CBP manuscript 21815 - Part A

Responses of insulin-like growth factor (IGF)-I and two IGF-binding protein-1 subtypes to fasting and re-feeding, and their relationships with individual growth rates in yearling masu salmon (*Oncorhynchus masou*)

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Abstract

Two subtypes of insulin-like growth factor binding protein (IGFBP)-1 are present in salmon blood and they are both up-regulated under catabolic conditions such as stress. The present study examined effects of fasting and re-feeding on IGFBP-1a (28-kDa form) and IGFBP-1b (22-kDa form) both at mRNA and protein levels along with IGF-I and RNA/DNA ratio in yearling masu salmon. Fish were individually tagged and assigned to one of three treatments: Fed, Fasted or Re-fed. Circulating IGF-I levels significantly decreased after fasting for 5 weeks and were positively correlated with individual growth rates. Liver *igf-1* mRNA levels were not affected by the treatment. Muscle RNA/DNA ratio did not respond to fasting nor showed correlations with growth rates. Circulating IGFBP-1a and IGFBP-1b increased during fasting and decreased after re-feeding. However, only serum IGFBP-1b levels were inversely correlated with growth rates presumably because IGFBP-1a was less sensitive to mild catabolic conditions. Fasting/re-feeding also affected their mRNA levels in the liver. These results suggest that circulating IGF-I and IGFBP-1b could serve as positive and negative indices of growth, respectively, in masu salmon. Different sensitivities of IGBP-1a and IGFBP-1b may be useful to assess a broad range of catabolic conditions when they are combined.

Keywords

insulin-like growth factor (IGF)-I, IGF-binding protein, growth, fasting, index, salmon

1. Introduction

Environmental factors such as food availability, water temperature, photoperiod, salinity and stress affect metabolism and growth of fish. These factors are integrated by fish and growth is adjusted to meet metabolic demands under a given environment. An accurate measurement of fish growth is important to understand how environment affects overall performance of fish and to improve fish farming and stock assessment. Body length and weight are sums of past growth but do not necessarily reflect recent growth, which gives a better estimate of fish performance in a short period of time under changing conditions/environment. However, measuring individual growth rate is often challenging since a direct measure of growth requires two sampling points of the same individual. Instead, indirect measures of growth are usually used to evaluate recent growth. Otolith and scale have been widely used to reconstruct the growth history of fish in population dynamics studies. Biochemical indices such as RNA/DNA ratio, enzymatic activities or hormone levels may be more reflective to recent growth or current growth status since they are closely related to its process (Bergeron, 1997; Couture et al., 1998; Chícharo and Chícharo, 2008; Picha et al., 2008b; Beckman, 2011).

The major hormones regulating animal growth are growth hormone (GH) and insulin-like growth factor (IGF)-I. GH from the pituitary gland can stimulate growth by directly acting on target tissues such as bone and muscle, but many of GH actions are believed to be mediated by liver-derived IGF-I in mammals (Daughaday and Rotwein, 1989; Le Roith et al., 2001; Ohlsson et al., 2009). IGF-I is also expressed in virtually all types of tissues and acts as a paracrine/autocrine growth factor (Daughaday and Rotwein, 1989; Le Roith et al., 2001; Ohlsson et al., 2009). Although the relative importance of endocrine and local IGF-I is under debate, a consensus is that IGF-I is critical for postnatal growth. IGF-I is relatively stable in the circulation due to the stabilization by multiple IGF-binding proteins (IGFBPs). The half-life of free IGF-I (not bound to IGFBP) in human circulation is about 10 min like insulin, but it is extended to several hours by associating with IGFBP (Guler et al., 1989). These features of

IGF-I appear to be conserved in teleosts (Wood et al., 2005; Reinecke, 2010) and make itself a candidate of growth index in fish.

Beckman et al. (1998, 2004a,b,c) conducted a series of studies using salmon to assess the response of IGF-I to different environments and its reliability as a growth index. When post-smolt coho salmon (*Oncorhynchus kisutch*) were reared under different feeding rations, plasma IGF-I levels were graded by feeding rations being highest with the highest ration (Beckman et al., 2004b). And even after changing feeding ration and thus growth rate, IGF-I levels generally showed good correlations with individual growth rates (Beckman et al., 2004b), suggesting that circulating IGF-I reflects nutritional status and recent growth. The positive relationship between IGF-I and growth rate were further confirmed in other fishes (Uchida et al., 2003; Dyer et al., 2004; Picha et al., 2006). On the other hand, some drawbacks using IGF-I as a growth index have been recognized; If maturing fish were included in the analysis or if water temperature were rapidly dropped (from 11°C to 7°C) and held for about a month, the relationship with growth rate was disturbed (Beckman et al., 2004b,c). Nevertheless, by taking account of these drawbacks, IGF-I is so far the most validated endocrine marker for recent growth (Picha et al., 2008b; Beckman, 2011).

IGFBPs are also candidates of growth indices. Besides prolonging half-life of IGF-I, IGFBPs regulate availability of IGF-I to target tissues, and either inhibiting or potentiating IGF-I actions (Jones and Clemmons, 1995; Rajaram et al., 1997; Firth and Baxter, 2002). In teleost circulation, three IGFBPs are typically detected at molecular ranges of 20-25, 28-32 and 40-45 kDa (Kelley et al., 1992, 2001, 2006). Since the levels of these IGFBPs in blood fluctuate in response to nutritional and physiological changes and hormonal treatments (Kelley et al., 1992; Siharath et al., 1996; Kajimura et al., 2003; Shimizu et al., 2003), fish IGFBPs likely participate in growth regulation through modulating the activity of IGF-I. Kelley et al. (2001, 2006) highlighted that two low-molecular-weight IGFBPs (i.e. 20-24- and 28-32-kDa forms) were induced under a variety of catabolic conditions such as fasting, handling/confinement stress and cortisol treatment, and proposed that they could be used as biomarkers of catabolic status.

In salmon circulation, three major IGFBPs at 22, 28 and 41-kDa have been detected (Shimizu et al., 2000). We have developed a radioimmunoassay for salmon 22-kDa IGFBP and found that this IGFBP levels in plasma inversely related to individual growth rates (Shimizu et al., 2006). In addition, we have recently shown that salmon 28- and 22-kDa IGFBPs are co-orthologs of mammalian IGFBP-1 (IGFBP-1a and -1b, respectively) (Shimizu et al., 2011).

Two subtypes of IGFBP-1 were first identified in zebrafish (*Danio rerio*) (Kamei et al., 2008). The presence of paralogs of IGFBP-1 is likely due to the teleost-speific third round of whole genome duplication since two IGFBP-1 sequences can be found in other fish genomes (Daza et al., 2011). IGFBP-1 is generally inhibitory to IGF actions by sequestering IGFs from receptors under catabolic conditions such as fasting, stress and hypoxia (Kajimura et al., 2007). Such inhibitory action may be adaptive to save energy and re-partition it to essential metabolism under catabolic conditions (Kajimura et al., 2005, 2007). Kamei et al. (2008) compared responses of two IGFBP-1 subtypes and found both were up-regulated by fasting and hypoxia. Moreover, functional analyses revealed that they inhibited zebrafish embryo growth *in vivo* and IGF-I induced cell division *in vitro* (Kamei et al., 2008). These findings suggest that two IGFBP-1 subtypes are growth inhibitors and may be useful as indices of negative growth. However, since two IGFBP-1 subtypes in fish circulation have been recently identified, no study has compared their responses to nutritional change both at protein and mRNA levels or correlated with individual growth rates. The present study examined responses of two IGFBP-1 subtypes along with IGF-I and RNA/DNA ratio to fasting and re-feeding using individually tagged yearling masu salmon, and analyzed their relationships with individual growth rates.

2. Materials and methods

2.1. Fish and fasting/re-feeding experiments

A captive brood stock of masu salmon (*Oncorhynchus masou*) from Shiribetsu River held at Nanae Freshwater Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Japan was used in the present study. In May 2011, one-year-old masu salmon were lightly anesthetized in water containing 2-phenoxy ethanol and individually marked with PIT tags (Biomark, Boise, ID). They were randomly placed into one of three 300L outdoor tanks, and allowed to recover and acclimate for 1 week with feeding. One week after tagging, their initial fork length and body weight were measured. During the experiment, one group was fed daily on a commercial diet (Marubeni Nisshin Feed Co. Ltd., Tokyo, Japan) to satiety for 6 weeks (Fed). Second group (Fasted) was fasted throughout the experimental period (6 weeks). Third group (Re-fed) was fasted for first 4 weeks and re-fed for following 2 weeks. They were reared using flow-through river water that ranged from 10.3°C to 18.0°C during the experiment. The experiment was carried out in accordance with the guidelines of Hokkaido University Field Science Center Animal Care and Use Committee.

Fork length (FL) and body weight (BW) of all fish were measured 4, 5 and 6 weeks after the beginning of the experiment. Hepato-somatic index (HSI) was calculated as follows: HSI (%) = liver weight (g) x 100/body weight (g). Condition factor (K) was calculated as follows: (body weight (g)) x 1000/(fork length (cm))^3. Specific growth rate (SGR) was calculated as follows: SGR (%/day) = ln ($s_2 - s_1$) x $(d_2 - d_1)^{-1}$ x 100, where s_2 is length or weight on day_2, s_1 is length or weight on day_1 and $d_2 - d_1$ is the number of days between measurements. At each time point, four to seven fish per treatment were sampled for blood and tissues. Blood was withdrawn by a syringe from the caudal vein, allowed to clot overnight at 4°C and centrifuged at 8,050*g* for 10 min. Serum was collected and stored at -30°C until use. A few small pieces of liver were dissected. One piece was placed in to 1.5 ml centrifuge tube, immediately frozen on dry ice and stored at -80°C until use. The other pieces were immersed in RNAlater (Ambion, Austin, TX, USA), sit at 4°C overnight and stored at -30°C until use. A piece of white muscle was also excised from the left side of fish body (between the lateral line and the front of dorsal fin), frozen on dry ice and stored at -80°C until use.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from livers as described in Shimizu et al. (2011). One and half µg RNA was reverse-transcribed using SuperScript VILO cDNA Synthesis kit (Invitrogen, Carlsbad, CA, USA) in a 10-µl reaction according to the manufacturer's instruction. cDNA was stored at -30°C until use. During the preparation, some RNA samples were lost due to an accident, that was why some time points had small numbers of samples.

2.3. Real-time quantitative PCR (qPCR)

Primer sets for qPCR of IGF-I and EF-1α were designed based on the cloned masu salmon cDNA sequences using MacVector Ver 9 (MacVector Inc., Cary, NC) (Shimomura et al., 2012) (Table 1). Open reading frames of masu salmon IGFBP-1a and IGFBP-1b were first cloned based on the sequences of Chinook salmon as described in Shimizu et al. (2011). Primers specific to each IGFBP-1 subtype were designed based on the cloned cDNAs (Table 1).

Reverse transcribed-PCRs using these primers were performed to prepare assay standards. PCR products run on 1.5% agarose gel were excised and purified using QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA, USA). Copy numbers of the purified amplicon were calculated from the molecular weight of the amplion and concentration. The standard cDNA were serially diluted from 1 x 10^7 to 3 x 10^2 copies.

qPCR was set up using Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) in a reaction volume of 20 µl with primer concentration of 100 nM. qPCR was run on a 7300 Sequence Detector (Applied Biosystems) using the manufacturer's recommended cycling conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles at 95°C for 15 se and 60°C for 1 min. Measured values were normalized to those of *ef1a* and further divided by liver RNA/DNA ratio to eliminate the strong effect of fasting on liver size, which could cause uneven RNA amount per similar-sized liver piece (Metzger et al., 2012).

2.4. Measurement of RNA/DNA ratio

RNA/DNA ratio was measured by a spectrofluorimetric method recommended by Grémare and Vétion (1994) with minor modifications. Frozen tissues in tubes received 0.5 ml 0.2 mg/ml Protease K (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS; 20 mM phosphate, 0.15 M NaCl, pH7.5) and were homogenized on ice. Fifty-six microliters of 0.1% sodium dodecyl sulfate (SDS) (Sigma-Aldrich) were added to the tubes and they were incubated on ice for 15 min with mixing every 3 min. After centrifugation at 4,500*g* for 15 min at 4°C, the supernatant was transferred to new tubes to measure total nucleic acids (DNA + RNA) or DNA. For measurement of total nucleic acids, 100 ul of the supernatant was diluted in PBS and reacted with 0.004 mg Thiazole orange (Sigma-Aldrich). Fluorescent was measured using a fluorometer (F-2000; Hitachi, Tokyo, Japan) with excitation wave-length at 509 nm and emission wave-length at 545 nm. Purified DNA from salmon sperm (Sigma-Aldrich) was used as a standard. Another set of the supernatants were mixed with 0.02 mg/ml Hoechst 33258 (Dojindo, Kumamoto, Japan) and incubated at 37°C for 30 min. Amount of DNA was measured with excitation wave-length at 352 nm and emission wave-length at 491 nm. Amount of RNA was calculated by subtracting DNA values from total nucleic acid value.

2.5. Time-resolved fluoroimuunoassay (TR-FIA) for IGF-I and IGFBP-1b

Prior to the assay for IGF-I, serum was extracted with an acid-ethanol as described in Shimizu et al. (2000). IGF-I was quantified by TR-FIA based on the method described in Small and Peterson (2005) using recombinant salmon/trout IGF-I (GroPep Bioreagents Pty Ltd., Adelaide, SA, Australia) as a standard.

A detailed protocol of TR-FIA for salmon IGFBP-1b is to be published elsewhere (Fukuda et al., unpublished data). Briefly, a competitive method was employed by following a procedure for DELFIA immunoassays (PerkinElmer, Waltham, MA, USA). Plasma samples were first incubated with antiserum against purified salmon IGFBP-1b overnight at 4°C in a 96-well microtiter plate coated with goat anti-rabbit IgG (PerkinElmer, Waltham, MA, USA). Biotinylated salmon IGFBP-1b was added to each well and incubated for 3 h at 4°C. After washing, each well received Eu-labeled streptavidin (Perkin Elmer) followed by DELFIA enhancement solution (PerkinElmer). Time-resolved fluorescence was measured using Wallac ARVO SX (PerkinElmer) at 615 nm.

2.6. Electrophoresis and Western ligand blotting

SDS-polyacrylamide gel electrophoresis with a 3% stacking gel and 12.5% or 10% separating gel was carried out. Samples were treated with an equal volume of the sample buffer containing 2% SDS and 10% glycerol at 85C for 5 min. Gels were run in a solution of 50 mM Tris, 400 mM glycine and 0.1% SDS at 50 V in the stacking gel and at 100 V in the separating gel until the bromophenol blue dye front reached the bottom of the gel. Gels were stained with 0.1% Coomassie Brilliant Blue R250 (Bio-Rad, Hercules, CA, USA). Molecular mass was estimated with Precision Marker (Bio-Rad).

Western ligand blotting with digoxigenin-labeled human IGF-I (DIG-hIGF-I) was carried out as described in Shimizu et al. (2000). After electroblotting, the nitrocellulose membrane was incubated with 10-50 ng/ml DIG-hIGF-I for 2h at room temperature and then incubated with antibody against DIG conjugated with horseradish peroxidase (Roche, Indianapolis, IN, USA) at a dilution of 1:1500-2500 for 1 h at room temperature. IGFBP was visualized by use of the enhanced chemiluminescence Western blotting reagents (Amersham Life Science, Arlington Heights, IL, USA).

2.7. Statistical analysis

Values from precociously maturing males were not included in the analysis since those disturb the IGF-I-growth relationship (Beckman et al., 2004b). Results of the experiments were first analyzed by two-way ANOVA (time x treatment) using the JMP program (SAS Institute Inc., Cary, NC, USA). When significant effects were found, differences were further identified by one-way ANOVA followed by the Fisher’s protected least significant difference (PLSD) test. Differences among groups were considered to be significant at $P < 0.05$. Correlation analysis was used to assess the relationships among endocrine/biochemical parameters and morphological/growth parameters.

3. Results

Average fork length, body weight, condition factor (K) and HSI values of each treatment and time point are shown in Table 2. During 6 weeks of experiment, there were no significant differences in fork length among three treatments. Fasting significantly reduced body weight and condition factor and 2 weeks of re-feeding did not fully restore them to fed control levels. HSI decreased after 4 weeks of fasting but returned to fed control levels 1 week after re-feeding.

Fasting for 4 weeks resulted in significantly lower SGR in length (Fig. 1a). Re-feeding for 1 week turned it positive. However, there were no significant differences among three treatments 2 weeks after re-feeding (Fig. 1a). SGR in weight in fasted fish were consistently lower than those of fed fish throughout the experiment and re-feeding for 1 week fully restored it to fed control levels (Fig. 1b).

RNA/DNA ratio in white muscle was not reduced even after 6 weeks of fasting (Fig. 2a). There was a significant increase in muscle RNA/DNA ratio in fish re-fed for 1 week, while no significant difference was found 2 weeks after re-feeding. Liver RNA/DNA ratio fluctuated even in fed controls (Fig. 2b). At week 5, fasted fish had the highest values of liver RNA/DNA ratio.

Relative liver *igf1* mRNA levels, which was normalized to *ef1a* and liver RNA/DNA ratio, in fed fish decreased in first 4 weeks compared to initial control levels and remained low thereafter (Fig. 3a). There were no significant differences among treatments. Serum IGF-I levels were significantly reduced after 5 weeks of fasting (Fig. 3b). Two weeks of re-feeding were not enough to restore its levels to those of fed controls. Liver *igfbp-1a* mRNA levels were significantly high in fasted fish than fed fish in week 5 and re-feeding for 1 week had a significant effect to reduce it (Fig. 3c). Serum IGFBP-1a band was invisible at the beginning of the experiment and rarely detected in fed fish throughout the experiment (Fig. 3d). Fasting consistently induced IGFBP-1a in blood but re-feeding was effective to diminish the induction. Liver *igfbp-1b* mRNA levels were increased by fasting despite of some variations (Fig. 3e). It returned to basal levels after 1 week of re-feeding. Serum IGFBP-1b was stably measured even in fed fish (Fig. 3f). Its levels increased after 4 weeks of fasting, showed a peak in week 5 and decreased in week 6. One week of re-feeding reduced IGFBP-1b to basal levels.

Data from week 6 were used for correlation analyses. There were no significant correlations between liver mRNA and circulating protein levels for IGF-I and IGFBP-1a (Table 3). In contrast, a relatively high correlation was found between liver *igfbp-1b* mRNA and serum

IGFBP-1b levels (Table 3). Unexpectedly, there was a strong positive relationship between serum IGFBP-1b and liver *igfbp-1a* mRNA levels (Table 3).

Serum IGF-I and IGFBP-1b had positive and negative correlations, respectively, with SGR both in length and weight, while correlation coefficient tended to be high for SGR in weight (Table 4). These parameters were also correlated with body weight, condition factor and HSI (Table 4). Liver *igf1* showed no correlation with SGR, whereas *igfbp-1a* and *-1b* had negative relationships with SGR in weight (Table 4).

4. Discussion

Circulating IGF-I has been proposed as a reliable index of recent growth in fish since its levels are generally well related to individual growth rates under different or/and changing nutritional conditions (Beckman et al. 2004a,b,c; Picha et al., 2008b; Beckman, 2011). Typically, circulating IGF-I levels are decreased when fish were restricted for feeding ration or deprived of feed, and restored after increasing feeding ration or re-feeding (Picha et al., 2008b; Beckman, 2011). In the present study, serum levels of IGF-I in yearling masu salmon showed patterns similar to those of other fishes. Moreover, there were positive correlations between serum IGF-I levels and individual growth rates (i.e. SGR) both in length and weight, which supports the notion that circulating IGF-I is a good growth index in a wide range of fish species. However, masu salmon at this stage appeared to be less sensitive to nutritional changes, since it required 5 weeks of fasting to show a significant reduction in circulating IGF-I levels and 2 weeks of re-feeding was not enough to restore the levels. In other salmonids, 4 days of fasting were sufficient to see a significant decrease in plasma IGF-I levels in Chinook salmon (Pierce et al., 2005) and 15 days of re-feeding restored IGF-I levels in rainbow trout (*Oncorhynchus mykiss*) (Gabillard et al., 2006). Time course of the responses may vary among species or experimental conditions (i.e. temperature, season and status of fish). Water temperature is known to affect the IGF system in fish (Gabillard et al., 2003). In the present study, water temperature fluctuated between 10.3°C to 18.0°C, which might mask for some extent the effect of fasting and/or re-feeding on circulating IGF-I levels. Nevertheless, circulating IGF-I in masu salmon reasonably reflected nutritional status and growth rate.

In contrast to circulating IGF-I, the response of liver *igf-1* mRNA was inconsistent to changes in nutritional status in masu salmon. After 4 weeks of fasting, liver *igf-1* mRNA levels tended to be higher in fed fish than in fasted fish. However, the basal *igf-1* levels gradually declined over time and no significant differences were found among treatments at week 6. As a result, there were no significant correlations between liver *igf-1* and serum IGF-I levels, or between liver *igf-1* and individual growth rates. These findings contrast to those observed in other fishes where liver *igf-1* mRNA levels are generally linked to circulating IGF-I levels (Gabillard et al., 2003; Pierce et al., 2005) and correlated with growth rates (Uchida et al., 2003). We have recently reported that liver *igf-1* mRNA levels were high in yearling masu salmon in March but continuously decreased in the course of smoltification (Shimomura et al., 2012). Masu salmon used in the present study might be at the late stage of smoltification and the decline of the basal levels of liver *igf-1* might be related to smoltification. Changing water temperature might also have an effect on liver *igf-1* since its influence has been reported in salmonid (Gabillard et al., 2003). In addition, there is a possibility that the reduction of hepatic *igf-1* mRNA during nutritional deficiency may not be a common response in teleosts. A few reports emphasize the response of muscle *igf-1* to nutritional changes. In yellowtail (*Seriola quinqueradiata*), fasting for 3 weeks had no effect on liver *igf-1* while muscle *igf-1* showed a decrease (Fukada et al., 2012). In one study using the tilapia (*Oreochromis mossambicus*), muscle *igf-1* rather than liver *igf-1* responded to fasting (Fox et al., 2010). A series of studies using a hybrid striped bass (*Morone chrysops x Morone saxatilis*) found that hepatic *igf-1* mRNA actually increased after 6 weeks of fasting while plasma IGF-I decreased (Picha et al., 2006, 2008a). However, the opposite pattern was obtained when mRNA levels were expressed as a whole liver. The same authors argued that since a dramatic decrease in the liver size caused uneven DNA amount (i.e. cell numbers) per similar-sized liver samples, it was therefore better be expressed as mRNA per liver (Picha et al., 2008a). The similar response was observed in coho salmon where *igf-1* mRNA levels showed no correlation with plasma IGF-I when mRNA was normalized to total RNA (Metzger et al., 2011). Metzger et al. (2012) introduced a new "biological normalization" by which mRNA levels were divided by RNA/DNA ratio to adjust a bias of DNA amount loaded into reverse-transcribe reaction. In the present study, *igf-1* mRNA values as well as other mRNA values were normalized by liver RNA/DNA ratio. But this normalization did not considerably change the *igf-1* mRNA values. Thus, the pattern of *igf-1* obtained in the present study reflected a biological response of masu salmon at least under the experimental conditions.

RNA/DNA ratio is most commonly used as a biochemical index of growth in marine biology (Chícharo and Chícharo, 2008). Given that RNA amount reflects capacity of protein synthesis in a cell and DNA amount per cell is relatively consistent, the assumption is that RNA/DNA ratio is related to growth of whole animal. White muscle may be the best part to collect the sample since muscle constitutes majority of body's mass. MacLean et al. (2008) found that muscle RNA/DNA ratio was strongly correlated with individual growth rates in weight in Atlantic salmon (*Salmo salar*) smolts. In contrast, no correlation between muscle RNA/DNA and individual growth rates was seen in the present study. Muscle RNA/DNA ratio was insensitive to fasting but showed a transient increase in fish that had been re-fed for 1 week. Muscle RNA/DNA may chiefly respond to accelerated growth in rapidly growing fish such as smolting salmon and larvae/juveniles. Despite of the popularity of the technique, its validity as a growth index depends on species, life-history stages and environments (Chícharo and Chícharo, 2008). Johnson et al. (2002) examined effect of variable rations on muscle RNA/DNA ratio in juvenile red drum (*Sciaenops ocellatus*) and found that fasting was effective to see significant reduction of RNA/DNA ratio but no significant differences were observed among different feeding rations. Thus, the sensitivity of muscle RNA/DNA ratio as a growth index needs to be carefully evaluated for each case. In the present study, liver RNA/DNA ratio fluctuated during the course of experiment both in fed and fasted fish presumably reflecting a sum of metabolic activities under different feeding status, and was not correlated with individual growth rates. However, as mentioned above, measuring liver RNA/DNA is important to normalize mRNA levels of genes. The ratio of liver size against body weight (i.e. HSI) is also a morphological index of growth in the hybrid striped bass (Picha et al., 2006). In the present study, however, HSI showed only a weak correlation with SGR in weight and thus not as good as the endocrine indices.

Although multiple IGFBPs have been detected in fish blood, identity of these IGFBPs has been a matter of debate/confusion (Kelley et al., 2001; Wood et al., 2005). Things are more complicated when duplicated copies of each of six IGFBPs are taken into account. The 22-kDa IGFBP in salmon circulation was assigned as an IGFBP-1 type and later named as IGFBP-1b (Shimizu et al., 2005, 2011). Quantification of plasma IGFBP-1b in coho salmon revealed that its levels increased depending on the length of fasting and responded well to changes in feeding ration (Shimizu et al., 2006, 2009). These changes could occur as fast as in several hours (Shimizu et al., 2009). In the present study, masu salmon IGFBP-1b levels increased after 4 weeks of fasting and returned to basal levels by re-feeding for 2 weeks, which agrees with the previous reports. In post-smolt coho salmon, there was a negative relationship between plasma IGFBP-1b levels and individual growth rates, although r^2 value was not as high as that of IGF-I (Shimizu et al., 2006). In the present study, serum IGFBP-1b levels showed a strong negative relationship with individual growth rates, which was indeed higher than that of

IGF-I. This finding suggests that circulating IGFBP-1b is a good candidate of negative growth index in masu salmon.

cDNAs for fish IGFBP-1 have been cloned in several species (Maures and Duan, 2002; Shimizu et al., 2005; Pedroso et al., 2009; Peterson and Waldbieser, 2009), and many of them likely belong to IGFBP-1b type except zebrafish and salmon/trout IGFBP-1a, and carp and channel catfish IGFBP-1 based on a phylogenetic analysis (Shimizu et al., 2011). These fish IGFBP-1 mRNA levels in the liver increased by fasting (Maures and Duan, 2002; Pedroso et al., 2009; Peterson and Waldbieser, 2009). In line with the previous reports, liver *igfbp-1b* in masu salmon responded to fasting and re-feeding. As in circulating IGFBP-1b, there were negative correlations between *igfbp-1b* and individual growth rates as well as other morphological parameters except length. However, the circulating protein levels showed higher correlation coefficient values with these parameters, suggesting that in masu salmon measuring circulating levels of IGFBP-1b may give a better estimation of negative growth.

The 28-32 kDa IGFBP in fish blood has been considered as IGFBP-1, -2 or -4 based on the molecular weight, response to stress or partial amino acid sequence (Bauchat et al., 2001; Kelley et al., 2001). We purified the 28-kDa IGFBP from serum of Chinook salmon, cloned its cDNA and identified it as IGFBP-1a (Shimizu et al., 2011). IGFBP-1a in salmon blood was usually undetectable but induced when fish suffered severe stress such as direct transfer of juvenile to full-seawater (Shimizu et al., 2011). Cortisol treatment induced IGFBP-1a as well as IGFBP-1b into blood of rainbow trout (Shimizu et al., 2011). These findings suggest that both IGFBP-1a and -1b respond to catabolic conditions. However, when Chinook salmon were fasted for 6 weeks, only IGFBP-1b was induced in plasma (Shimizeu et al., 2005). In the present study, IGFBP-1a band was hardly detected in initial controls and fed fish, but was induced in fish fasted for 4 weeks. Two weeks of re-feeding were sufficient to reduce it below undetectable levels. These changes were similar to those of IGFBP-1b, but a clear contrast is that while IGFBP-1b was constantly detected even in well-fed fish, IGFBP-1a was detected virtually only in fasted fish. This implies that in masu salmon IGFBP-1a is less sensitive to catabolic state than IGFBP-1b, and there may be a threshold level of catabolic state at which IGFBP-1a is induced. This on-and-off character of circulating IGFBP-1a resulted in no linear correlation with growth rates since many of samples had undetectable levels. However, if detectable IGFBP-1a values from all time points were used and log-transformed for analysis, there was a negative relationship with growth rates in weight ($r^2 = 0.64$, $n = 23$). Thus, although IGFBP-1a does not show a linear response to a wide range of catabolic conditions, it may be useful to detect severe stressful conditions.

In the present study, *igfbp-1a* mRNA was similar to the circulating protein in terms of being induced by fasting but different by showing a linear inverse relationship with growth rates in weight. Moreover, there was no correlation between *igfbp-1a* mRNA and circulating protein levels. There are a few possibilities to explain this. First, the transcript does not simply reflect translation or release, or clearance from the circulation is important to regulate the protein level. Alternatively, since IGFBP-1a is expressed in many tissues besides liver (Shimizu et al., 2011), peripheral tissues may significantly contribute as sources of circulating IGFBP-1a. It is worth mentioning that there was a strong positive correlation between circulating IGFBP-1b levels and *igfbp-1a* mRNA levels (r = 0.89). Given that IGFBP-1b appears to be more sensitive to changes in nutritional input, it may be possible that IGFBP-1b could influence expression of *igfbp-1a* in the liver directly or indirectly through blocking IGF-I action which might be inhibitory to *igfbp-1a*. In zebrafish, IGFBP-1a is a more potent inhibitor of IGF-I action than IGFBP-1b (Kamei et al., 2008). If this is also the case in masu salmon, the sequential induction of two IGFBP-1 subtypes could provide a broad degree of inhibitory actions on IGF-I under increasing catabolic state. However, this is totally a speculation at present and awaits future studies.

In conclusion, the present study examined responses of two IGFBP-1 subtypes both at hepatic mRNA and circulating protein levels to fasting and re-feeding in yearling masu salmon. Our results suggest that circulating IGFBP-1b is an index of negative growth. IGFBP-1a may be less sensitive to mild catabolic conditions but could be a marker of severe stress. Combining IGFBP-1a and -1b may allow us to assess a broad range of catabolic conditions. In addition, IGF-I is an index of positive growth in masu salmon as seen in other fish species. These endocrine parameters should provide an accurate measure of salmon growth.

Acknowledgments

We thank Etsuro Yamaha, Nanae Freshwater Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, for providing masu salmon and allowing us to use the rearing facility. We also thank Takahiro Shimomura, Faculty of Fisheries Sciences, Hokkaido University for his technical help in qPCR. This work was supported by Grant-in-Aids for Scientific Research from Japan Society for the Promotion of Science (#21580214), and grant from Northern Advancement Center for Science & Technology (Noastec).

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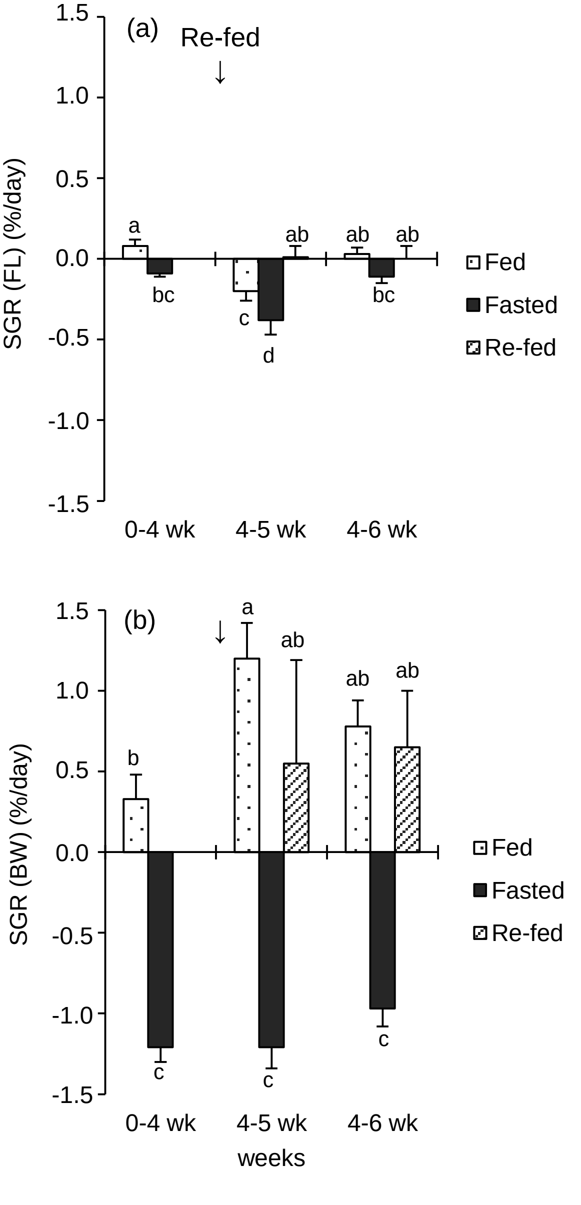
Figure legends

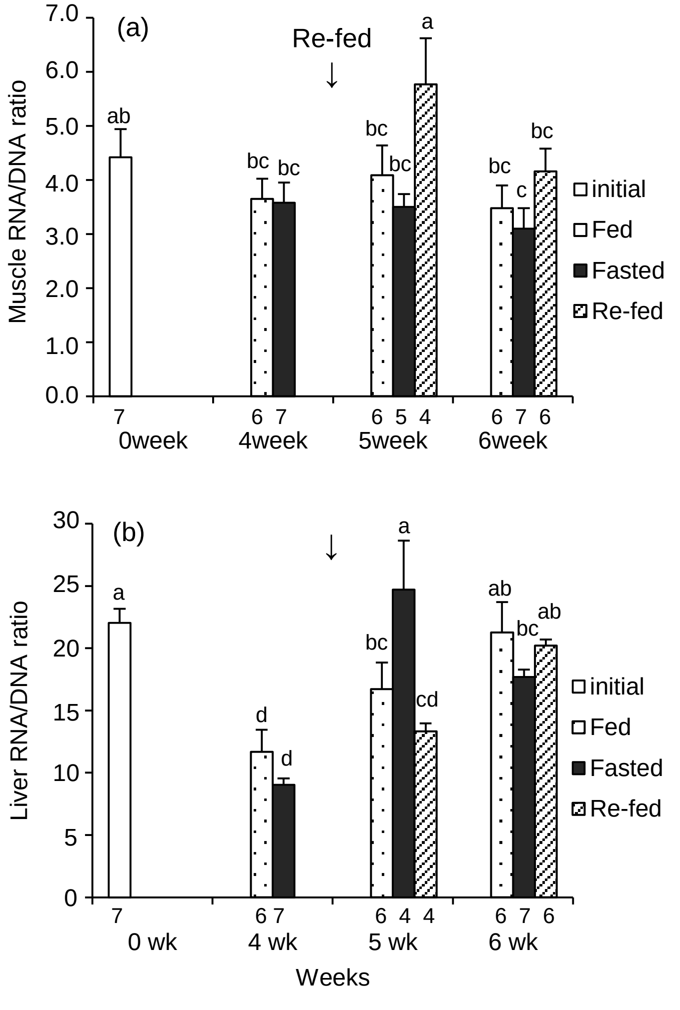
Fig. 1. Specific growth rates (SGR) in fork length (FL) (a) and body weight (BW) (b) of individuals. Fish were fed or fasted for 6 weeks, or fasted for first 4 weeks and then re-fed for following 2 weeks. Values are expressed as means ± SE (n = 4-7). Symbols sharing the same letters are not significantly different from each other.

Fig. 2. Effects of fasting and re-feeding on RNA/DNA ratios in muscle (a) and liver (b). Values are expressed as means ± SE (n are indicated below bars). Symbols sharing the same letters are not significantly different from each other.

Fig. 3. Effects of fasting and re-feeding on IGF-I (a,b), IGFBP-1a (c,d) and IGFBP-1b (e,f) at mRNA levels in the liver (a,c,e) and at protein levels in serum (b,d,f). Values are expressed as means ± SE (n are indicated below bars). Symbols sharing the same letters are not significantly different from each other.



(a)
Re-fed
SGR (FL) (%/day)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
a
bc
c
d
ab
ab
bc
ab
0-4 wk
4-5 wk
4-6 wk
Fed
Fasted
Re-fed
(b)
SGR (BW) (%/day)
b
c
a
c
ab
ab
c
ab
weeks



(a)
Muscle RNA/DNA ratio
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Re-fed
ab
bc
bc
bc
bc
a
bc
c
bc
bc
initial
Fed
Fasted
Re-fed
7
6 7
6 5 4
6 7 6
0week
4week
5week
6week
(b)
Liver RNA/DNA ratio
30
25
20
15
10
5
0
a
d
d
bc
a
cd
ab
bc
ab
initial
Fed
Fasted
Re-fed
7
6 7
6 4 4
6 7 6
0 wk
4 wk
5 wk
6 wk
Weeks

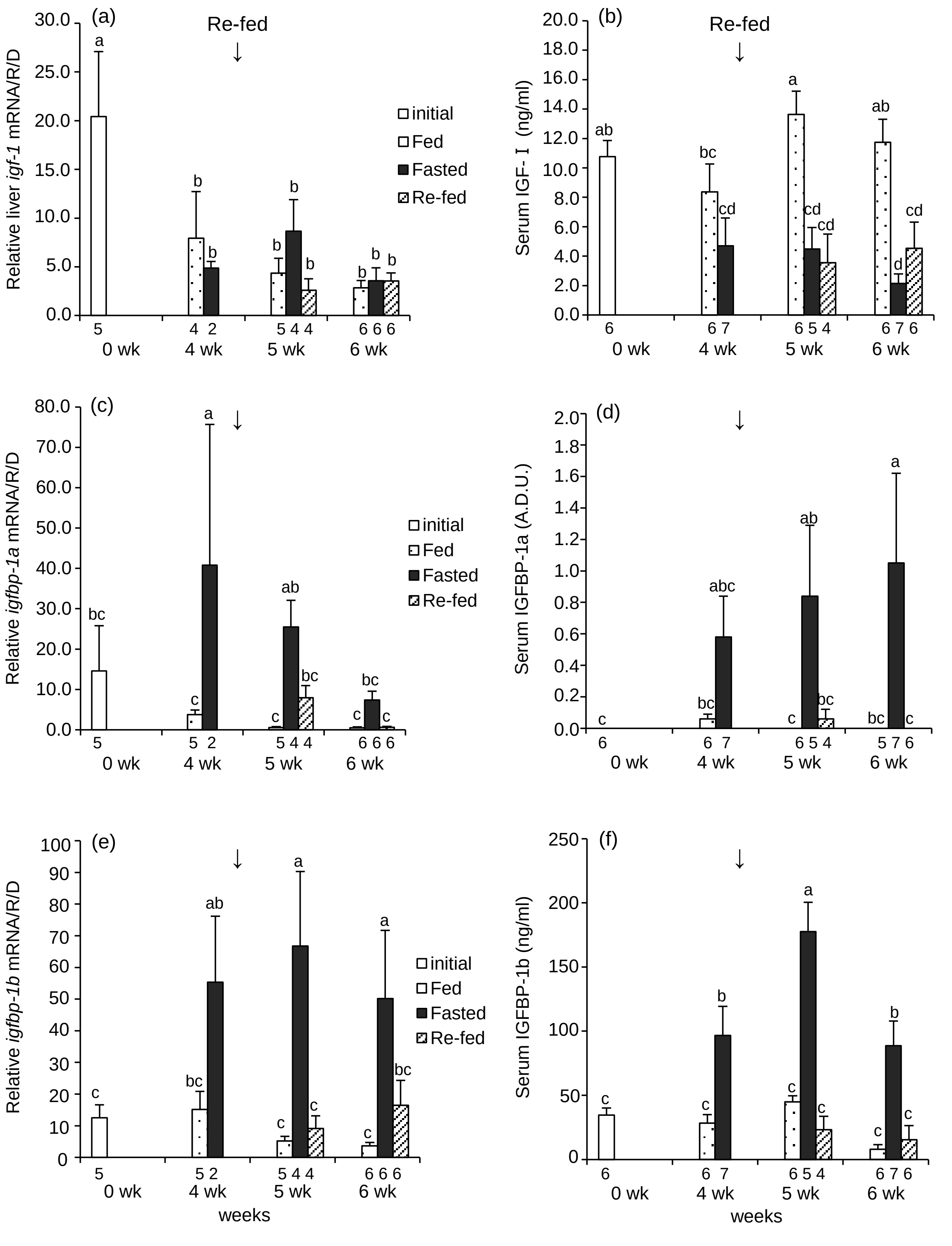

(a)
Re-fed
Relative liver igf-1 mRNA/R/D
initial
Fed
Fasted
Re-fed
0 wk
4 wk
5 wk
6 wk
(b)
Serum IGF- I (ng/ml)
(c)
Relative igfbp-1a mRNA/R/D
(d)
Serum IGFBP-1a (A.D.U.)
(e)
Relative igfbp-1b mRNA/R/D
weeks
(f)
Serum IGFBP-1b (ng/ml)
weeks

Table 1
Primer sequences used for real-time PCR analysis

Primer name	Primer sequence (5'-3')	Direction	Product size (bp)
IGF-Ⅰ(F) RT-PCR	TCTCCAAAACGAGCCTGCG	Forward	207 bp
IGF-Ⅰ R	CACAGCACATCGCACTCTTGA	Reverse	
IGFBP-1aF	AAGGAGCGGCGGACAATG	Forward	83 bp
IGFBP-1aR	CTGTGGCCGTGGAGATAGAG	Reverse	
IGFBP-1bF	GACAAGGGACAAGAGGTAGTAGAAT	Forward	108 bp
IGFBP-1bR	GCTCTCCTGATTCCCCTCAT	Reverse	
EF-1a F	GAATCGGCCATGCCCGGTGAC	Forward	142 bp
EF1a-qR1	GGATGATGACCTGAGCGGTG	Reverse	

Table 2
Comparison of morphological parameters among treatments.

		0 wk	4 wk	5 wk	6 wk
FL	Fed	19.5±0.50	21.05 ±0.50	20.08 ±0.34	20.97 ±0.59
	Fasted		18.93 ±0.53	19.54 ±0.27	20.24 ±0.56
	Re-fed			18.88 ±0.90	19.82 ±0.51
BW	Fed	70.5 ±4.79 [bc]	88.58 ±6.41 [ab]	87.18 ±6.76 [ab]	91.73 ±10.09 [a]
	Fasted		47.74 ±5.15 [d]	54.46 ±3.33 [cd]	58.6 ±6.84 [cd]
	Re-fed			57.88 ±13.23 [cd]	62.97 ±4.28 [cd]
K	Fed	0.94 ±0.03 [b]	0.94 ±0.02 [b]	1.06 ±0.04 [a]	0.97 ±0.03 [b]
	Fasted		0.69 ±0.02 [d]	0.73 ±0.02 [cd]	0.69 ±0.02 [d]
	Re-fed			0.82 ±0.07 [c]	0.81 ±0.02 [c]
HSI	Fed	0.85 ±0.08 [c]	1.33 ±0.11 [b]	1.62 ±0.10 [a]	1.65 ±0.08 [a]
	Fasted		0.91 ±0.11 [c]	0.74 ±0.04 [c]	0.98 ±0.08 [c]
	Re-fed			1.57 ±0.05 [ab]	1.45 ±0.12 [ab]

FL: fork length; BW: body weight; K: condition factor; HSI: hepato-somatic index. Values are expressed as mean ±SE (n = 5-7). Symbols sharing the same letters are not significantly different from each other.

Table 3
Correlation coefficients (r) among endocrine and biochemical parameters.

	igf-1	*igfbp-1a*	*igfbp-1b*	Muscle R/D	Liver R/D	IGF- I	IGFBP-1a	IGFBP-1b
Liver *igf-1*		-	-	-	-	-	-	-
Liver *igfbp-1a*	-		0.72	-	-	-0.49	-	0.89
Liver *igfbp-1b*	-	0.72		-	-	-	-	0.76
Muscle R/D	-	-	-		-	-	-	-
Liver R/D	-	-	-	-		-	-	-
Serum IGF- I	-	-0.49	-	-	-		-	-0.60
Serum IGFBP-1a	-	-	-	-	-	-		0.79
Serum IGFBP-1b	-	0.89	0.76	-	-	-0.60	0.79	

(-): not significant.

Table 4.
Correlation coefficients (r) between endocrine/biochemical parameters and morphological/growth parameters.

	FL	BW	K	HSI	SGR(FL)	SGR(BW)
HSI					0.22	0.40
Liver *igf-1*	-	-	-	-	-	-
Liver *igfbp-1a*	-	-	-0.64	-0.63	-	-0.70
Liver *igfbp-1b*	-	-0.49	-0.58	-0.48	-0.51	-0.59
Muscle R/D	-	-	-	-	-	-
Liver R/D	-	-	-	-0.54	-	-
Serum IGF- I	-	0.57	0.83	0.55	0.51	0.71
Serum IGFBP-1a	-	-	-	-	-	-
Serum IGFBP-1b	-	-0.54	-0.75	-0.59	-0.60	-0.84

HSI: hepato-somatic index. Blanks: not analyzed; (-): not significant.

Supplementary Table 1
Correlation coefficients (r) between endocrine/biochemical parameters and morphological/growth parameters in week 5.

	FL	BW	K	HSI	SGR(FL)	SGR(BW)
HSI					0.56	0.78
Liver *igf-1*	-	-	-	-	-	-
Liver *igfbp-1a*	-	-	-0.65	-0.78	-	-0.75
Liver *igfbp-1b*	-	-	-	-0.71	-0.62	-0.63
Muscle R/D	-0.63	-	-	-	-	-
Liver R/D	-	-	-	-0.65	-	-
Serum IGF-Ⅰ	0.53	0.70	0.74	-	-	0.62
Serum IGFBP-1a	-	-	-0.52	-0.57	-0.60	-0.62
Serum IGFBP-1b	-	-	-0.59	-0.83	-0.59	-0.79

HSI: hepato-somatic index. Blanks: not analyzed; (-): not significant.