF-I implants stimulates growth (McCormick et al., 1992). *In vitro* proliferation studies using isolated rainbow trout (*Oncorhynchus mykiss*) muscle cells demonstrated different responses to fasting and re-feeding (Fauconneau and Paboeuf, 2000), as well as different effects from both IGF-I and insulin in cell proliferation and glucose and alanine metabolism (Castillo et *al*., 2004). The cellular responses to IGFs in muscle are mediated primarily by the IGF-I receptor, which is a member of the tyrosine kinase growth factor receptors (Zorzano et *al*., 1988; Stewart and Rotwein, 1996; Le Roith 2001; Yakar et *al*., 2005). The binding of the ligand to its receptor initiates various signalling cascades in primary cultures of isolated trout muscle cells (Castillo et *al*., 2004; 2006), thereby mediating cellular proliferation and several metabolic effects, as has been previously reported in trout skeletal muscle (Baños et *al*., 1998; Méndez et *al*., 2001).

Nevertheless, studies on the interaction between the IGF system and other myogenic factors in growth regulation under different physiological situations remain scarce. The aim of the present work was to study changes in gene muscle expression, as well as in growth parameters and plasma data, in rainbow trout (*Oncorhynchus mykiss*) during compensatory growth induced by re-feeding after a fasting period.

2. Materials and methods

2.1 Animals and experimental design

Three hundred and fifty alevins of rainbow trout (*Oncorhynchus mykiss*) were obtained from the "Truchas del Segre" fish farm (Oliana, Spain). Fish were randomly distributed in eight rectangular 430 m^3 outdoor tanks, with alevins of rainbow acclimatized to environmental conditions under natural temperatures (ranging from 7 to 12 ºC) and photoperiods. The experiment was performed form October to December 2002.

In the first phase of the trial, three fasting conditions were induced: 1 week (W1), 2 weeks (W2) and 4 weeks (W4). Controls (C) were fed until satiety with commercial pellets throughout the experiment. In the second phase of the trial the W1, W2 and W4 groups were fed to satiation twice a day over 4 weeks. The W groups were deprived of food in such a way that all of the groups finished their fasting period at the same time; they were also re-fed simultaneously during this same 4-week period. Two tanks for each experimental condition were used.

2.2 Sample collection

At the beginning of the experiment, 24 fish (3 animals per tank) were weighed (23, 05± 5 g) under moderate anesthesia (3-aminobenzoic acid ethyl ester, MS-222; 100 ppm).

At the end of each experiment, 8 fish per tank (16 fish per condition) were rapidly anaesthetized and killed by a blow to the head. Blood samples were taken from the caudal vessels with heparinised syringes. Plasma samples were obtained following blood centrifugation (700 g, 10 min, 4º C) and stored at -20 ºC until further glucose and insulin analysis. The viscera were examined and the livers were dissected for individual determinations of IGF-I, IGF-II and eIFα mRNA levels.

White lateral muscle was sampled for binding studies and individual determinations of IGF-I, IGF-II, IGFRIa, IGFRIb, myostatin, myogenin, FGF2, FGF6, myoD1 and myoD2 mRNA were carried out.

The standard length (cm) and body weight (g) were measured, with specific growth rates (SGR) for fish under the different treatments calculated as follows: 100*ln (W2-W1)/ T2-T1 (W2: weight on time 2; W1: initial weight before running the experiment). The condition factor (an indicator of body shape) was calculated as follows: CF=body weight * 100/body length ^{3}.

2.3 RNA purification and gene expression

Total RNA was extracted from 100 mg of tissue sample using TRI Reagent® (Molecular Research Center, Cincinnati, OH, USA) as specified by the manufacturer. Total RNA was quantified based on the absorbance at 260 nm, with the integrity checked on 1% agarose gel stained with ethidium bromide.

cDNA was generated with 5µg total RNA using a commercial kit (Applied Biosystems, P/N 4322171, Foster City, California). Briefly, 5 µg of total RNA was incubated in 25 µl mixture (10XRT buffer, 25X dNTPs, 10X random primers, 50 U/µl MultiScribe ™ Reverse Transriptase, Nuclease-free water) at 25ºC for 10 min and then at 37ºC for 120 min. The reaction was set at 200 µl adding nuclease free water. Reverse transcription was performed in duplicate for each individual sample.

Real-time PCR was performed using iCycleiQ ™ (Bio-Rad). Quantitative PCR analyses for eIFα, IGF1, IGF2, IGFra, IGFrb, myostatin, myogenin, FGF2, FGF6, myoD1 and myoD2 mRNA were performed with 10 µl of the RT reaction using the SYBR® Green I with fluorescein qPCR kit (EUROGENTEC, RT-SN2X-03+NRFL, Belgium). The total volume was 25 µl, containing 300 nM of primers. For each target gene, primers designed by Chauvigné et al. (2003) were used.

Amplification and detection of samples were performed using the following thermal conditions: 50º C for 2 min, 95º C for 15 s, and 60º C for 1 min (66º C for myogenin) for 35-40 cycles.

PCR data were normalized using ef1α transcript abundance as follows:

Correcting factor = eF1 α value/mean of eF1α values for a given group (eight groups).

Normalized data = raw data/CF.

With this method, the correcting factor includes variations due to RT efficiency independently to change of eF1α due to treatment and (or) time. In our experiments, PCR results without normalization gave exactly the same tendency to what we obtained following our method of normalization with eF1a but with a lower variability. Moreover, no correlation between eF1a and others parameters was observed, which confirm the reliability of our method.

2.4 Partial purification of receptors and ligand-binding assays

Partial purification of solubilized insulin and IGF-I receptors from white muscle was performed at 4 °C, as described by Párrizas et al. (1995) by affinity chromatography on wheat-germ agglutinin (WGA) bound to agarose (Vector Laboratories, Burlingame, USA). The glycoproteins obtained were measured following the method described by Bradford (1972). Binding assays were performed as in Párrizas et al., 1994. A volume of 30–40 µl of the WGA eluate (approximately 30 µg of glycoproteins) was incubated for 14–16 h at 4 °C with increasing concentrations of cold hormone (from 0 to 100 nM, final dilution) and the

radio-labelled ligand as tracer (25 pM). Semi-purified receptors were precipitated by addition of 0.08% bovine γ-globulin and 10.4% polyethylene glycol (final concentrations), followed by centrifugation at 14,000×*g* for 7 min at 4 °C. Binding data were analysed in Scatchard plots and only the high-affinity, low-capacity binding sites were considered in the analysis. Porcine insulin was obtained from Lilly (Indianapolis, USA) and human recombinant IGF-I from Chiron (Emeryville, CA, USA). Human Tyr A14 ^{125}I-monoiodoinsulin and human recombinant 3-^{125}I-IGF-I, both with 2000 Ci/mmol specific activity, were purchased from Amersham Life Sci. (Arlington Heights, IL). All other chemicals used were purchased from Sigma (St. Louis, MO, USA)

2.5. Insulin and glucose plasma levels

Plasma glucose concentration was determined by the glucose oxidase colorimetric method (GLUCOFIX; Menarini Diagnostics, Firenze, Italy) (Huggett and Nixon, 1957; Sala-Rabanal et al., 2003). Plasma insulin levels were measured by radio-immunoassay (RIA) using bonito insulin as a standard and rabbit anti-bonito insulin as an antiserum (Gutiérrez et al., 1984).

2.6. Statistical analysis

Data are reported as the mean values ± standard error of mean (S.E.M). The fasting/re-feeding effects were analysed with one-way ANOVA using the non-parametric Wilcoxon/Kruskal-Wallis rank test. If a significant regime effect was found, the differences between the two means were determined using the non-parametric Mann-Whitney *U* test ($P<0.05$).

3. **Results**

3.1 *Growth performance*

Table 1 shows final mean weights (W), condition factors (CF) and specific growth rates (SGR) of fish at the end of both experimental periods (fasting and re-feeding). Food deprivation induced a progressive reduction in W when comparing W1, W2 and W4 to the control group (C) ($P < 0.05$). After 1 month of re-feeding, only the W1 group reached the final body weight of C group. The strategy of weight recovery of the W1, W2 and W4 groups is indicated by higher SGR, suggesting accelerated growth in those groups.

TABLE 1. Mean weight (W), Specific Growht Rate for Weight (SGRW) and Condition Factor (CF) of rainbow trout reared at the different regime treatments : control (C), 1 (1W), 2 (2W) or 4 (4W) weeks fasting during fasting or after the refeeding trial.

	W (g)		SGRW (%)	CF (%)
	Fasted	Fed	Fed	Fed
C	45,32±5,7[a]	72,01±5,0[a]	1,4± 0,23[a]	1,5±0,0,7
W1	38,3±2,03[b]	72, ±4,2[a]	2,05± 0,2[b]	1,75±0,1
W2	27,8±5,5[b]	55,3±4[b]	2,1± 0,21[b]	1,54±0,06
W4	19,4±1,6[c]	33,2±1,52[c]	1,73± 0,1[c]	1,52±0,06

Note. W, SGR and CF means(±SEM) correspond to measurement of at least 16 fish at the end of each regime trial. The *P* value corresponds to the results of the one way (fasting/refeeding) analysis of variance (Kruskal-Wallis rank test).Where there is a significant regime effect, different letters indicate differences ($P<0.05$) between means.

3.2 *Insulin and glucose plasma levels*

Food deprivation caused a progressive and significant ($P< 0.05$) reduction of plasma insulin levels when comparing C with W groups, while remaining undetectable in the group W4 (Table 2). Insulin levels after the re-feeding period were similar in all the experimental groups.

Glucose plasma levels decreased significantly in W groups ($P < 0.05$) during the different periods of fasting when compared to C group. After one month of re-feeding, C group values had recovered.

TABLE 2. Changes on insulin and glucose plasma levels of rainbow trout reared at the different regime treatments : control (C), 1 (1W), 2 (2W) or 4 (4W) weeks fasting during fasting or after the refeeding trial.

	Insulin (ng/ml)		**Glucose (mg/dl)**	
	Fasted	**Fed**	**Fasted**	**Fed**
C	**9,06±1,1[a]**	**12,±0,8**	**90,2±4,35[a]**	**131,5±7,9**
W1	**3,5±0,34[b]**	**10,7±1,06**	**63,5±4,55[b]**	**132±8,87**
W2	**1,84±0,12[c]**	**9,6±1,03**	**60,24±2,6[b]**	**120,7±13**
W4	**N/D**	**12,2±0,7**	**66,47±2,5[b]**	**116,3±8**

Note. Insulin (ng/ml) and glucose (mg/dL) means(±SEM) correspond to measurement of at least 16 fish at the end of each regime trial. The *P* value corresponds to the results of the one way (fasting/refeeding) analysis of variance (Kruskal-Wallis rank test). Where there is a significant regime effect, different letters indicate differences (*P*<0.05) between means.

3.3 *Insulin and IGF-I binding in white muscle*

Table 3 shows muscle insulin and IGF-I receptor number (Ro) and affinity constant (Kd) throughout the experiment. In all groups, the number of IGF-I receptors was between one and a half and two times higher than the number of insulin receptors.

Neither fasting nor re-feeding modified the number of insulin receptors (Table 3A). At the end of the experiment, values were lower probably as a consequence of seasonal effects.

The number of IGF-I receptors (Table 3.B) and the percentage of specific binding (Fig. 1) increased with fasting, being statistically ($P < 0.05$) significant for the W4 group. After one month of re-feeding, all of the values were comparable to the C group.

TABLE 3. Characteristics of insulin (A) and IGF-I (B) binding to semipurified receptor preparations of rainbow trout white skeletal muscle from control (C), 1 (1W), 2 (2W) or 4 (4W) weeks fasting during fasting or refeeding trials

	Kd (nM)		Ro (fmol/mg)	
A	**Fasted**	**Fed**	**Fasted**	**Fed**
C	**0,04±0,014**	**0,05±0,091**	**29,4±0,011**	**11±0,001**
W1	**0,12±0,107**	**0,03±0,0096**	**32,6±0,006**	**13±0,06**
W2	**0,05±0,015**	**0,03±0,01**	**35±0,017**	**16,52±0,01**
W4	**0,06±0,008**	**0,03±0,02**	**24±0,001**	**14,3±0,0122**

	Kd (nM)		Ro (fmol/mg)	
B	**Fasted**	**Fed**	**Fasted**	**Fed**
C	**0,15±0,05**	**0,03±0,008**	**40±0,012[a]**	**49,3±0,008**
W1	**0,19±0,015**	**0,06±0,013**	**41,25±0,02[a]**	**57,66±0,031**
W2	**0,20±0,085**	**0,03±0,002**	**52±0,015[b]**	**51,25±0,023**
W4	**0,16±0,081**	**0,20±0,029***	**60±0,009[c]**	**52,75±0,014**

Note. Kd, dissociation constant (1/affinity of receptors, nM); Ro, receptor number (binding capacity fmol per mg glycoprotein eluted). The means (±SEM) of at least five different semipurifications, each performed in duplicated are shown. Differences between letters indicate significantly different means at $P<0.05$.

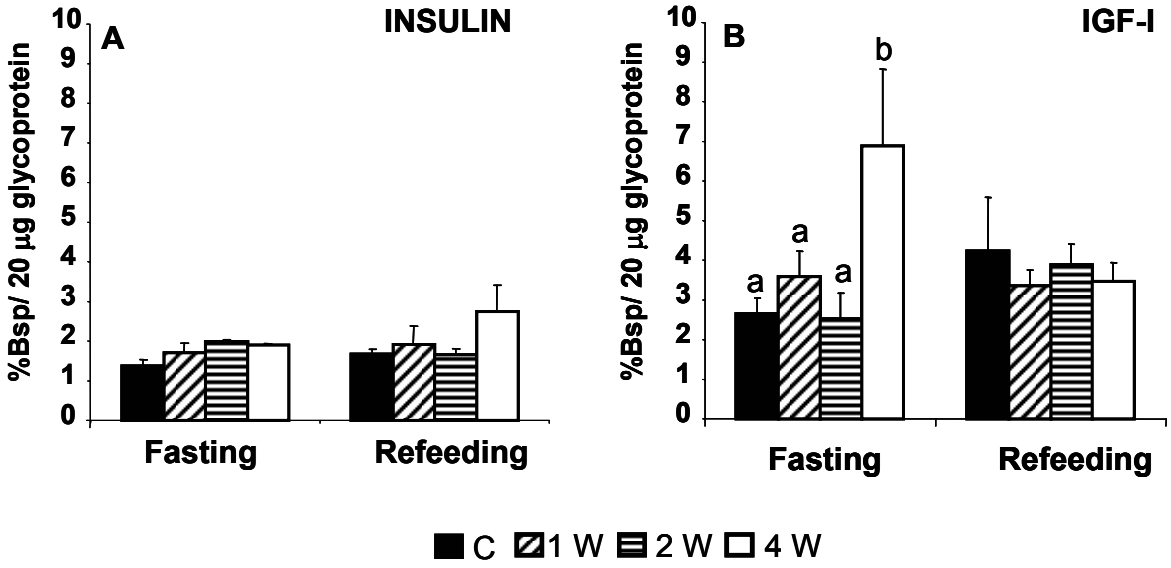

Fig. 1.-Changes in percentage binding of insulin (A) and IGF-I (B) in white muscle of rainbow trout from control (C), 1 (1W), 2 (2W) or 4 (4W) weeks fasting trout during fasting or re-feeding trials. The means (±SEM) of at least five different semipurifications, each performed in duplicated are shown. Differences were determined by the non-parametric Mann-Withney U test. Asterisk indicates significantly different means at $P<0.05$.

3.4 *Hepatic and white muscle expression of IGF1 and IGF2 mRNA*

Hepatic IGF-I mRNA gene expression decreased ($P < 0.001$) after 2 and 4 weeks of fasting (Fig. 2A). After re-feeding, while the W1 and W2 groups had already reached C values, W4 had not (Fig. 2B).

White muscle expression of IGF-I mRNA decreased during fasting in the W1, W2 and W4 groups ($P < 0.001$) (Fig. 3A). After one month re-feeding, all of the groups recovered the C group values.

IGF-II mRNA levels in liver (Fig. 2B) or muscle (Fig. 3B) were not affected either by fasting or re-feeding.

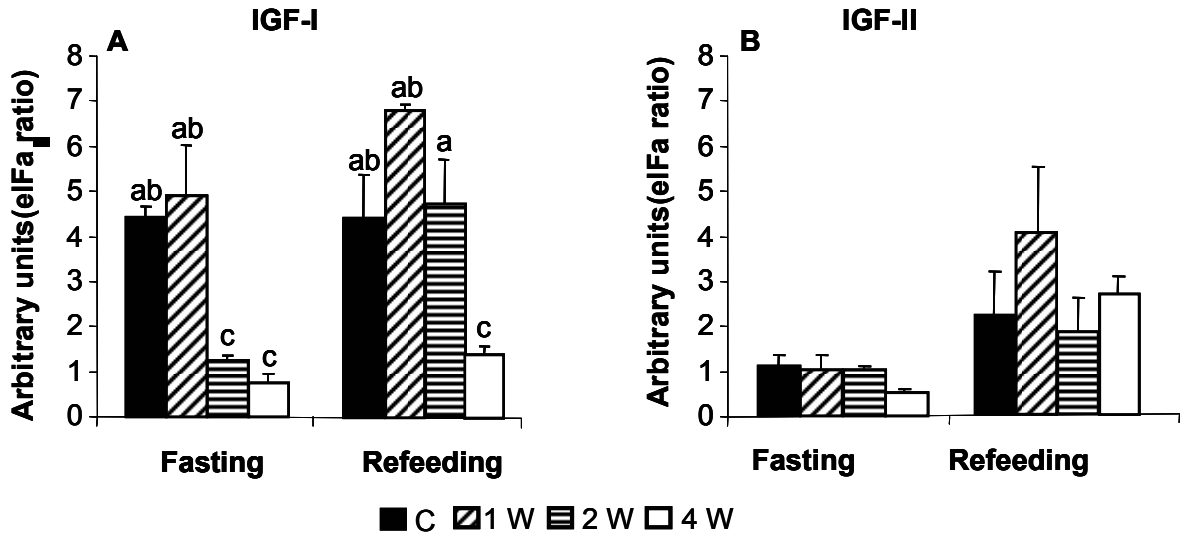

Fig.2. IGF-I (A) and IGF-II (B) mRNA quantification in liver of rainbow trout from control (C), 1 (1W), 2 (2W) or 4 (4W) weeks fasting during fasting or refeeding trials. The means (±SEM) of seven animals in each group are shown.Differences were determined by the non-parametric Mann–Whitney *U* test. Differences between letters indicate significantly different means at $P<0.05$.

Fig.2. - IGF-I (A) and IGF-II (B) mRNA quantification in liver of rainbow trout from control (C), 1 (1W), 2 (2W) or 4 (4W) weeks fasting during fasting or re-feeding trials.The means (±SEM) of seven animals in each group are shown. Differences were determined by the non-parametric Mann–Whitney *U* test. Differences between letters indicate significantly different means at $P<0.05$.

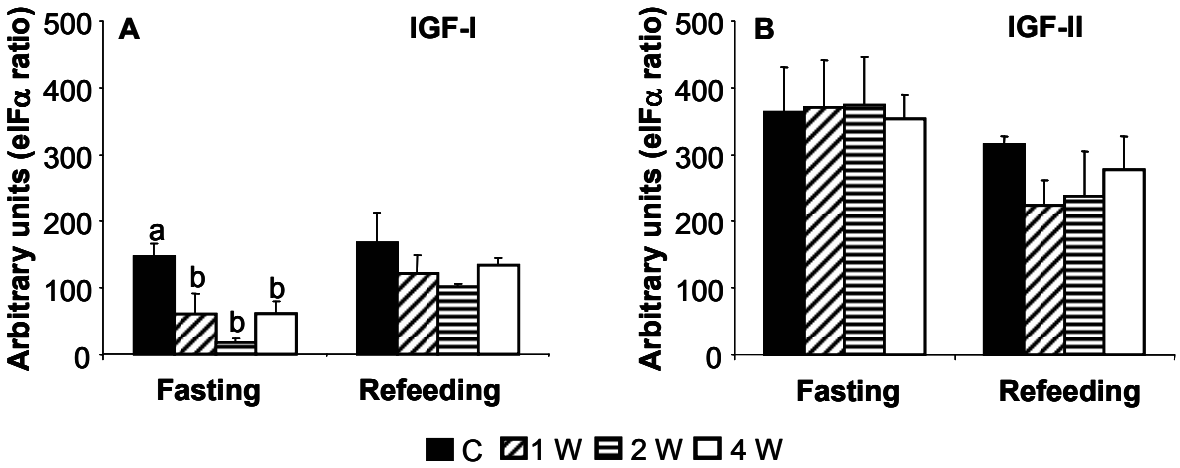

Fig.3. - IGF-I (A) and IGF-II (B) mRNA quantification in fast myotomal muscle of rainbow trout from control (C), 1 (1W), 2 (2W) or 4 (4W) weeks fasting during fasting or re-feeding trials. The means (±SEM) of seven animals in each group are shown. Differences were determined by the non-parametric Mann-Withney U test. Asterisk indicates significantly different means.

3.5 *IGF type I receptor expression*

White muscle IGFRIa mRNA expression (Fig. 4A) was not affected during the fasting period, but after re-feeding, the group W4 showed an increased expression ($P < 0.05$) when compared to C values. In contrast, IGFRIb mRNA levels increased in group W4 ($P < 0.05$) during fasting compared to C group. After re-feeding for one month, IGFRIb mRNA levels in all W groups were of the same magnitude as the C values (Fig. 4B).

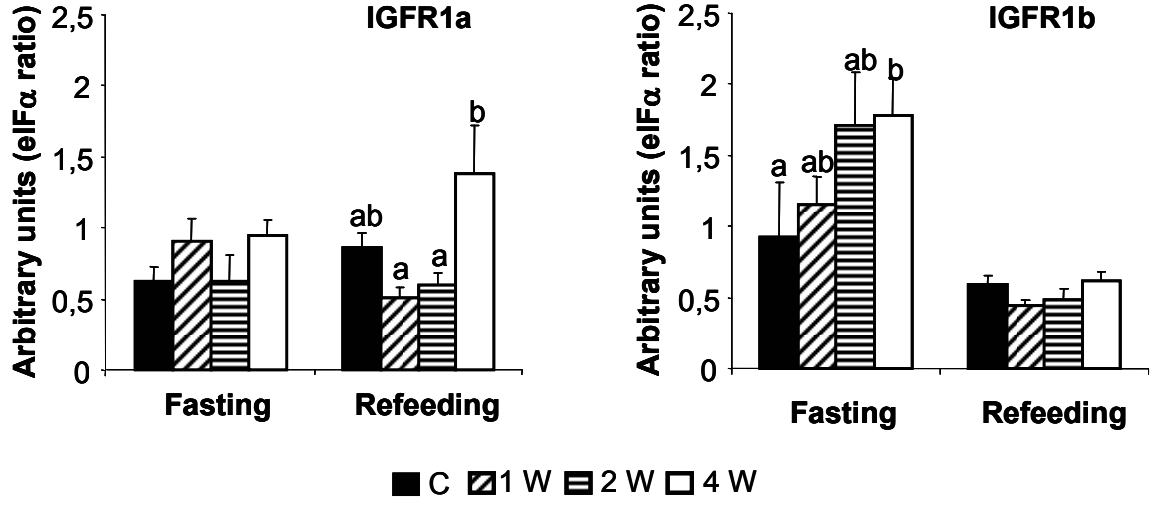

Fig.4. - IGF-I receptor (a,b) mRNA quantification in fast myotomal muscle of rainbow trout from control (C), 1 (1W), 2 (2W) or 4 (4W) weeks fasting during fasting or re-feeding trials. The means (±SEM) of seven animals in each group are shown. Differences were determined by the non-parametric Mann–Whitney *U* test. Differences between letters indicate significantly different means at $P<0.05$.

3.6 *FGF2, FGF6, MYOD1 and MYOD2 expression in white muscle*

FGF2, FGF6 (Fig. 6A, B), MYOD1 and MYOD2 (Fig. 7A, B) mRNAs levels in white muscle were not significantly affected (P > 0.05) by the feeding conditions of the animals.

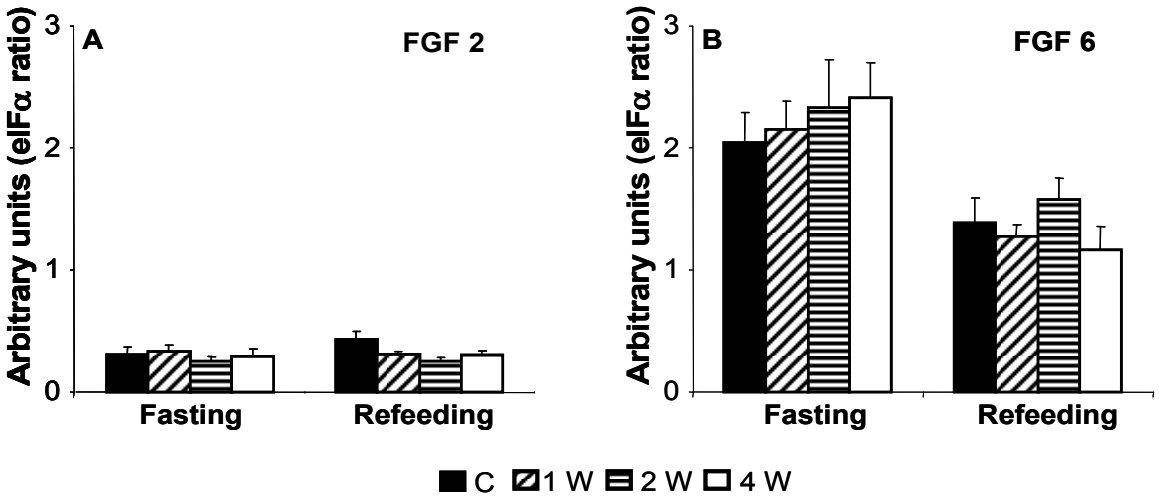

Fig.5.- FGF2 (A) and FGF6 (B) mRNA quantification in fast myotomal muscle of rainbow trout from control (C), 1 (1W), 2 (2W) or 4 (4W) weeks during fasting or re-feeding trials. The means (±SEM) of seven animals in each group are shown. Differences were determined by the non-parametric Mann–Whitney *U* test. Differences between letters indicate significantly different means at $P<0.05$.

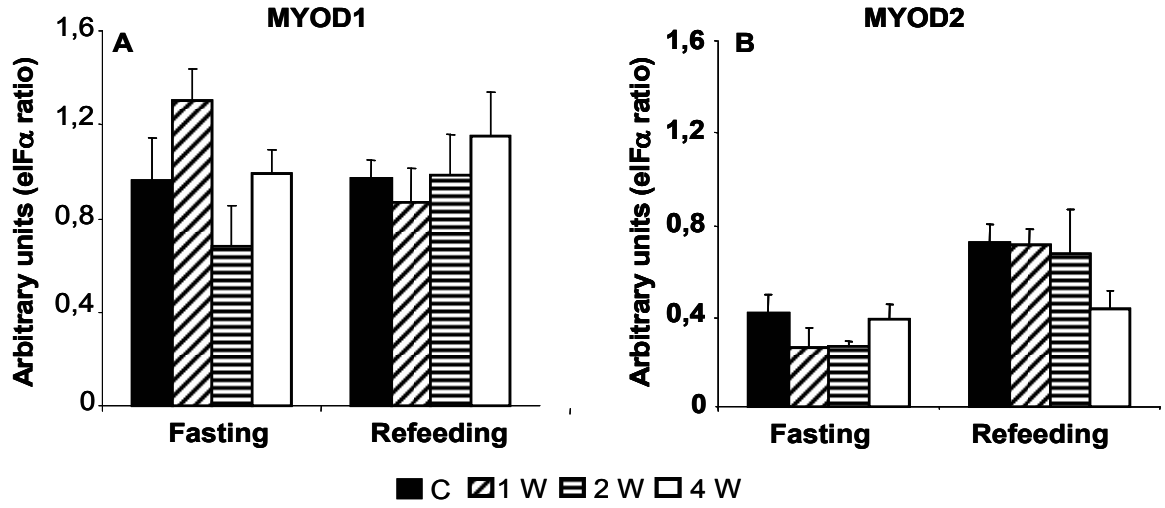

Fig.6.- MYOD1 (A) and MYOD2 (B) mRNA quantification in fast myotomal muscle of rainbow trout from control (C), 1 (1W), 2 (2W) or 4 (4W) weeks fasting during fasting or re-feeding trials. The means (±SEM) of seven animals in each group are shown. Bars indicate error of the mean. Differences were determined by the non-parametric Mann–Whitney *U* test. Differences between letters indicate significantly different means at $P<0.05$.

3.7 *Myogenin mRNA expression in white muscle*

A significant decrease in the expression of mean myogenin mRNA (Fig. 8) was apparent in the W1 and W2 fasting groups ($P < 0.05$), while W4 showed a significant increase with respect to those groups. After re-feeding, myogenin mRNA expression showed and inverse profile than that observed during fasting periods, experimental and control groups showed same levels.

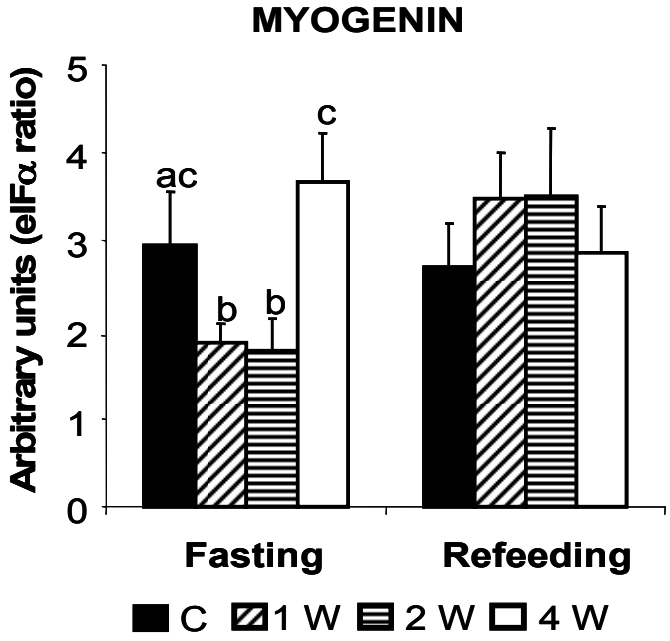

Fig.7. - Myogenin mRNA quantification in fast myotomal muscle of rainbow trout from control from control (C), 1 (1W), 2 (2W) or 4 (4W) weeks fasting trout during fasting or re-feeding trials. The means (±SEM) of seven animals in each group are shown. Differences were determined by the non-parametric Mann–Whitney *U* test. Differences between letters indicate significantly different means at $P<0.05$.

3.8 *Myostatin expression in white muscle*

Myostatin mRNA levels in muscle were not significantly affected ($P > 0.05$) during the fasting period, whereas in re-feeding their expression decreased in W groups. This result showed an opposite relationship between growth and myostatin expression (Fig. 7).

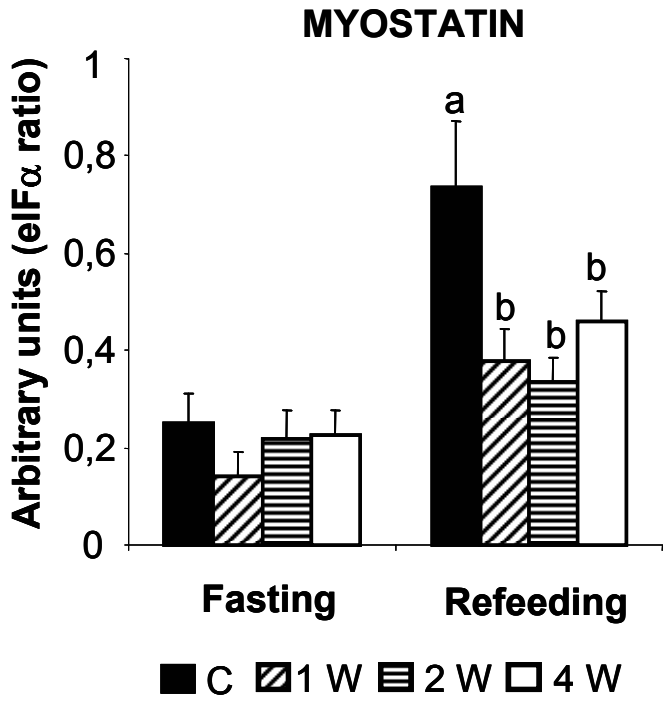

Fig.8.-Myostatin mRNA quantification in fast myotomal muscle of rainbow trout from control (C), 1 (1W), 2 (2W) or 4 (4W) weeks fasting during fasting or re-feeding trials. The means (±SEM) of seven animals in each group are shown. Differences were determined by the non-parametric Mann-Withney U test. Asterisk indicates significantly different means at $P< 0.05$.

4. **Discussion**

The development and growth of vertebrate skeletal muscle result from well-characterized events (Johansen et *al.*, 2005). Moreover, nutritional patterns exert important effects on muscle growth and composition (Navarro and Gutiérrez, 1995; Johnston et *al*., 2001).

In order to investigate the effects of compensatory growth on *Oncorhynchus mykiss*, we designed an experimental protocol in which, following different fasting periods (1, 2 or 4 weeks), animals were re-fed *ad libitum* for one month. The aim of our study was to examine whether compensatory growth by re-feeding modulates growth and plasma insulin and glucose in tandem with the gene expression of major muscle growth regulators. This is the first experiment on fish growth in which IGF-I receptor mRNA expression has been analyzed simultaneously with binding studies in skeletal muscle.

Different degrees of compensation have been described as a measure of the effectiveness of compensatory growth (Jobling et *al*., 1994). As expected, we observed that animals deprived of food showed a significant diminution in final weight when compared to the simultaneously maintained control group. After re-feeding for one month *ad libitum*, the group deprived of food for one week (W1) achieved the same weight as the control animals and also showed higher specific growth rates (SGR), indicating full compensation in this group. However, although W2 and W4 showed higher SGR after re-feeding, their final weight was less than that of the control group, suggesting partial compensation in growth. The increase in SGR in W groups after re-feeding indicates that the fish had a tendency to achieve the control weight and growth. It is generally consistent with increases in food intake, conversion efficiency, and protein and energy retention. (Jobling et *al*., 1994 ; Nicieza and Metcalfe, 1997 ; Quian et *al*., 2000; Boujard et *al*., 2000; Zhu et *al*., 2003).

Due to the normal life cycle of fish, numerous species can starve for long periods and then recover fully after re-feeding (Navarro and Gutiérrez, 1995). The degree of glucose homeostasis during fasting varies with each species. In some studies, the maintenance of glycaemia during food deprivation has been directly related with the capacity of mobilization of tissue reserves (Moon and Foster, 1995; Navarro and Gutiérrez, 1995; Figueredo-Garutti et *al.*, 2002; Larsen et *al*., 2001; Mommsen et *al*., 1980; French et *al*., 1983). The complete preservation of hepatic glycogen reserves during fasting periods has also been reported (Vijayan et *al*., 1995; Sheridan and Mommsen, 1991). We found that glucose plasma levels decreased after one week of fasting, which is in agreement with previous data in other studies involving *Salmo trutta fario,* wherein the decrease of glycaemia began after 10 days fasting

(Navarro et *al*., 1992) and after 7 days fasting in *Onchorynchus mykiss* (Pottinger et *al*., 2003). After one month re-feeding, blood glucose levels were comparable to those of control fed values. These results are consistent with those found in re-fed fish that rapidly recovered normal glycaemia after different fasting periods (Blasco et *al*., 1992 (a); Böhm et *al*., 1994; Pottinger et *al.*, 2002) and demonstrates that, as in mammals, glucose is an effective indicator of the animal's metabolic stage (Reviewed by Moon, 2004).

Insulin plasma levels throughout the experiment showed a rapid diminution, proving undetectable after 4 weeks of fasting and this is consistent with previous studies (Patent et *al*., 1971; Plisetskaya et *al*., 1976; Sheridan and Mommsem 1991; Blasco et *al*., 1992 b; Navarro and Gutierrez, 1995; Larsen et *al*., 2001). This observed decrease is entirely reversed after re-feeding (Blasco et *al*., 1992 (a); Navarro et *al*., 1995; Larsen et *al*., 2001). In our case, even in the W4 group, insulin was recovered and showed the highest levels of the fourth experimental groups; suggesting that fish were eating sufficiently to recover the weight (Reviewed in Navarro et *al*., 2004)

Although the response of insulin binding in muscle to fasting conditions seems to vary between species, after fifteen days of food deprivation a decrease in insulin receptor numbers and binding has been described in *Cyprinus carpio*, while in *Salmo trutta* the decrease in receptor numbers was not significant (Párrizas et 1994 et *al*.). This is in agreement with the present study in which neither fasting nor re-feeding affected insulin binding. This response could suggest that during fasting in trout muscle, insulin receptors are poorly regulated. In contrast, IGF-I receptors responded to fasting with a significant increase, suggesting that the IGF system plays a different role in fish muscle. The antiapoptotic effects of IGF-I have been extensively reported in mammals (Yakar, 2005).

Hepatic mRNA IGF-I levels decreased in starved fish to varying degree, depending on the species (Duan and Hirano, 1992; Matthews et *al.*, 1997; Pierce et *al.*, 2005; Small and Peterson, 2005). Less information on the effect of re-feeding on IGF-I hepatic expression is available (Duan and Plisetskaya 1993; Meton et *al*., 1999; Pedroso et *al*., 2005). In the present study, two weeks of fasting provoked a significant decrease in hepatic IGF-I mRNA expression; this result was also observed in eel (Duan and Hinaron, 1992) and indicates the dependency of IGF-I liver production on the alimentary regime. It is well known that growth is interrupted if food supply is terminated, since the maintenance of basal metabolism remains of paramount importance. After 1 month of re-feeding, a clear increase and recovery of IGF-I mRNA expression in liver was observed, except in the W4 group, which was unable to achieve the control group mRNA IGF-I levels. Four weeks of fasting seems to have a strong

effect in liver, which needs longer time to recover control levels. This corresponds well with the fact that the W4 group was unable to reach the weight of the control group. This delay in recovering IGF-I liver expression in comparison with previous studies (Duan and Plisetskaya 1993; Pedroso et *al*., 2005) could also stem from the fact that the experiment was carried out when water temperature was decreasing (late autumn).

No effects of fasting on IGF-II mRNA expression were observed, either in liver or in muscle, in accordance with the more focused role IGF-II has been observed to play in embrionary growth regulation (Méndez et *al*., 2002, Gabillard et *al*., 2003).

It is interesting to note that muscle IGF-I expression showed the same fasting response to that observed in liver, with a fall already apparent at 1 week of food deprivation and low levels persisting through 4 weeks fasting, in agreement with the study by Chauvigné et *al*. (2003). Re-feeding restored control muscle IGF-I expression even in the W4 group. Chauvigné et *al*. (2003) also found a rapid recovery in muscle IGF-I expression that was already significant at 4 days of re-feeding, and which showed maximum values at 12 days, while at 34 days of re-feeding expression returned to levels recorded at day 4. All of these findings indicate that in terms of IGF-I expression, muscle possesses a faster response capacity to re-feeding than does liver. In that study, Chauvigné et *al*. (2003) also found muscle IGF-II expression increased progressively during re-feeding, proving significant after 34 days. The authors suggested that both IGFs are differently regulated by the nutritional status and probably have a differential role in promoting muscle growth recovery.

IGF-1 receptors were shown to be present in high amounts in the skeletal muscle of the brown trout *Salmo trutta* throughout its life cycle (Méndez et *al*, 2001); in fact, they were found at higher levels than that of insulin in the same tissue (Párrizas et *al*., 1995; Gutiérrez et *al*., 1995; Baños et *al*., 1998). This would indicate that in trout, IGF-I might contribute more to the regulation of muscle function than insulin, in contrast to the dynamic found in mammals (Reviewed by Gutiérrez et *al*., 2005). IGF-1 binding and IGF-I effects have been also studied in primary cultures of *Oncorhynchus mykiss* muscle cells, indicating that both peptides play a key role in proliferation, differentiation, and metabolic processes (Castillo et *al*., 2002)

We found that IGFRIa expression was not affected by fasting. It is interesting to note that the effects on the other receptor IGFRIb isoform were different in both fasting and re-feeding conditions. Thus, IGFRIb expression responded to food deprivation with a progressive increase until reaching 4 weeks of fasting. This increase via fasting was also found in IGF-I total binding. Similar results were observed in mammals and birds (Bayol et

al., 2004; Matsumura et *al*., 1996; Tomita et *al*., 2001), suggesting that dietary restrictions could augment the local tissue response to IGF-I by increasing the number of IGF-I receptors expressed in muscle. Therefore, similar to that in mammals, decreases in circulating IGF-I might up-regulate isoform b receptor gene expression (Hernandez-Sánchez 1997). During re-feeding, the isoform IGFRIb did not show significant changes in expression in agreement with that observed at 4, 12 or 34 weeks of re-feeding in trout (Chauvigné et *al*., 2003).

All these findings suggest that the two IGF-I receptor isoforms play different roles depending on nutritional status, with isoform b responding to fasting as isoform a does to re-feeding. We also found the respective influence of each receptor isoform on binding results, with isoform b proving to be more highly correlated.

It is well known that hyperplasia occurs in some fish species during adulthood (Johansen et *al*., 2005), as well as in phases of rapid growth described in teleost fish (Reviewed by Rowerson and Veggetti, 2001). Due to the fact that compensatory growth has been defined as a "phase of acceleration in growth" after a period of food restriction (Amali et al., 2003), it was interesting to analyze the expression of certain MRFs.

Different MyoD genes have been reported in *Oncorhynchus mykiss* (Delande and Rescan, 1999; Rescan and Gauvry 1996) and seem to play a positive role in myogenic lineage and muscle differentiation (Rescan, 2001). It has been postulated, based on mammalian responses to muscle damage (Johansen and Overturf, 2005), that the expression of MRFs takes place when muscle damage or decreases in weight muscle occur (Aksens et *al*., 1986). We did not find a clear change in the expression of both MyoD trout isoforms during our experiment. This could be due to the fact that, as

Johansen and Overturf (2005) found in rainbow trout at an age similar to the fish in our study, very low levels of MyoD2 persist compared with those found in embrionary development or in spawning periods. As far as we know, expression data for MyoD in fasting or re-feeding processes in fish remains non-existent. Muscle MyoD expression did not change in fetal rats that underwent gestational under-nutrition (Bayol, et *al*., 2004).

Myogenin plays a role in initiating and stabilising the differentiation muscle program (Megeny and Rudnicki 1995), as well as in hypertrophic processes throughout the post-embryonic life in trout (Rowerlson and Veggetti, 2001). However, the rebound effect at 4 weeks fasting indicated that a new stage has been reached whereby the role of myogenin could be necessary for muscles maintenance. Johansen and Overturf (2005) found in rainbow trout a peak in myogenin levels during the sexual maturation period, when muscle atrophy and overall decreases in growth occur. This condition could be similar to that found in trout

subjected to 4 weeks fasting, but not for groups fasted for 1 or 2 weeks, in which myogenin expression had decreased. After fasting, re-feeding recovered control values, a response similar to that observed by Chauvigné et *al*. (2003), with a peak in trout myogenin expression occurring after twelve days re-feeding. Here, the authors suggest that such an increase is subsequent to the proliferation of myoblast formations in myogenic cell population during muscle recovery.

It has been proposed that in the skeletal muscle of rats, myogenin and myoD expression may help to regulate protein synthesis and degradation (Loughna and Brownson 1996). Little remains understood regarding myogenin expression in fasting conditions or during re-feeding. In fact, it is known that IGF-II stimulates myogenin expression in rats (Florini 1991) and in our study this growth factor was not diminished in the liver or muscle of fasted trout. Thus, in IGF-II it should be interesting to verify whether the myogenin peak observed at 4 weeks fasting similarly occurs.

The expression of FGFs did not show clear changes, either in fasting or in re-feeding conditions. The expression of FGF2 was found to be up-regulated after 4 and 12 days re-feeding, (Chauvigné et *al*., 2002); however, at 34 days FGF2 expression had already normalized, which agrees with our finding in the W4 group after 1 month re-feeding. It is also known that FGF2 has been implicated in processes of skeletal reconstruction in *Carassius aurata* (Santos-Ruiz et *al*., 2001). Most likely we found no changes in FGF factor expression due to the fact that we measured it after one month of re-feeding. Previous data suggest that FGF2, more than FGF6, is a critical modulator of myotomal muscle growth during the first days of re-feeding (Chauvigné et *al*., 2003). Rescan (1998) suggested that prolonged expression of FGF6 is involved in the continuous proliferation of myogenic cells during fish growth. We did not find any change in its expression under any experimental conditions. Thus, our data are also in agreement with those found in *Dicentrarchus labrax L* (Terova et *al*., 2005), in which no changes in FGF6 expression were observed, either after one month fasting or during the re-feeding period.

Myostatin has been described as a negative regulator of skeletal muscle development and growth in mammals and it seems to be critical to the proper regulation of skeletal muscle mass (McPherron et *al*., 1997). In fish, information remains less abundant; myogenin was recently postulated to play a major role in *Danio rerio* during myogenesis, apart from inhibiting proliferation and differentiation (Amali et *al*., 2005). Additionally, body mass increases due to hyperplasia or hypertrophy after myostatin dsRNA injection have been described in *Danio rerio* (Acosta et *al*., 2005). As in previous studies involving

Oncorhynchus mykiss (Chauvigné et *al*., 2003), we did not find any change in myostatin expression during fasting periods. Terova et *al.* (2005) did not find any effects on myostatin expression in sea bass resulting from 4 weeks fasting. However, they did find that re-feeding also induced a decrease in myostatin expression very similar to that observed in our experiment. Terova et *al*. (2005) suggested that this observed decrease during re-feeding represents an indirect regulatory mechanism allowing the hypertrophic and hyperplasic processes to re-start and stimulate muscle growth. All of these findings correlate well with the fact that inactivation of myostatin expression in mice is associated with muscle hypertrophy in McPherron gene knock-out mice (McPherron et *al*., 1997).

In conclusion, we have found that during fasting IGF-I expression in liver and muscle decreased, but IGF-I binding and IGF-IRb expression were up regulated in muscle. At the same time, IGF-IRa, which was not affected in fasting, increased in re-feeding, suggesting a complementary role for both receptor isoforms in muscle growth. Whereas myogenin was affected by fasting, recovery of muscle growth during re-feeding was accompanied by myostatin depression. Thus, the regulation of fish muscle growth is a complex process involving many molecules, which merits further study.

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Regulació coordinada dels gens dels sistema GH/IGF durant la realimentació de la truita irisada (*Oncorhynchus mykiss*)

Coordinated regulation of the GH/IGF system genes during refeeding in rainbow trout (*Oncorhynchus mykiss*)

Regulació coordinada dels gens dels sistema GH/IGF durant la realimentació de la truita irisada (Oncorhynchus mykiss).

Resum

El sistema GH/IGF és una xarxa complexa regulada per la disponibilitat de l'aliment. Tot i que l'efecte del dejuni sobre l'eix GH/IGF s'ha estudiat àmpliament, els esdeveniments que tenen lloc durant diferents dies en el transcurs de la realimentació sobre la recuperació del sistema GH/IGF són encara desconeguts.

S'han analitzat els nivells plasmàtics de GH, IGF-I i d'IGF-II, així com l'expressió dels components del sistema GH/IGF en el fetge i el múscul.

El dejuni ha provocat increments en els nivells plasmàtics de GH. Per contra, 1 dia de realimentació va ser suficient per recuperar els nivells plasmàtics de GH als nivells detectats en el grup control (1.10±0.27 *versus* 1.12±0.28 ng/ml).Tot i això, es va trobar que després de 7 dies de realimentació els nivells de GH circulant van tornar a decaure, i que no va ser un altre cop fins al dia 15, quan els valors de GH van ser completament restablerts.

El dejuni ha provocat disminucions en els nivells plasmàtics d'IGF-I i d'IGF-II. La realimentació ha incrementat els nivells d'IGF-I circulant únicament a partir del dia 4.Per contra els nivells plasmàtics d'IGF-II es van duplicar després d'1 dia de realimentació (26.5±1.9 *versus* 44.0±3.4 ng/ml; $P<0.01$).

Els peixos dejunats han exhibit una quantitat més elevada del RNAm de GHR1 en el fetge i el múscul que els grups control, mentre que la quantitat del RNAm de GHR2 només es va veure incrementada en el múscul. En el fetge un dia de realimentació va provocar una disminució en l'expressió del RNAm de GHR1 i un increment en l'expressió del RNAm de GHR2. Posteriorment es va trobar una recuperació en l'expressió del RNAm dels dos receptors al llarg de la realimentació.

L'expressió del RNAm d'IGFBP4 va disminuir durant el dejuni i es va recuperar progressivament durant la realimentació. El dejuni no ha tingut cap efecte en l'expressió hepàtica del RNAm d'IGFBP2 ni d'IGFBP6, mentre que després de 7 dies de realimentació s'ha observat un pic en l'expressió d'IGFBP2 i d'IGFBP6. En el múscul blanc el dejuni ha provocat una disminució en l'expressió del RNAm d'IGFBP2 que es va recuperar només després de 7 dies de realimentació. L'expressió del RNAm d'IGFBP4 va disminuir després del dejuni i partir del dia 1 de la realimentació es va observar un increment transitori.

L'expressió dels RNAm d'IGF1, d'IGFBP5 i d'IGFBP-rP1 va mostrar un perfil similar. Durant el dejuni es va observar un pic en la seva expressió. Durant la realimentació es va observar un primer pic de recuperació en el dia 2, un segon pic en el dia 7 i una recuperació total comparable als nivells d'expressió del grup control en el dia 15. De fet, l'expressió dels RNAm d'IGF-I, d'IGFBP5 i d'IGFBP-rP1 mostra una correlació positiva ($r = 0.6\text{-}0.8$; $P<0.0001$).

El present treball, doncs, mostra que els nivells plasmàtics d'IGF-I es recuperen més tard que els de la GH, fet que suggereix que l'IGF-I plasmàtic no és el responsable dels canvis plasmàtics de la GH. La regulació coordinada de l'expressió d' d' IGFBP5, I d'IGFBP-rP1 és una indicació de la recuperació de l'activitat miogènica durant la realimentació.

Coordinated regulation of the GH/IGF system genes during refeeding in rainbow trout (*Oncorhynchus mykiss*)

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Short title: Regulation of the GH/IGF system during refeeding

Abstract

The GH/IGF system is a complex regulation network strongly dependent on nutrient availability. While the effect of starvation on the GH/IGF system has been extensively studied, the time course of events leading to the restoration of GH/IGF system activity after starvation is largely unknown. We therefore measured the plasma levels of GH, IGF1 and IGF2 and the expression of the GH/IGF system in liver and muscle. Starvation increased the plasma GH level and one day of refeeding completely restored it (1.10±0.27 *versus* 1.12±0.28 ng/ml). Thereafter, plasma GH continued to decrease until day 7 and then returned to control values from day 15. Starvation decreased plasma IGF1 and IGF2 and refeeding raised plasma IGF1 only from day 4. In contrast, the plasma IGF2 level doubled after one day's refeeding (26.5±1.9 *versus* 44.0±3.4 ng/ml; $P<0.01$). Starved fish exhibited higher GHR1 mRNA abundance in liver and muscle than in controls, whereas GHR2 mRNA abundance was increased only in muscle. In liver, one day of refeeding decreased GHR1 (2-fold) but increased GHR2 mRNA abundance (2-fold). Thereafter, a progressive return to normal values was observed. Liver IGFBP4 mRNA abundance was lowered in starved fish followed by a progressive restoration during refeeding. Starvation had no effect on liver IGFBP2 and IGFBP6 mRNA abundance, whereas refeeding provoked a peak of IGFBP2 and IGFBP6 expression at day 7. In muscle, starvation led to a drop of the IGFBP2 mRNA level, which was restored only from day 7. IGFBP4 mRNA abundance in starved fish was lower than in the controls and refeeding led to a transient up-regulation (7-fold) of IGFBP4 gene at day 1. IGF1, IGFBP5, and IGFBP-rP1 expression profiles were similar, showing a decrease of expression after starvation, a first peak of expression at day 2, a second peak at day 7, and a return to normal value from day 15. Moreover, IGF1, IGFBP5, and IGFBP-rP1 mRNA abundance were positively correlated ($r = 0.6$-0.8; $P<0.0001$). In conclusion, plasma IGF1 was restored later than plasma GH level, which suggests that plasma IGF1 cannot account for plasma GH changes. The coordinated regulation of IGF1, IGFBP5, and IGFBP-rP1 expression would be a signature for the resumption of myogenic activity.

1. Introduction

Fish growth is a complex function mostly regulated by the GH/IGF system (Gabillard *et al.* 2005; Reinecke *et al.* 2005; Wood *et al.* 2005). The general organisation of this system is well conserved between higher vertebrates and fish, including growth hormone (GH), GH receptor (GHR), Insulin-like growth factors (IGF1 and IGF2), IGF receptor (IGFRI and IGFRII) and IGF binding proteins (IGFBPs). Resulting from their higher affinity for IGFs than that of the IGF receptor itself, the IGFBPs act not only as carriers of IGFs, but also function as modulators of IGF availability and activity. In addition to the 6 IGFBPs, closely related proteins (IGFBP-rPs) sharing structural similarities with IGFBPs have been described in mammals (Hwa *et al.* 1999). Recently, a cDNA homologous to IGFBP-related Protein 1 (IGFBP-rP1) was cloned in rainbow trout (Kamangar *et al.* 2006). In fish, the function of the IGFBP-rP1 is unknown but in mammals, it has been shown that IGFBP-rP1 may function as an autocrine/paracrine factor that specifies the proliferative response to the IGFs in myogenesis (Haugk *et al.* 2000). Finally, within the GH/IGF system most of the components interact with each other forming a complex regulation network (Duan & Xu 2005; Gabillard *et al.* 2005).

Fish growth depends for the mostly on nutrient availability, which is known to modulate the GH/IGF system activity. Starvation increases plasma GH levels (Duan & Plisetskaya 1993; Marchelidon *et al.* 1996; Pérez-Sánchez *et al.* 1995; Pierce *et al.* 2005; Sumpter *et al.* 1991; Weber & Grau 1999) and pituitary GH content (Marchelidon *et al.* 1996; Weber & Grau 1999). In contrast, hepatic GH-binding capacities are lowered after several weeks of starvation (Gray *et al.* 1992; Pérez-Sánchez *et al.* 1994; Pérez-Sánchez *et al.* 1995). Recently, it was shown that starvation decreased the expression of GHR mRNA level in the liver (Deng *et al.* 2004; Fukada *et al.* 2004), but the effect of starvation on the gene expression of the two GHRs present in salmonids is unknown. It has been hypothesized that the hepatic resistance to GH would partly account for the low plasma levels of IGF1 observed in starved fish (Baños *et al.* 1999; Pérez-Sánchez *et al.* 1995; Shimizu *et al.* 1999) and that the increase in GH levels results from the decline of IGF1 feedback on GH cells. Nevertheless, evidence for this hypothesis is still lacking and the possible role of IGF2 has never been explored.

IGFBPs are important components of the GH/IGF system, since they modulate IGF activity and availability, but the regulation of their expression by nutritional status

is scarce. Starvation increases plasma IGFBP1 (Kelley *et al.* 2001; Shimizu *et al.* 1999; Siharath *et al.* 1996) and IGFBP2 mRNA levels in zebrafish (Duan *et al.* 1999a; Maures & Duan 2002). Plasma IGFBP3 is decreased in starved salmon (Shimizu *et al.* 2003) but no difference of IGFBP3 mRNA abundance is observed in starved zebrafish (Chen *et al.* 2004). To date, nutritional regulation of IGFBP4-6 and IGFBP-rP1 is completely unknown in fish and their possible implication in muscle growth recovery has never been studied.

Although the effect of starvation on the GH/IGF system has been extensively studied, the time course of events leading to the restoration of the GH/IGF system activity after starvation is largely unknown. We therefore studied the plasma level of GH, IGF1 and IGF2 during refeeding as well as the expression profiles of the GH/IGF system genes in liver and muscle. This approach allowed us to show that plasma IGF1 was restored later than plasma GH and that muscular expression of IGF1, IGFBP5 and IGFBP-rP1 were coordinated and associated with muscle growth recovery.

2. Materials and Methods

2.1. Animals and experimental design

Two groups of female immature trout (*Oncorhynchus mykiss*), with a mean weight of 136±10 g, were constituted. Three experimental groups (S1, S2 and S3) reared in triplicate, (9 different tanks, 50 fish/tank) were starved for 1 month then refed for 1 month *ad libitum*. The control group (reared in triplicate) was fed *ad libitum* throughout the experiment. Fish were reared in freshwater tanks (PEIMA-INRA, Sizun, France) under a natural photoperiod and fed with a commercial diet (BioMar). The water temperature was 18°C before starvation (day -30), 15.3°C at day 0 and 12.3°C at day 29.

2.2. Sample collection

Investigations were conducted according to the guiding principles for the use and care of laboratory animals and in compliance with French and European regulations on animal welfare. Before starvation (day - 30) and at day 0, 1, 2, 4, 7, 15 and 29 days following refeeding, 12 fish (4 per tank) were sampled for the experimental groups. To limit the stress caused by repeated sampling, fish were collected from group S1 on days 0, 4 and 15, from group S2 on days 1 and 7 and from group S3 on days 2 and 29. For the control group 12 fish were sampled (4 per tank) on days 0 and 29. Fish were anaesthetised with eugenol (10 ml/l), killed by a blow on the head and blood was collected within 10 min.

2.3. Sample analysis

Plasma GH, IGF1 and IGF2 levels were measured by homologous radioimmunoassay as previously described (Gabillard *et al.* 2003; Le Bail *et al.* 1991).

Total RNA was extracted from 100 mg of tissue (muscle or liver) using TRIzol (Gibbco BRL). Total RNA was quantified, based on absorbance at 260 nm (NanoDrop® ND-1000 spectrophotometer), and the integrity checked for all RNA samples on 1% agarose gel, stained with ethidium bromide. Then, 5 µg of total RNA was used to perform the reverse transcription reaction (Applied Biosystems kit #N808-0234).

Quantitative PCR analyses were carried out with 5 µl of the RT reaction using a real-time PCR kit provided with a SYBR Green fluorophore (Eurogentec, Belgium) according to the manufacturer's instructions and using 600 nM of each primer (Table 1).

After a 2 min incubation step at 50°C and a 10 min incubation step at 95°C, the amplification was performed using the following cycle: 95°C, 20 sec; 62°C, 1 min for 40 times. The relative abundance of target cDNA within the sample set was calculated from a serially diluted (standard curve) liver cDNA pool using the I-Cycler IQ software. Subsequently, real-time PCR data were normalized using ef1α transcript abundance as follows:

Correcting factor = eF1a value/mean of eF1a values for a given group (10 groups).

Normalized data = raw data/CF.

With this method, the correcting factor includes variations due to RT efficiency independently to change of eF1a due to treatment and (or) time. In our experiments, PCR results without normalization gave exactly the same tendency to what we obtained following our method of normalization with eF1a but with a lower variability. Moreover, no correlation between eF1a and others parameters was observed, which confirm the reliability of our method.

Table 1: Primers used for real-time PCR (qPCR) study. Forward and reverse primer sequences of rainbow trout IGFBP1-6, IGFBP-rP1, GHR1, GHR2, IGF1 and EF1α genes are shown.

Gene	Forward	Reverse
IGF1	TGGACACGCTGCAGTTTGTGTGT	CACTCGTCCACAATACCACGGT
GHR1	CGTCCTCATCCTTCCAGTTTTA	GTTCTGTGAGGTTCTGGAAAAC
GHR2	TCTCCTCATCCTAACAGTTGTG	GCTCTGTGAGGTTCTGGAATGT
IGFBP1	AGTTCACCAACTTCTACCTACC	GACGACTCACACTGCTTGGC
IGFBP2	GTGCTGGAGAGGATATCTAAGA	AGACATCTTACACTGTTTGAGGT
IGFBP3	TTCCATGATAACAGGGGACATG	GACCGTGGGTGGACATGTGG
IGFBP4	TGTCGTGCTGAGCTGCAGAG	TGGCTGGCACTGCTTGGCAT
IGFBP5	ACTTCACGCGCTTCTCCATGGCA	CGAGACTCATGATCTATGGGTGGA
IGFBP6	GCTCAATAGTGTTCTGCGTGG	CTTGGAGGAACGACACTGCTT
IGFBP-rP1	GCTCCGATGGAGTGACCTATA	ACAATGACAGGTGCTGTTGCG
EF1α	AGCGCAATCAGCCTGAGAGGTA	GCTGGACAAGCTGAAGGCTGAG

2.4. Statistical analysis

The refeeding effect was analysed with a one-way ANOVA using the non-parametric Wilcoxon/Kruskal-Wallis rank test. If a significant effect was found, the

significance between two means was determined by the non-parametric Mann-Whitney U-test. All the data are presented as the mean±SEM. All parameters measured were subjected to a Principal Component Analysis (PCA) using PAST software (Version 1.18) (Hammer *et al.* 2001). Pearson' linear regression was used to assess the relation between IGF1, IGFBP5, IGFBP-rP1 mRNA abundance.

3. Results

3.1. Hormonal profile of GH, IGF1 and IGF2 after refeeding

Fish of the fed group grew rapidly during the experiment, whereas starved fish lost weight (136±2.9 g *versus* 125.9±3.5 g; $P<0.05$). Thereafter, fish grew rapidly as in the control group (Fig 1).

Plasma GH levels were increased significantly (1.12±0.28 *versus* 3.4±0.7 ng/ml; $P<0.01$) because of food deprivation (Fig. 1). Refeeding for 1 day restored plasma GH levels to those of the control fish. Thereafter, plasma GH continued to decrease and at day 4 and 7, GH levels were significantly lower than those measured on days 1 or 2. After 15 and 29 days of refeeding, GH levels were again similar to the values observed in the control fish.

Plasma IGF1 levels were significantly decreased by 1 month's starvation, leading to a 3-fold drop in IGF1 levels in starved fish compared to the controls (9.9±0.7 *versus* 34.8±2.8 ng/ml; $P<0.001$). Two days after refeeding, plasma IGF1 remained low. After 4 days, plasma IGF1 started to increase slightly but it needed 2 weeks for the level to be restored to that of the control group. One month after refeeding, no difference in plasma IGF1 was seen between the controls and starved fish. The expression profile of IGF1 in the liver was similar to what was observed at plasma level. Nevertheless, it is noteworthy that after refeeding, IGF1 mRNA abundance continued to decrease up to 4 days.

Plasma IGF2 levels (Fig. 1) were also significantly decreased by 1 month's starvation. At day 0, the IGF2 levels of starved fish were 3-fold lower than in control fish (26.5±1.9 *versus* 86.8±9.2 ng/ml; $P<0.001$). At day 1, the plasma IGF2 level in refed fish had nearly doubled and thereafter increased progressively but never reached the levels measured in the control fish for a given time. Indeed, even after 1 month of refeeding plasma IGF2 levels in the control group were always higher (128±9 *versus*

162±16 ng/ml; P<0.01). In control fish, plasma IGF2 levels increased throughout the experiment.

The expression profile of IGF2 in the liver was close to that observed at the plasma level. Indeed, it is noteworthy that a transitory peak of IGF2 expression occurred at day 2. Thereafter, as observed for plasma IGF2 level, IGF2 mRNA abundance increased progressively without reaching the IGF2 mRNA abundance measured in the control fish for a given date.

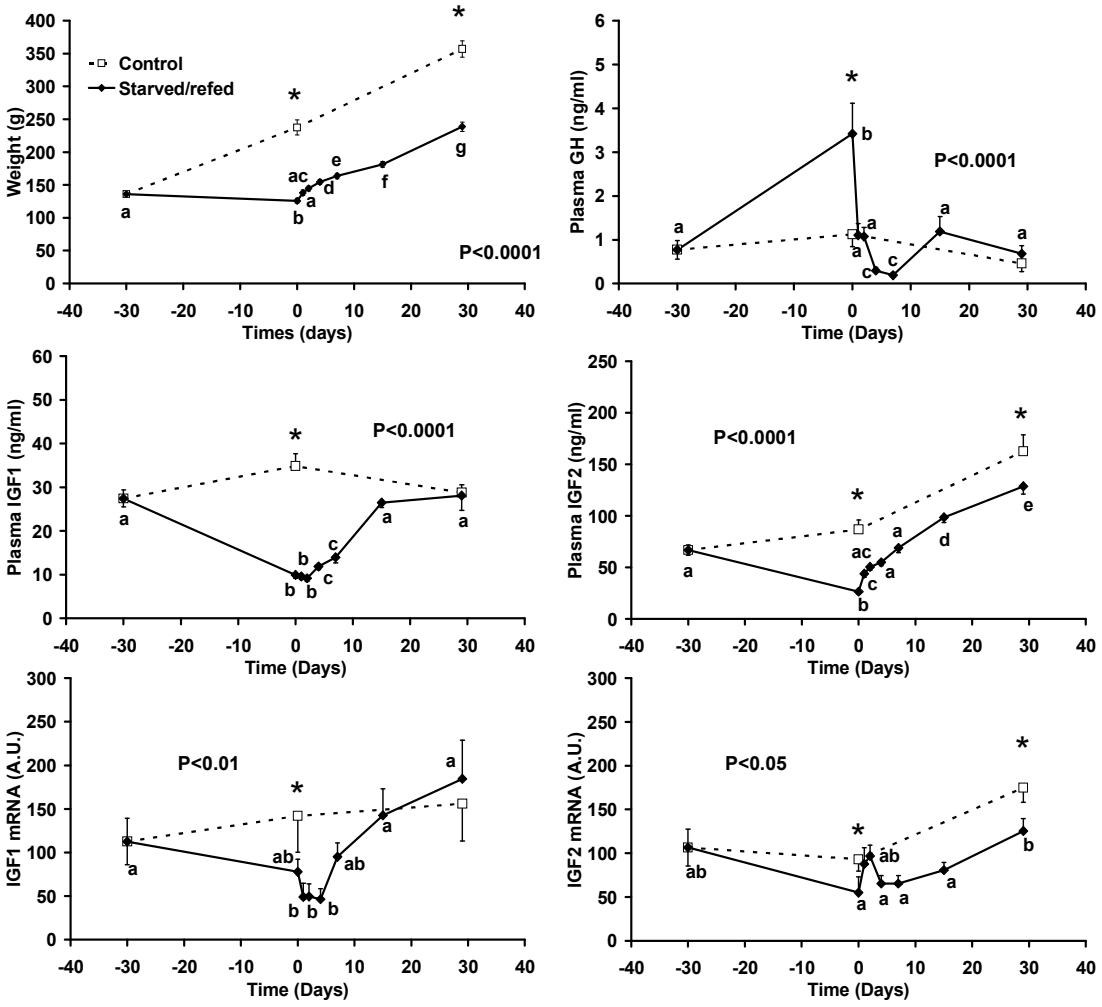

Figure 1: Body weights, plasma GH, plasma IGF1, plasma IGF2, and IGF1 and IGF2 mRNA level in liver of rainbow trout fasted and refed. Fish were fasted for one month (day -30 to day 0) and then refed (day 1 to day 29). Means (±SEM) correspond to 12 fish. The P value corresponds to the results of the one-way analysis of variance Kruskal-Wallis rank test. different letters indicate a significant difference between groups (P<0.05). Stars indicate a significant difference between control and starved/refed groups (P<0.05).

3.2. *Expression profile of the GH receptor*

Changes of GHR1 and GHR2 mRNA abundance in liver and muscle are shown in Fig. 2. In liver, one month of fasting stimulated expression of the GHR1 gene compared to control fish (49±9 *versus* 136±26 A.U.; P<0.01). By contrast, no obvious effect of starvation was observed on GHR2 gene expression in the liver. Whereas 1 day of refeeding decreased GHR1 mRNA abundance, an increase in GHR2 mRNA abundance was simultaneously observed. Thereafter, GHR1 mRNA dropped continuously until day 7 while GHR2 mRNA abundance returned to a similar level to that of the controls as soon as day 7. In muscle, one month of food deprivation raised both GHR mRNA abundances to the same extent (2-fold) followed by a rapid return to control values after 4 days. At day 29 there was no longer any difference between refed fish and the control groups.

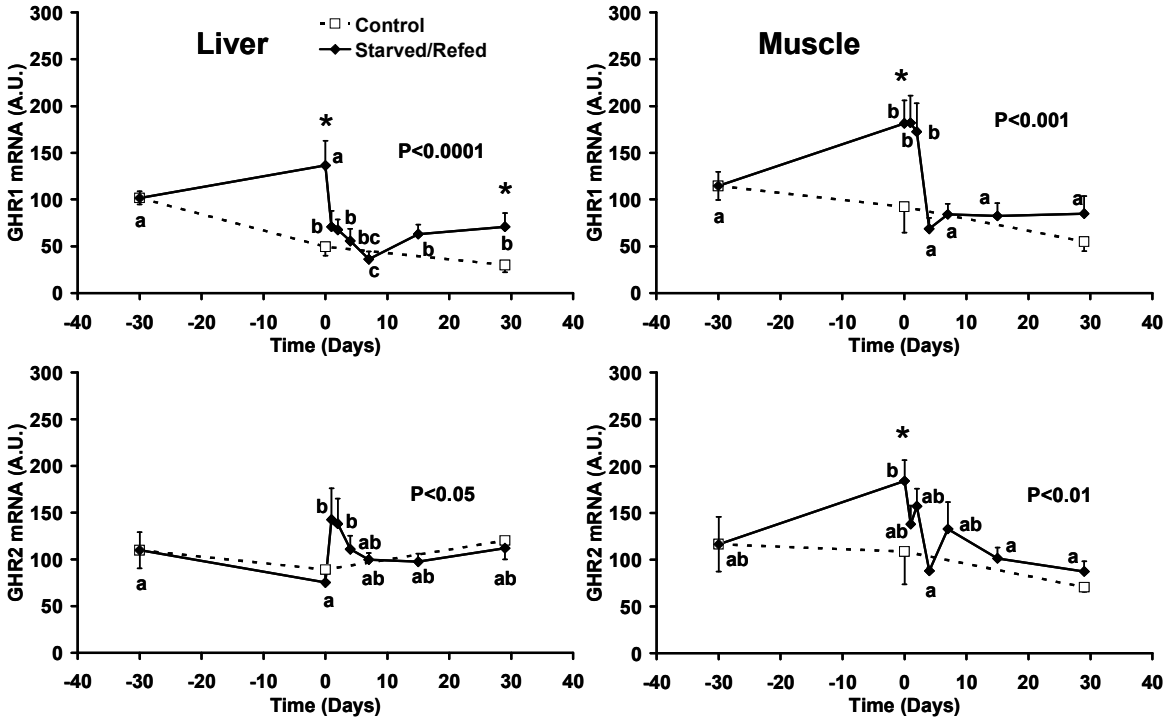

Figure 2: Expression profiles of GHR1 and GHR2 transcripts in liver and muscle (means ± SEM). Fish were fasted for one month (day -30 to day 0) and then refed (day 1 to day 29). Each means corresponds tot 6-7 fish. The P value corresponds to the results of the one-way analysis of variance Kruskal-Wallis rank test. Different letters indicate a significant difference between groups (P<0.05). Stars indicate a significant difference between control and starved/refed groups (P<0.05).

3.3. Expression profiles of IGFBPs and IGFBP-rP1

Changes of IGFBP1-6 and IGFBP-rP1 mRNA abundance in the liver are presented in Fig. 3. Among the 7 genes studied, IGFBP1, IGFBP3, IGFBP5, IGFBP-rP1 did not exhibit obvious changes either after starvation or refeeding. IGFBP2 and IGFBP6 mRNA abundance was similar in starved and fed fish. Next, the mRNA abundances of both IGFBPs rose up to day 7 and then returned to values similar to those of control fish. After 29 days of refeeding IGFBP2 and IGFBP6 mRNA abundances were similar to those of control fish. One month of food deprivation decreased IGFBP4 mRNA abundance leading to 7-fold less mRNA in starved than in control fish (21±6 *versus* 147±32 A.U.; $P<0.01$). Thereafter, from day 2, IGFBP4 mRNA levels increased progressively and no difference between refed and control fish was any longer observed at day 29.

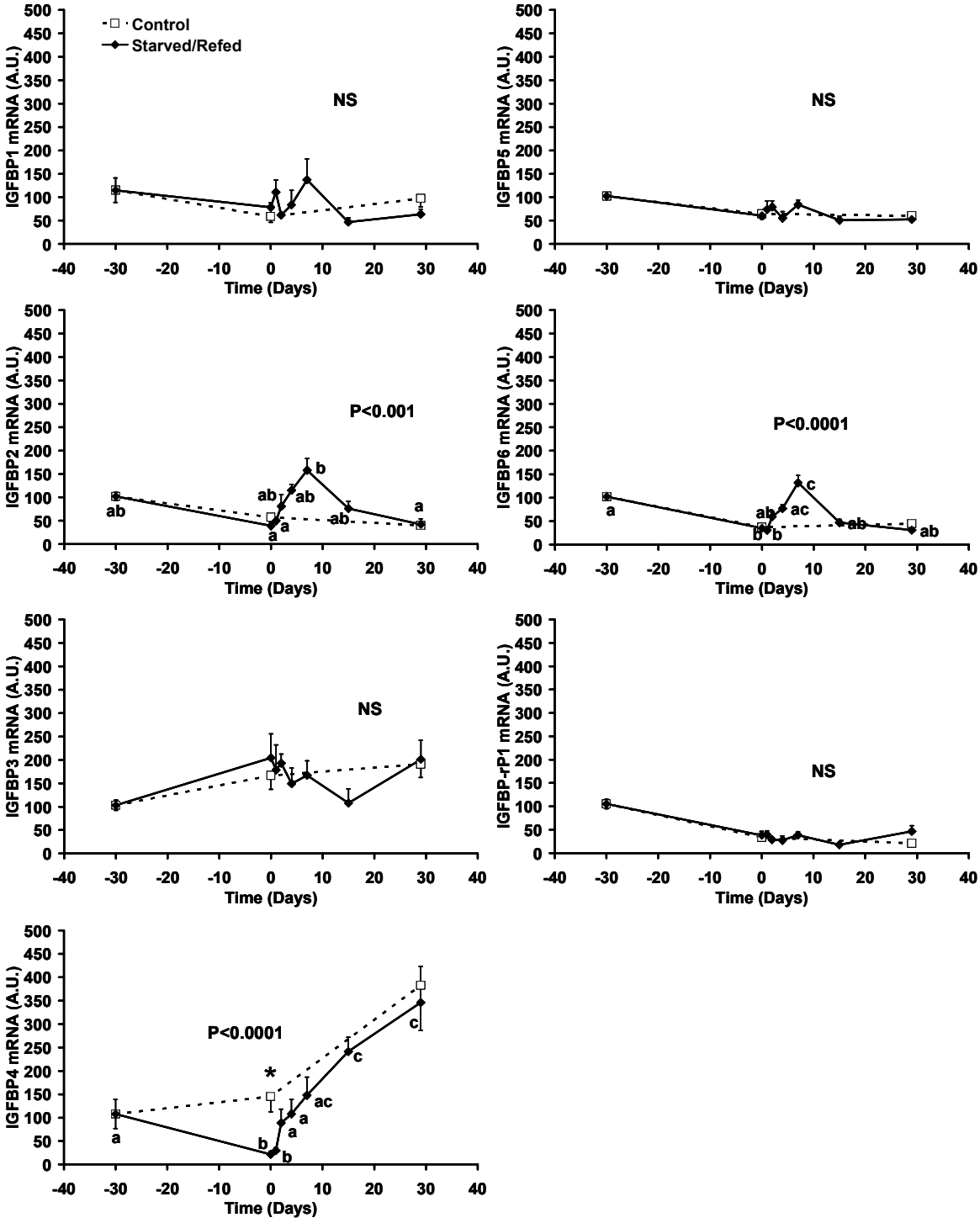

Figure 3: Expression profiles of IGFBP1-6 and IGFBP-rP1 transcripts in liver (means ± SEM). Fish were fasted for one month (day -30 to day 0) and then refed (day 1 to day 29). Each mean corresponds to 6-7 fish. The P value corresponds to the results of the one-way analysis of variance Kruskal-Wallis rank test. Different letters indicate a significant difference between groups ($P<0.05$). Stars indicate a significant difference between control and starved/refed groups ($P<0.05$).

Changes of IGFBP2-6 and IGFBP-rP1 mRNA abundance in muscle are shown in Fig. 4. Among the 7 IGFBP/IGFBP-rP studied, only the IGFBP1 expression profile could not be monitored above background level in the muscle. Starvation or refeeding influenced all IGFBP/IGFBP-rP gene expressions except IGFBP3 where no obvious effect on mRNA abundance was seen. After one month of starvation, IGFBP2 mRNA abundance was lowered (69±10 *versus* 152±18 A.U.; $P>0.05$) compared to the control group. A low level of IGFBP2 mRNA was maintained until day 4 but from day 7 it was restored and was similar to that of the controls. IGFBP4 mRNA abundance was also lower (2-fold) in starved fish than in fed fish. Surprisingly, a 7-fold increase of IGFBP4 mRNA abundance was monitored as soon as 1 day after refeeding, following from day 2 to a return to a level comparable to those of control fish. The abundance of IGFBP5 mRNA was also lower in starved than in control fish. A first peak of its mRNA abundance was observed at day 2 following by a second one at day 7. Next, from day 15, IGFBP5 mRNA abundance returned to a level similar to the control fish. It is noteworthy that the IGFBP5 mRNA abundance at day 7 was above the level observed in the control fish. For IGFBP6, the treatment had a weak but significant ($P<0.05$) effect on the mRNA level. One day of refeeding led to a significant drop (130±30 *versus* 60±12 A.U.; $P<0.05$), leading to a quick and complete restoration of IGFBP6 mRNA levels. IGFBP-rP1 mRNA abundance was lower in starved than in control fish. A first peak of its mRNA abundance was observed at day 2 following by a second one at day 7. Next, from day 15, IGFBP-rP1 mRNA abundance returned to a level similar to the control fish. While no obvious effect of the treatment was observed for expression of IGF2 in muscle (Fig. 4), the response of IGF1 was very strong. Starvation led to a 5-fold drop of IGF1 mRNA abundance compared to control fish. As soon as 1 day of refeeding, the IGF1 mRNA level was restored. Thereafter, a peak of IGF1 expression (7-fold higher compared to starved fish) was monitored at day 7 following to a return to normal value from day 15.

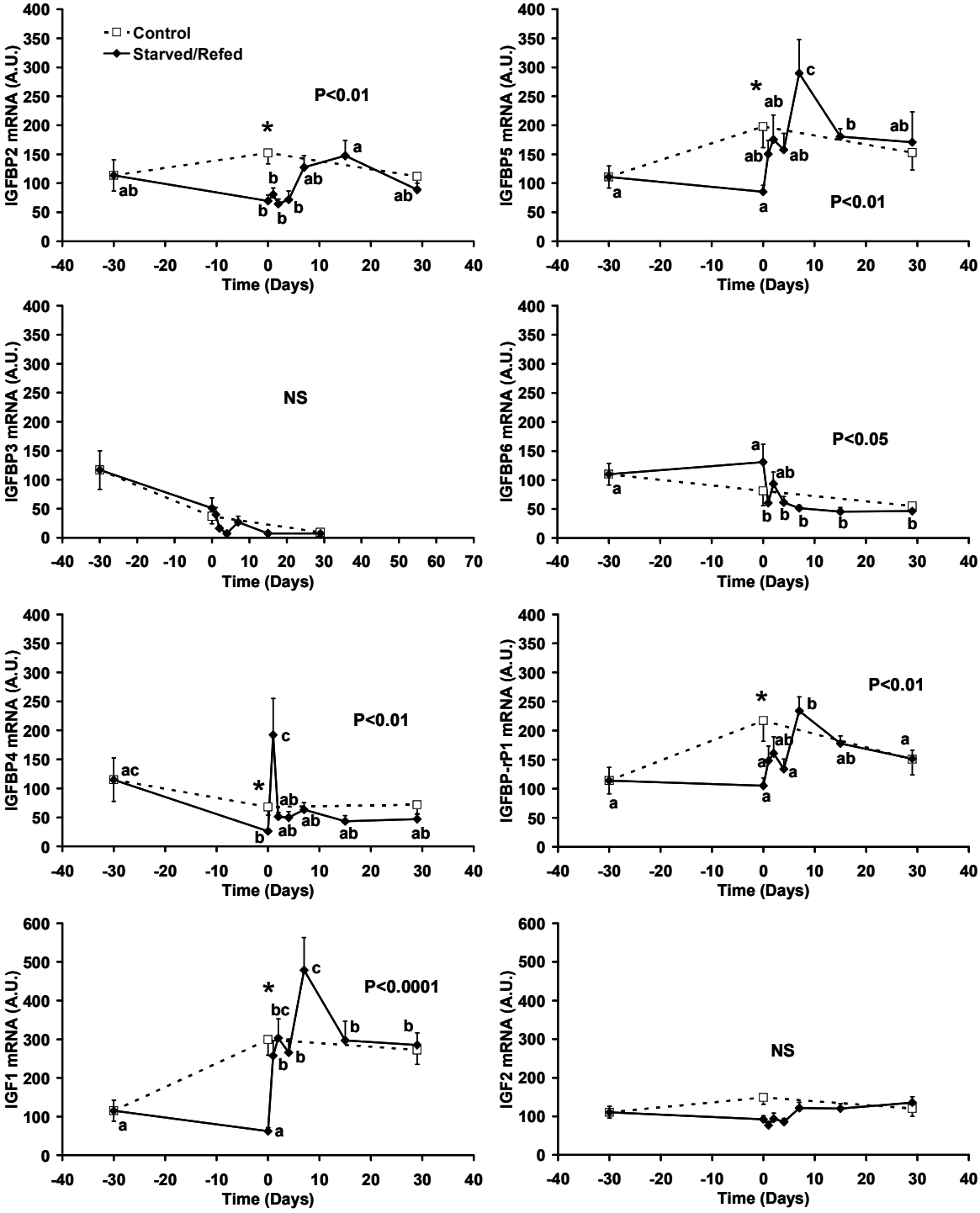

Figure 4: Expression profiles of IGFBP2-6, IGFBP-rP1, IGF1 and IGF2 transcripts in muscle (means ± SEM). The IGFBP1 expression profile could not be monitored above background level in the muscle. Fish were fasted for one month (day -30 to day 0) and then refed (day 1 to day 29). Each means corresponds to 6-7 fish. The P value corresponds to the results of the one-way analysis of variance Kruskal-Wallis rank test. Different letters indicate a significant difference between groups ($P<0.05$). Stars indicate a significant difference between control and starved/refed groups ($P<0.05$).

Myogenin mRNA abundance (Fig. 5) was measured as marker of myogenesis activity. Starvation induced a 3-fold drop of myogenin mRNA abundance compare to control fish. Thereafter, a peak of myogenin expression (5-fold compared to starved fish) was monitored at day 7 following to a return to normal value from day 15.

Principal component analysis did not allow identifying strong determining factor since factor 1, 2 and 3 explain only 19.6, 15.5 and 11.9 % of the total variability. Nevertheless, factor 1 links together parameters that decreased after 1 month of fasting and then increased during refeeding where muscle IGF1, IGFBP5, IGFBP-rP1 and myogenin mRNA were the major descriptors (data not shown). Pearson's linear regression analysis indicated that transcript abundances of IGF1, IGFBP5, IGFBP-rP1 and myogenin were significantly correlated between each other ($r = 0.68$, $P<0.0001$; $r = 0.71$, $P<0.0001$; $r = 0.77$, $P<0.0001$; $r = 0.7$, $P<0.0001$ respectively).

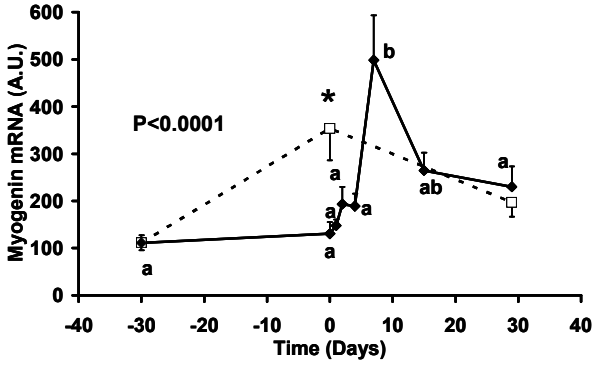

Figure 5: Expression profiles of myogenin transcripts in muscle (means ± SEM). Further details as in figure 4.

4. Discussion

For the first time, this study reports the time-course expression profiles of the GH/IGF system genes during refeeding in rainbow trout. This approach allows us to identify the coordinated regulations between the GH/IGF system genes, which leads to a progressive restoration of this endocrine system.

In order to produce a strong disruption of the GH/IGF system, we starved rainbow trout for one month. As expected food deprivation increased plasma GH level and lowered plasma IGF1 and IGF2 levels, as previously reported in trout (Gentil et al. 1996; Shimizu et al. 1999; Sumpter et al. 1991). Since plasma IGF1 inhibits GH release (Blaise et al. 1995), it is often considered that the drop of IGF1 leads to the rise in the GH level. Likewise, when hepatic production of IGF1 was deleted in the mouse, plasma IGF1 was only 25% of the normal value and these mice exhibited a 3-fold higher GH level (Sjogren et al. 1999). In our study, refeeding rapidly restored plasma GH, since the plasma GH of refed fish was identical to that of the controls after 1 day. Surprisingly, plasma IGF1 started to rise only from day 4. Therefore, plasma IGF1 cannot account for the restoration of plasma GH during refeeding. This is reminiscent of a recent study on the time course response of the GH/IGF system to fasting in salmon (Pierce et al. 2005). Indeed, during fasting, it has been observed that plasma GH increases before the plasma IGF1 drops. Together, these results show that plasma IGF1 is not a key regulator of plasma GH level during starvation and refeeding. On the other hand, our results indicated that plasma IGF2 doubled at day 1 when plasma GH decreased. Given that IGF2 can inhibit GH release (Duval et al. 2002; Blaise et al. 1995) and that plasma IGF2 levels are 2-5 fold higher than plasma IGF1 in the trout, it seems likely that plasma IGF2 could exert an in vivo negative control on GH release. Likewise, we previously reported a negative correlation between plasma GH and plasma IGF2 when fish were subjected to a moderate food restriction (Gabillard et al. 2003). In the present study, we also found a negative correlation between plasma GH and plasma IGF2 from day 0 to day 4 ($r = - 0.68$; $P<0.0001$) whereas no relation was found with plasma IGF1. Therefore, the precocious rise in plasma IGF2 in addition to its high plasma concentration (compared to IGF1) suggest that plasma IGF2 instead of IGF1 may be implicated in the plasma GH regulation during refeeding and perhaps during fasting. Our data also indicated that in the liver, the nutritional state differentially regulated

IGF1 and IGF2 genes suggesting they have a distinct function in endocrine control of growth.

As a result of the recent genome duplication, two genes coding for the GH receptor are present in rainbow trout and our study provides the first data on their regulations during starvation and refeeding. One month of food deprivation increased the hepatic GHR1 mRNA abundance while GHR2 mRNA remained unchanged. In muscle, the levels of the two GHR transcripts were higher in starved fish than in control. One day of refeeding is sufficient to restore GHR1 mRNA abundance in liver, whereas a peak of GHR2 mRNA was observed at day 1. In muscle both GHR mRNA abundance were restored after 4 days. A recent study performed in black seabream (Acanthopagrus schlegeli) reported a lower GHR mRNA level in liver of starved fish (Deng et al. 2004). This apparent discrepancy between these data and ours could be related to species differences as well as to the environmental conditions since water temperature and salinity were not tightly controlled in the Deng et al., study. In salmon, the level of hepatic GHR mRNA was slightly lower in starved fish compared to fed fish (Fukada et al. 2004). However, in this latest study, primers were designed to amplify simultaneously both GHR mRNA, which prevents conclusion about specific regulation of both GHR genes. Moreover, given that we observed an opposite effect of starvation on both GHR genes expression, measurement of each gene appears essential for precisely understanding the regulation GHR genes. Together, our results show that in trout both GHR genes are differentially regulated during starvation and refeeding. In starved fish, the low plasma IGF1 level whereas plasma GH level is high is considered as the result of hepatic GH resistance in accordance with the low GH-binding capacities of the liver in starved fish (Pérez-Sánchez et al. 1994; Pérez-Sánchez et al. 1995). Given that the GHR mRNA level in liver rapidly returned to the normal control value, it cannot explain the late recovery of plasma IGF1 level. There is an apparent discrepancy between previous study on GH-binding and our study on GHR gene expression in response to fasting. However, we recently reported in trout that high temperatures decrease hepatic GH-binding, but increase GHR mRNA level (Gabillard et al. 2006). Furthermore, hepatic GH-binding is strongly influenced by circulating GH independently to the GHR protein expression (Yao et al. 2006). In salmon, Fukada et al., (2004) showed that as soon as 1 week of fasting plasma IGF1 level decreases despite an increase of circulating GH. In this typical situation of hepatic GH resistance, GHR mRNA was unaffected within the first 2 weeks suggesting that GHR mRNA in

such situation cannot explain hepatic GH resistance. Indeed, in mammals it has been shown that fasting does not change the content of GHR protein, and that GH resistance is rather due to the impairment of the signal transduction pathway (Beauloye et al. 2002).

IGFBPs are important components of the GH/IGF system, since they modulate IGF activity and availability, but the regulation of their expression by nutritional status is largely unknown. Our data indicated that after 4 weeks of starvation, hepatic mRNA abundance of IGFBP1 was similar between starved and fed fish. In zebrafish, starvation increased IGFBP1 mRNA abundance (Maures & Duan 2002) to a lesser extent after 3 weeks (2-fold) than after 2 weeks (4-fold). Therefore, it cannot be excluded that prolonged starvation (4 weeks in the present study) allowed the recovery of IGFBP1 mRNA abundance. Starvation and refeeding did not alter IGFBP3, IGFBP5, and IGFBP-rP1 mRNA abundance in the liver suggesting that nutrient availability is not a key regulator of the expression of these genes in the liver. Together these data suggest that expressions of IGFBP1, IGFBP3, IGFBP5, and IGFBP-rP1 in the liver are not key regulators in growth recovery after starvation. We observed that IGFBP4 expression decreased after 4 weeks of starvation following a progressive recovery. This observation is reminiscent to what is observed in mammals (Chen & Arnqvist 1994) but the physiological significance is still largely unknown. Interestingly, IGFBP2 and IGFBP6 exhibited a similar expression profile i.e. no effect of starvation followed by a peak of mRNA abundance after 7 days of refeeding. The significance of the IGFBP2 and IGFBP6 peaks is not known but IGFBP2 is known to inhibit IGF1 action in zebrafish (Duan et al. 1999a) and it is considered to be a marker of the catabolic state in fish (Kelley et al. 2001). Moreover in humans, refeeding may lead to a transitory diabetes (Marinella 2005) which is known to increase IGFBP2 mRNA level in the liver (Chen & Arnqvist 1994). Therefore, during refeeding, changes of IGFBP2 and IGFBP6 mRNA levels could result from metabolic changes in the liver (Navarro & Gutierrez 1995; Pottinger et al. 2003) within the first weeks of refeeding.

In muscle, IGFBP1 mRNA expression was not detected and IGFBP3 mRNA was not influenced by the nutritional status. In zebrafish, 3 weeks of food deprivation did not significantly modify the IGFBP3 mRNA abundance (Chen et al. 2004). Together, this suggests that IGFBP1 and IGFBP3 do not participate in muscle growth recovery. Starvation decreased IGFBP2 mRNA level as observed in zebrafish (Duan et al. 1999a) and refeeding started to restore IGFBP2 mRNA level from day 7, suggesting

that it is not involved in the precocious events leading to muscle growth recovery. Starvation slightly increased IGFBP6 mRNA abundance and refeeding quickly restored it. In terms of their differences in expression profile in liver and muscle, it appears that IGFBP2 and IGFBP6 should not have similar function in muscle and liver. IGFBP4 has a unique expression profile consisting of a lower mRNA abundance in starved fish, followed by a transitory but strong increase one day after refeeding. Given that in muscle, IGFBP4 mRNA has been detected in connective tissue instead of muscle fibers (Jennische & Hall 2000), our observations therefore suggest that the transitory increase of IGFBP4 mRNA does not participate in myogenesis resumption.

IGFBP5, IGFBP-rP1, and IGF1 exhibited similar response to fasting and refeeding. Their expression profile involved a decrease of mRNA abundance after starvation, then refeeding induced a first peak of expression at day 2, a second at day 7 and a complete restoration from day 15. Moreover, the mRNA abundance of these genes were highly correlated ($r = 0.6\text{-}0.8$; $P<0.0001$). Therefore, our observation indicates that IGFBP5, IGFBP-rP1, and IGF1 are co-ordinately regulated during refeeding. In this experiment and other (Chauvigné et al. 2003), we observed a peak of IGF1 mRNA expression, which was accompanied by a peak of myogenin. Given that myogenin is involved in the differentiation of myoblast (Florini et al. 1996), it is likely that the peak of IGF1 expression is associated with the resumption of myogenesis. Likewise, IGFBP5 expression that is up-regulated by IGF1 in mammals (Duan et al. 1999b), is also stimulated during myoblast differentiation (James et al. 1993). Moreover, it has been shown that after injury IGFBP5 expression is restricted to the regenerating muscle cell (Jennische & Hall 2000). In contrast, IGFBP-rP1 is up-regulated in proliferative myoblast and down-regulated in differentiating myoblast (Damon et al. 1997). Therefore, elevation of IGFBP5 mRNA would arise from differentiating myoblast whereas IGFBP-rP1 mRNA would arise from proliferating myoblasts. Together, our observations strongly suggest that the expression profiles of IGF1, IGFBP5, and IGF-rP1 are a signature of the resumption of myogenic activity.

In conclusion, the rapid return to a control value of the plasma GH and liver GHR1 expression is the first step of the restoration of GH/IGF system activity after starvation. Plasma IGF1 level is restored later than plasma GH level, which does not support the current model according to which elevation of plasma GH would result from the decrease of plasma IGF1. Given that the precocious restoration of plasma IGF2 level, our study rather suggests a role of IGF2 instead of IGF1 in plasma GH level

restoration. In muscle, our observations indicate that fasting and refeeding induced a coordinated regulation of IGF1, IGFBP5 and IGFBP-rP1 that are probably involved in a strong myogenesis resumption. Nevertheless, functional analysis of IGFBP5 and IGFBP-rP1 remains to be performed to fully understand their role in myogenesis.

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