Intact rather than total circulating insulin-like growth factor binding protein-1a is a negative indicator of growth in masu salmon

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Running title

Immunoassays for salmon IGFBP-1a

Abstract

Insulin-like growth factor binding protein (IGFBP)-1a is one of three major circulating forms in salmon and induced under catabolic conditions. However, there is currently no immunoassay available for this form due to lack of standard and specific antibodies. We developed a time-resolved fluoroimmunoassay (TR-FIA) for salmon IGFBP-1a using recombinant protein for labelling, an assay standard, and production of antiserum. The TR-FIA had a low cross-reactivity (3.6%) with IGFBP-1b, another major form in the circulation. Fasting for 4 weeks had no effect on serum immunoreactive (total) IGFBP-1a levels in yearling masu salmon, whereas 6-week fasting significantly increased it. There was a significant, but weak, negative relationship between serum total IGFBP-1a level and individual growth rate (r^2 = 0.12, P = 0.01). We next developed a ligand immuno-functional assay (LIFA) using europium-labelled IGF-I to quantify intact IGFBP-1a. In contrast to total IGFBP-1a, serum intact IGFBP-1a levels increased after 4 weeks of fasting, and refeeding for 2 weeks restored it to levels similar to those of the fed control. Serum intact IGFBP-1a level showed a significant negative correlation with individual growth rate (r^2 = 0.52, $P < 0.001$), which was as good as that of IGFBP-1b. Our findings using newly developed TR-FIA and LIFA suggest that regulation of intact IGFBP-1a level has an important effect on growth in salmon and that intact IGFBP-1a is a negative index of salmon growth.

Keywords

Insulin-like growth factor binding protein-1a; Immunoassay; Salmon; Growth; Fasting

1. Introduction

Insulin-like growth factor binding proteins (IGFBPs) are important modulators of the actions of insulin-like growth factors (IGFs) (1, 2, 11). IGFBPs in the circulation prolong the half-lives of IGFs and carry them to target tissues (3, 42, 57). In target tissues, IGFBPs can either potentiate or inhibit the availability of IGFs to the receptor, depending on the type of IGFBP, post-translational modification such as phosphorylation, enzymatic degradation, or/and the cellular microenvironment (1, 15, 22, 23).

In mammals, six types of IGFBP, termed -1 to -6, have been identified (22, 43). IGFBP-1 is one of the major circulating forms and its levels are increased when animals are under catabolic conditions, such as nutritional deficiency and stress (36, 37, 56). IGFBP-1 is usually unoccupied with endogenous IGFs and thus can act as an inhibitor of IGFs by sequestering free IGFs from the circulation (36, 37, 56). IGFBP-1 is highly phosphorylated, resulting in an IGF-binding affinity six-fold higher than that of non-phosphorylated forms (24). Under certain situations where IGFBP-1 is non-phosphorylated or/and partially degraded, it can potentiate the action of IGF-I (24, 55). In addition, Brandt et al. (8) reported that phosphorylated IGFBP-1 promoted the IGF-I stimulated proliferation of human dermal fibroblasts when cells were sparsely plated at approximately 30% confluence. Thus, IGFBP-1 is an important regulator of IGF action.

In teleosts, there are two subtypes of IGFBP-1, which are products of the teleost-specific third round of the whole genome duplication event (13, 27, 38). Kamei et al. (27) was the first to identify two co-orthologs of IGFBP-1 in zebrafish (*Danio rerio*). The authors highlighted that two IGFBP-1s overlapped their function but were different in terms of temporal/spatial expression patterns, responses to fasting, IGF-binding affinity, and thus inhibitory actions on the IGF-induced cell proliferation (27). Such subfunction partitioning should increase the complexity of IGF regulation and enable fine-tuning of growth in teleosts.

These two subtypes are present in the circulation of salmon. In salmon circulation, three IGFBPs are detected at 22-, 28- and 41-kDa (45) and the 22- and 28-kDa forms have been identified as IGFBP-1b and -1a, respectively (48, 51). Two low-molecular-weight IGFBPs, presumably corresponding to IGFBP-1s, are also consistently detected in circulation in other fish and reported to increase by fasting, osmotic stress, hypoxia, handling, and cortisol injection (26, 31-33, 39, 51, 52). Kelley et al. (31-33) proposed that they are markers of catabolic status in fish. To use fish IGFBPs for catabolic markers, unraveling their regulation and relation to IGFs and growth is essential.

Fish IGFBPs in the circulation are usually detected and semi-quantified by ligand blotting using labeled IGF-I. The ligand blotting is useful because it detects different types of IGFBPs simultaneously based on their ability to bind IGF-I and does not require specific antisera. In addition, the ligand blotting does not detect IGFBP fragments that have lost the IGF-binding ability. Such a feature is important when enzymatic degradation of IGFBPs is a concern (8, 9, 18, 55). However, the ligand blotting has the limitations of being semi-quantitative and not being able to process a large number of samples. Thus, development of immunoassay for circulating fish IGFBPs is crucial to facilitate analyses of physiological regulation of fish IGFBPs in the circulation.

We have previously developed a radioimmunoassay (RIA) and a time-resolved fluoroimmunoassay (TR-FIA) for salmon IGFBP-1b and showed that circulating IGFBP-1b increased in fasting fish, and negatively correlated with individual growth rates in immature coho salmon (*Onocorhynchus kisutch*), masu salmon (*O. masou*), and chum salmon (*O. keta*) (19, 29, 30, 49, 50). These findings led us to propose its utility as a quantitative negative index of growth. On the one hand, circulating IGFBP-1a semi-quantified by ligand blotting also increased under long-term fasting or osmotic stress but appeared to be less sensitive to catabolic states than IGFBP-1b (30, 51). On the other hand, circulating IGFBP-1a still showed a negative correlation with growth rate in masu salmon, suggesting that it is also useful as a negative index of growth (30). However, there is currently no immunoassay available for fish IGFBP-1a due to lack of purified protein and specific antiserum.

We have recently produced recombinant masu salmon IGFBP-1a using a bacterial expression system (54). By using the recombinant protein for assay components and antiserum production, the present study developed a TR-FIA for salmon IGFBP-1a. However, our initial analyses revealed that serum IGFBP-1a levels measured by the newly developed TR-FIA showed little response to fasting while it clearly increased in the analysis using ligand blotting (30). Such discrepancy between the immunoassay and ligand blotting has been recognized in human clinical research and suggested enzymatic digestion of IGFBP (20). We thus hypothesized that the TR-FIA detected "total" immunoreactive IGFBP-1a, a mixture of the fragmented form and "intact" form capable of binding to IGF-I. Measuring both "total" and "intact" IGFBP-3 in humans suggested their different biological roles (34). Therefore, we converted the TR-FIA to a ligand immuno-functional assay (LIFA) that detects only intact IGFBP-1a. Comparison of these two immunoassays for IGFBP-1a indicates that intact, rather than total, IGFBP-1a is a reliable negative index of growth in salmon.

2. Materials and methods

2.1. Production and purification of recombinant salmon IGFBP-1a

Recombinant salmon (rs) IGFBP-1a was produced using a bacterial expression system as described in Tanaka et al. (54). Briefly, a pET-32(+) expression vector (Novagen, Madison, WI) carrying the mature masu salmon *igfbp-1a* cDNA was transformed into a strain of *Escherichia coli* [Rosetta-gami™ B(DE3)pLysS (Novagen)], and rsIGFBP-1a was expressed as a fusion protein with a histidine tag and thioredoxin. The fusion protein was solubilized and isolated by Ni-affinity chromatography. The fusion partners were cleaved by enterokinase, and rsIGFBP-1a was purified by reversed-phase high-pressure liquid chromatography (HPLC) on a Vydac C-4 column (Separation Group, Hesperia, CA, USA). Purified rsIGFBP-1a was aliquoted into low-absorption tubes (PGC Scientifics, Frederick, MD, USA), and stored at −80°C until use.

2.2. Production of antiserum against rsIGFBP-1a

Polyclonal antiserum against purified rsIGFBP-1a (anti-IGFBP-1a) was raised in a rabbit. Immunization of the rabbit was conducted at a designated facility in accordance with the guidelines of the Animal Care Committee of Hokkaido University. Purified rsIGFBP-1a in phosphate buffered saline (pH 7.0) was emulsified in an equal volume of TiterMax Gold Research Adjuvant (TiterMax USA, Inc., Norcross, GA, USA). A rabbit was first immunized with 50 μg antigen by lymph node injection followed by subcutaneous boost injections with 100 and 150 μg antigen 2 and 3 weeks after the first injection, respectively. One week after the last boost, the blood was withdrawn from the ear vein and antiserum was collected after centrifugation. The antiserum was stored at −80°C until use.

2.3. Other assay components

IGFBP-1b and IGFBP-2b purified from serum of spawning Chinook salmon (*O. tshawytscha*; 47, 48) were used to examine the cross-reactivity.

To examine the parallelism of serum dilution with a standard curve, pooled sera were obtained from yearling masu salmon and rainbow trout (*O. mykiss*) reared in freshwater at the Nanae Freshwater Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Japan. Blood was withdrawn by a syringe from the caudal vein, allowed clotting overnight at 4°C and centrifuged at 10,000 rpm for 15 min. Serum was collected and stored at −30°C until use.

2.4. TR-FIA for "total" IGFBP-1a

In the present study, "total" IGFBP-1a is all immunoreactive components recognized by the antiserum, which include "intact" (to be defined below) IGFBP-1a and its fragments. A competitive method was employed in the assay for quantifying total IGFBP-1a. Purified rsIGFBP-1a was labeled with biotin (EZ-link Sulfo-NHS-Biotin, Thermo Scientific, Rockford, IL, USA). Thirty-four micrograms of purified protein was reacted with 31 µl of 1 mM NHS-Biotin at a molar ratio of 1:25. The mixture was incubated for 2 hours on ice under dark. The reaction was stopped by adding 0.1 M Tris-HCl, pH 7.5, and incubation for 30 min. Biotinylated rsIGFBP-1a was dialyzed against 0.05 M Tris-HCl and 0.15 M NaCl, pH 7.5, using Slide-A-Lyzer 3.5K dialysis cassette (Thermo Scientific). After dialysis, aliquots of the biotinylated rsIGFBP-1a were stored at −80°C until use.

A 96-well strip assay plate (DELFIA strip plate) or yellow plate (DELFIA yellow plate) coated with goat anti-rabbit IgG (PerkinElmer, Turku, Finland) was first washed with 200 µl DELFIA Wash Buffer (PerkinElmer). Each well received 80 µl DELFIA Assay Buffer (PerkinElmer), 20 µl anti-IGFBP-1a (1:8,000), and 40 µl standard (purified rsIGFBP-1a) or serum diluted with Assay Buffer. The plate was sealed and incubated at 4°C overnight with shaking at 600 rpm on a shaker. The plate was flash centrifuged and each well received 20 µl biotinylated rsIGFBP-1a (1:8,000) and incubated at 4°C overnight with shaking at 600 rpm on a shaker. After the plate was washed three times with 160 µl Wash Buffer, each well received 160 µl europium (Eu)-labeled streptavidin (1:2,000, PerkinElmer) and incubated at room temperature for 1 hour with shaking at 600 rpm. The plate was washed with 160 µl five times and 200 µl three times with Wash Buffer. Each well received 200 µl DELFIA Enhancement Solution (PerkinElmer), which acclimated to room temperature before use, and the plate was shaken without sealing for 10 min at room temperature. Time-resolved fluorescence was measured using the Wallac ARVO X4 multilabel counter (PerkinElmer) or SPARK multimode microplate reader (TECAN Group Ltd., Männedorf, Switzerland).

2.5. LIFA for "intact" IGFBP-1a

In the present study, "intact" indicates immunoreactive IGFBP-1a with IGF-binding ability in the reaction mixture. A LIFA for intact IGFBP-1a was developed by modifying the method for human IGFBP-3 (34); unextracted IGFBP-1a was first captured by anti-IGFBP-1a, reacted with Eu-labeled salmon IGF-I (GroPep Bioreagents Pty Ltd., Adelaide, SA, Australia) and quantified

for its binding capacity. A 96-well strip assay plate (DELFIA strip plate) or yellow plate (DELFIA yellow plate) coated with goat anti-rabbit IgG (PerkinElmer) was first washed with 200 µl DELFIA Wash Buffer (PerkinElmer). Each well received 80 µl DELFIA Assay Buffer (PerkinElmer), 20 µl IGFBP-1a antiserum (1:250), and 40 µl standard (purified rsIGFBP-1a) or serum diluted with Assay Buffer. The plate was sealed and incubated at 4°C overnight on a shaker at 600 rpm. On the next day, each well received 20 µl Eu-labeled IGF-I (25 ng/ml), and incubated at 4°C overnight on a shaker at 600 rpm. The plate was washed six times with 200 µl Wash Buffer, and each well received 200 µl DELFIA Enhancement Solution (PerkinElmer) that had been acclimated to room temperature before use. The plate was shaken without sealing for 10 min at room temperature. Time-resolved fluorescence was measured using the Wallac ARVO X4 multilabel counter (PerkinElmer) or SPARK multimode microplate reader (TECAN Group Ltd.). Intact IGFBP-1a levels were expressed as binding unit (BU) relative to purified rsIGFBP-1a instead of absolute unit (ng/ml). This was due to the possibility that phosphorylation of IGFBP-1a in serum/plasma might change its affinity to IGF-I and thus measured value. In the present study, 1 BU was equivalent to the IGF binding capacity of 1 ng/ml non-phosphorylated rsIGFBP-1a.

2.6. TR-FIA for IGF-I and IGFBP-1b

For measuring IGF-I, serum was first extracted with an acid-ethanol, as described in Shimizu et al. (45). IGF-I was quantified by TR-FIA using the method described in Small and Peterson (53), with recombinant salmon/trout IGF-I (GroPep Bioreagents Pty, Ltd.) as a standard. Time-resolved fluorescence was measured using the Wallac ARVO X4 multilabel counter (PerkinElmer).

Serum IGFBP-1b levels were quantified by TR-FIA, as described in Fukuda et al. (19). Briefly, a competitive method was employed by following a procedure for DELFIA immunoassays (PerkinElmer). Serum samples were first incubated with antiserum against purified salmon IGFBP-1b (49), overnight at 4°C, in a 96-well microtiter plate coated with goat anti-rabbit IgG (PerkinElmer). Biotinylated salmon IGFBP-1b was added to each well and incubated overnight at 4°C. After washing with DELFIA Wash Buffer (PerkinElmer), each well received Eu-labeled streptavidin (PerkinElmer) followed by DELFIA Enhancement Solution (PerkinElmer). Time-resolved fluorescence was measured using the Wallac ARVO X4 multilabel counter (PerkinElmer).

206 *2.7. Fasting/refeeding experiment*

207 A captive brood stock of yearling masu salmon from the Shiribetsu River in southern Hokkaido
208 held at Nanae Freshwater Laboratory, Field Science Center for Northern Biosphere, Hokkaido
209 University, Japan (41°54'N, 140°41'E), was used in the present study. In June 2012, fish were
210 lightly anesthetized in water containing 2-phenoxyethanol and individually marked with passive
211 integrated transponder tags (Biomark, Boise, ID, USA). They were randomly placed into one of
212 three 350-l outdoor tanks (φ95 × 70 cm), and allowed to recover and acclimate for 1 week with
213 feeding. One week after tagging, their initial standard length (SL) and body weight (BW) were
214 measured. During the experiment, one group was fed daily with a commercial diet (Marubeni
215 Nisshin Feed Co. Ltd., Tokyo, Japan) to satiety for 6 weeks (Fed). The second group (Fasted)
216 was fasted throughout the experimental period (6 weeks). The third group (Refed) was fasted
217 for first 4 weeks and re-fed for the following 2 weeks. They were reared using flow-through
218 river water that ranged from 11.0°C to 16.0°C during the experiment (Suppl. Fig. 1;
219 https://doi.org/10.6084/m9.figshare.7957250.v1). The experiment was carried out in accordance
220 with the guidelines of Hokkaido University Field Science Center Animal Care and Use
221 Committee.

222 The SL and BW of all fish were measured 4 and 6 weeks after the beginning of the
223 experiment. The condition factor (K) was calculated as follows: BW (g) × 100 / SL (cm)^3. The
224 hepato-somatic index (HSI) was calculated as follows: HSI (%) = liver weight (g) × 100/BW (g).
225 The specific growth rate (SGR) was calculated as follows: SGR (%/day) = $\ln(s_2 - s_1) \times (d_2 -$
226 $d_1)^{-1} \times 100$, where s_2 is length or weight on day$_2$, s_1 is length or weight on day$_1$, and $d_2 - d_1$ is
227 the number of days between measurements. Blood samples were collected from 8 fish per
228 treatment at 0 and 4 weeks, and 18-21 fish per treatment at 6 weeks. Blood was withdrawn by a
229 syringe from the caudal vein, allowed to clot overnight at 4°C, and centrifuged at 10,000 rpm
230 for 15 min. Serum was collected and stored at −80°C until use.

231

232 *2.8. Statistical analyses*

233 Results were first analyzed by two-way analysis for variance (ANOVA) (time × treatment)
234 using the JMP software (SAS Institute Inc., Cary, NC, USA). When significant effects were
235 found, differences were further identified by one-way ANOVA followed by Fisher's protected
236 least significant difference (LSD) test. Differences between groups were considered to be
237 significant at $P < 0.05$. Simple regression analysis was also conducted using JMP software and
238 the relations were considered to be significant at $P < 0.05$. When analyzing the regression,

values of total, intact IGFBP-1a, and IGFBP-1b in the circulation were transformed to natural-log form to obtain a normal distribution.

3. Results

Specific binding of the biotinylated IGFBP-1a was displaced by increasing amounts of unlabeled IGFBP-1a in the TR-FIA. Sera from masu salmon and rainbow trout were serial diluted in parallel with that of the standard (Fig. 1). The half-maximal displacement (ED_{50}) occurred at 61.5 ± 2.3 ng/ml (n = 8). The ED_{80} and ED_{20} were 18.1 ± 2.5 ng/ml (n = 8) and 226.9 ± 23.8 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 9.2 ng/ml (n = 6). The intra- and inter-assay coefficients of variation estimated using the control sample were 5.3% (n = 4) and 8.1% (n = 4), respectively. The recovery of purified rsIGFBP-1a (50 ng/ml) added to rainbow trout serum was 96.3% (n = 9).

Cross-reactivity of the polyclonal antibodies with other IGFBP subtypes purified from Chinook salmon serum was examined in the TR-FIA (Fig. 2). Both IGFBP-2b (41-kDa form) and IGFBP-1b (22-kDa form) showed some displacement at higher concentrations and their cross-reactivity was calculated as 1.5% and 3.6%, respectively. Adding salmon IGF-I at a 1:10 molar ratio to masu salmon and rainbow trout sera did not considerably alter the displacement curves (Fig. 3).

Specific binding of Eu-labeled IGF-I was increased by increasing amounts of the IGFBP-1a standard in LIFA. Serial dilutions of sera from masu salmon and rainbow trout were parallel with that of the standard, which was not affected by feeding status in masu salmon (Fig. 4). The minimum detection limit of the assay, defined as the mean count of the zero standard plus two standard deviations, was 1.2 BU (n = 4). The intra- and inter-assay coefficients of variation estimated using the control sample were 6.4% (n = 4) and 10.1% (n = 4), respectively. The recovery of purified rsIGFBP-1a (10 ng/ml) added to Atlantic salmon plasma was 107.5% (n = 8).

Responses to fasting and refeeding of serum IGF-I, IGFBP-1b, total IGFBP-1a, and intact IGFBP-1a in yearling masu salmon were examined (Fig. 5). Serum IGF-I levels in fasted fish were significantly lower than those in fed fish at week 4 ($P < 0.0001$; Fig. 5a). Refeeding for 2 weeks significantly increased serum IGF-I ($P < 0.0001$), but its levels were intermediate between fed and fasted fish (Fig. 5a). Fasting for 4 weeks had no significant effect on serum IGFBP-1b levels ($P = 0.0620$; Fig. 5b). It became significantly higher in fasted fish than fed and

refed fish at week 6 ($P < 0.0001$; Fig. 5b). Total IGFBP-1a levels tended to be higher in fasted fish but were not different among groups ($P = 0.2615$; Fig. 5c). On the one hand, intact IGFBP-1a levels significantly increased in fish fasted for 4 weeks ($P = 0.0132$) and maintained higher values until 6-week (Fig. 5b). On the other hand, refeeding for 2 weeks restored it to a level similar to that in the fed control ($P = 0.9288$; Fig. 5d).

Data from week 6 were used for correlation analyses. There was a positive correlation between serum IGF-I and SGR in weight while serum IGFBP-1b showed a negative correlation with SGR in weight (Fig. 6a, b). Both total and intact IGFBP-1a levels negatively correlated with SGR in weight, although the correlation coefficient of total IGFBP-1a was much weaker than that of intact IGFBP-1a (Fig. 6c, d). There were no significant relationships between body size (SL and BW) and IGFBP-1b or total IGFBP-1a, whereas IGF-I and intact IGFBP-1a were positively and negatively correlated, respectively (Table 1). IGF-I showed a positive correlation with K, while IGFBP-1b and total and intact IGFBP-1a showed negative correlations (Table 1). Total and intact IGFBP-1a were positively correlated, but only intact IGFBP-1a showed a negative relationship with IGF-I (Table 2).

4. Discussion

We developed two immunoassays to quantify "total" and "intact" IGFBP-1a in salmon as defined earlier. The findings of the present study are relevant to other teleosts because IGFBP-1a is most likely present as a major circulating form in fish (44). In addition, availability of immunoassays for both IGFBP-1a and -1b (19) enables us to further investigate how these duplicated IGFBP-1s are regulated and related to growth in salmon.

The present study is the first to report the development of immunoassay for IGFBP-1a in teleosts. Availability of an immunoassay for quantifying fish IGFBPs is limited to salmon IGFBP-1b and -2b (19, 46, 49). One of the challenges in establishing an immunoassay for fish IGFBPs is to prepare enough purified IGFBP as antigen for immunization because circulating levels of IGFBPs are low, being approximately 300 ng/ml or less (47, 48). Purifying salmon IGFBP-1a from serum was possible, but the final yield was less than 25 μg from 1 l of serum (51), making protein purification an impractical method for antigen preparation. We have recently produced recombinant masu salmon IGFBP-1a using a bacterial expression system (54). The present study used the recombinant protein for antiserum production, assay tracer, and a standard to establish a TR-FIA for salmon IGFBP-1a.

The TR-FIA was validated for its stability using recombinant protein and sera from

masu salmon and rainbow trout, as well as specificity and cross-reactivity using other subtypes of IGFBP purified from Chinook salmon serum. Purified rsIGFBP-1a was biotinylated and used as a tracer. This labeling appeared to have little effect on binding to the antiserum and possible interaction with IGFs in the assay. The cross-reactivity of the TR-FIA with other IGFBPs, particularly IGFBP-1b, was a concern because IGFBP-1a and -1b are paralogs sharing 61% sequence homology (51). Indeed, antiserum against IGFBP-1b showed cross-reactivity with IGFBP-1a in the TR-FIA (3.1%; 19). In the present study, anti-IGFBP-1a also cross-reacted with IGFBP-1b but showed little cross-reactivity with IGFBP-2b. Because the cross-reactivity with IGFBP-1b was low (3.6%), as is the case for the TR-FIA for IGFBP-1b, it should not severely affect the quantification of IGFBP-1a. The TR-FIA using components from masu salmon IGFBP-1a can be used for quantifying IGFBP-1a in other salmonids, such as rainbow trout, because a serial dilution of trout serum was conducted in parallel with the masu salmon standard.

Macqueen and colleagues identified up to 22 genes for IGFBPs in salmonids (14, 38). A larger number of IGFBP repertoires came from an additional round of whole-genome duplication among salmonids. As a result, four IGFBP-1 paralogs are present and recombinant IGFBP-1a used in the present study corresponds to IGFBP-1a1. Because IGFBP-1a1 and -1a2 share 79% sequence homology (38), the TR-FIA using antiserum against IGFBP-1a1 should cross-react with IGFBP-1a2. However, it is not known whether IGFBP-1a2 is present in the circulation and, if so, how much. In any case, we assume it is possible to measure both IGFBP-1a subtypes in our TR-FIA.

Because IGFs are tightly associated with IGFBPs in the circulation, IGFBPs interfere with an accurate measurement of IGFs in immunoassay (12, 17). Therefore, separation of IGF-I from IGFBPs by acid-ethanol extraction is important for measuring salmon IGF-I (44). In contrast, IGFs generally do not interfere the performance of IGFBP assays, although in some RIAs, the interference by IGF-I was reported (4, 46). In the present study, the effect of IGF-I on the serial dilution curves of masu salmon and rainbow trout sera was examined by adding excess IGF-I. As a result, the serum dilution curves were not affected by the presence of IGF-I, indicating that interference by IGF-I in the TR-FIA is minimal.

Despite the validity of the TR-FIA for IGFBP-1a, measurement of total IGFBP-1a level did not provide useful information on the catabolic status or growth retardation of masu salmon. IGFBP-1 is believed to be an inhibitor of IGF-I actions in fish by increasing under catabolic conditions and sequestering IGF-I from the circulation (25). There have been attempts

to utilize circulating protein level or hepatic mRNA level of IGFBP-1 as an index of negative growth and/or stress (30, 31, 33, 41). Kawaguchi et al. (30) semi-quantified serum IGFBP-1a levels by ligand blotting using labeled IGF-I and found a negative correlation with SGR in weight in yearling masu salmon. Our assumption was that quantification of circulating IGFBP-1a by TR-FIA should establish a consistent negative relationship between serum IGFBP-1a and growth rate. However, total IGFBP-1a was less sensitive to fasting, and only a weak negative relationship with growth rate was observed, which conflicts with the findings by ligand blotting (30). Ligand blotting detects IGFBPs based on the ability to bind to labeled IGF, while TR-FIA measures immunoreactive components regardless of their IGF-binding ability. A possibility is that IGFBP-1a in serum of fed fish was partly degraded by enzymes and that of fasted fish remained intact. We thus hypothesized that selective measurement of intact IGFBP-1a that retained IGF-binding ability was more reflective of the degree of growth retardation and developed a LIFA.

LIFA is a combination of immunoassay and ligand-binding assay, as reported by Lassarre and Binoux (34), for measuring intact IGFBP. In a LIFA for human IGFBP-3, immunoreactive IGFBP-3 containing both fragments and intact protein was first captured by a monoclonal antibody, and its ability to bind to ^{125}I-IGF-I was quantified, which made accurate and sensitive measurement of intact IGFBP-3 possible (34). In the present study, we modified the protocol (34) to avoid a risk of denaturation of intact IGFBP-1a during acid-ethanol extraction, and used Eu-labeled IGF-I instead of radio-labeled IGF-I. The IGF-I binding by the standard rsIGFBP-1a increased in a dose-dependent manner and serial dilutions of masu salmon and rainbow trout sera showed parallelism with the standard, indicating that the LIFA quantifies intact IGFBP-1a in salmonids. It is of note that the LIFA does not distinguish intact IGFBP-1a that is occupied or unoccupied with endogenous IGF since the incubation time with excess Eu-IGF-I may be long enough to bring it an equilibration state and displace with endogenous IGFs for intact IGFBP-1a. In human, a complex of IGFBP-1 and IGF-I was formed by incubation for 2.5 h at 37°C (16) and overnight at 4°C (10). Despite its limitation, intact IGFBP-1a measured by the LIFA showed biologically meaningful responses to fasting and refeeding in masu salmon.

As we expected, intact IGFBP-1a was more sensitive to fasting treatment by increasing after 4 weeks of fasting when total IGFBP-1a was unchanged. Intact IGFBP-1a also responded to refeeding, and its level was restored to a level comparable to that in the fed control. Such changes were in good agreement with those reported by Kawaguchi et al. (30). The

371 difference in the responses between total and intact IGFBP-1a suggests that measurement of
372 both fractions is of biological value. Although the significance of different patterns of total and
373 intact IGFBP-1a is unknown at present, our finding invites future study on the regulation of
374 circulating IGFBP-1a in salmon.

375 The result that intact IGFBP-1b levels were higher than those of total IGFBP-1a in
376 fasted fish is puzzling, but a different degree of phosphorylation of IGFBP-1a under feeding and
377 fasting conditions may account for it. The LIFA developed in the present study relies both on
378 the immunoreactivity to antiserum against recombinant masu salmon IGFBP-1a and on the
379 affinity to Eu-labeled IGF-I. If the affinity of IGFBP-1a to IGF-I was increased by fasting, it
380 would result in a higher binding of the label and thus higher measured value. The results of the
381 LIFA were thus expressed as a binding unit (BU) relative to recombinant masu salmon
382 IGFBP-1a in the present study. Indeed, phosphorylation of human IGFBP-1, but not rat
383 IGFBP-1, is known to increase the affinity for IGF-I (24, 40). There are potential
384 phosphorylation sites in the deduced amino acid sequences of Chinook and masu salmon
385 IGFBP-1a (51, 54). Recombinant masu salmon IGFBP-1a used as a standard is unlikely to be
386 phosphorylated because it was produced by bacterial cells (54). Thus, a hypothesis is that
387 fasting altered the phosphorylation status of IGFBP-1a, increased its affinity to IGF-I, and
388 resulted in apparent high levels in LIFA. However, whether salmon IGFBP-1a is actually
389 phosphorylated needs to be confirmed.

390 Correlation analysis on the relationships between circulating total and intact
391 IGFBP-1a with individual growth rate suggests that intact IGFBP-1a but not total IGFBP-1a can
392 be used as a negative index of salmon growth. The utility of circulating IGF-I and IGFBP-1b as
393 positive and negative growth indices, respectively, has been reported in salmonid species (5-7,
394 28-30, 49) and confirmed in the present study. Correlation analysis suggests that intact
395 IGFBP-1a is comparable to IGFBP-1b as a growth index. However, it is not known whether
396 dual measurements of IGFBP-1a and -1b would give a better estimate of growth retardation
397 because a stepwise regression model that combines intact IGFBP-1a or/and IGFBP-1b with
398 IGF-I provided an improved but similar coefficient of regression to explain growth variance
399 (Data not shown). Comparing responses of intact IGFBP-1a and -1b under other developmental
400 and physiological situations such as smoltification, maturation, disease, and stress may reveal
401 differential regulation of these two subtypes and their relations with growth rate.

402 We provide data showing that intact IGFBP-1a levels increased in fasting fish and
403 suggest that a fraction of circulating IGFBP-1a is degraded under normal feeding situation.

Although there is no empirical evidence of fragmentation of IGFBP-1a in fish, specific enzymes play an important role in controlling the binding affinity of IGFBPs for IGFs in mammals (9, 18, 55). For instance, a significant portion of IGFBP-3 in the serum of pregnant women is enzymatically degraded and has a reduced IGF-binding affinity, so that more IGFs are readily available to the receptor (21, 35). Examining the presence of specific enzymes for IGFBP-1a in the circulation is a subject for future study.

5. Perspective and significance

The present study developed two immunoassays: TR-FIA for total immunoreactive IGFBP-1a (fragments+intact) and LIFA for intact IGFBP-1a with IGF-binding ability in salmon. Intact IGFBP-1a was more sensitive to fasting and refeeding treatment than total IGFBP-1a and had a higher negative relationship with individual growth rate, suggesting that degradation of IGFBP-1a is an important mechanism to regulate IGF-I activity in the circulation. Thus, intact rather than total IGFBP-1a is useful as a negative index of salmon growth. In addition, the availability of immunoassays for both IGFBP-1a and IGFBP-1b will help us unravel how circulating IGF-I is regulated by two subtypes of IGFBP-1 in salmon.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author Contributions

N.K., H.T., A.H. and M.S. performed experiments; N.K. and M.S. analyzed data; N.K., T.O.N., A.H. and M.S. interpreted results of experiments; N.K. prepared figures; N.K. drafted the manuscript, N.K., T.O.N. and M.S. edited and revised the manuscript; and N.K., T.O.N., H.T., A.H. and M.S. approved the final version of the manuscript.

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Figure legends

Fig. 1. Displacement of biotinylated salmon IGFBP-1a with purified IGFBP-1a and serum dilutions from masu salmon (a) and rainbow trout (b). Binding (B/B_0) is expressed as a percentage of specific binding.

Fig. 2. Cross-reactivity of the antiserum against salmon IGFBPs. Displacement of the tracer was assessed by adding increasing amounts of purified salmon IGFBP-1a, -1b, and -2b to the assay. Binding (B/B_0) is expressed as a percentage of specific binding.

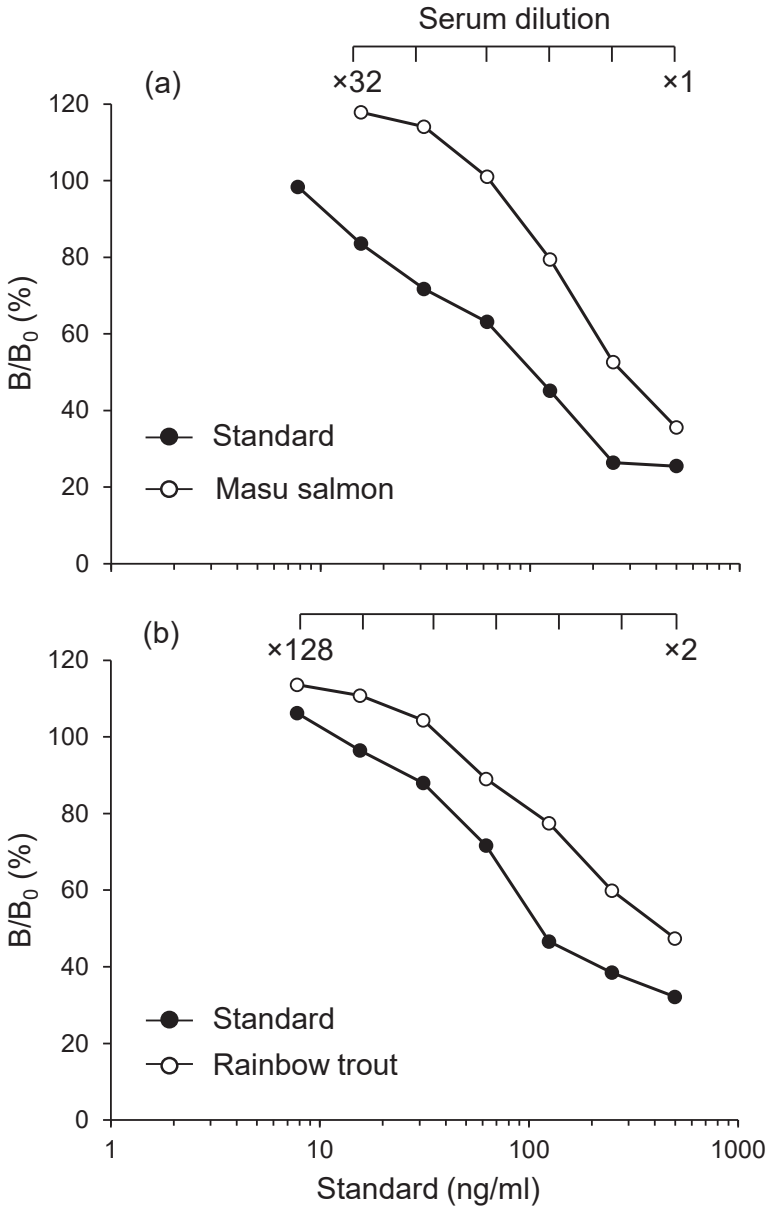
Fig. 3. Effects of exogenous salmon IGF-I on the displacement curves of masu salmon (a) and rainbow trout (b) serum dilutions in TR-FIA. Salmon IGF-I was added to serum at a molar ratio of 1:10. Binding (B/B_0) is expressed as a percentage of specific binding.

Fig. 4. Standard curves in the LIFA for intact IGFBP-1a and dilution curves of serum dilutions from fed, fasted, and refed masu salmon (a) and raibow trout (b). The count is the time-resolved fluorescence of Eu-labeled IGF-I bound to the IGFBP-1a standard or serum. BU: binding unit.

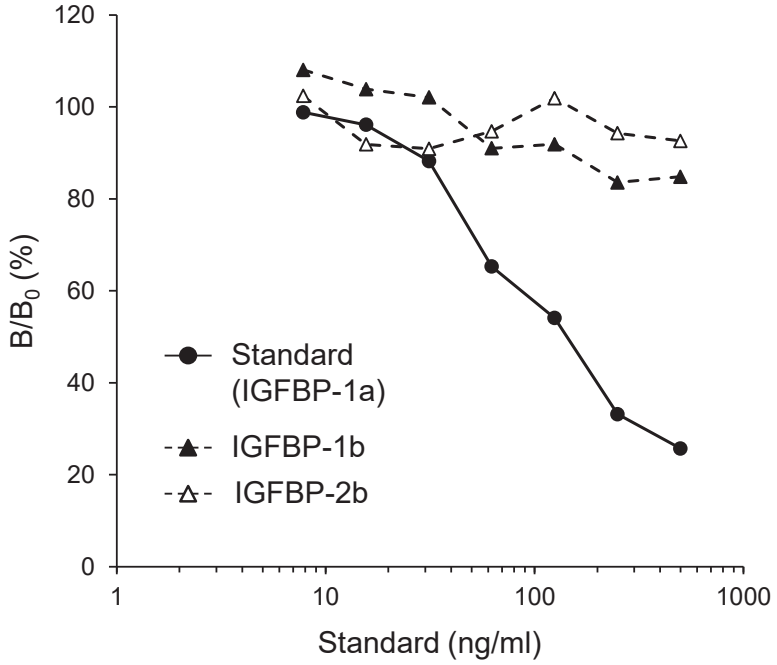
Fig. 5. Effects of fasting and refeeding on serum IGF-I (a), IGFBP-1b (b), total IGFBP-1a (c), and intact IGFBP-1a (d) in yearling masu salmon. Values are expressed as mean ±SE. The number of fish sampled in each group and time point is shown under the corresponding bar. Symbols sharing the same letter are not significantly different each other (Fisher's LSD, $P < 0.05$). BU: binding unit.

Fig. 6. Correlations between specific growth rate (SGR) in body weight and serum IGF-I (a), IGFBP-1b (b), total IGFBP-1a (c), and intact IGFBP-1a (d). Peason's correlation coefficients and sample size were shown in each figure ($P < 0.05$). Values of IGFBP-1a and -1b are natural-log transformed.

Kaneko et al., Fig. 1



Kaneko et al., Fig. 2



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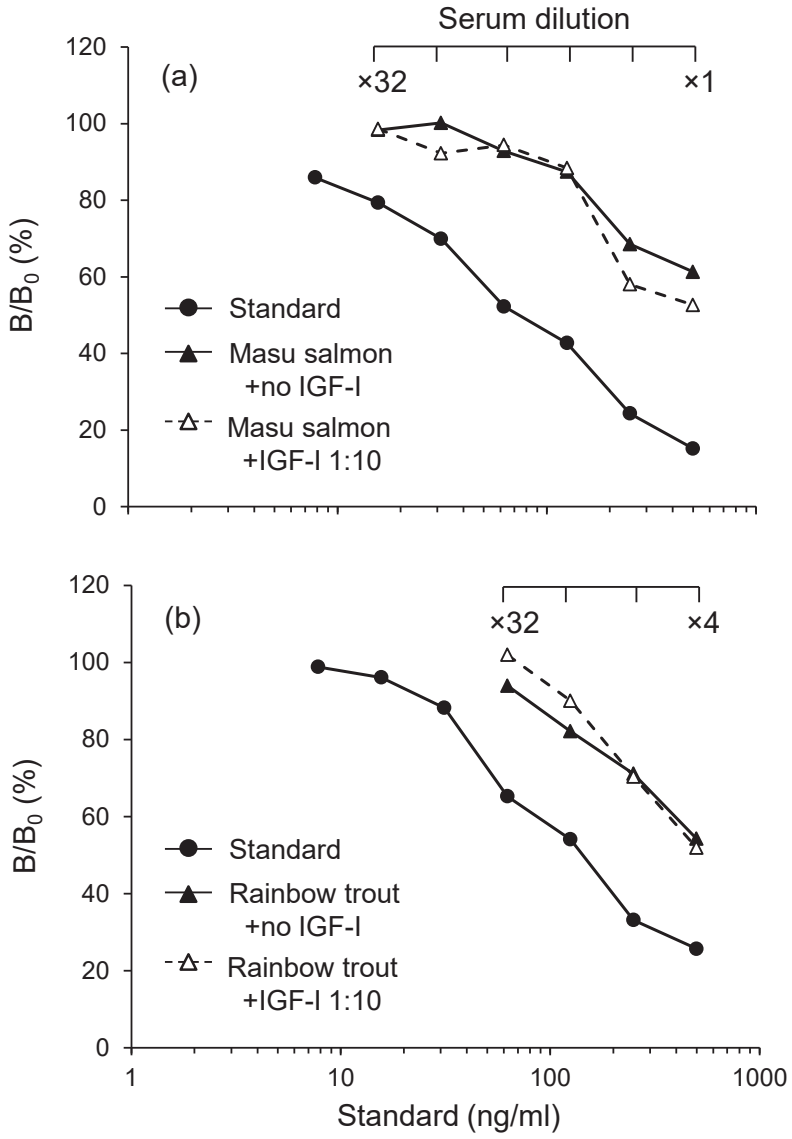

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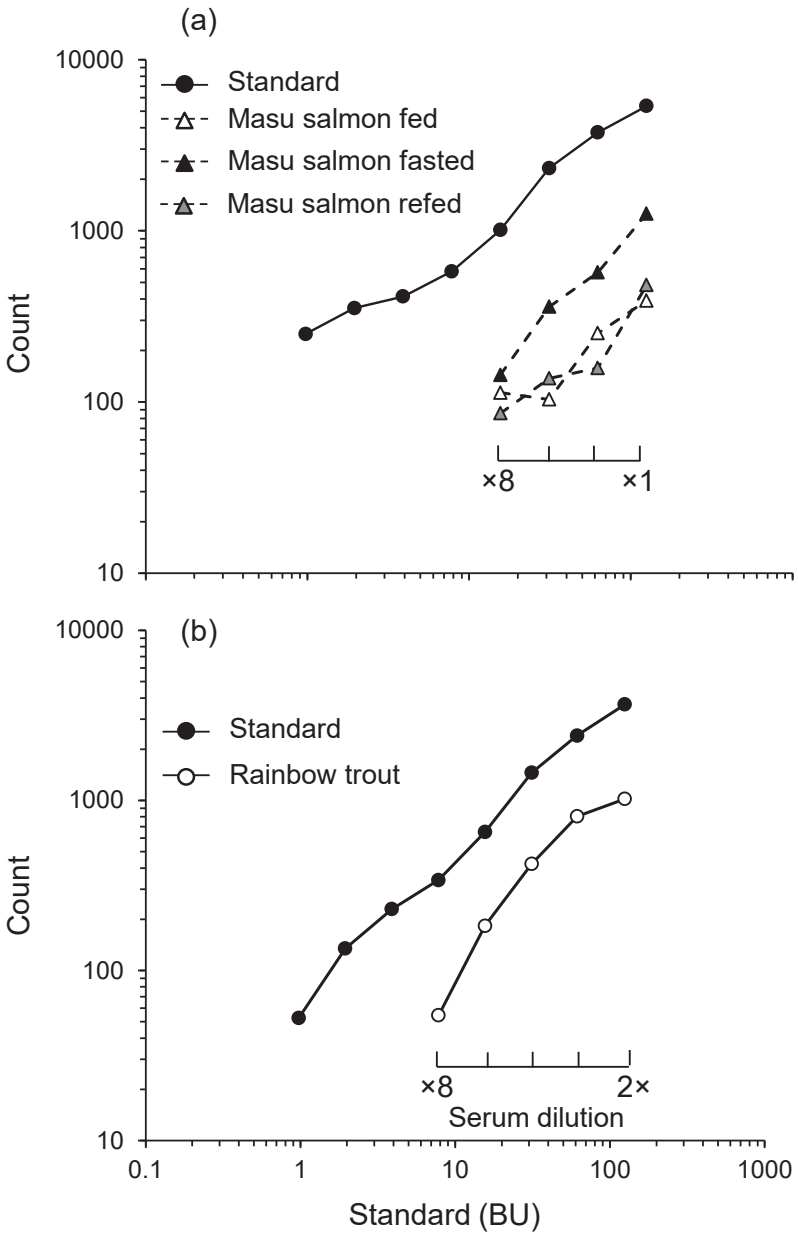

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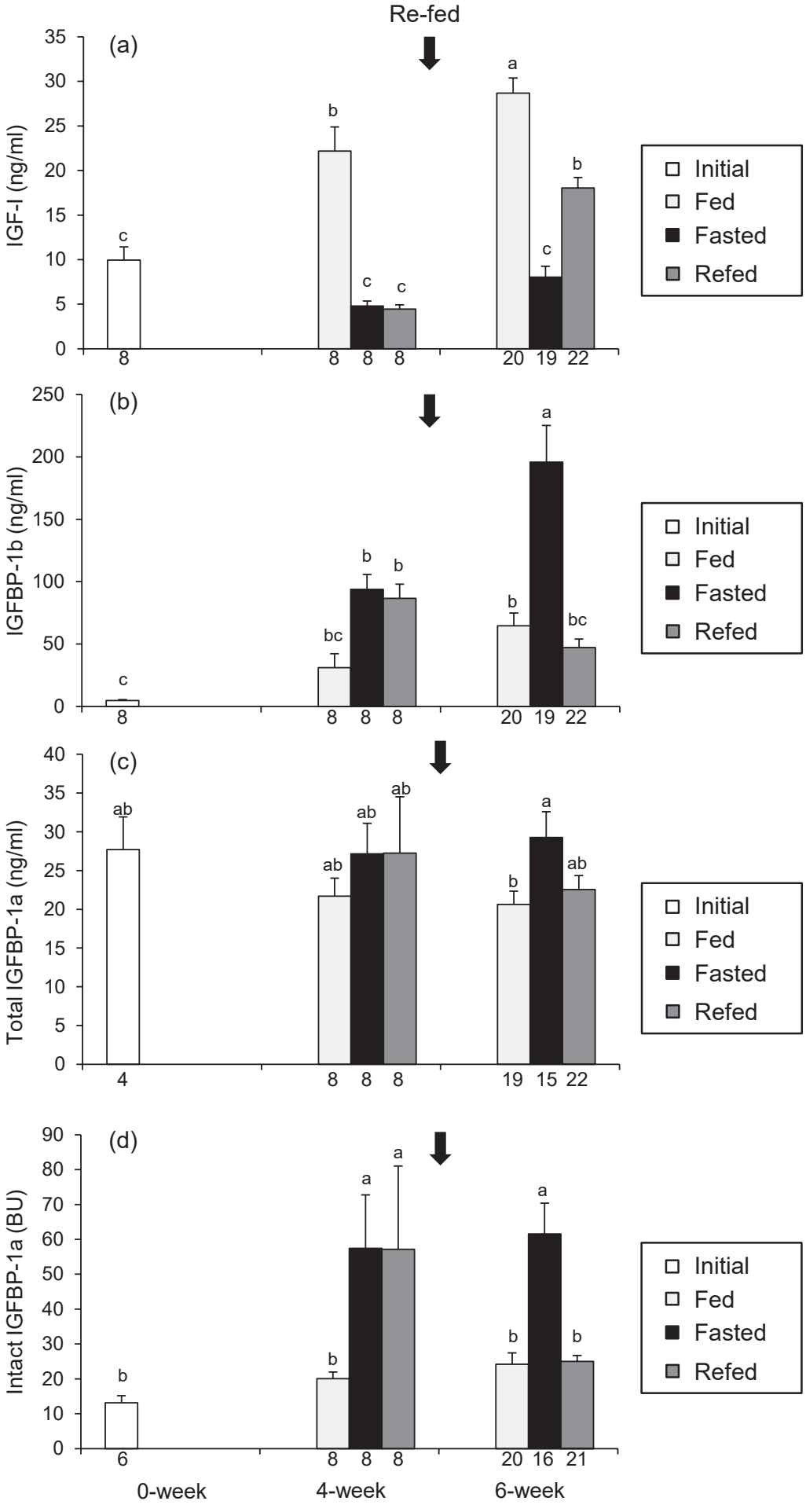

Kaneko et al., Fig. 6

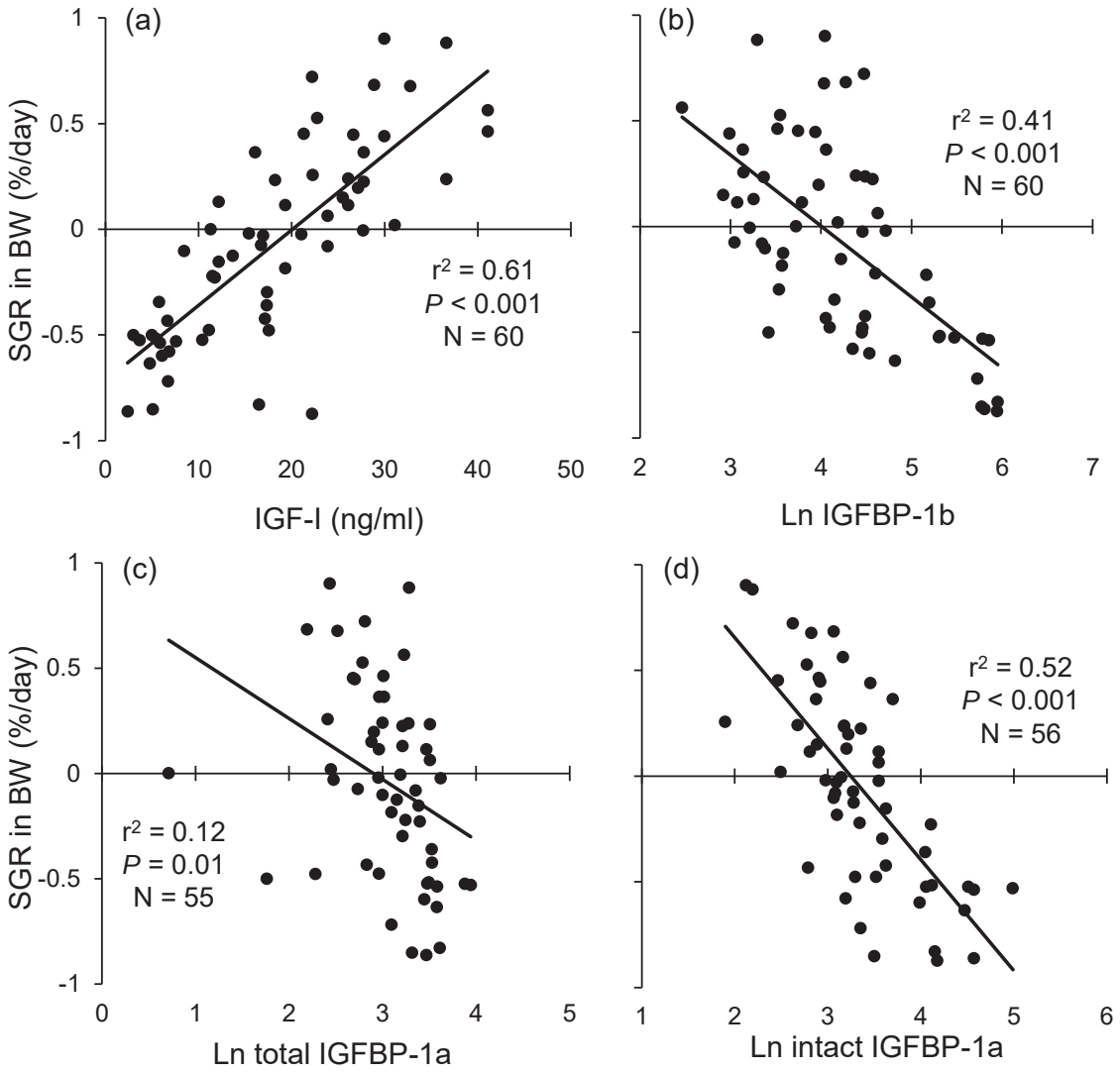

Table 1. Correlation coefficients (r) between endocrine parameters and morphological parameters in week 6.

		SL	BW	K	HSI	SGR-SL
IGF-I	r	0.53	0.63	0.59	–	0.82
	P value	< 0.001	< 0.001	< 0.001	0.083	< 0.001
	N	60	60	60	60	60
BP-1b	r	–	–	-0.63	-0.54	-0.51
	P value	0.798	0.123	< 0.001	< 0.001	< 0.001
	N	60	60	60	60	60
Total BP-1a	r	–	–	-0.41	–	-0.27
	P value	0.705	0.600	0.002	0.250	0.045
	N	55	55	55	55	55
Intact BP-1a	r	-0.48	-0.63	-0.73	-0.36	-0.68
	P value	< 0.001	< 0.001	< 0.001	0.007	< 0.001
	N	56	56	56	56	56

(–) : not significant. IGFBP-1a and -1b values were natural log-transformed.

Table 2. Correlation coefficients (r) among endocrine parameters in week 6.

		IGF-I	BP-1b	Total BP-1a	Intact BP-1a
IGF-I	r		-0.42	–	-0.57
	P value		0.001	0.251	< 0.001
	N		61	56	57
BP-1b	r	-0.42		0.38	0.60
	P value	0.001		0.004	< 0.001
	N	61		56	57
Total BP-1a	r	–	0.38		0.75
	P value	0.251	0.004		< 0.001
	N	56	56		54
Intact BP-1a	r	-0.57	0.60	0.75	
	P value	< 0.001	< 0.001	< 0.001	
	N	57	57	57	

(–) : not significant. IGFBP-1a and -1b values were natural log-transformed.