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Title:

Development of a time-resolved fluoroimmunoassay for salmon insulin-like growth factor binding protein-1b

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Abstract

In salmon plasma/serum, three major insulin-like growth factor binding proteins (IGFBPs) are consistently detected at 22-, 28- and 41-kDa. The 22-kDa form has been identified as IGFBP-1b and shown to increase under catabolic conditions. We developed a competitive time-resolved fluoroimmunoassay (TR-FIA) for salmon IGFBP-1b. Purified salmon IGFBP-1b was used for biotin-labeling, assay standard and antiserum production. The TR-FIA did not cross-react with the 41-kDa form (IGFBP-2b) but showed 3% cross-reactivity with the 28-kDa form (IGFBP-1a). It measured IGFBP-1b levels as low as 0.4 ng/ml, and ED_{80} and ED_{20} were 0.9 and 24.6 ng/ml, respectively. There appears to be little interference by IGF-I. Using the TR-FIA, serum IGFBP-1b levels were measured in individually-tagged underyearling masu salmon fed or fasted for 5 weeks, or fasted for 3 weeks followed by refeeding for 2 weeks. Fasting for 3 weeks significantly increased circulating IGFBP-1b levels, while it returned to the basal levels after prolonged fasting for additional 2 weeks. Serum IGFBP-1b level negatively correlated with condition factor, specific growth rate in weight and serum IGF-I level. During parr-smolt transformation of masu salmon, average circulating IGFBP-1b levels were highest in May. There was a positive correlation between serum IGFBP-1b and IGF-I, which is in contrast to that in the fasting/feeding experiment. IGFBP-1b also showed a positive relationship with gill Na^+,K^+-ATPase activity. These results suggest that the relationship between circulating IGFBP-1b and IGF-I during smoltification differs from that during fasting and IGFBP-1b may play a role in the development of hypoosmoregulatory ability.

Keywords: fasting, growth, immunoassay, insulin-like growth factor binding protein, salmon, smoltfication

Introduction

Insulin-like growth factor binding proteins (IGFBPs) are a family of cysteine-rich proteins that are not structurally related to receptors for IGFs (Hwa et al., 1999). Despite the lack of sequence homology with the receptor, IGFBPs have high affinity for IGFs and are capable of regulating availability of IGF to target tissues (Hwa et al., 1999; Forbes et al., 2012). IGFBPs prolong the half-life of IGFs in the circulation, target them to certain tissues and either potentiate or inhibit action of IGFs depending on the type, physiological conditions and/or cellular environment (Rajaram et al., 1997; Firth and Baxter, 2002). They can also influence cell growth independent of IGFs through interacting on the cell surface receptors or translocating into the nucleus (Forbes et al., 2012; Wheatcroft and Kearney, 2009).

There are six members of the IGFBP family (IGFBP-1 to -6). They arose from local and whole genome duplications throughout the vertebrate evolution. A possible scenario is that an ancestral IGFBP gene was first duplicated locally followed by two rounds of whole-genome duplication (WGD) creating eight genes and two of them were lost (Daza et al., 2011). Supporting this, pairs of IGFBP-1 and -3, and IGFBP-2 and -5 are located on the same chromosomes, respectively, and IGFBP-4 and -6 are found on separate chromosomes (Daza et al., 2011). In teleosts, because of the 3rd round of WGD, there are two paralogs of each member of IGFBPs except IGFBP-4 (Daza et al., 2011). In addition, salmonids underwent an extra round of WGD and thus have four paralogs (Macqueen et al., 2013). Fate of duplicated genes depends on how selective pressure acts on them. One of duplicated gene is often lost (nonfunctionalization) but in rare case, gains new function(s) (neofunctionalization) (Postlethwait et al., 2004). Beside these fates, certain portion of duplicated genes undergo partitioning of their original functions (subfunctionalization) (Postlethwait et al., 2004). Subfunction partitioning can be spatial and temporal. Duan and colleagues conducted a series of functional studies using developing zebrafish (*Danio rerio*) embryos and proved subfunctional partitioning of duplicated IGFBPs (Kamei et al., 2008; Zhou et al., 2008; Wang et al., 2009; Dai et al., 2010). Moreover, expression patterns of duplicated IGFBPs in response to changes in feeding status have been investigated in Atlantic salmon (Bower et al., 2008), Arctic charr (*Salvelinus alpinus*; Macqueen et al., 2011), adult zebrafish (Amaral and Johnston, 2011, 2012) and Mozambique tilapia (*Oreochromis mossambicus*; Breves et al., 2014). These studies suggest that responses of dulplicated IGFBPs are generally similar but differ under certain tissues and physiological conditions.

IGFBP-1 is the first member to be identified in humans and is one of major

72 circulating IGFBPs (Lee et al., 1988; Lee et al., 1993,1997). It shows diurnal fluctuation often
73 reflective of meal and insulin status (Lee et al., 1993,1997). Insulin is a potent inhibitor of
74 IGFBP-1. Fasting induces IGFBP-1 in the circulation probably due to relaxation of the
75 suppressive effect of insulin. An amino acid deficiency also leads to an induction of IGFBP-1 in
76 the hepatocytes through affecting mRNA stability (Jousse et al., 1998, Averous et al., 2005).
77 IGFBP-1 is unsaturated in circulation and usually inhibits IGF actions by sequestering free IGFs
78 from the circulation (Lee et al., 1997; Wheatcroft and Kearney, 2009). This response is believed
79 to be protective from energy expenditure by the actions of IGFs during shift from anabolism to
80 catabolism. Cortisol, on the other hand, induces IGFBP-1 while its effect is not as strong as
81 insulin (Unterman et al., 1991). IGFBP-1 is thus a fine tuner of energy partitioning and growth
82 velocity under fluctuating metabolic status (Kajimura and Duan, 2007).
83 Candidates of IGFBP-1 in fish circulation have been detected by ligand blotting
84 using labeled human IGF-I (Kelley et al., 1992, 2001). In fish blood, three major IGFBPs are
85 consistently detected at 20-25 kDa, 28-32 kDa and 40-50 kDa and the two
86 low-molecular-weight forms have been thought to be IGFBP-1, -2 or -4 (Kelley et al., 1992,
87 2001). Analyses of these fish IGFBPs using ligand blotting revealed that they are up-regulated
88 under catabolic conditions such as fasting, stress and cortisol treatment as seen in mammals
89 (Siharath et al., 1996; Park et al., 2000; Kelley et al., 2001, 2006; Kajimura et al., 2003;
90 Peterson and Small, 2004, 2005; Hevrøy et al., 2011). We have identified 22- and 28-kDa
91 IGFBPs in salmon plasma as co-orthologs of IGFBP-1 through protein purification and cDNA
92 cloning (Shimizu et al., 2011a). Our finding suggests that other fish IGFBPs with similar
93 molecular weights and physiological responses are also IGFBP-1s.
94 We previously developed a radioimmunoassay (RIA) for salmon IGFBP-1b (22-kDa
95 form) and reported that it was increased by fasting or restricted ration of feed and negatively
96 correlated with individual growth rate (Shimizu et al., 2006). Plasma IGFBP-1b also showed a
97 consistent negative relationship with condition factor and quick response to a single meal
98 (Shimizu et al., 2009). Availability of RIA has thus provided important information on
99 physiological regulation and possible roles of IGFBP-1b in salmon. However, there is a trend to
100 switch from RIA to non-radioisotopic (RI) immunoassay because of safety, stableness and ease
101 of use. Indeed, RIA for fish IGF-I is being replaced by time-resolved fluoroimmunoassay
102 (TR-FIA) (Ando 2005; Small and Peterson, 2005). In contrast, there is no non-RI immunoassay
103 available for fish IGFBP. The present study reports development of a competitive TR-FIA for
104 salmon IGFBP-1b.

105

106 **Materials and methods**

107 *Fish and blood collection*

108 Yearling masu salmon (*Oncorhynchus masou*, Shiribetsu River strain) were reared in freshwater
109 at Nanae Freshwater Laboratory, Field Science Center for Northern Biosphere, Hokkaido
110 University, Japan. Blood was withdrawn by a syringe from the caudal vein, allowed to clot
111 overnight at 4°C and centrifuged at 8,050*g* for 10 min. Serum was collected and stored at -30°C
112 until use.

113 Underyearling Atlantic salmon (*Salmo salar*, NLA strain) and rainbow trout (*O.*
114 *mykiss*, NLA strain) were reared in seawater at Matre Research Station, Institute of Marine
115 Research, Matredal., Norway. Blood was collected from the caudal vein with the use of a
116 heparinised syringe. Plasma was collected after centrifugation at 1,250*g* for 10 min. Plasma was
117 stored at -80°C until use.

118

119 *Assay components*

120 IGFBP-1b was purified from serum of spawning Chinook salmon (*O. tshawytscha*; Shimizu *et*
121 *al*., 2005). Briefly, salmon serum was first fractionated by ammonium sulfate precipitation
122 and loaded onto an IGF-affinity column. IGFBP-1b was eluted from the column with 0.5M
123 acetic acid and further purified by reversed-phase high pressure liquid chromatography (HPLC)
124 on a Vydac C-4 column (Separation Group, Hesperia, CA). Purified IGFBP-1 was aliquoted
125 into prelubricated microcentrifuge tubes (PGC Scientifcs, Frederick, MD) and stored at -80°C
126 until use. Chinook salmon IGFBP-1a and -2b were also purified as described in Shimizu et al.
127 (2011a) and Shimizu et al. (2003b), respectively. Polyclonal antiserum against purified
128 IGFBP-1b (anti-IGFBP-1b) was raised in a rabbit as described in Shimizu et al. (2006).

129

130 *Ligand blotting and western blotting*

131 Sodium dodecyl suphate polyacrylamide gel eletrophoresis (SDS-PAGE) with a 3% stacking
132 gel and 12.5% separating gel was carried. Purified salmon IGFBPs (50 ng) were treated with an
133 equal volume of a sample buffer containing 2% SDS, 10% glycerol at 85°C for 5 min. Gels
134 were run in a solution of 50 mM Tris, 400 mM glycine and 0.1% SDS at 50V in the stacking gel
135 and at 100V in the separating gel until the bromophenol blue dye front reached the bottom of
136 the gel. For western blotting, an electroblotted nitrocellulose membrane was incubated with
137 anti-IGFBP-1b serum at a dilution of 1:200 for 2 hr at room temperature. The membrane was

then incubated with goat anti-rabbit IgG-HRP conjugate (Bio-Rad, Hercules, CA) at a dilution of 1:2,000 for 1 hr at room temperature. Immunoreactive bands were visualized on an X-ray film by use of ECL western blotting reagents.

The result of western ligand blotting using digoxigenin-labled human IGF-I (DIG-hIGF-I) of purified salmon IGFBPs was reproduced from Shimizu et al., (2003a) with permission.

Biotinylation of IGFBP-1b

Purified IGFBP-1b was labeled with a biotin (EZ-link sulfo-NHS-LC-biotin, Thermo Scientific, Rockford, IL). Two micrograms of purified protein were mixed with 15 µl 0.5 M phosphate buffer, pH7.4 and reacted with 2 µl 1mM NHS-LC-biotin at a molar ratio of 1:25. A low-adsorption 0.5 ml tube (PGC Scientifics) containing the mixture was incubated at room temperature under dark with occasional flipping. Reaction was stopped by adding 32 µl 0.1 M Tris-HCl, pH8.0 for 15 min and 64 µl 0.05 M Tris-HCl, 0.15 M NaCl, 0.2% BSA (Nacalai tesque, Kyoto, Japan) were added to the tube. The biotinylated IGFBP-1b was dialyzed against 0.05 M Tris-HCl, 0.15 M NaCl, pH7.5 using Slyde-A-Lyzer 3.5K dialysis cassette (Thermo Scientific). After dialysis, aliquots of the biotinylated IGFBP-1b were stored at -80°C until use.

TR-FIA for IGFBP-1b

A competitive method was employed in the assay. A 96-well assay plate coated with goat anti-rabbit IgG (DELFIA® Yellow Plate; Perkin Elmer, Turku, Finland) was first washed with 200 µl DELFIA® Wash Buffer (Perkin Elmer) and each well received 40 µl DELFIA® Assay Buffer (Perkin Elmer), 40 µl anti-IGFBP-1b (1:1,250-5,000) and 40 µl standard (purified IGFBP-1b) or serum diluted with Assay Buffer. The plate was sealed by Thermal Seal RT™ (Excel Scientific, Victorville, CA) and incubated at 4°C overnight with shaking at 150 rpm on a shaker. The plate was flash centrifuged, and each well received 40 µl biotinylated IGFBP-1b (1.3-10 ng/ml) and incubated at 4°C overnight at 150 rpm or at room temperature for 3 hr at 600 rpm. After plate was washed three times, each well received 100 µl europium-avidin conjugated with HRP (Perkin Elmer) and incubated at room temperature for 1 hr with shaking at 600 rpm. The plate was washed six times and room-temperature acclimated 200 µl DELFIA® Enhancement Solution (Perkin Elmer) was added to each plate. The plate was shook without sealing for 10 min at room temperature. Time-resolved fluorescence was measured by a fluorometer (Victor3; Perkin Elmer) with emission and read wavelengths at 340 nm and 615 nm,

171 respectively.
172
173 *TR-FIA for IGF-I*
174 Prior to the assay, serum IGF-I was extracted with an acid-ethanol as described in Shimizu et al.
175 (2000). IGF-I was quantified by TR-FIA based on the method described in Small and Peterson
176 (2005) using recombinant salmon/trout IGF-I (GroPep, Adelaide, SA, Australia) for standard
177 and labeling with europium, and anti-barramundi IGF-I (GroPep) as a primary antiserum.
178
179 *Effect of fasting and refeeding*
180 A captive brood stock of masu salmon from the Shiribetsu River held at Nanae Freshwater
181 Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Japan was used
182 in the present study. In May 2010, underyearling masu salmon were lightly anesthetized in
183 water containing 2-phenoxy ethanol (Kanto Chemical, Tokyo, Japan) and individually marked
184 with PIT tags (Biomark, Boise, ID). They were randomly placed into one of three 300L outdoor
185 tanks, and allowed to recover and acclimate for 1 week with feeding. One week after tagging,
186 their initial fork length and body weight were measured. During the experiment, one group was
187 fed daily on a commercial diet (Marubeni Nisshin Feed Co. Ltd., Tokyo, Japan) to satiety for 5
188 weeks (Fed). Second group (Fasted) was fasted throughout the experimental period (5 weeks).
189 Third group (Re-fed) was fasted for first 3 weeks and re-fed for following 2 weeks. They were
190 reared using flow-through river water that ranged from 10.3°C to 18.0°C during the experiment.
191 The experiment was carried out in accordance with the guidelines of Hokkaido University Field
192 Science Center Animal Care and Use Committee.
193 Fork length (FL) and body weight (BW) of all fish were measured 3, 4 and 5 weeks
194 after the beginning of the experiment. Condition factor (K) was calculated as follows: (body
195 weight (g)) x 1000/(fork length (cm))^3. Specific growth rate (SGR) was calculated as follows:
196 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
197 or weight on day_1 and d_2 – d_1 is the number of days between measurements. At each time point,
198 seven fish per treatment were sampled for blood. Blood was withdrawn by a syringe from the
199 caudal vein, allowed to clot overnight at 4°C and centrifuged at 8,050*g* for 10 min. Serum was
200 collected and stored at -30°C until use.
201
202 *Changes during parr-smolt transformation*
203 Yearling masu salmon reared in freshwater at the South Branch of Salmon and Freshwater

Fisheries Institute, Hokkaido Research Organization (42°N, 140°E) (Nikai-gun, Hokkaido, Japan) were sampled from February to June as described in Shimomura et al. (2012). These fish were for stock enhancement and released to the river in May 2010. Some fish were kept in the same pond and reared until June in freshwater. Seven fish were sampled monthly. Fish were anesthetized by 3.3% 2-phenoxyethanol and measured for FL and BW. K was calculated as described above. 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.

Statistical analysis

Results of the fasting/refeeding experiment 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 $p < 0.05$. Simple linear reguression analysis was used to assess the relationships between endocrine parameters and morphological/growth parameters.

For the seasonal samples, data from the June samples were not included in the analysis because fish held in freshwater in June were under quite different physiological state and disturbed the IGF-I-gill NKA relationship (Shimomura et al., 2012). Data from February through May were analyzed by one-way ANOVA with time as a factor followed by the PLSD test as described above.

Results

In ligand blotting using digoxigenin-labeled human IGF-I, purified salmon IGFBP-2b, -1a and -1b were detected at corresponding molecular weights of 41, 28 and 22 kDa, respectively (Fig. 1). Other minor bands were also visualized. Western blotting using anti-IGFBP-1b visualized the 22-kDa band in the purified IGFBP-1b fraction as well as a band at 42 kDa (Fig. 1). The 42-kDa band was not immunostained by antiserum against IGFBP-2b (Shimizu et al., 2003a), suggesting it is homodimer of IGFBP-1b. No band was detected in the purified IGFBP-2b faction whereas the 28-kDa IGFBP was also visualized but weakly in ligand blotting.

Specific binding of the biotinylated IGFBP-1 was displaced by increasing amounts of cold IGFBP-1b (Fig. 2). Serial dilutions of serum/plasma from masu salmon, rainbow trout and Atlantic salmon were parallel with that of the standard (Fig. 2). The half-maximal displacement

237 (ED_{50}) occurred at 3.8 ± 0.2 ng/ml (n = 8). The ED_{80} and ED_{20} were 0.9 ± 0.1 ng/ml (n = 8) and
238 24. 6 ± 2.7 ng/ml (n = 8), respectively. The minimum detection limit of the assay, defined as the
239 mean count of the zero standard minus two standard deviations, was 0.4 ng/ml. The intra- and
240 inter-assay coefficients of variation estimated using a control serum were 9.7% (n = 4) and
241 12.5% (n = 3), respectively. The recovery of purified IGFBP-1 (10 ng/ml) added to masu
242 salmon serum was 114.6% (n = 8).
243 Cross-reactivity of other salmon IGFBPs in the TR-FIA was examined (Fig. 3). The
244 41-kDa form (IGFBP-2b) had no effect on displacing the binding. The 28-kDa form
245 (IGFBP-1a) showed some displacement at higher concentrations and its cross-reactivity was
246 calculated as 3.1%. Adding salmon IGF-I at 1:1 or 1:10 molar ratio to masu salmon serum did
247 not considerably alter its displacement curve (Fig. 4).
248 Using the TR-FIA, responses of circulating IGF-I and IGFBP-1b to fasting/refeeding
249 were examined in underyearling masu salmon (Fig. 5). Serum IGF-I levels were similar
250 between fed and fasted groups 3 weeks after fasting but became significantly lower in fasted
251 fish in week 4 (Fig. 5a). Refeeding for 1 week partly restored reduced IGF-I levels. However,
252 IGF-I levels in all groups were not significantly different in week 5. There was an overall effect
253 of fasting on IGFBP-1b (Fig. 5b). Serum IGFBP-1b levels were significantly higher in fasted
254 fish than in fed fish, while its levels became not significantly different among groups in weeks 4
255 and 5. Serum IGF-I level positively correlated with SGRs both in length and weight (Table 1,
256 Supplementary Fig. 1). On one hand, log-transformed serum IGFBP-1b level negatively
257 correlated with K and SGRs both in length and weight (Table 1, Fig. 6). There was a negative
258 correlation between serum IGFBP-1b and IGF-I (r = -0.51, p = 0.0325) in the fasting/feeding
259 experiment. IGF-I:ln IGFBP-1b ratio was calculated and plotted against SGRs (Supplementary
260 Fig. 1). Albeit not significant, IGF-I:ln IGFBP-1b ratio had a higher regression coefficient value
261 against SGRs than IGF-I alone (Supplementary Fig. 1).
262 Changes in circulating IGF-I and IGFBP-1b levels during smoltification in masu
263 salmon were measured by TR-FIA (Fig. 7). Serum IGF-I levels were steadily increased from
264 March to May and continued to increase even during the desmoltification period (June) (Fig.
265 7a). Serum IGFBP-1 levels were relatively stable from February to April, sharply increased in
266 May and were kept high in June (Fig. 7b). During February and May, serum IGF-I showed
267 positive relationships with FL, BW and gill Na^+,K^+-ATPase (NKA) activity (Table 2). Serum
268 IGFBP-1 levels were also positively correlated with FL, BW and gill NKA activity (Table 2,
269 Fig. 8). A negative correlation between serum IGFBP-1b and K was seen (Table 2). There was a

positive correlation between serum IGFBP-1b and IGF-I ($r = 0.52$, $p = 0.0067$).

Discussion

The present study developed a TR-FIA for salmon IGFBP-1b which can be used as an alternative to the RIA. The assay components employed for the TR-FIA were the same as used in the RIA (Shimizu et al., 2006) and showed the similar specificity. In the present study, purified IGFBP-1b was biotinylated and used as tracer. This labeling appeared to have little effects on the binding to the antiserum and the interference by IGFs in the assay. In salmon blood, there are three major IGFBPs and two of them have been identified as IGFBP-1a (28-kDa form) and -1b (22-kDa form) whereas the other as IGFBP-2b (41-kDa form) (Shimizu et al., 2005, 2011a,b). Since IGFBP-1a and -1b share 61% sequence homology (Shimizu et al., 2011a), the antiserum against IGFBP-1b could cross-react with IGFBP-1a. Indeed, the antiserum visualized the IGFBP-1a band in western blotting and showed cross-reactivity to IGFBP-1a in the TR-FIA. However, since its cross-reactivity is low (3.1%), it should not severely affect the quantification of IGFBP-1b in TR-FIA. The assay components of the TR-FIA were originally from Chinook salmon but serial dilutions of serum/plasma from masu and Atlantic salmon and rainbow trout showed parallelism to the standard, indicating that this assay can be used for salmonids as is the case for the RIA (Shimizu et al., 2006).

The establishment of the TR-FIA enabled us to investigate an effect of fasting/refeeding on masu salmon IGFBP-1b (Kawaguchi et al., 2013) and an effect of increased rearing temperature on Atlantic salmon and rainbow trout IGFBP-1b (Hevrøy et al., umpublished data). In the latter study, a high temperature (19°C) significantly affected IGFBP-1b levels in both species but different timing and direction, suggesting that species-difference in catabolic response to elevated temperature exists between Atlantic salmon and rainbow trout (Hevrøy et al., unpublished data). At present, availability of immunoassay for quantifying fish IGFBPs is limited to salmon IGFBP-1b and -2b (Shimizu et al., 2003a, 2006). One of the challenges in establishing immunoassay for fish IGFBP is to prepare enough amount of IGFBP for antigen since circulating levels of IGFBPs are approximately 300 ng/ml at the highest (Shimizu et al., 2003a, 2006). The present study used IGFBP-1b purified from Chinook salmon but the final yield was very low (22 μg from 600 ml serum; Shimizu et al., 2005), making protein purification not a practical method for antigen preparation. In order to facilitate studies on physiological regulation of fish IGFBPs, production of recombinant IGFBP and its use as antigen is desired.

Induction of circulating IGFBP-1 by fasting is a well-known response in mammals and fish (Lee et al., 1993, 1997; Siharath et al., 1996; Kelley et al., 2001; Peterson and Small, 2004; Shimizu et al., 2006, 2009). In post-smolt coho salmon, plasma IGFBP-1b was graded by the period of fasting (1, 2 or 3 weeks) and a single meal after fasting for 3 weeks restored increased IGFBP-1 to the basal level (Shimizu et al., 2009). The present study subjected underyearling masu salmon to 5-week fasting or 3-week fasting followed by refeeding for 2 weeks. Fasting for 3 weeks significantly increased serum IGFBP-1b levels. However, after week 3 serum IGFBP-1b levels in fasted fish decreased and became insignificant from those in fed and refed groups. The same trend of the decrease in IGFBP-1b levels during prolonged fasting was observed in post-smolt masu salmon (Kawaguchi et al., 2013). In contrast, IGFBP-1 levels continued to increase for 60 days in striped bass (*Morone saxatilis*; Siharath et al., 1996) or kept high until 45 days in channel catfish (*Ictalurus punctatus*; Peterson and Small, 2004). These differences may be species-dependent but the presence of IGFBP-1a needs to be considered since there is a possibility that IGFBP-1a could take over the IGFBP-1b role under certain conditions. Kamei et al. (2008) reported subfunction partitioning of duplicated IGFBP-1s in developing zebrafish embryos. In their study, both *igfbp-1a* and *-1b* were induced by hypoxia but their timing and responsiveness to hypoxia were different, which was termed as inducible or regulation subfunctioning partitioning (Kamei et al., 2008). On the other hand, only *igfbp-1b* was induced by chronic low oxygen stress in the liver of Atlantic salmon (Olsvik et al., 2013). However, in the same species hepatic *igfbp-1a* and *-1b* were increased when rearing temperature was increased from 13°C to 17°C (Hevrøy et al., 2013; Olsvik et al., 2013). Quantifying IGFBP-1a and comparing its response with that of IGFBP-1b will be useful to understand how the inducible/regulation subfunction partitioning of these paralogs is operated in fish.

IGFBP-1 is believed to be an inhibitor of IGF-I actions in fish (Kajimura and Duan, 2007). There are attempts to utilize its circulating protein levels or hepatic mRNA levels as an index of negative growth and/or stress (Kelley et al., 2001, 2006; Picha et al., 2008; Kawaguchi et al., 2013). However, few studies correlated *igfbp-1b*/IGFBP-1b levels with SGR. In post-smolt masu salmon, serum IGFBP-1b and its hepatic mRNA levels were negatively correlated with SGRs in both FL and BW (Kawaguchi et al., 2013). In the present study, serum IGFBP-1b was negatively correlated with SGR in both FL and BW. However, its relationships with SGRS were as not high as those in post-smolt masu salmon (Kawaguchi et al., 2013). The reason for the weaker correlation with SGRs in underyearling masu salmon is not known but

care should be taken to use IGFBP-1b as a negative growth index in fish at different age, or/and physiological conditions.

Simple correlation analyses on the relationships between circulating IGFBP-1b and other growth-related traits and endocrine factors suggest possible negative effects of IGFBP-1b in growth (Shimizu et al., 2006, 2009). In coho salmon (*O. kisutch*), IGFBP-1b was consistently, negatively correlated with K (Shimizu et al., 2006, 2009). A negative correlation between IGFBP-1 and body mass index is also evident in humans, which is likely explained by the effect of body fatness on insulin sensitivity (Travers et al., 1998). In the present study, the same negative relationship with K was found in the fasting/feeding experiment and during smoltification. Whether insulin sensitivity is involved in the link between IGFBP-1b with K in salmon is not known at present, but it is of note that insulin had little effect on hepatic expression of *igfbp-1b* in coho salmon (Pierce et al., 2006), suggesting another physiological mechanism. A direct effect of circulating IGFBP-1b on adipose tissue might be a possible pathway. Weil et al. (2011) characterized expression patterns of the IGF/IGFBP system including the IGF-receptor in adipose tissue in rainbow trout and suggested its involvement in adipose tissue growth. How circulating IGFBP-1b affects availability of circulating IGFs to the adipose tissue in fish is a subject to future study.

Our analysis also revealed that there was a negative correlation between circulating IGFBP-1b and IGF-I in the feeding/fasting experiment using underyearling masu salmon. This is in accordance with the result from the similar experiment using post-smolt masu salmon (Kawaguchi et al., 2013) but differs from the result in coho salmon where IGFBP-1b levels show no correlation with IGF-I under different feeding status (Shimizu et al., 2006, 2009). These findings suggest a species-difference between coho and masu salmon. In humans, a weak negative correlation between serum IGFBP-1 and IGF-I has been reported (Travers et al., 1998). Given that salmon IGFBP-1b is potentially an inhibitor of IGF-I action, a ratio of IGF-I to IGFBP-1b may reflect a fraction of circulating IGF-I available for promoting growth. In order to test this hypothesis, we calculated the ratio and correlated it with SGR in the present study. As a result, although IGF-I:ln IGFBP-1b ratio did not statistically improve the regression coefficient of variation but was higher than that of IGF-I alone. This kind of analysis may be worthy trying when more data points are available

Parr-smolt transformation (smoltification) is a series of changes pre-adaptive for salmon to ocean life (Stefansson et al., 2008; McCormick et al., 2013). Acquisition of hypoosmoregulatory ability (seawater adaptability) mainly through activation of gill NKA is

one of the characteristic changes occurring during smoltification. A rapid lean growth, generally reflected by a reduction of K, is also seen during this period. Circulating IGF-I levels have shown to change during smoltification in several salmonid species (Aas-Hansen et al., 2003; Àugustsson et al., 2001; Shimizu et al., 2003a), which could improve seawater adaptability and/or promote body growth (McCormick et al., 2013). In masu salmon, IGF-I typically shows a peak or high values when gill NKA was activated, suggesting that circulating IGF-I is involved in the NKA activation (Shimomura et al., 2012; Kaneko et al., in press). On one hand, there is only one study reporting changes in circulating IGFBP-1b during smoltification in coho salmon (Shimizu et al., 2006). In that study, plasma IGFBP-1b sharply increased from the end of March through late April. IGF-I levels in the same study increased prior to that of IGFBP-1b and there was no significant relation between IGFBP-1b and IGF-I. In contrast, there was a positive correlation between them in the present study. In yellowtail kingfish (*Seriola lalandi*), albeit at the mRNA level, a strong positive correlation was seen between liver *igf-1* and *igfbp-1*, which might buffer the hypoglycemic action of circulating IGF-I (Collins et al., 2014). Thus, IGFBP-1b might have a role in preventing IGF-I from interacting with the insulin receptor during smoltification. Alternatively, IGFBP-1b may target IGF-I to certain tissues. In the present study, IGFBP-1b positively correlated with gill NKA activity. Given that circulating IGF-I is believed to be important for activating branchial NKA (Reinecke, 2010; Shimomura et al., 2012), IGFBP-1b could have a positive effect on the IGF-I action by delivering IGF-I to the gill or protecting it from degradation. Although this is totally a speculation at present but the present study is the first to report a positive correlation between circulating IGFBP-1b and gill NKA, and suggests a possible involvement of IGFBP-1b in the regulation of gill NKA activity.

In summary, the present study established a TR-FIA for salmon IGFBP-1b. IGFBP-1b levels were increased by fasting but it might be transient in underyearling masu salmon. During smoltification of yearling masu salmom, circulating IGFBP-1b was positively correlated with circulating IGF-I and gill NKA activity, suggesting its possible involvement in the development of hypoosmoregulatory ability in salmon.

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585

585 **Figure legends**

586 Fig. 1. Ligand blotting (LB) and western blotting (WB) of purified salmon IGFBPs. Samples
587 were run on 12.5% gel under non-reducing conditions, transferred to nitrocellulose membranes.
588 The membranes were probed by either digoxigenin-labled human IGF-I for ligand blotting or
589 anti-salmon IGFBP-1b for western blotting. Arrowheads indicate molecular weights of IGFBPs.
590

591 Fig. 2. Displacement of biotinylated-salmon IGFBP-1b with purified IGFBP-1b and sera from
592 masu salmon and rainbow trout (a) and Atlantic salmon (b).
593

594 Fig. 3. Cross-reactivity of salmon IGFBPs in the TR-FIA. Displacement of tracer was assessed
595 by adding increasing amounts of purified salmon IGFBP-1a, -1b and -2b to the assay.
596

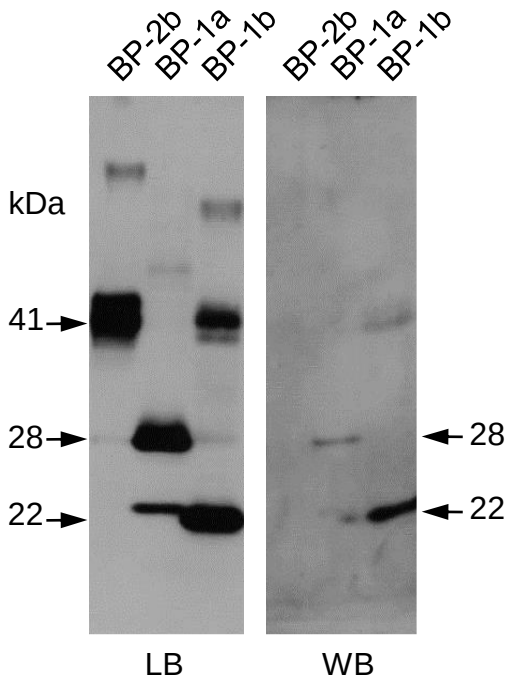
597 Fig. 4. Effect of exogenous salmon IGF-I on the displacement of masu salmon serum in TR-FIA.
598 Salmon IGF-I was added to masu salmon serum at molar ratio of 1:1 or 1:10.
599

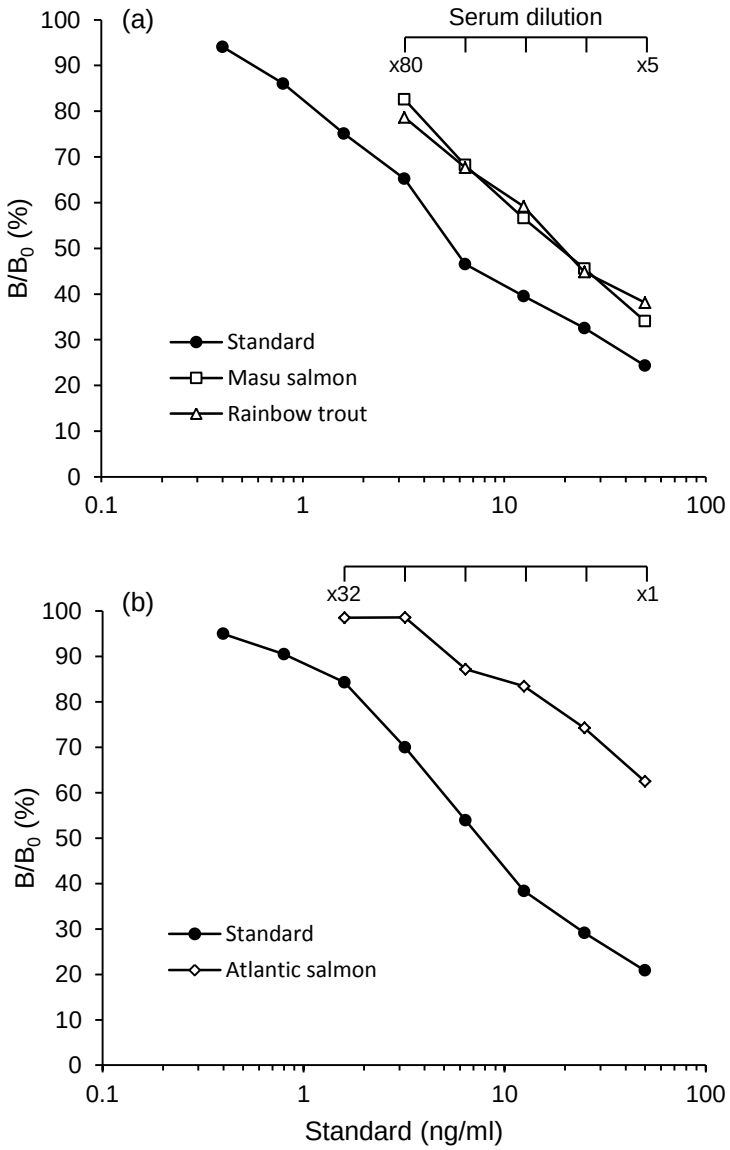
600 Fig. 5. Effects of fasting and re-feeding on circulating IGF-I (a) and IGFBP-1b (b). Values are
601 expressed as means ± SE (n = 6-7). An asterisk indicates an overall effect. Symbols sharing the
602 same letters are not significantly different from each other.
603

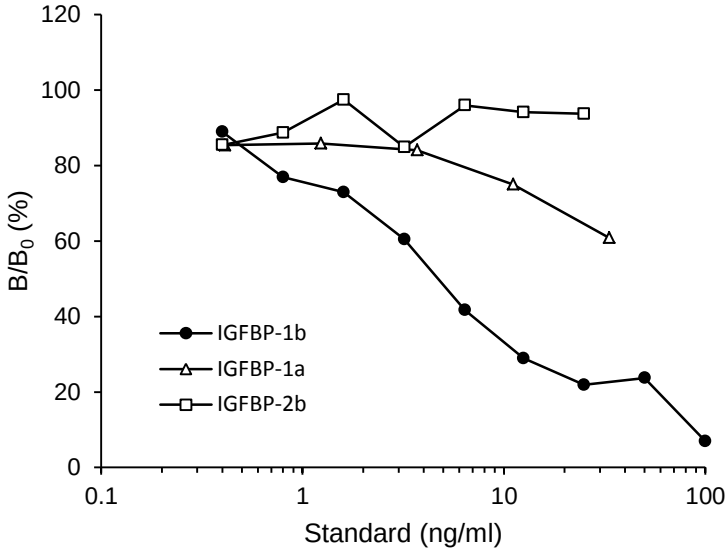
604 Fig. 6. Correlations between natural-log transformed IGFBP-1b level and condition factor (a),
605 specific growth rate (SGR) in fork length (FL) (b) and SGR in body weight (BW) (c).
606

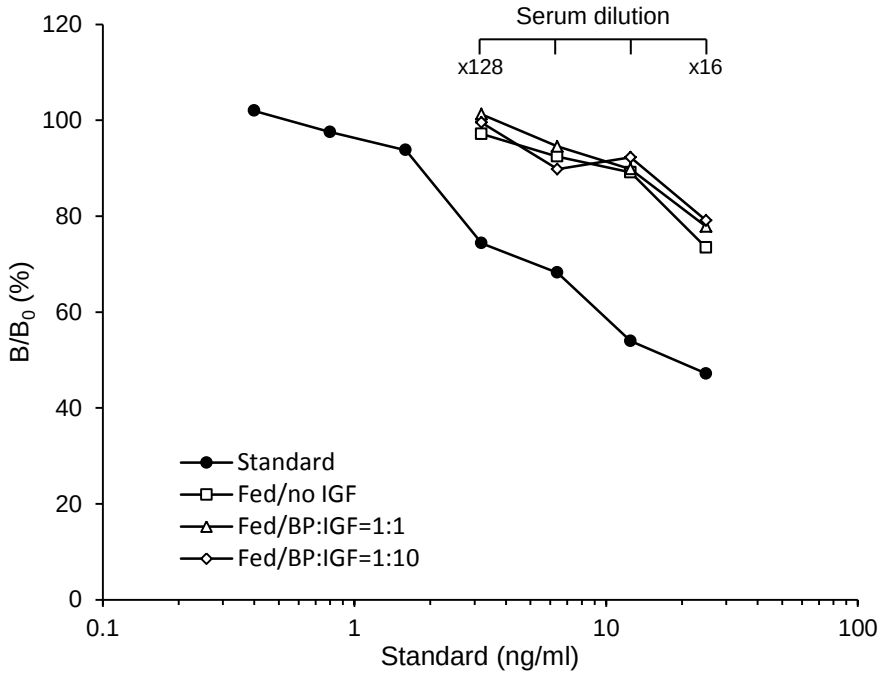
607 Fig. 7. Changes in serum IGF-I (a) and IGFBP-1b (b) levels during smoltification of masu
608 salmon. Values are expressed as means ± SE (n = 6-7). Data on serum IGF-I were from
609 Shimomura et al. (2012) with permission. Symbols sharing the same letters are not significantly
610 different from each other.
611

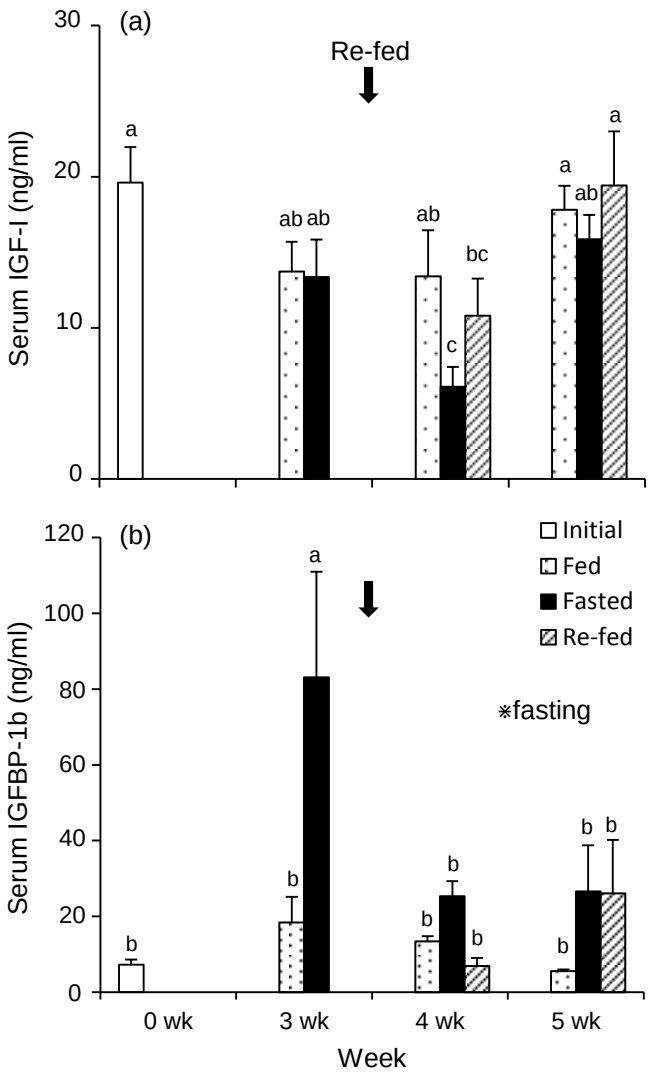
612 Fig. 8. Correlation between natural-log transformed IGFBP-1b level and gill Na+,K+-ATPase
613 (NKA) activity during smoltification of masu salmon (March to May, n = 19). Data on gill
614 NKA activity were from Shimomura et al. (2012) with permission.

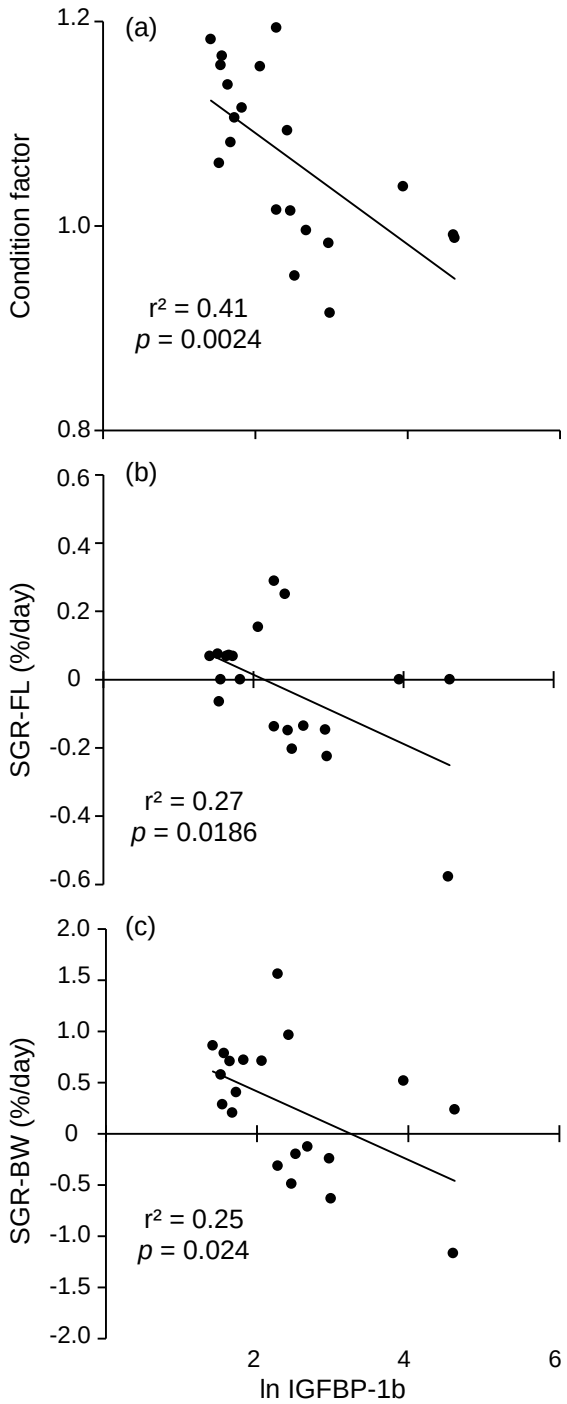

BP-2b
BP-1a
BP-1b
BP-2b
BP-1a
BP-1b
kDa
41
28
22
28
22
LB
WB

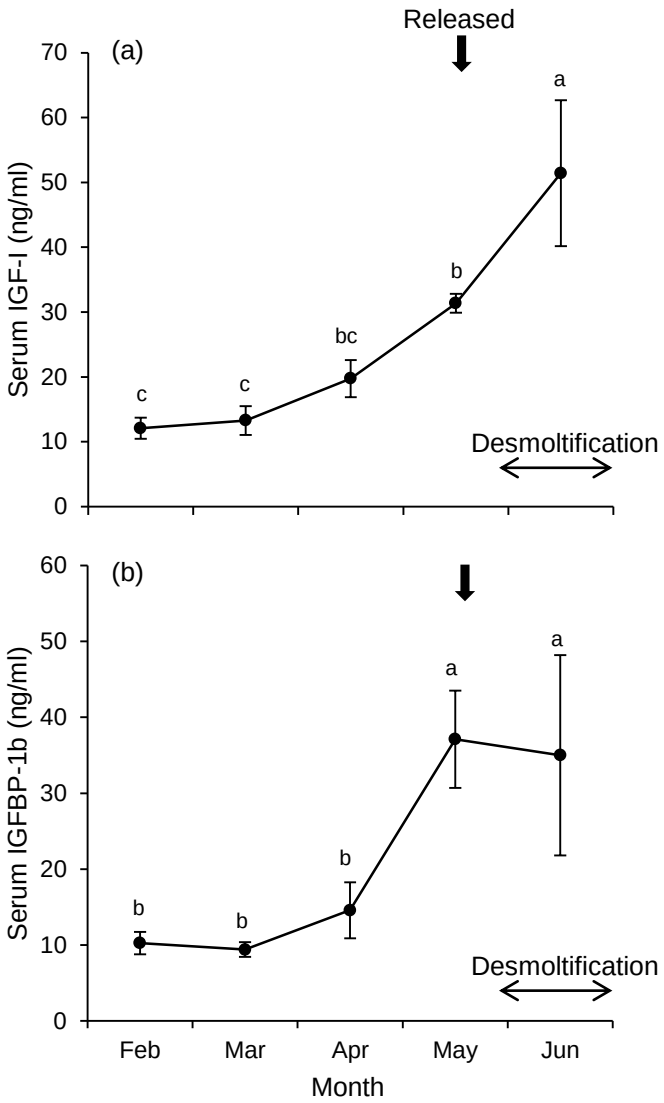

(a)
Serum dilution
x80
x5
100
90
80
70
60
50
40
30
20
10
0
B/B0 (%)
Standard
Masu salmon
Rainbow trout
0.1
1
10
100
(b)
x32
x1
Standard
Atlantic salmon
Standard (ng/ml)


120
100
80
60
40
20
0
B/B0 (%)
0.1
1
10
100
Standard (ng/ml)
IGFBP-1b
IGFBP-1a
IGFBP-2b


Serum dilution
x128
x16
120
100
80
60
40
20
0
B/B0 (%)
0.1
1
10
100
Standard (ng/ml)
Standard
Fed/no IGF
Fed/BP:IGF=1:1
Fed/BP:IGF=1:10


(a)
Re-fed
Serum IGF-I (ng/ml)
30
20
10
0
a
ab ab
ab
c
bc
a
ab
a
(b)
Initial
Fed
Fasted
Re-fed
∗fasting
Serum IGFBP-1b (ng/ml)
120
100
80
60
40
20
0
b
a
b
b
b
b
b
b
0 wk
3 wk
4 wk
5 wk
Week


(a)
Condition factor
1.2
1.0
0.8
r² = 0.41
p = 0.0024
(b)
SGR-FL (%/day)
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
r² = 0.27
p = 0.0186
(c)
SGR-BW (%/day)
2.0
1.5
1.0
0.5
0
-0.5
-1.0
-1.5
-2.0
r² = 0.25
p = 0.024
2
4
6
ln IGFBP-1b


Released
(a)
Serum IGF-I (ng/ml)
70
60
50
40
30
20
10
0
a
b
bc
c
c
Desmoltification
(b)
Serum IGFBP-1b (ng/ml)
60
50
40
30
20
10
0
a
a
b
b
b
Desmoltification
Feb
Mar
Apr
May
Jun
Month

Fukuda *et al.*, Fig. 8

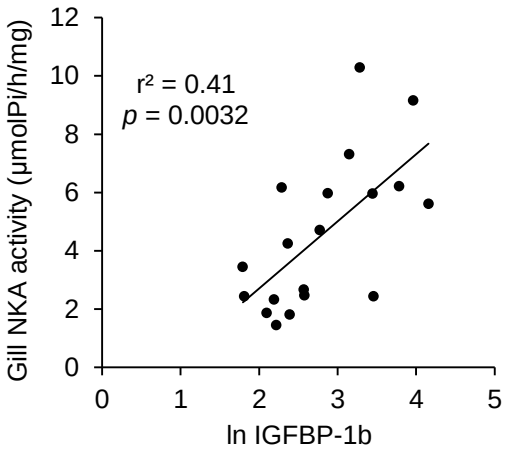


Table 1
Correlation coefficients (r) between endocrine parameters and morphological/ growth parameters.

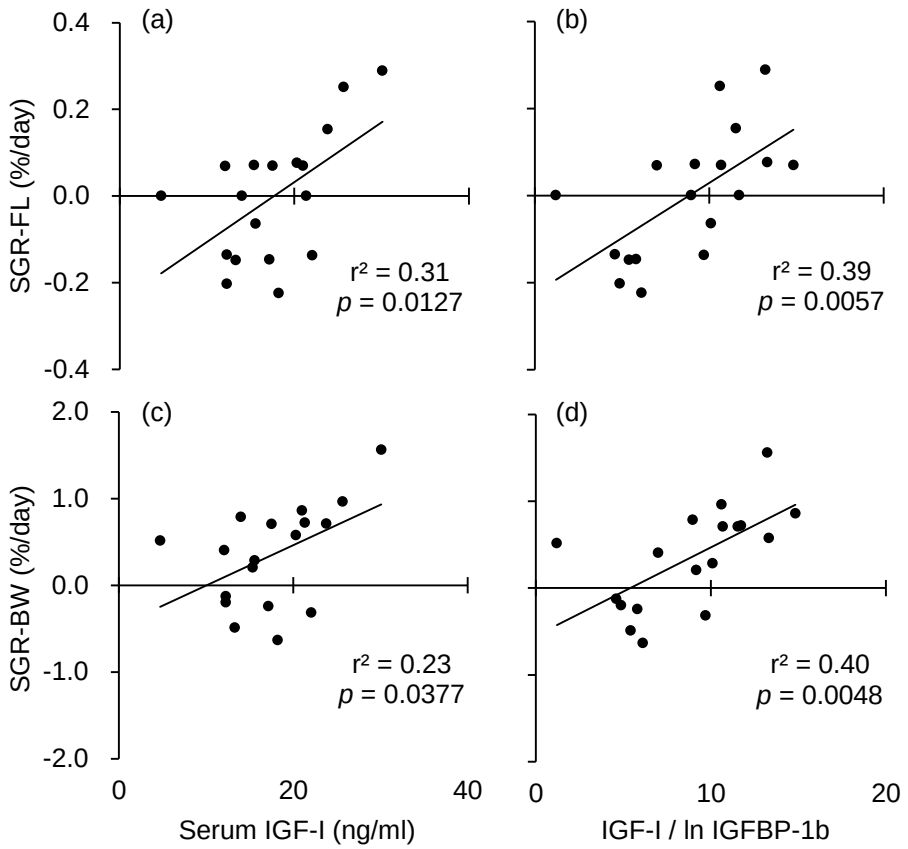
	FL	BW	K	SGR-FL	SGR-BW
IGF-I	-	-	-	0.56	0.48
ln IGFBP-1b	-	0.48	-0.64	-0.52	-0.50

(-): not significant. Analysis was used in 5th week.

Table 2
Correlation coefficients (r) between endocrine parameters and morphological parameters and gill NKA activity during February to May.

	FL	BW	K	Gill NKA activity
IGF-I	0.77	0.76	-	0.86
ln IGFBP-1b	0.61	0.55	-0.42	-0.64

(-): not significant. Data on serum IGF-I were from Shimomura et al. (2012) with permission.



Supplementary Fig. 1. Correlations between IGF-I level and specific growth rate (SGR) in fork length (FL) (a) and body weight (BW) (c), and between IGF-I/ln IGFBP-1b and SGR-FL (b) and -BW (d). Regression coefficents were statistically not different between IGF-I and IGF-I/ln IGFBP-1b for both SGR-FL and -BW. Note that data points (n = 18) are not identical to those in Fig. 6 (n = 19) due to a small serum volume in one sample.

Table
Comparison of morphological parameters among treatments.

		0 wk	3 wk	4 wk	5 wk
FL	Fed	9.6±0.18	Data lost	9.9 ±0.17	10.2 ±0.23
	Fasted		10.0 ±0.26	10.0 ±0.19	9.9 ±0.18
	Re-fed			9.9 ±0.28	9.8 ±0.22
BW	Fed	10.1 ±0.54^{ab}	Data lost	9.8 ±0.52^{b}	11.0 ±0.86^{a}
	Fasted		9.7 ±0.83^{b}	9.6 ±0.53^{b}	11.4 ±0.55^{b}
	Re-fed			10.8 ±1.03^{ab}	10.4 ±0.78^{ab}
K	Fed	1.14 ±0.02^{a}	Data lost	1.02 ±0.02^{b}	1.04 ±0.02^{a}
	Fasted		0.97 ±0.02^{b}	0.97 ±0.02^{b}	1.18 ±0.04^{b}
	Re-fed			1.09 ±0.02^{a}	1.11 ±0.02^{a}
HSI	Fed	2.21 ±0.22^{a}	Data lost	1.56 ±0.24^{b}	1.40 ±0.10^{b}
	Fasted		1.19 ±0.07^{b}	1.22 ±0.17^{b}	0.89 ±0.04^{b}
	Re-fed			1.30 ±0.24^{b}	1.41±0.21^{b}

Values are expressed as mean ±SE (n = 6-7). Symbols sharing the same letters are not significantly different from each other.