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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, smoltification

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 duplicated 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

circulating IGFBPs (Lee et al., 1988; Lee et al., 1993,1997). It shows diurnal fluctuation often reflective of meal and insulin status (Lee et al., 1993,1997). Insulin is a potent inhibitor of IGFBP-1. Fasting induces IGFBP-1 in the circulation probably due to relaxation of the suppressive effect of insulin. An amino acid deficiency also leads to an induction of IGFBP-1 in the hepatocytes through affecting mRNA stability (Jousse et al., 1998, Averous et al., 2005). IGFBP-1 is unsaturated in circulation and usually inhibits IGF actions by sequestering free IGFs from the circulation (Lee et al., 1997; Wheatcroft and Kearney, 2009). This response is believed to be protective from energy expenditure by the actions of IGFs during shift from anabolism to catabolism. Cortisol, on the other hand, induces IGFBP-1 while its effect is not as strong as insulin (Unterman et al., 1991). IGFBP-1 is thus a fine tuner of energy partitioning and growth velocity under fluctuating metabolic status (Kajimura and Duan, 2007).

Candidates of IGFBP-1 in fish circulation have been detected by ligand blotting using labeled human IGF-I (Kelley et al., 1992, 2001). In fish blood, three major IGFBPs are consistently detected at 20-25 kDa, 28-32 kDa and 40-50 kDa and the two low-molecular-weight forms have been thought to be IGFBP-1, -2 or -4 (Kelley et al., 1992, 2001). Analyses of these fish IGFBPs using ligand blotting revealed that they are up-regulated under catabolic conditions such as fasting, stress and cortisol treatment as seen in mammals (Siharath et al., 1996; Park et al., 2000; Kelley et al., 2001, 2006; Kajimura et al., 2003; Peterson and Small, 2004, 2005; Hevrøy et al., 2011). We have identified 22- and 28-kDa IGFBPs in salmon plasma as co-orthologs of IGFBP-1 through protein purification and cDNA cloning (Shimizu et al., 2011a). Our finding suggests that other fish IGFBPs with similar molecular weights and physiological responses are also IGFBP-1s.

We previously developed a radioimmunoassay (RIA) for salmon IGFBP-1b (22-kDa form) and reported that it was increased by fasting or restricted ration of feed and negatively correlated with individual growth rate (Shimizu et al., 2006). Plasma IGFBP-1b also showed a consistent negative relationship with condition factor and quick response to a single meal (Shimizu et al., 2009). Availability of RIA has thus provided important information on physiological regulation and possible roles of IGFBP-1b in salmon. However, there is a trend to switch from RIA to non-radioisotopic (RI) immunoassay because of safety, stableness and ease of use. Indeed, RIA for fish IGF-I is being replaced by time-resolved fluoroimmunoassay (TR-FIA) (Ando 2005; Small and Peterson, 2005). In contrast, there is no non-RI immunoassay available for fish IGFBP. The present study reports development of a competitive TR-FIA for salmon IGFBP-1b.

Materials and methods

Fish and blood collection

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

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

Assay components

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

Ligand blotting and western blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with a 3% stacking gel and 12.5% separating gel was carried. Purified salmon IGFBPs (50 ng) were treated with an equal volume of a sample buffer containing 2% SDS, 10% glycerol at 85°C for 5 min. Gels were run in a solution of 50 mM Tris, 400 mM glycine and 0.1% SDS at 50V in the stacking gel and at 100V in the separating gel until the bromophenol blue dye front reached the bottom of the gel. For western blotting, an electroblotted nitrocellulose membrane was incubated with 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-labeled 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 (DELFI[®]A Yellow Plate; Perkin Elmer, Turku, Finland) was first washed with 200 µl DELFI[®]A Wash Buffer (Perkin Elmer) and each well received 40 µl DELFI[®]A 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 DELFI[®]A 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,

respectively.

TR-FIA for IGF-I

Prior to the assay, serum IGF-I 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, Adelaide, SA, Australia) for standard and labeling with europium, and anti-barramundi IGF-I (GroPep) as a primary antiserum.

Effect of fasting and refeeding

A captive brood stock of masu salmon from the Shiribetsu River held at Nanae Freshwater Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Japan was used in the present study. In May 2010, underyearling masu salmon were lightly anesthetized in water containing 2-phenoxy ethanol (Kanto Chemical, Tokyo, Japan) 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 5 weeks (Fed). Second group (Fasted) was fasted throughout the experimental period (5 weeks). Third group (Re-fed) was fasted for first 3 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 3, 4 and 5 weeks after the beginning of the experiment. Condition factor (K) was calculated as follows: $(\text{body weight (g)}) \times 1000 / (\text{fork length (cm)})^3$. Specific growth rate (SGR) was calculated as follows: $\text{SGR (\%/day)} = \ln(s_2 - s_1) \times (d_2 - d_1)^{-1} \times 100$, where s_2 is length or weight on day₂, s_1 is length or weight on day₁ and $d_2 - d_1$ is the number of days between measurements. At each time point, seven fish per treatment were sampled for blood. Blood was withdrawn by a syringe from the caudal vein, allowed to clot overnight at 4°C and centrifuged at 8,050g for 10 min. Serum was collected and stored at -30°C until use.

Changes during parr-smolt transformation

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 8,050g 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 regression 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 fraction 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

(ED₅₀) occurred at 3.8 ± 0.2 ng/ml ($n = 8$). The ED₈₀ and ED₂₀ were 0.9 ± 0.1 ng/ml ($n = 8$) and 24.6 ± 2.7 ng/ml ($n = 8$), respectively. The minimum detection limit of the assay, defined as the mean count of the zero standard minus two standard deviations, was 0.4 ng/ml. The intra- and inter-assay coefficients of variation estimated using a control serum were 9.7% ($n = 4$) and 12.5% ($n = 3$), respectively. The recovery of purified IGFBP-1 (10 ng/ml) added to masu salmon serum was 114.6% ($n = 8$).

Cross-reactivity of other salmon IGFBPs in the TR-FIA was examined (Fig. 3). The 41-kDa form (IGFBP-2b) had no effect on displacing the binding. The 28-kDa form (IGFBP-1a) showed some displacement at higher concentrations and its cross-reactivity was calculated as 3.1%. Adding salmon IGF-I at 1:1 or 1:10 molar ratio to masu salmon serum did not considerably alter its displacement curve (Fig. 4).

Using the TR-FIA, responses of circulating IGF-I and IGFBP-1b to fasting/refeeding were examined in underyearling masu salmon (Fig. 5). Serum IGF-I levels were similar between fed and fasted groups 3 weeks after fasting but became significantly lower in fasted fish in week 4 (Fig. 5a). Refeeding for 1 week partly restored reduced IGF-I levels. However, IGF-I levels in all groups were not significantly different in week 5. There was an overall effect of fasting on IGFBP-1b (Fig. 5b). Serum IGFBP-1b levels were significantly higher in fasted fish than in fed fish, while its levels became not significantly different among groups in weeks 4 and 5. Serum IGF-I level positively correlated with SGRs both in length and weight (Table 1, Supplementary Fig. 1). On one hand, log-transformed serum IGFBP-1b level negatively correlated with K and SGRs both in length and weight (Table 1, Fig. 6). There was a negative correlation between serum IGFBP-1b and IGF-I ($r = -0.51$, $p = 0.0325$) in the fasting/feeding experiment. IGF-I:ln IGFBP-1b ratio was calculated and plotted against SGRs (Supplementary Fig. 1). Albeit not significant, IGF-I:ln IGFBP-1b ratio had a higher regression coefficient value against SGRs than IGF-I alone (Supplementary Fig. 1).

Changes in circulating IGF-I and IGFBP-1b levels during smoltification in masu salmon were measured by TR-FIA (Fig. 7). Serum IGF-I levels were steadily increased from March to May and continued to increase even during the desmoltification period (June) (Fig. 7a). Serum IGFBP-1 levels were relatively stable from February to April, sharply increased in May and were kept high in June (Fig. 7b). During February and May, serum IGF-I showed positive relationships with FL, BW and gill Na⁺,K⁺-ATPase (NKA) activity (Table 2). Serum IGFBP-1 levels were also positively correlated with FL, BW and gill NKA activity (Table 2, 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., unpublished 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; Augustsson 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 salmon, 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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References

- Aas-Hansen, O., Johnsen, H.K., Vijayan, M.M., Jorgensen, E.H., 2003. Development of seawater tolerance and concurrent hormonal changes in fed and fasted Arctic charr at two temperature regimes. *Aquaculture* 222, 135-148.
- Ágústsson, T., Sundell, K., Sakamoto, T., Johansson, V., Ando, M., Th Björnsson, B., 2001. Growth hormone endocrinology of Atlantic salmon (*Salmo salar*): pituitary gene expression, hormone storage, secretion and plasma levels during parr-smolt transformation. *J. Endocrinol.* 170, 227-234.
- Amaral, I.P.G., Johnston, I.A., 2011. Insulin-like growth factor (IGF) signalling and genome-wide transcriptional regulation in fast muscle of zebrafish following a single-satiating meal. *J. Exp. Biol.* 214, 2125-2139.
- Amaral, I.P.G., Johnston, I.A., 2012. Experimental selection for body size at age modifies early life-history traits and muscle gene expression in adult zebrafish. *J. Exp. Biol.* 215, 3895-3904.
- Andoh, T., 2005. Development of non-radioisotopic immunoassay systems for measuring flounder IGF-I. *Zool. Sci.* 22, 1023-1030.
- Averous, J., Maurin, A.-C., Bruhat, A., Jousse, C., Arliguie, C. Fafournoux, P., 2005. Induction of IGFBP-1 expression by amino acid deprivation of HepG2 human hepatoma cells involves both a transcriptional activation and an mRNA stabilization due to its 3'UTR. *FEBS Lett.* 579, 2609-2614.
- Bower, N.I., Li, X., Taylor, R., Johnston, I.A., 2008. Switching to fast growth: the insulin-like growth factor (IGF) system in skeletal muscle of Atlantic salmon. *J. Exp. Biol.* 211, 3859-3870.
- Breves, J.P., Tipsmark, C.K., Stough, B.A., Seale, A.P., Flack, B.R., Moorman, B.P., Lerner, D.T., Grau, E.G., 2014. Nutritional status and growth hormone regulate insulin-like growth factor binding protein (*igfbp*) transcripts in Mozambique tilapia. *Gen. Comp. Endocr.* 207, 66-73.
- Collins, G.M., Ball, A.S., Qin, J.G., Bowyer, J.N., Stone, D.A.J., 2014. Effect of alternative lipids and temperature on growth factor gene expression in yellowtail kingfish (*Seriola lalandi*). *Aqua. Res.* 45, 1236-1245.
- Dai, W., Kamei, H., Zhao, Y., Ding, J., Du, Z., Duan, C., 2010. Duplicated zebrafish

435 insulin-like growth factor binding protein-5 genes with split functional domains:
 436 evidence for evolutionarily conserved IGF binding, nuclear localization, and
 437 transactivation activity. *FASEB J.* 24, 2020-2029.

438 Daza, D.O., Sundstrom, G., Bergqvist, C.A., Duan, C.M., Larhammar, D., 2011. Evolution of
 439 the Insulin-Like Growth Factor Binding Protein (IGFBP) Family. *Endocrinology* 152,
 440 2278-2289.

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

443 Forbes, B.E., McCarthy, P., Norton, R.S., 2012. Insulin-like growth factor binding proteins: a
 444 structural perspective. *Front. Endocrinol.* 3, 38.

445 Hevrøy, E.M., Azpeleta, C., Shimizu, M., Lanzén, A., Kaiya, H., Espe, M., Olsvik, P.A. 2011.
 446 Effects of short-term starvation on ghrelin, GH-IGF system, and IGF-binding proteins
 447 in Atlantic salmon. *Fish Physiol. Biochem.* 37, 217-232.

448 Hevrøy, E.M., Hunskaar, C., de Gelder, S., Shimizu, M., Waagbø, R., Breck, O., Takle, H.,
 449 Sussort, S., Hansen, T., 2013. GH-IGF system regulation of attenuated muscle growth
 450 and lipolysis in Atlantic salmon reared at elevated sea temperatures. *J. Comp. Physiolol.*
 451 B 183, 243-259.

452 Hwa, V., Oh, Y., Rosenfeld, R.G., 1999. The insulin-like growth factor-binding protein
 453 (IGFBP) superfamily. *Endocr. Rev.* 20, 761-787.

454 Jousse, C., Bruhat, A., Ferrara, M., Fafournoux, P., 1998. Physiological concentration of amino
 455 acids regulates insulin-like-growth-factor-binding protein 1 expression. *Biochem. J.* 334,
 456 147-153.

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

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

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

466 Kaneko, N., Iijima, A., Shimomura, T., Nakajima, T., Shimura, H., Oomori, H., Urabe, H., Hara,
 467 A., Shimizu, M. Profiles of circulating insulin-like growth factor-I during smoltification

468 of masu salmon reared under different conditions. Fish. Sci. (in press).

469 Kawaguchi, K., Kaneko, N., Fukuda, M., Nakano, Y., Kimura, S., Hara, A., Shimizu, M., 2013.

470 Responses of insulin-like growth factor (IGF)-I and two IGF-binding protein-1 subtypes

471 to fasting and re-feeding, and their relationships with individual growth rates in yearling

472 masu salmon (*Oncorhynchus masou*). Comp. Biochem. Physiol. A 165, 191-198.

473 Kelley, K.M., Siharath, K., Bern, H.A., 1992. Identification of insulin-like growth

474 factor-binding proteins in the circulation of four teleost fish species. J. Exp. Zool. 263,

475 220-224.

476 Kelley, K.M., Haigwood, J.T., Perez, M., Galima, M.M., 2001. Serum insulin-like growth factor

477 binding proteins (IGFBPs) as markers for anabolic/catabolic condition in fishes. Comp.

478 Biochem. Physiol. B 129, 229-236.

479 Kelley, K.M., Price, T.D., Galima, M.M., Sak, K., Reyes, J.A., Zepeda, O., Hagstrom, R.,

480 Truong, T.A., Lowe, C.G., 2006. Insulin-like growth factor-binding proteins (IGFBPs)

481 in fish: beacons for (disrupted) growth endocrine physiology. In: Reinecke, M.,

482 Zaccane, G., Kapoor, B.G. (Eds.), Fish Endocrinology. Science Publishers, Enfield,

483 New Hampshire, pp. 167-195.

484 Lee, P.D., Conover, C.A., Powell, D.R., 1993. Regulation and function of insulin-like growth

485 factor-binding protein-1. Proc. Soc. Exp. Biol. Med. 204, 4-29.

486 Lee, P.D., Giudice, L.C., Conover, C.A., Powell, D.R., 1997. Insulin-like growth factor binding

487 protein-1: recent findings and new directions. Proc. Soc. Exp. Biol. Med. 216, 319-357.

488 Lee, Y.-L., Hintz, R.L., James, P.M., Lee, P.D.K., Shively, J.E., Powell, D.R., 1988.

489 Insulin-like growth factor (IGF) binding protein complementary deoxyribonucleic acid

490 from human HEP G2 hepatoma cells: predicted sequence suggests an IGF binding

491 domain different from those of the IGF-I and IGF-II receptors. Mol. Endocrinol. 2,

492 401-411.

493 Macqueen, D.J., Kristjansson, B.K., Paxton, C.G.M., Vieira, V.L.A., Johnston, I.A., 2011. The

494 parallel evolution of dwarfism in Arctic charr is accompanied by adaptive divergence in

495 mTOR-pathway gene expression. Mol. Ecol. 20, 3167-3184.

496 Macqueen, D.J., de la Serrana, D.G., Johnston, I.A., 2013. Evolution of ancient functions in the

497 vertebrate insulin-like growth factor system uncovered by study of duplicated salmonid

498 fish genomes. Mol. Biol. Evol. 30, 1060-1076.

499 McCormick, S.D., 2013. Smolt physiology and endocrinology. In: McCormick, S.D., Farrell,

500 A.P., Brauner, C.J. (Eds.), Euryhaline Fishes. Academic Press, Oxford, UK, pp.

199-251.

Olsvik P.A., Vikeså, V., Lie, K.K., Hevrøy, E.M., 2013. Transcriptional responses to temperature and low oxygen stress in Atlantic salmon studied with next-generation sequencing technology. BMC Genom. 14, 817.

Park, R., Shepherd, B.S., Nishioka, R.S., Grau, E.G., Bern, H.A., 2000. Effects of homologous pituitary hormone treatment on serum insulin-like growth-factor-binding proteins (IGFBPs) in hypophysectomized tilapia, *Oreochromis mossambicus*, with special reference to a novel 20-kDa IGFBP. Gen. Comp. Endocrinol. 117, 404-412.

Peterson, B.C., Small, B.C., 2004. Effects of fasting on circulating IGF-binding proteins, glucose, and cortisol in channel catfish (*Ictalurus punctatus*). Domest. Anim. Endocrinol. 26, 231-240.

Peterson, B.C., Small, B.C., 2005. Effects of exogenous cortisol on the GH/IGF-I/IGFBP network in channel catfish. Domest. Anim. Endocrinol. 28, 391-404.

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

Pierce, A.L., Shimizu, M., Felli, L., Swanson, P., Dickhoff, W.W., 2006. Metabolic hormones regulate insulin-like growth factor binding protein-1 mRNA levels in primary cultured salmon hepatocytes; lack of inhibition by insulin. J. Endocrinol. 191, 379-386.

Postlethwait, J., Amores, A., Cresko, W., Singer, A., Yan, Y.L., 2004. Subfunction partitioning, the teleost radiation and the annotation of the human genome. Trends Genet. 20, 481-490.

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

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

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

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

Shimizu, M., Swanson, P., Hara, A., Dickhoff, W.W., 2003b. Purification of a 41-kDa

534 insulin-like growth factor binding protein from serum of chinook salmon,
 535 *Oncorhynchus tshawytscha*. Gen. Comp. Endocrinol. 132, 103-111.

536 Shimizu, M., Dickey, J.T., Fukada, H., Dickhoff, W.W., 2005. Salmon serum 22 kDa
 537 insulin-like growth factor-binding protein (IGFBP) is IGFBP-1. J. Endocrinol. 184,
 538 267-276.

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

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

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

549 Shimizu, M., Suzuki, S., Horikoshi, M., Hara, A., Dickhoff, W.W., 2011b. Circulating salmon
 550 41-kDa insulin-like growth factor binding protein (IGFBP) is not IGFBP-3 but an
 551 IGFBP-2 subtype. Gen. Comp. Endocrinol. 171, 326-331.

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

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

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

563 Stefansson, S.O., Bjornsson, B.T., Ebbesson, L.O.E., McCormick, S.D., 2008. Smoltification.
 564 in: Finn, R.N., Kapoor, B.G. (Eds.), Fish Larval Physiology. Science Publishers,
 565 Enfield, NH, pp. 639-681.

566 Travers, S.H., Labarta, J.I., Gargosky, S.E., Rosenfeld, R.G., Jeffers, B.W., Eckel, R.H., 1998.

567 Insulin-like growth factor binding protein-I levels are strongly associated with insulin
 568 sensitivity and obesity in early pubertal children. *J. Clin. Endocrinol. Metab.* 83,
 569 1935-1939.

570 Unterman, T.G., Oehler, D.T., Murphy, L.J., Lacson, R.G., 1991. Multihormonal regulation of
 571 insulin-like growth factor-binding protein-1 in rat H4IIE hepatoma cells: the dominant
 572 role of insulin. *Endocrinology* 128, 2693-2701.

573 Wang, X., Lu, L., Li, Y., Li, M., Chen, C., Feng, Q., Zhang, C., Duan, C., 2009. Molecular and
 574 functional characterization of two distinct IGF binding protein-6 genes in zebrafish. *Am.*
 575 *J. Physiol. Regul. Integr. Comp. Physiol.* 296, R1348-1357.

576 Weil, C., Lebret, V., Gabillard, J.C., 2011. The IGF/IGFBP system in rainbow trout
 577 (*Oncorhynchus mykiss*) adipose tissue: expression related to regional localization and
 578 cell type. *Fish Physiol. Biochem.* 37, 843-852.

579 Wheatcroft, S.B., Kearney, M.T., 2009. IGF-dependent and IGF-independent actions of
 580 IGF-binding protein-1 and-2: implications for metabolic homeostasis. *Trends*
 581 *Endocrinol. Metab.* 20, 153-162.

582 Zhou, J., Li, W., Kamei, H., Duan, C., 2008. Duplication of the IGFBP-2 gene in teleost fish:
 583 protein structure and functionality conservation and gene expression divergence. *PLoS*
 584 *One.* 3, e3926.

585

Figure legends

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

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

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

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

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

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

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

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

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 morpho-
logical 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 coefficients 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	$10.1 \pm 0.54^{\text{ab}}$	Data lost	$9.8 \pm 0.52^{\text{b}}$	$11.0 \pm 0.86^{\text{a}}$
	Fasted		$9.7 \pm 0.83^{\text{b}}$	$9.6 \pm 0.53^{\text{b}}$	$11.4 \pm 0.55^{\text{b}}$
	Re-fed			$10.8 \pm 1.03^{\text{ab}}$	$10.4 \pm 0.78^{\text{ab}}$
K	Fed	$1.14 \pm 0.02^{\text{a}}$	Data lost	$1.02 \pm 0.02^{\text{b}}$	$1.04 \pm 0.02^{\text{a}}$
	Fasted		$0.97 \pm 0.02^{\text{b}}$	$0.97 \pm 0.02^{\text{b}}$	$1.18 \pm 0.04^{\text{b}}$
	Re-fed			$1.09 \pm 0.02^{\text{a}}$	$1.11 \pm 0.02^{\text{a}}$
HSI	Fed	$2.21 \pm 0.22^{\text{a}}$	Data lost	$1.56 \pm 0.24^{\text{b}}$	$1.40 \pm 0.10^{\text{b}}$
	Fasted		$1.19 \pm 0.07^{\text{b}}$	$1.22 \pm 0.17^{\text{b}}$	$0.89 \pm 0.04^{\text{b}}$
	Re-fed			$1.30 \pm 0.24^{\text{b}}$	$1.41 \pm 0.21^{\text{b}}$

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