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

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20
21 **Abstract**

22 Two subtypes of insulin-like growth factor binding protein (IGFBP)-1 are present in salmon
23 blood and they are both up-regulated under catabolic conditions such as stress. The present
24 study examined effects of fasting and re-feeding on IGFBP-1a (28-kDa form) and IGFBP-1b
25 (22-kDa form) both at mRNA and protein levels along with IGF-I and RNA/DNA ratio in
26 yearling masu salmon. Fish were individually tagged and assigned to one of three treatments:
27 Fed, Fasted or Re-fed. Circulating IGF-I levels significantly decreased after fasting for 5 weeks
28 and were positively correlated with individual growth rates. Liver *igf-1* mRNA levels were not
29 affected by the treatment. Muscle RNA/DNA ratio did not respond to fasting nor showed
30 correlations with growth rates. Circulating IGFBP-1a and IGFBP-1b increased during fasting
31 and decreased after re-feeding. However, only serum IGFBP-1b levels were inversely correlated
32 with growth rates presumably because IGFBP-1a was less sensitive to mild catabolic conditions.
33 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-specific 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.

132 Fork length (FL) and body weight (BW) of all fish were measured 4, 5 and 6 weeks
133 after the beginning of the experiment. Hepato-somatic index (HSI) was calculated as follows:
134 $HSI (\%) = \text{liver weight (g)} \times 100 / \text{body weight (g)}$. Condition factor (K) was calculated as
135 follows: $(\text{body weight (g)}) \times 1000 / (\text{fork length (cm)})^3$. Specific growth rate (SGR) was
136 calculated as follows: $SGR (\%/day) = \ln(s_2 - s_1) \times (d_2 - d_1)^{-1} \times 100$, where s_2 is length or weight
137 on day₂, s_1 is length or weight on day₁ and $d_2 - d_1$ is the number of days between measurements.
138 At each time point, four to seven fish per treatment were sampled for blood and tissues. Blood
139 was withdrawn by a syringe from the caudal vein, allowed to clot overnight at 4°C and
140 centrifuged at 8,050g for 10 min. Serum was collected and stored at -30°C until use. A few
141 small pieces of liver were dissected. One piece was placed in to 1.5 ml centrifuge tube,
142 immediately frozen on dry ice and stored at -80°C until use. The other pieces were immersed in
143 RNAlater (Ambion, Austin, TX, USA), sit at 4°C overnight and stored at -30°C until use. A
144 piece of white muscle was also excised from the left side of fish body (between the lateral line
145 and the front of dorsal fin), frozen on dry ice and stored at -80°C until use.

146

147 2.2. RNA extraction and cDNA synthesis

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

153

154 2.3. Real-time quantitative PCR (qPCR)

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

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

165 qPCR was set up using Power SYBR Green PCR Master Mix (Applied Biosystems,
166 Carlsbad, CA, USA) in a reaction volume of 20 µl with primer concentration of 100 nM. qPCR
167 was run on a 7300 Sequence Detector (Applied Biosystems) using the manufacturer's
168 recommended cycling conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles at
169 95°C for 15 se and 60°C for 1 min. Measured values were normalized to those of *efla* and
170 further divided by liver RNA/DNA ratio to eliminate the strong effect of fasting on liver size,
171 which could cause uneven RNA amount per similar-sized liver piece (Metzger et al., 2012).

172

173 *2.4. Measurement of RNA/DNA ratio*

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

189

190 *2.5. Time-resolved fluoroimmunoassay (TR-FIA) for IGF-I and IGFBP-1b*

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

195 A detailed protocol of TR-FIA for salmon IGFBP-1b is to be published elsewhere
196 (Fukuda et al., unpublished data). Briefly, a competitive method was employed by following a
197 procedure for DELFIA immunoassays (PerkinElmer, Waltham, MA, USA). Plasma samples

198 were first incubated with antiserum against purified salmon IGFBP-1b overnight at 4°C in a
199 96-well microtiter plate coated with goat anti-rabbit IgG (PerkinElmer, Waltham, MA, USA).
200 Biotinylated salmon IGFBP-1b was added to each well and incubated for 3 h at 4°C. After
201 washing, each well received Eu-labeled streptavidin (Perkin Elmer) followed by DELFIA
202 enhancement solution (PerkinElmer). Time-resolved fluorescence was measured using Wallac
203 ARVO SX (PerkinElmer) at 615 nm.

204

205 *2.6. Electrophoresis and Western ligand blotting*

206 SDS-polyacrylamide gel electrophoresis with a 3% stacking gel and 12.5% or 10% separating
207 gel was carried out. Samples were treated with an equal volume of the sample buffer containing
208 2% SDS and 10% glycerol at 85°C for 5 min. Gels were run in a solution of 50 mM Tris, 400
209 mM glycine and 0.1% SDS at 50 V in the stacking gel and at 100 V in the separating gel until
210 the bromophenol blue dye front reached the bottom of the gel. Gels were stained with 0.1%
211 Coomassie Brilliant Blue R250 (Bio-Rad, Hercules, CA, USA). Molecular mass was estimated
212 with Precision Marker (Bio-Rad).

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

220

221 *2.7. Statistical analysis*

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

230

231 3. Results

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

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

242 RNA/DNA ratio in white muscle was not reduced even after 6 weeks of fasting (Fig.
243 2a). There was a significant increase in muscle RNA/DNA ratio in fish re-fed for 1 week, while
244 no significant difference was found 2 weeks after re-feeding. Liver RNA/DNA ratio fluctuated
245 even in fed controls (Fig. 2b). At week 5, fasted fish had the highest values of liver RNA/DNA
246 ratio.

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

260 Data from week 6 were used for correlation analyses. There were no significant
261 correlations between liver mRNA and circulating protein levels for IGF-I and IGFBP-1a (Table
262 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

296 result, there were no significant correlations between liver *igf-1* and serum IGF-I levels, or
297 between liver *igf-1* and individual growth rates. These findings contrast to those observed in
298 other fishes where liver *igf-1* mRNA levels are generally linked to circulating IGF-I levels
299 (Gabillard et al., 2003; Pierce et al., 2005) and correlated with growth rates (Uchida et al., 2003).
300 We have recently reported that liver *igf-1* mRNA levels were high in yearling masu salmon in
301 March but continuously decreased in the course of smoltification (Shimomura et al., 2012).
302 Masu salmon used in the present study might be at the late stage of smoltification and the
303 decline of the basal levels of liver *igf-1* might be related to smoltification. Changing water
304 temperature might also have an effect on liver *igf-1* since its influence has been reported in
305 salmonid (Gabillard et al., 2003). In addition, there is a possibility that the reduction of hepatic
306 *igf-1* mRNA during nutritional deficiency may not be a common response in teleosts. A few
307 reports emphasize the response of muscle *igf-1* to nutritional changes. In yellowtail (*Seriola*
308 *quinqueradiata*), fasting for 3 weeks had no effect on liver *igf-1* while muscle *igf-1* showed a
309 decrease (Fukada et al., 2012). In one study using the tilapia (*Oreochromis mossambicus*),
310 muscle *igf-1* rather than liver *igf-1* responded to fasting (Fox et al., 2010). A series of studies
311 using a hybrid striped bass (*Morone chrysops* x *Morone saxatilis*) found that hepatic *igf-1*
312 mRNA actually increased after 6 weeks of fasting while plasma IGF-I decreased (Picha et al.,
313 2006, 2008a). However, the opposite pattern was obtained when mRNA levels were expressed
314 as a whole liver. The same authors argued that since a dramatic decrease in the liver size caused
315 uneven DNA amount (i.e. cell numbers) per similar-sized liver samples, it was therefore better
316 be expressed as mRNA per liver (Picha et al., 2008a). The similar response was observed in
317 coho salmon where *igf-1* mRNA levels showed no correlation with plasma IGF-I when mRNA
318 was normalized to total RNA (Metzger et al., 2011). Metzger et al. (2012) introduced a new
319 “biological normalization” by which mRNA levels were divided by RNA/DNA ratio to adjust a
320 bias of DNA amount loaded into reverse-transcribe reaction. In the present study, *igf-1* mRNA
321 values as well as other mRNA values were normalized by liver RNA/DNA ratio. But this
322 normalization did not considerably change the *igf-1* mRNA values. Thus, the pattern of *igf-1*
323 obtained in the present study reflected a biological response of masu salmon at least under the
324 experimental conditions.

325 RNA/DNA ratio is most commonly used as a biochemical index of growth in marine
326 biology (Chícharo and Chícharo, 2008). Given that RNA amount reflects capacity of protein
327 synthesis in a cell and DNA amount per cell is relatively consistent, the assumption is that
328 RNA/DNA ratio is related to growth of whole animal. White muscle may be the best part to

329 collect the sample since muscle constitutes majority of body's mass. MacLean et al. (2008)
330 found that muscle RNA/DNA ratio was strongly correlated with individual growth rates in
331 weight in Atlantic salmon (*Salmo salar*) smolts. In contrast, no correlation between muscle
332 RNA/DNA and individual growth rates was seen in the present study. Muscle RNA/DNA ratio
333 was insensitive to fasting but showed a transient increase in fish that had been re-fed for 1 week.
334 Muscle RNA/DNA may chiefly respond to accelerated growth in rapidly growing fish such as
335 smolting salmon and larvae/juveniles. Despite of the popularity of the technique, its validity as a
336 growth index depends on species, life-history stages and environments (Chícharo and Chícharo,
337 2008). Johnson et al. (2002) examined effect of variable rations on muscle RNA/DNA ratio in
338 juvenile red drum (*Sciaenops ocellatus*) and found that fasting was effective to see significant
339 reduction of RNA/DNA ratio but no significant differences were observed among different
340 feeding rations. Thus, the sensitivity of muscle RNA/DNA ratio as a growth index needs to be
341 carefully evaluated for each case. In the present study, liver RNA/DNA ratio fluctuated during
342 the course of experiment both in fed and fasted fish presumably reflecting a sum of metabolic
343 activities under different feeding status, and was not correlated with individual growth rates.
344 However, as mentioned above, measuring liver RNA/DNA is important to normalize mRNA
345 levels of genes. The ratio of liver size against body weight (i.e. HSI) is also a morphological
346 index of growth in the hybrid striped bass (Picha et al., 2006). In the present study, however,
347 HSI showed only a weak correlation with SGR in weight and thus not as good as the endocrine
348 indices.

349 Although multiple IGFBPs have been detected in fish blood, identity of these
350 IGFBPs has been a matter of debate/confusion (Kelley et al., 2001; Wood et al., 2005). Things
351 are more complicated when duplicated copies of each of six IGFBPs are taken into account. The
352 22-kDa IGFBP in salmon circulation was assigned as an IGFBP-1 type and later named as
353 IGFBP-1b (Shimizu et al., 2005, 2011). Quantification of plasma IGFBP-1b in coho salmon
354 revealed that its levels increased depending on the length of fasting and responded well to
355 changes in feeding ration (Shimizu et al., 2006, 2009). These changes could occur as fast as in
356 several hours (Shimizu et al., 2009). In the present study, masu salmon IGFBP-1b levels
357 increased after 4 weeks of fasting and returned to basal levels by re-feeding for 2 weeks, which
358 agrees with the previous reports. In post-smolt coho salmon, there was a negative relationship
359 between plasma IGFBP-1b levels and individual growth rates, although r^2 value was not as high
360 as that of IGF-I (Shimizu et al., 2006). In the present study, serum IGFBP-1b levels showed a
361 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 (Shimizu 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.

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428 **References**

- 429 Bauchat, J.R., Busby, W.H., Jr., Garmong, A., Swanson, P., Moore, J., Lin, M., Duan, C., 2001.
430 Biochemical and functional analysis of a conserved IGF-binding protein isolated from
431 rainbow trout (*Oncorhynchus mykiss*) hepatoma cells. J. Endocrinol. 170, 619-628.
- 432 Beckman, B.R., 2011. Perspectives on concordant and discordant relations between insulin-like
433 growth factor 1 (IGF1) and growth in fishes. Gen. Comp. Endocrinol. 170, 233-252.
- 434 Beckman, B.R., Larsen, D.A., Moriyama, S., Lee-Pawlak, B., Dickhoff, W.W., 1998.
435 Insulin-like growth factor-I and environmental modulation of growth during
436 smoltification of spring chinook salmon (*Oncorhynchus tshawytscha*). Gen. Comp.
437 Endocrinol. 109, 325-335.
- 438 Beckman, B.R., Fairgrieve, W., Cooper, K.A., Mahnken, C.V.W., Beamish, R.J., 2004a.
439 Evaluation of endocrine indices of growth in individual postsmolt coho salmon. Trans.
440 Am. Fish. Soc. 133, 1057-1067.
- 441 Beckman, B.R., Shimizu, M., Gadberry, B.A., Cooper, K.A., 2004b. Response of the
442 somatotrophic axis of juvenile coho salmon to alterations in plane of nutrition with an
443 analysis of the relationships among growth rate and circulating IGF-I and 41 kDa
444 IGFBP. Gen. Comp. Endocrinol. 135, 334-344.
- 445 Beckman, B.R., Shimizu, M., Gadberry, B.A., Parkins, P.J., Cooper, K.A., 2004c. The effect of
446 temperature change on the relations among plasma IGF-I, 41-kDa IGFBP, and growth
447 rate in postsmolt coho salmon. Aquaculture 241, 601-619.
- 448 Bergeron, J.P., 1997. Nucleic acids in ichthyoplankton ecology: a review, with emphasis on
449 recent advances for new perspectives. J. Fish Biol. 51, 284-302.
- 450 Chícharo, M.A., Chícharo, L., 2008. RNA : DNA ratio and other nucleic acid derived indices in
451 marine ecology. Int. J. Mol. Sci. 9, 1453-1471.
- 452 Couture, P., Dutil, J.D., Guderley, H., 1998. Biochemical correlates of growth and condition in
453 juvenile Atlantic cod (*Gadus morhua*) from Newfoundland. Can. J. Fish. Aquat. Sci. 55,
454 1591-1598.
- 455 Daughaday, W.H., Rotwein, P., 1989. Insulin-like growth factors I and II. Peptide, messenger
456 ribonucleic acid and gene structures, serum, and tissue concentrations. Endocr. Rev. 10,
457 68-91.

458 Daza, D.O., Sundström, G., Bergqvist, C.A., Duan, C.M., Larhammar, D., 2011. Evolution of
 459 the Insulin-Like Growth Factor Binding Protein (IGFBP) Family. *Endocrinology* 152,
 460 2278-2289.

461 Dyer, A.R., Barlow, C.G., Bransden, M.P., Carter, C.G., Glencross, B.D., Richardson, N.,
 462 Thomas, P.M., Williams, K.C., Carragher, J.F., 2004. Correlation of plasma IGF-I
 463 concentrations and growth rate in aquacultured finfish: a tool for assessing the potential
 464 of new diets. *Aquaculture* 236, 583-592.

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

467 Fox, B.K., Breves, J.P., Davis, L.K., Pierce, A.L., Hirano, T., Grau, E.G., 2010. Tissue-specific
 468 regulation of the growth hormone/insulin-like growth factor axis during fasting and
 469 re-feeding: Importance of muscle expression of IGF-I and IGF-II mRNA in the tilapia.
 470 *Gen. Comp. Endocrinol.* 166, 573-580.

471 Fukada, H., Murashita, K., Furutani, T., Masumoto, T., 2012. Yellowtail insulin-like growth
 472 factor 1: molecular cloning and response to various nutritional conditions. *Domest.*
 473 *Anim. Endocrinol.* 42, 220-229.

474 Gabillard, J.C., Weil, C., Rescan, P.Y., Navarro, I., Gutierrez, J., Le Bail, P.Y., 2003. Effects of
 475 environmental temperature on IGF1, IGF2, and IGF type I receptor expression in
 476 rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* 133, 233-242.

477 Gabillard, J.C., Kamangar, B.B., Montserrat, N., 2006. Coordinated regulation of the GH/IGF
 478 system genes during refeeding in rainbow trout (*Oncorhynchus mykiss*). *J. Endocrinol.*
 479 191, 15-24.

480 Grémare, A., Vétion, G., 1994. Comparison of several spectrofluorimetric methods for
 481 measuring RNA and DNA concentrations in the deposit-feeding bivalve *Abra ovata*.
 482 *Comp. Biochem. Physiol.* 107B, 297-308.

483 Guler, H.P., Zapf, J., Schmid, C., Froesch, E.R., 1989. Insulin-like growth factors I and II in
 484 healthy man. Estimations of half-lives and production rates. *Acta Endocrinol. (Copenh).*
 485 121, 753-758.

486 Johnson, M.W., Rooker, Jr., Gatlin, D.M., Holt, G.J., 2002. Effects of variable ration levels on
 487 direct and indirect measures of growth in juvenile red drum (*Sciaenops ocellatus*). *J*
 488 *Exp. Mar. Biol. Ecol.* 274, 141-157.

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

491 Kajimura, S., Duan, C., 2007. Insulin-like growth factor-binding protein-1: an evolutionarily
 492 conserved fine tuner of insulin-like growth factor action under catabolic and stressful
 493 conditions. *J. Fish Biol.* 71, 309-325.

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

497 Kajimura, S., Hirano, T., Visitacion, N., Moriyama, S., Aida, K., Grau, E.G., 2003. Dual mode
 498 of cortisol action on GH/IGF-I/IGF binding proteins in the tilapia, *Oreochromis*
 499 *mossambicus*. *J. Endocrinol.* 178, 91-99.

500 Kamei, H., Lu, L., Jiao, S., Li, Y., Gyrupe, C., Laursen, L.S., Oxvig, C., Zhou, J., Duan, C., 2008.
 501 Duplication and diversification of the hypoxia-inducible IGFBP-1 gene in zebrafish.
 502 *PLoS One* 3, e3091.

503 Kelley, K.M., Siharath, K., Bern, H.A., 1992. Identification of insulin-like growth
 504 factor-binding proteins in the circulation of four teleost fish species. *J. Exp. Zool.* 263,
 505 220-224.

506 Kelley, K.M., Haigwood, J.T., Perez, M., Galima, M.M., 2001. Serum insulin-like growth factor
 507 binding proteins (IGFBPs) as markers for anabolic/catabolic condition in fishes. *Comp.*
 508 *Biochem. Physiol.* 129B, 229-236.

509 Kelley, K.M., Price, T.D., Galima, M.M., Sak, K., Reyes, J.A., Zepeda, O., Hagstrom, R.,
 510 Truong, T.A., Lowe, C.G., 2006. Insulin-like growth factor-binding proteins (IGFBPs)
 511 in fish: beacons for (disrupted) growth endocrine physiology. in: Reinecke, M., Zaccone,
 512 G., Kapoor, B.G. (Eds.), *Fish Endocrinology*. Science Publishers, Enfield, New
 513 Hampshire, pp. 167-195.

514 Le Roith, D., Bondy, C., Yakar, S., Liu, J.L., Butler, A., 2001. The somatomedin hypothesis:
 515 2001. *Endocr. Rev.* 22, 53-74.

516 MacLean, S.A., Caldarone, E.M., 2008. Estimating Recent Growth Rates of Atlantic Salmon
 517 Smolts Using RNA-DNA Ratios from Nonlethally Sampled Tissues. *Trans. Am. Fish.*
 518 *Soc.* 137, 1279-1284.

519 Maures, T.J., Duan, C., 2002. Structure, developmental expression, and physiological regulation
 520 of zebrafish IGF binding protein-1. *Endocrinology* 143, 2722-2731.

521 Metzger, D.C., Luckenbach, J.A., Shimizu, M., Beckman, B.R., 2012. Normalizing for biology:
 522 Accounting for technical and biological variation in levels of reference gene and

523 insulin-like growth factor 1 (igf1) transcripts in fish livers. Comp. Biochem. Physiol.
 524 163A, 7-14.

525 Ohlsson, C., Mohan, S., Sjogren, K., Tivesten, A., Isgaard, J., Isaksson, O., Jansson, J.O.,
 526 Svensson, J., 2009. The role of liver-derived insulin-like growth factor-I. Endocr. Rev.
 527 30, 494-535.

528 Pedroso, F.L., Fukada, H., Masumoto, T., 2009. Molecular characterization, tissue distribution
 529 patterns and nutritional regulation of IGFBP-1, -2, -3 and -5 in yellowtail, *Seriola*
 530 *quinqueradiata*. Gen. Comp. Endocrinol. 161, 344-353.

531 Peterson, B.C., Waldbieser, G.C., 2009. Effects of fasting on IGF-I, IGF-II, and IGF-binding
 532 protein mRNA concentrations in channel catfish (*Ictalurus punctatus*). Domest. Anim.
 533 Endocrinol. 37, 74-83.

534 Picha, M.E., Silverstein, J.T., Borski, R.J., 2006. Discordant regulation of hepatic IGF-I mRNA
 535 and circulating IGF-I during compensatory growth in a teleost, the hybrid striped bass
 536 (*Morone chrysops* x *Morone saxatilis*). Gen. Comp. Endocrinol. 147, 196-205.

537 Picha, M.E., Turano, M.J., Tipsmark, C.K., Borski, R.J., 2008a. Regulation of endocrine and
 538 paracrine sources of Igfs and Gh receptor during compensatory growth in hybrid striped
 539 bass (*Morone chrysops* X *Morone saxatilis*). J. Endocrinol. 199, 81-94.

540 Picha, M.E., Turano, M.J., Beckman, B.R., Borski, R.J., 2008b. Endocrine biomarkers of
 541 growth and applications to aquaculture: A minireview of growth hormone, insulin-like
 542 growth factor (IGF)-I, and IGF-Binding proteins as potential growth indicators in fish.
 543 N. Am. J. Aquacult. 70, 196-211.

544 Pierce, A.L., Shimizu, M., Beckman, B.R., Baker, D.M., Dickhoff, W.W., 2005. Time course of
 545 the GH/IGF axis response to fasting and increased ration in chinook salmon
 546 (*Oncorhynchus tshawytscha*). Gen. Comp. Endocrinol. 140, 192-202.

547 Rajaram, S., Baylink, D.J., Mohan, S., 1997. Insulin-like growth factor-binding proteins in
 548 serum and other biological fluids: regulation and functions. Endocr. Rev. 18, 801-831.

549 Reinecke, M., 2010. Influences of the environment on the endocrine and paracrine fish growth
 550 hormone-insulin-like growth factor-I system. J. Fish Biol. 76, 1233-1254.

551 Shimizu, M., Hara, A., Dickhoff, W.W., 2003. Development of an RIA for salmon 41 kDa
 552 IGF-binding protein. J. Endocrinol. 178, 275-283.

553 Shimizu, M., Swanson, P., Fukada, H., Hara, A., Dickhoff, W.W., 2000. Comparison of
 554 extraction methods and assay validation for salmon insulin-like growth factor-I using
 555 commercially available components. Gen. Comp. Endocrinol. 119, 26-36.

556 Shimizu, M., Dickey, J.T., Fukada, H., Dickhoff, W.W., 2005. Salmon serum 22 kDa
 557 insulin-like growth factor-binding protein (IGFBP) is IGFBP-1. J. Endocrinol. 184,
 558 267-276.

559 Shimizu, M., Beckman, B.R., Hara, A., Dickhoff, W.W., 2006. Measurement of circulating
 560 salmon IGF binding protein-1: assay development, response to feeding ration and
 561 temperature, and relation to growth parameters. J. Endocrinol. 188, 101-110.

562 Shimizu, M., Cooper, K.A., Dickhoff, W.W., Beckman, B.R., 2009. Postprandial changes in
 563 plasma growth hormone, insulin, insulin-like growth factor (IGF)-I, and IGF-binding
 564 proteins in coho salmon fasted for varying periods. Am. J. Physiol. Regul. Integr. Comp.
 565 Physiol. 297, R352-361.

566 Shimizu, M., Kishimoto, K., Yamaguchi, T., Nakano, Y., Hara, A., Dickhoff, W.W., 2011.
 567 Circulating salmon 28- and 22-kDa insulin-like growth factor binding proteins
 568 (IGFBPs) are co-orthologs of IGFBP-1. Gen. Comp. Endocrinol. 174, 97-106.

569 Shimomura, T., Nakajima, T., Horikoshi, M., Iijima, A., Urabe, H., Mizuno, S., Hiramatsu, N.,
 570 Hara, A., Shimizu, M., 2012. Relationships between gill Na⁺,K⁺-ATPase activity and
 571 endocrine and local insulin-like growth factor-I levels during smoltification of masu
 572 salmon (*Oncorhynchus masou*). Gen. Comp. Endocrinol. 178, 427-435.

573 Siharath, K., Kelley, K.M., Bern, H.A., 1996. A low-molecular-weight (25-kDa) IGF-binding
 574 protein is increased with growth inhibition in the fasting striped bass, *Morone saxatilis*.
 575 Gen. Comp. Endocrinol. 102, 307-316.

576 Small, B.C., Peterson, B.C., 2005. Establishment of a time-resolved fluoroimmunoassay for
 577 measuring plasma insulin-like growth factor I (IGF-I) in fish: effect of fasting on
 578 plasma concentrations and tissue mRNA expression of IGF-I and growth hormone (GH)
 579 in channel catfish (*Ictalurus punctatus*). Domest. Anim. Endocrinol. 28, 202-215.

580 Uchida, K., Kajimura, S., Riley, L.G., Hirano, T., Aida, K., Grau, E.G., 2003. Effects of fasting
 581 on growth hormone/insulin-like growth factor I axis in the tilapia, *Oreochromis*
 582 *mossambicus*. Comp. Biochem. Physiol. 134A, 429-439.

583 Wood, A.W., Duan, C., Bern, H.A., 2005. Insulin-like growth factor signaling in fish. Int. Rev.
 584 Cytol. 243, 215-285.

585 **Figure legends**

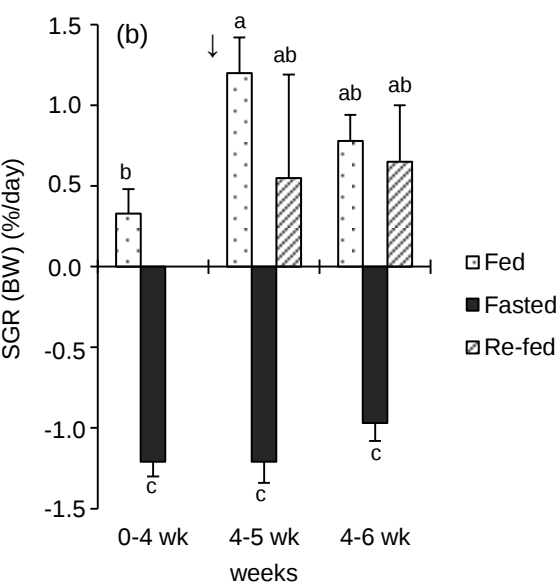
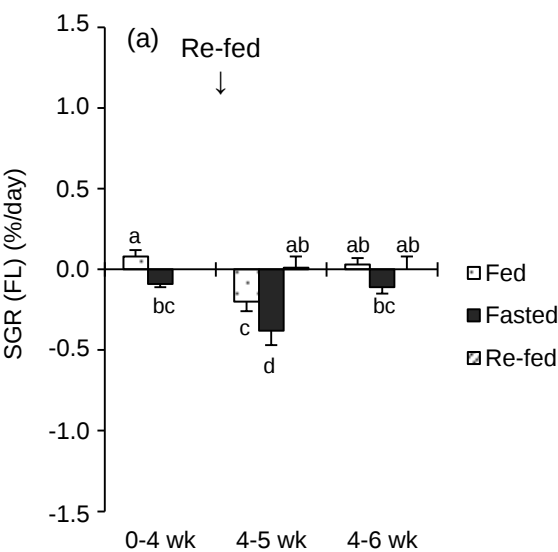
586 Fig. 1. Specific growth rates (SGR) in fork length (FL) (a) and body weight (BW) (b) of
587 individuals. Fish were fed or fasted for 6 weeks, or fasted for first 4 weeks and then re-fed for
588 following 2 weeks. Values are expressed as means $\pm$ SE (n = 4-7). Symbols sharing the same
589 letters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

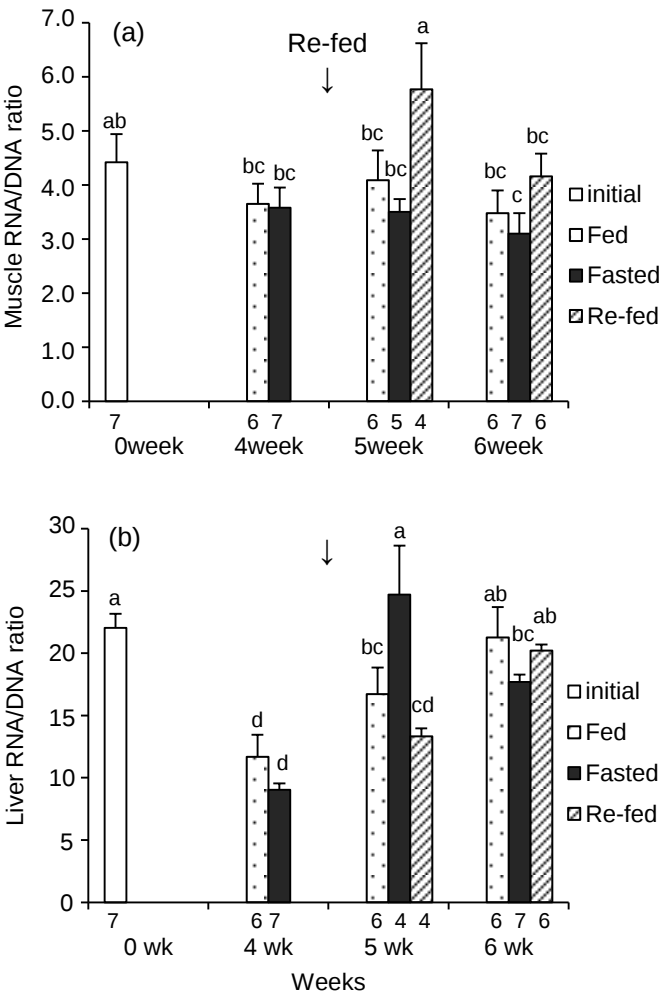
590

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

594

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





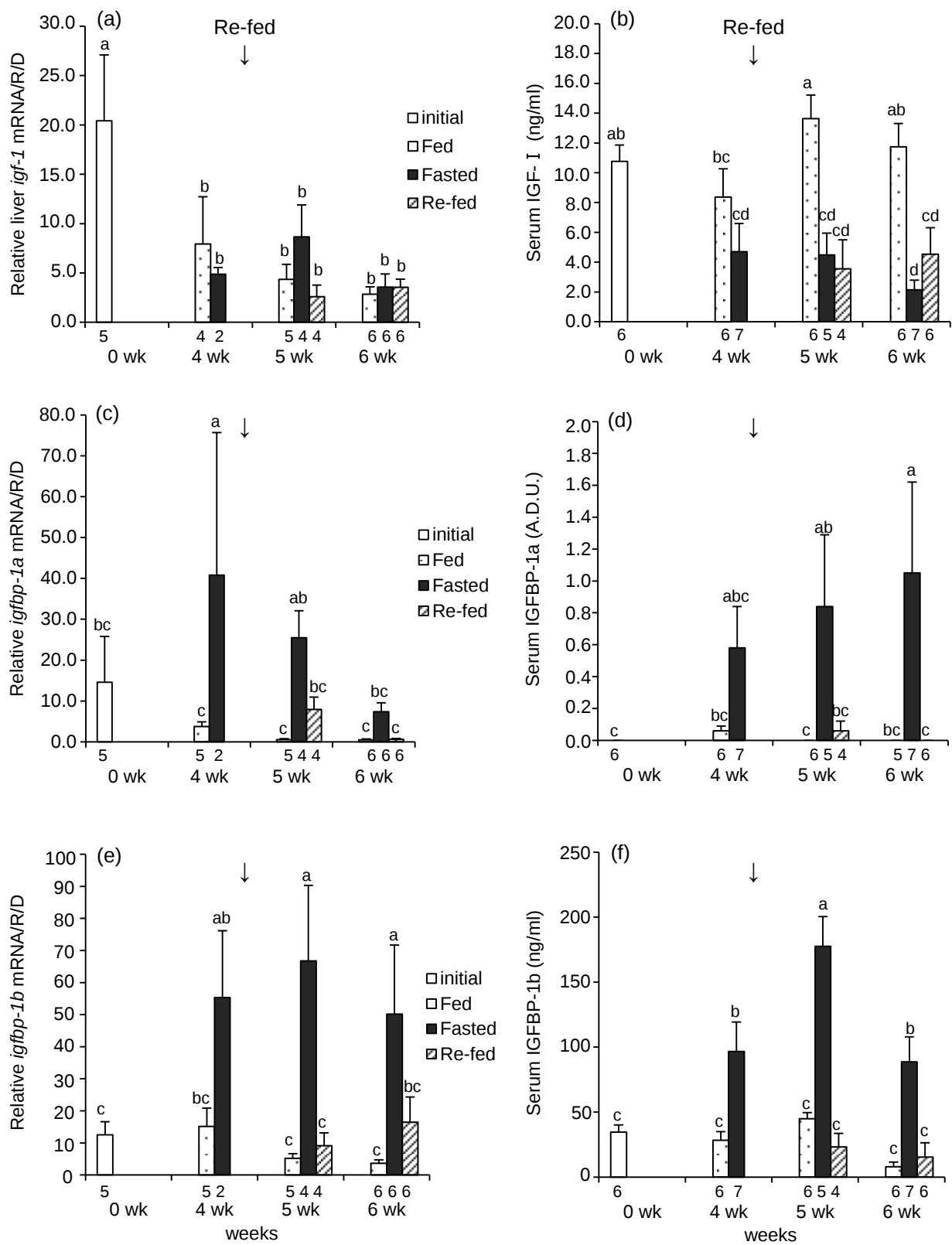


Table 1
Primer sequences used for real-time PCR analysis

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	GCTCTCCTGATTCCTCAT	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.

	<i>igf-1</i>	<i>igfbp-1a</i>	<i>igfbp-1b</i>	Muscle R/D	Liver R/D	IGF- I	IGFBP-1a	IGFBP-1b
Liver <i>igf-1</i>		-	-	-	-	-	-	-
Liver <i>igfbp-1a</i>	-		0.72	-	-	-0.49	-	0.89
Liver <i>igfbp-1b</i>	-	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 <i>igf-1</i>	-	-	-	-	-	-
Liver <i>igfbp-1a</i>	-	-	-0.64	-0.63	-	-0.70
Liver <i>igfbp-1b</i>	-	-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 <i>igf-1</i>	-	-	-	-	-	-
Liver <i>igfbp-1a</i>	-	-	-0.65	-0.78	-	-0.75
Liver <i>igfbp-1b</i>	-	-	-	-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.