

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

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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, IGF-I and IGF-II and the expression of the GH/IGF system in liver and muscle. Starvation increased the plasma GH level and 1 day of refeeding completely restored it (1.10 ± 0.27 vs 1.12 ± 0.28 ng/ml). Thereafter, plasma GH continued to decrease until day 7 and returned to control values from day 15. Starvation decreased plasma IGF-I and IGF-II and refeeding raised plasma IGF-I only from day 4. In contrast, the plasma IGF-II level doubled after 1 day's refeeding (26.5 ± 1.9 vs 44.0 ± 3.4 ng/ml; $P < 0.01$). Starved fish exhibited higher GH receptor (GHR)1 mRNA abundance in liver and muscle than in controls, whereas GHR2 mRNA abundance was increased only in muscle. In liver, 1 day of refeeding, decreased GHR1 (twofold), but increased GHR2 mRNA abundance (twofold). Thereafter, a progressive return to normal values was observed. Liver IGFBP-4 mRNA

abundance was lowered in starved fish followed by a progressive restoration during refeeding. Starvation had no effect on liver IGFBP-2 and IGFBP-6 mRNA abundance, whereas refeeding provoked a peak of IGFBP-2 and IGFBP-6 expression at day 7. In muscle, starvation led to a decrease of the IGFBP-2 mRNA level, which was restored only from day 7. IGFBP-4 mRNA abundance in starved fish was lower than in the controls and refeeding led to a transient upregulation (sevenfold) of *IGFBP-4* gene at day 1. IGF-I, IGFBP-5, and IGFBP-related protein 1 (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, IGF-I, IGFBP-5, and IGFBP-rP1 mRNA abundance were positively correlated ($r = 0.6-0.8$; $P < 0.0001$). In conclusion, plasma IGF-I was restored later than plasma GH level, which suggests that plasma IGF-I levels cannot account for plasma GH changes. The coordinated regulation of IGF-I, IGFBP-5, and IGFBP-rP1 expression would be a signature for the resumption of myogenic activity.

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Introduction

Fish growth is a complex function mostly regulated by the growth hormone (GH)/insulin-like growth factor (IGF) system (Gabillard *et al.* 2005, Reinecke *et al.* 2005, Wood *et al.* 2005). The general organization of this system is well conserved between higher vertebrates and fish, including GH, GH receptor (GHR), IGF-I and IGF-II, IGF receptors (IGFRI and IGFRII), and IGF binding proteins (IGFBPs). Resulting from their higher affinity for IGFs than that of the IGF receptor itself, the IGFBPs not only act as carriers of IGFs, but also function as modulators of IGF availability and activity. In addition to the six 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 (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 mostly on nutrient availability, known to modulate the GH/IGF system activity. Starvation increases plasma GH levels (Sumpter *et al.* 1991, Duan & Plisetskaya 1993, Pérez-Sánchez *et al.* 1995, Marchelidon *et al.* 1996, Weber & Grau 1999, Pierce *et al.* 2005) 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, 1995). Recently, it was shown that starvation decreased the expression of GHR mRNA 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 IGF-I observed in starved fish (Pérez-Sánchez et al. 1995, Baños et al. 1999, Shimizu et al. 1999) and that the increase in GH levels results from the decline of IGF-I feedback on GH cells. Nevertheless, the evidence for this hypothesis is still lacking and the possible role of IGF-II 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 rare. Starvation increases plasma IGFBP-1 (Siharath et al. 1996, Shimizu et al. 1999, Kelley et al. 2001) and IGFBP-2 mRNA levels in zebrafish (Duan et al. 1999a, Maures & Duan 2002). Plasma IGFBP-3 is decreased in starved salmon (Shimizu et al. 2003), but no difference of IGFBP-3 mRNA abundance is observed in starved zebrafish (Chen et al. 2004). To date, nutritional regulation of IGFBP-4 to IGFBP-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 levels of GH, IGF-I, and IGF-II 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 IGF-I was restored after plasma GH and that the muscular expressions of IGF-I, IGFBP-5, and IGFBP-rP1 were coordinated and associated with muscle growth recovery.

Materials and Methods

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 (nine

different tanks, 50 fish/tank) were starved for 1 month and re-fed for 1 month with food available *ad libitum*. The control group (reared in triplicate) had food available *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, Nersac, France). The water temperature was 18 °C before starvation (day 30), 15.3 °C at day 0, and 12.3 °C at day 29.

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 days 0, 1, 2, 4, 7, 15 and 29 days following refeeding, 12 fish (four 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 (four per tank) on days 0 and 29. Fish were anesthetized with eugenol (10 ml/l), killed by a blow on the head and blood was collected within 10 min.

Sample analysis

Plasma GH, IGF-I and IGF-II levels were measured by homologous RIA as previously described (Le Bail et al. 1991, Gabillard et al. 2003).

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

Quantitative PCR analyses were carried out with 5 µl reverse transcriptase (RT) reaction using a real-time PCR kit

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

Gene	Forward	Reverse
<i>IGF-I</i>	TGGACACGCTGCAGTTTGTGTG	CACTCGTCCACAATACCACGGT
<i>GHR1</i>	CGTCCTCATCCTCCAGTTT	GTTCTGTGAGGTTCTGGAAAAC
<i>GHR2</i>	TCTCCTCATCCTAACAGTTGTG	GCTCTGTGAGGTTCTGGAAATGT
<i>IGFBP-1</i>	AGTTCACCAACTTCTACCTACC	GACGACTCACACTGCTTGCC
<i>IGFBP-2</i>	GTGCTGGAGAGGATATCTAAGA	AGACATCTTACACTGTTTGAGGT
<i>IGFBP-3</i>	TTCCATGATAACAGGGGACATG	GACCTGGGTGGTGGACATGTGG
<i>IGFBP-4</i>	TGTCGTGCTGAGCTGCAGAG	TGGCTGGCACTGCTTGGCAT
<i>IGFBP-5</i>	ACTTACGCGCTTCTCCATGGCA	CGAGACTCATGATCTATGGGTGGA
<i>IGFBP-6</i>	GCTCAATAGTGTCTGCGTGG	CTTGGAGGAACGACACTGCTT
<i>IGFBP-rP1</i>	GCTCCGATGGAGTGACCTATA	ACAATGACAGGTGCTGTTCGG
<i>EF1α</i>	AGCGCAATCAGCCTGAGAGGTA	GCTGGACAAGCTGAAGGCTGAG

provided with an SYBR Green fluorophore (Eurogentec, Seraing, 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 s; 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 iCycler iQTM software (Bio-Rad, Hercules, CA, USA). Subsequently, real-time PCR data were normalized using eF1a transcript abundance as follows:

$$\text{Correcting factor} = \frac{\text{eF1a value}}{\text{mean of eF1a values for a given group (ten groups)}}$$

Normalized data = raw data/correcting factor.

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

Statistical analysis

The refeeding effect was analyzed 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's *U*-test. All the data are presented as the mean \pm S.E.M. All parameters measured were subjected to a principal component analysis (PCA) using PAST software, version 1.18, <http://folk.uio.no/ohammer/past/>; Hammer *et al.* 2001). Pearson's linear regression was used to assess the relationship between IGF-I, IGFBP-5, and IGFBP-rP1 mRNA abundance.

Results

Hormonal profile of GH, IGF-I and IGF-II after refeeding

Fish of the fed group grew rapidly during the experiment, whereas starved fish lost weight (136 ± 2.9 vs 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 vs 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 days 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 IGF-I levels were significantly decreased by 1 month's starvation, leading to a threefold decrease in IGF-I levels in starved fish compared with the controls (9.9 ± 0.7 vs 34.8 ± 2.8 ng/ml; $P < 0.001$). Two days after refeeding, plasma IGF-I remained low. After 4 days, plasma IGF-I started to increase slightly, but it needed 2 weeks for the level to be restored compared with the control group. One month after refeeding, no difference in plasma IGF-I was seen between the controls and starved fish. The expression profile of IGF-I in the liver was similar to what was observed at plasma level. Nevertheless, it is noteworthy that after refeeding, IGF-I mRNA abundance continued to decrease up to 4 days.

Plasma IGF-II levels (Fig. 1) were also significantly decreased by 1 month's starvation. At day 0, the IGF-II levels of starved fish were threefold lower than in control fish (26.5 ± 1.9 vs 86.8 ± 9.2 ng/ml; $P < 0.001$). At day 1, the plasma IGF-II level in refeed 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 IGF-II levels in the control group were always higher (128 ± 9 vs 162 ± 16 ng/ml; $P < 0.01$). In control fish, plasma IGF-II levels increased throughout the experiment.

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

Expression profile of the GH receptor

Changes of GHR1 and GHR2 mRNA abundance in liver and muscle are shown in Fig. 2. In liver, 1 month of fasting stimulated the expression of the *GHR1* gene compared with control fish (136 ± 26 vs 49 ± 9 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 decreased continuously until day 7, while *GHR2* mRNA abundance returned to a level similar to that of the controls, as early as day 7. In muscle, 1 month of food deprivation raised both GHR mRNA abundances to the same extent (twofold) followed by a rapid return to control values after 4 days. At day 29, there was no longer any difference between the refeed fish and the control groups.

Expression profiles of IGFFBPs and IGFBP-rP1

Changes of IGFBP-1 to IGFBP-6 and IGFBP-rP1 mRNA abundance in the liver are presented in Fig. 3. Among the seven genes studied, *IGFBP-1*, *IGFBP-3*, *IGFBP-5*, and

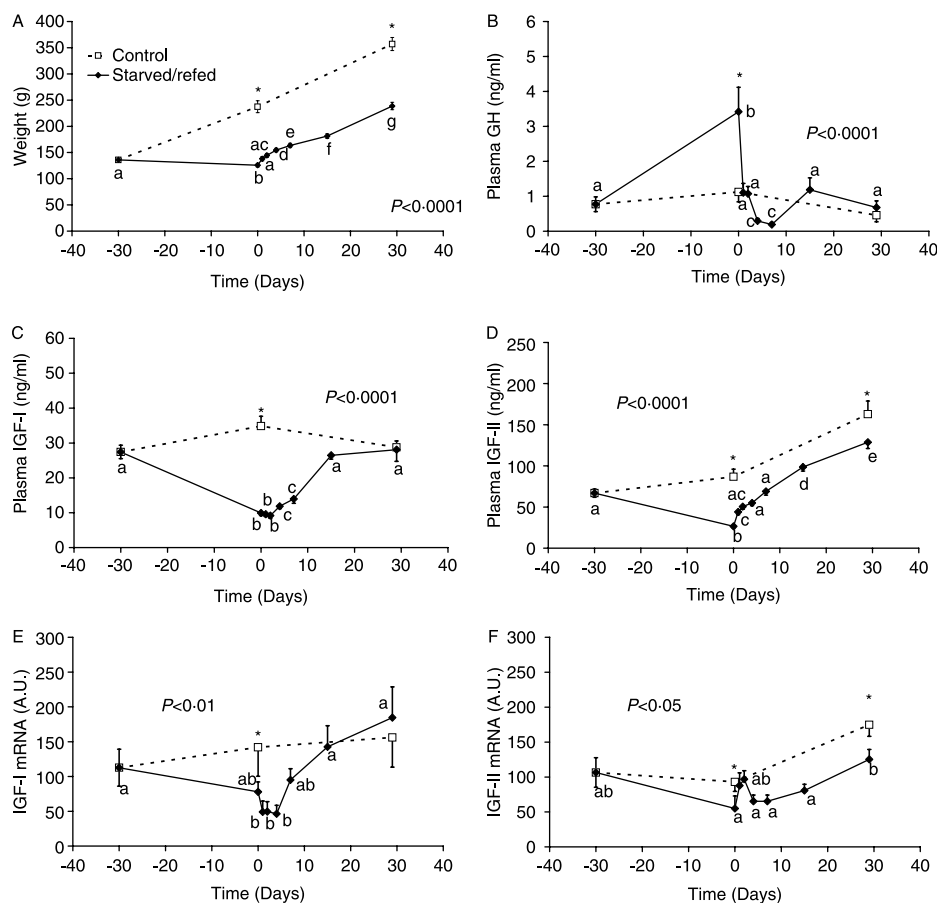


Figure 1 Body weights (A), plasma GH (B), plasma IGF-I (C), plasma IGF-II (D), and IGF-I mRNA (E) and IGF-II (F) mRNA level in liver of rainbow trout fasted and refed. Fish were fasted for 1 month (days -30 to 0) and then refed (days 1 to 29). Means (\pm S.E.M.) correspond to 12 fish. The P value corresponds 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 ($P < 0.05$).

IGFBP-rP1 did not exhibit obvious changes either after starvation or refeeding. *IGFBP-2* and *IGFBP-6* mRNA abundances were 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, *IGFBP-2* and *IGFBP-6* mRNA abundances were similar to those of control fish. One month of food deprivation decreased *IGFBP-4* mRNA abundance leading to sevenfold less mRNA in starved than in control fish (21 ± 6 vs 147 ± 32 A.U.; $P < 0.01$). Thereafter, from day 2, *IGFBP-4* mRNA levels increased progressively and no difference between refed and control fish was any longer observed at day 29.

Changes of *IGFBP-2* to *IGFBP-6* and *IGFBP-rP1* mRNA abundance in muscle are shown in Fig. 4. Among the seven *IGFBPs/IGFBP-rP* studied, only the *IGFBP-1* expression profile could not be monitored above background level in the muscle. Starvation or refeeding influenced all

IGFBP/IGFBP-rP gene expressions, except *IGFBP-3* where no obvious effect on mRNA abundance was seen. After 1 month of starvation, *IGFBP-2* mRNA abundance was lowered (69 ± 10 vs 152 ± 18 A.U.; $P > 0.05$) compared with the control group. A low level of *IGFBP-2* mRNA was maintained until day 4, but from day 7, it was restored and was similar to that of the controls. *IGFBP-4* mRNA abundance was also lower (twofold) in starved fish than in fed fish. Surprisingly, a sevenfold increase of *IGFBP-4* mRNA abundance was monitored as soon as 1 day after refeeding, followed from day 2 by a return to a level comparable to that of control fish. The abundance of *IGFBP-5* mRNA was also lower in starved than in control fish. A first peak of its mRNA abundance was observed at day 2 followed by a second one at day 7. Next, from day 15, *IGFBP-5* mRNA abundance returned to a level similar to the control fish. It is noteworthy that the *IGFBP-5* mRNA abundance at day 7 was above the level observed in the

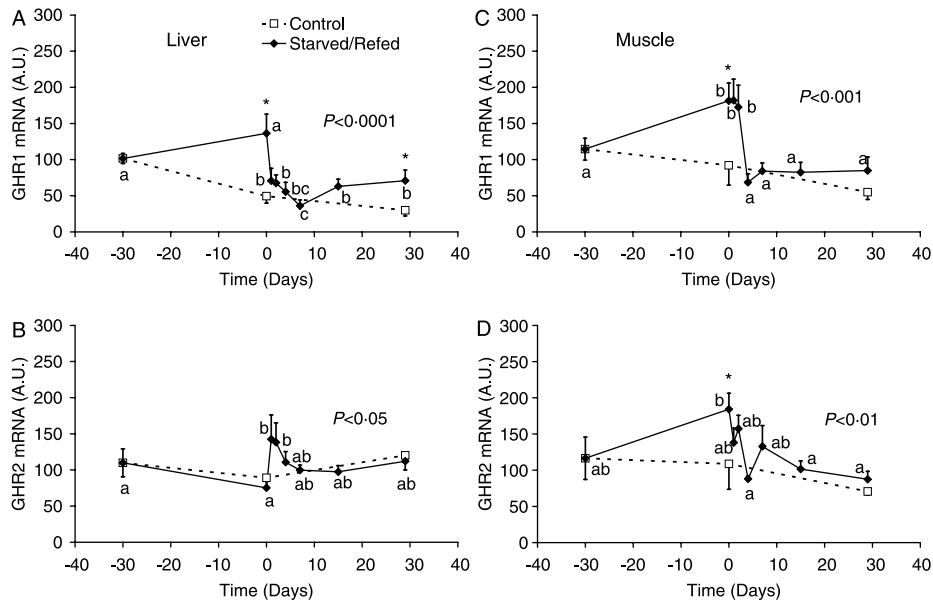


Figure 2 Expression profiles of GHR1 and GHR2 transcripts in liver (A and B) and muscle (C and D) (mean \pm S.E.M.). Fish were fasted for 1 month (days -30 to 0) and then refeed (days 1 to 29). Each mean corresponds to six or seven fish. The P value corresponds to the results of the one-way analysis 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 ($P < 0.05$).

control fish. For IGFBP-6, the treatment had a weak, but significant ($P < 0.05$) effect on the mRNA level. One day of refeeding led to a significant decrease (60 ± 12 vs 130 ± 30 A.U.; $P < 0.05$), leading to a quick and complete restoration of IGFBP-6 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 followed 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 on the expression of IGF-II in muscle (Fig. 4), the response of IGF-I was very strong. Starvation led to a fivefold decrease of IGF-I mRNA abundance compared with control fish. As soon as 1 day of refeeding, the IGF-I mRNA level was restored. Thereafter, a peak of IGF-I expression (sevenfold higher compared with starved fish) was monitored at day 7 followed by a return to normal value from day 15. Myogenin mRNA abundance (Fig. 5) was measured as a marker of myogenesis activity. Starvation induced a threefold decrease of myogenin mRNA abundance compared with control fish. Thereafter, a peak of myogenin expression (fivefold compared with starved fish) was monitored at day 7 followed by a return to normal values from day 15.

PCA did not allow the identification of a strong determining factor, since factors 1, 2, and 3 explain only 19.6, 15.5, and 11.9% of the total variability. Nevertheless, factor 1 links parameters together that decreased after 1 month of fasting and then increased during refeeding, where muscle IGF-I, IGFBP-5, IGFBP-rP1, and myogenin mRNA were the major

descriptors (data not shown). Pearson's linear regression analysis indicated that the transcript abundances of IGF-I, IGFBP-5, 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).

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 regulation 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 1 month. As expected, food deprivation increased plasma GH levels and lowered plasma IGF-I and IGF-II levels, as previously reported in trout (Sumpter *et al.* 1991, Gentil *et al.* 1996, Shimizu *et al.* 1999). Since plasma IGF-I inhibits GH release (Blaise *et al.* 1995), it is often considered that the decrease of IGF-I leads to the increase in the GH levels. Likewise, when hepatic production of IGF-I was deleted in the mouse, plasma IGF-I was only 25% of the normal value and these mice exhibited a threefold higher GH level (Sjogren *et al.* 1999). In our study, refeeding rapidly restored plasma GH, since the plasma GH of refeed fish was identical to that of the controls after 1 day.

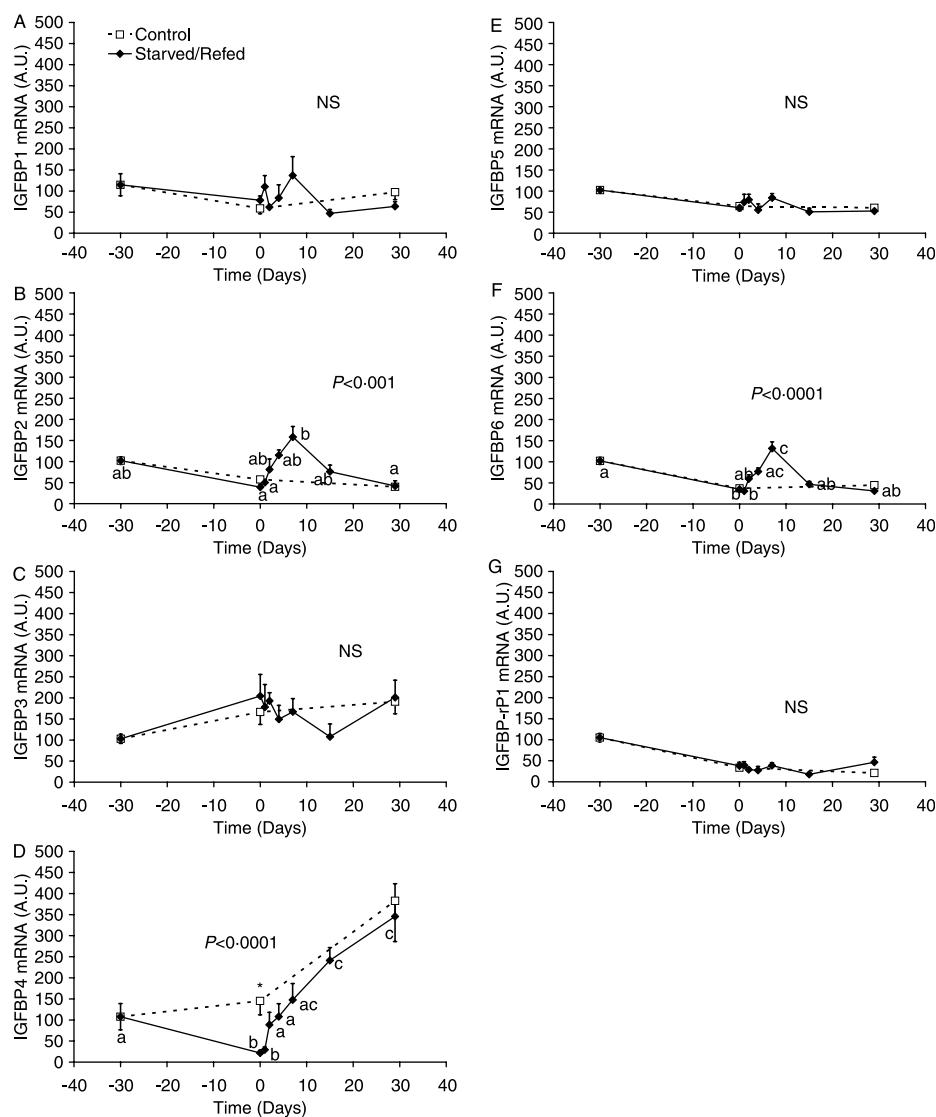


Figure 3 Expression profiles of IGFBP-1 to IGFBP-6 (A–F) and IGFBP-rP1 (G) transcripts in liver (mean \pm S.E.M.). Fish were fasted for 1 month (days -30 to 0) and refed (days 1 to 29). Each mean corresponds to six to seven fish. The P value corresponds to the results of the one-way analysis of variance Kruskal–Wallis rank test. NS, not significant. Different letters indicate a significant difference between groups ($P < 0.05$). Stars indicate a significant difference between control and starved/refed groups ($P < 0.05$).

Surprisingly, plasma IGF-I started to increase only from day 4. Therefore, plasma IGF-I 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 IGF-I decreases. Together, these results show that plasma IGF-I is not a key regulator of plasma GH level during starvation and refeeding. On the other hand, our results indicated that the plasma IGF-II doubled at day 1 when plasma GH decreased. Given that IGF-II can inhibit GH release (Blaise *et al.* 1995, Duval *et al.* 2002) and that plasma

IGF-II levels are two- to fivefold higher than plasma IGF-I in the trout, it seems likely that plasma IGF-II could exert an *in vivo* negative control on GH release. Likewise, we previously reported a negative correlation between plasma GH and plasma IGF-II 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 IGF-II from days 0 to 4 ($r = -0.68$; $P < 0.0001$), whereas no correlation was found with plasma IGF-I. Therefore, the precocious increase in plasma IGF-II in addition to its high plasma concentration (compared with IGF-I) suggest that plasma IGF-II instead of IGF-I may be

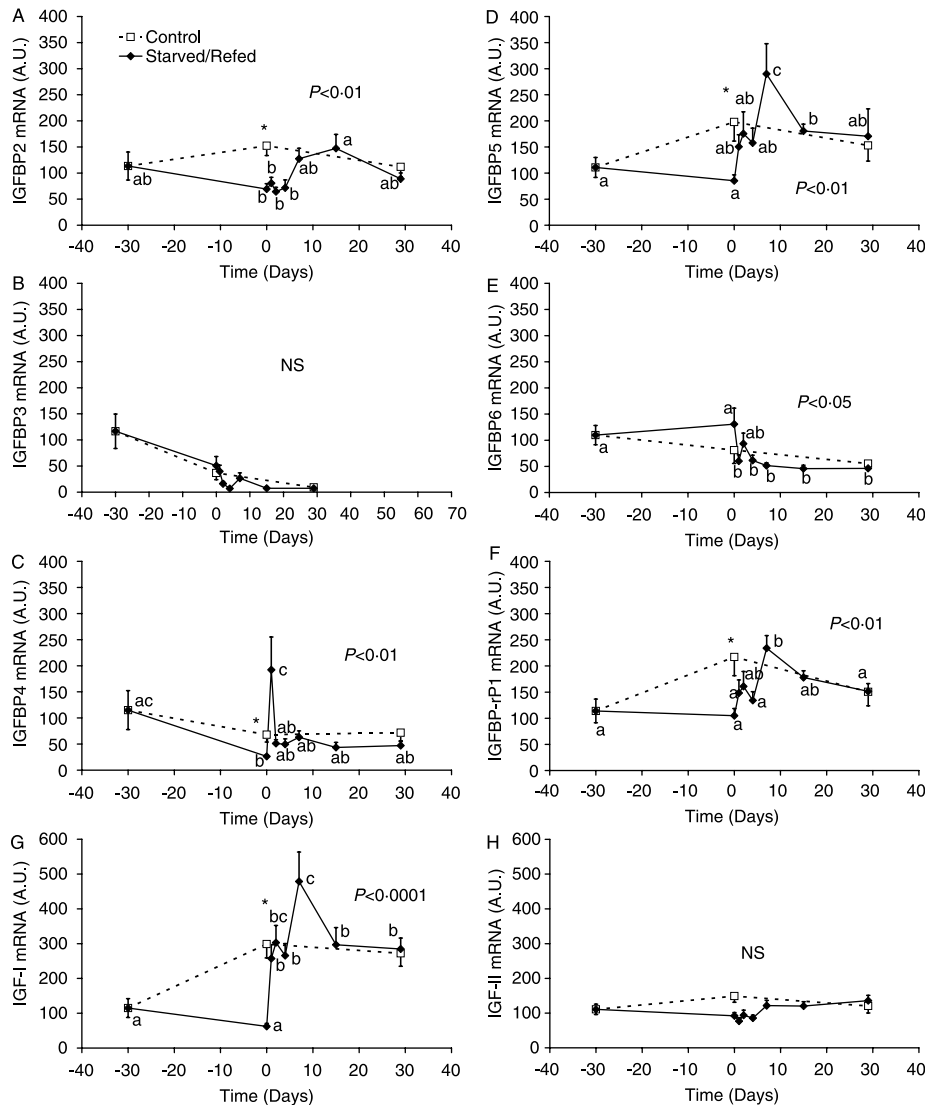


Figure 4 Expression profiles of IGFBP-2 to IGFBP-6 (A–E), IGFBP-rP1 (F), IGF-I (G) and IGF-II (H) transcripts in muscle (mean \pm S.E.M.). The IGFBP-1 expression profile could not be monitored above background level in the muscle. Fish were fasted for 1 month (days -30 to 0) and refed (days 1 to 29). Each means correspond to six to seven 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 ($P < 0.05$).

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 *IGF-I* and *IGF-II* genes suggesting that 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 regulation 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 controls. 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 abundances were restored after 4 days. A recent study performed in black seabream (*Acanthopagrus schlegelii*) 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.

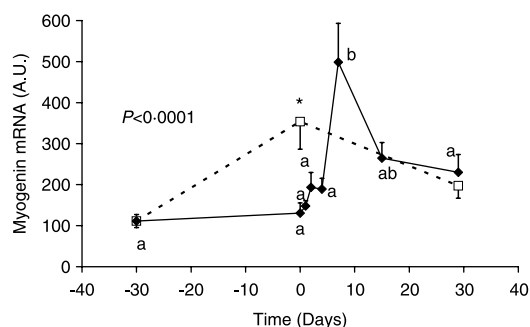


Figure 5 Expression profiles of myogenin transcripts in muscle (mean \pm S.E.M.). Further details are provided in the legend to Fig. 4.

In salmon, the level of hepatic GHR mRNA was slightly lower in starved fish compared with fed fish (Fukada *et al.* 2004). However, in this latest study, primers were designed to amplify simultaneously the mRNA of both GHRs, 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, the measurement of each gene appears essential for precisely understanding the regulation of *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 IGF-I level, with high plasma GH level, is considered to be the result of hepatic GH resistance in accordance with the low GH-binding capacities of the liver (Pérez-Sánchez *et al.* 1994, 1995). Given that the GHR mRNA level in liver rapidly returned to the normal control value, it cannot explain the late recovery of plasma IGF-I level. There is an apparent discrepancy between previous studies 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 levels (Gabillard *et al.* 2006). Furthermore, hepatic GH-binding is strongly influenced by circulating GH independent of the GHR protein expression (Yao *et al.* 2006). In salmon, Fukada *et al.* (2004) showed that as little as 1 week of fasting, decreased plasma IGF-I levels 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 a 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 IGFBP-1 was similar between starved and fed fish. In zebrafish, starvation increased IGFBP-1 mRNA abundance (Maures & Duan 2002) to a lesser extent after 3 weeks (twofold) than after 2 weeks (fourfold).

Therefore, it cannot be excluded that prolonged starvation (4 weeks in the present study) allowed the recovery of IGFBP-1 mRNA abundance. Starvation and refeeding did not alter IGFBP-3, IGFBP-5, 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 the expressions of IGFBP-1, IGFBP-3, IGFBP-5, and IGFBP-rP1 in the liver are not key regulators in growth recovery after starvation. We observed that IGFBP-4 expression decreased after 4 weeks of starvation following a progressive recovery. This observation is reminiscent of that observed in mammals (Chen & Arnqvist 1994), but the physiological significance is still largely unknown. Interestingly, IGFBP-2 and IGFBP-6 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 IGFBP-2 and IGFBP-6 peaks is not known but IGFBP-2 is known to inhibit IGF-I 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 IGFBP-2 mRNA levels in the liver (Chen & Arnqvist 1994). Therefore, during refeeding, changes of IGFBP-2 and IGFBP-6 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, IGFBP-1 mRNA expression was not detected and IGFBP-3 mRNA was not influenced by the nutritional status. In zebrafish, 3 weeks of food deprivation did not significantly modify the IGFBP-3 mRNA abundance (Chen *et al.* 2004). Together, this suggests that IGFBP-1 and IGFBP-3 do not participate in muscle growth recovery. Starvation decreased IGFBP-2 mRNA levels as observed in zebrafish (Duan *et al.* 1999a) and refeeding started to restore IGFBP-2 mRNA levels from day 7, suggesting that it is not involved in the precocious events leading to muscle growth recovery. Starvation slightly increased IGFBP-6 mRNA abundance and refeeding quickly restored it. In terms of their differences in expression profile in liver and muscle, it appears that IGFBP-2 and IGFBP-6 should not have similar functions in muscle and liver. IGFBP-4 has a unique expression profile consisting of a lower mRNA abundance in starved fish, followed by a transitory but strong increase 1 day after refeeding. Given that in muscle, IGFBP-4 mRNA has been detected in connective tissue instead of muscle fibers (Jennische & Hall 2000), our observations suggest that the transitory increase of IGFBP-4 mRNA does not participate in myogenesis resumption.

IGFBP-5, IGFBP-rP1, and IGF-I exhibited similar responses to fasting and refeeding. Their expression profiles involved a decrease of mRNA abundance after starvation, and 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 was highly correlated ($r = 0.6 - 0.8$; $P < 0.0001$). Therefore, our observation indicates that IGFBP-5, IGFBP-rP1, and IGF-I are

coordinately regulated during refeeding. In this experiment and others by Chauvigné *et al.* (2003), we observed a peak of IGF-I 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 IGF-I expression is associated with the resumption of myogenesis. Likewise, IGFBP-5 expression, which is upregulated by IGF-I in mammals (Duan *et al.* 1999b), is also stimulated during myoblast differentiation (James *et al.* 1993). Moreover, it has been shown that after injury IGFBP-5 expression is restricted to the regenerating muscle cell (Jennische & Hall 2000). In contrast, IGFBP-rP1 is upregulated in proliferative myoblast and downregulated in differentiating myoblast (Damon *et al.* 1997). Therefore, the elevation of IGFBP-5 mRNA would arise from differentiating myoblasts, whereas IGFBP-rP1 mRNA would arise from proliferating myoblasts. Together, our observations strongly suggest that the expression profiles of IGF-I, IGFBP-5, 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 IGF-I levels are restored after plasma GH levels, which does not support the present model according to which the elevation of plasma GH would result from the decrease of plasma IGF-I. Given the precocious restoration of plasma IGF-II levels, our study suggests a role of IGF-II instead of IGF-I in plasma GH level restoration. In muscle, our observations indicate that fasting and refeeding induced a coordinated regulation of IGF-I, IGFBP-5, and IGFBP-rP1 all of which are probably involved in a strong myogenesis resumption. Nevertheless, the functional analysis of IGFBP-5 and IGFBP-rP1 remains to be performed to fully understand their role in myogenesis.

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