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Title:

Development of a time-resolved fluoroimmunoassay for salmonid insulin-like growth factor binding protein-2b

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Abstract

Insulin-like growth factor (IGF)-1 promotes the growth of vertebrates, and its binding proteins (IGFBPs) regulate the activity of circulating IGF-1. Three IGFBPs, IGFBP-2b, -1a, and -1b, were consistently detected in the circulatory system of salmonids. IGFBP-2b is thought to be the main carrier of IGFs and promoter of IGF-1-mediated growth in salmonids. Currently, there are no immunoassays for detecting IGFBP-2b. In this study, we developed a time-resolved fluoroimmunoassay (TR-FIA) for IGFBP-2b detection in salmonid fishes. To establish TR-FIA, we produced two recombinant trout (rt) IGFBP-2bs expressed, one with thioredoxin (Trx) and a histidine (His) tag, and the other with His-tag only. We labeled both recombinant proteins with Europium (Eu). Only Eu-Trx.His.rsIGFBP-2b cross-reacted with anti-IGFBP-2b, and the addition of increasing amounts of Trx.His.rsIGFBP-2b replaced the binding, indicating its utility as a tracer and assay standard. The addition of unlabeled salmon IGF-1 did not affect the binding of the standard or sample. Serial dilution curves of sera from rainbow trout, Chinook salmon, and chum salmon were parallel to those of the standard. The assay range (ED_{80}–ED_{20}) of the TR-FIA was 60.4 to 251.3 ng/ml, and its minimum detection limit of this assay was 21 ng/ml. The intra- and inter-assay coefficients of variation were 5.68% and 5.65%, respectively. Circulating IGFBP-2b levels in fed rainbow trout were higher than those in fasted fish and were correlated with individual growth rates. This TR-FIA is useful for further exploring the physiological responses of circulating IGFBP-2b and evaluating the growth status of salmonids.

Keywords

growth index, hormone, immunoassay, salmonids

1. Introduction

Insulin-like growth factor (IGF)-1 is a 7.5 kDa polypeptide structurally similar to proinsulin and plays an important role in the promotion of growth in vertebrates. IGF-1 exerts its effect through endocrine, paracrine, and autocrine activities (Daughaday and Rotwein, 1989; Wood et al., 2005). Endocrine IGF-1 is mainly secreted from the liver upon stimulation by growth hormone (GH) and mediates the growth-promoting actions of GH on skeletal muscle and other tissues (Daughaday and Rotwein, 1989; Ohlsson et al., 2009). Unlike insulin, IGF-1 in the circulation is stabilized by IGF-binding proteins (IGFBPs). IGFBPs prolong the half-life of IGF-1 by protecting it from glomerular filtration and enzymatic degradation and modulate the action of IGF-1 by regulating its availability to the receptor (Rajaram et al., 1997; Bach, 2018; Clemmons, 2018).

Six IGFBPs, IGFBP-1–6, have been identified in mammals (Shimasaki and Ling, 1991; Rajaram et al. 1997; Bach, 2018). IGFBPs can either inhibit or promote IGF action, depending on the type of IGFBP, post-translational modification, and cellular environment (Firth and Baxter, 2002). Besides regulating IGF-1 availability, the IGF-independent action of IGFBPs on cell growth has been reported in various mammalian cell types (Mohan and Baylink, 2002). Among them, IGFBP-3 is a major carrier of IGFs in the mammalian circulatory system (Rajaram et al., 1997). IGFBP-3 carries approximately 80% of the circulating IGF-1 by forming a ternary complex with IGF-1 and acid-labile subunit (ALS) (Baxter and Martin, 1989). The ternary complex creates a large pool of circulating IGF-1, and when needed, specific enzymes, such as metalloproteinases, partially degrade IGFBP-3 and make IGF-1 available to the receptor (Ranke, 2015). IGFBP-3 is a promoter of IGF-1-mediated growth.

All six types of IGFBPs have been identified in teleosts (Ocampo Daza et al., 2011; Macqueen et al., 2013; de la Serrana and Macqueen, 2018). Due to an extra 3rd round of whole-genome duplication (WGD) in teleosts, there are two paralogs of each member of the IGFBP family, except IGFBP-4 (Ocampo Daza et al., 2011). In addition, salmonids experience autotetraploidization and possess up to four subtypes of IGFBPs (Macqueen et al., 2013). The retention of these gene repertoires may have a selective advantage by partitioning their original functions and fine-tuning their growth pace (de la Serrana and Macqueen, 2018). Three IGFBP bands have been consistently detected in the circulatory system of teleosts by ligand blotting using labeled human IGF-1 (Kelley et al., 1992, 2001; Upton et al., 1993; Shimizu and Dickhoff, 2017). One of the bands at 40–50 kDa was thought to be fish IGFBP-3 because its molecular weight, post-translational modification (i.e., *N*-glycosylation), and physiological regulation were similar to those of mammalian IGFBP-3. However, in Chinook salmon (*Oncorhynchus tshawytscha*), it

has been identified as a subtype of IGFBP-2 (i.e., IGFBP-2b) while two others being IGFBP-1a and -1b (Shimizu et al., 2003, 2011a,b). In contrast, there is no evidence of the ternary complex despite the presence of genes and transcripts for *igfbp-3* and *als* in teleosts (Shimizu and Dickhoff, 2017). These findings suggest that IGFBP-2b, and not IGFBP-3, is a major carrier of circulating IGF-1, at least in salmonids (Shimizu et al., 2003, 2011b; Shimizu and Dickhoff, 2017).

A radioimmunoassay (RIA) for salmon IGFBP-2b was developed using purified proteins as antigens, radiolabeled tracers, and assay standards (Shimizu et al., 2003). Quantification of circulating IGFBP-2b in salmonids has revealed that its level is high in fish fed a high ration or treated with GH, as seen in mammalian IGFBP-3 (Shimizu et al., 2003). Moreover, there is a positive correlation between circulating IGFBP-2b levels and individual growth rates in post-smolt coho salmon (*O. kisutch*) in freshwater (Beckman et al., 2004a,b). These studies suggest that IGFBP-2b can be used as a positive index of growth in salmonids. However, RIA is currently not being operated because of the shortage of purified protein for use as a tracer and assay standard (Shimizu, personal communication). Moreover, RIAs, in general, have been replaced by non-radioactive assays.

Rainbow trout (*O. mykiss*) is an important species for aquaculture and the second most cultured salmonid in the world (FAO, 2020). This species is cultured under a variety of rearing conditions, including salinity, water temperature, photoperiod, and fish density. In addition, alternative diets such as plant protein-based diets are being developed for sustainable salmon aquaculture (Jalili et al., 2013; Hua et al., 2019). These rearing conditions and new diets may cause rainbow trout to grow under suboptimal conditions. Therefore, monitoring the growth status of rainbow trout is particularly important in aquaculture.

Our previous study using ligand blotting revealed that circulating IGFBP-2b in immature rainbow trout was positively correlated with individual growth rate, as was the case for circulating IGF-1, supporting its utility as a growth index in rainbow trout (Izutsu et al., 2022). The objective of this study was to develop a time-resolved fluoroimmunoassay (TR-FIA) for trout/salmon IGFBP-2b using recombinant rainbow trout IGFBP-2b.

2. Materials and Methods

2.1. Production of recombinant rainbow trout IGFBP-2b1

A cDNA encoding rainbow trout IGFBP-2b1 (GenBank Accession No. NM_ 001124649) was used to express recombinant protein. The procedures followed for vector construction and protein expression were as described by Tanaka et al. (2018). Briefly, the pET-32a(+) vector (Novagen,

Madison, WI, USA), which adds thioredoxin (Trx) and histidine (His)-tag (6 × His) at the N-terminal region of the expressed recombinant protein, and the pET-16b vector (Novagen), which adds only a His-tag, were used. The constructed plasmids (pET32a-Trx. His.rtIGFBP-2b1 and pET16b-His. rtIGFBP-2b1) were transformed into the *Escherichia coli* strain Rosetta-gami B(DE3)pLysS (Novagen). Recombinant protein expression was induced by adding isopropyl-β-D-thiogalactoside (IPTG). Bacterial proteins were separated into soluble and insoluble fractions using Bugbuster Protein Extraction Reagent (Novagen). The insoluble fractions containing recombinant proteins were solubilized in urea and subjected to affinity chromatography using a His-Bind Resin (Novagen) column. The eluate from the column was dialyzed against 20 mM Tris-HCl, pH 8.0, containing 2% NaCl and urea was stepwise removed to facilitate correct refolding.

2.2. Electrophoresis, ligand blotting and immunoblotting

Four micrograms of affinity-purified Trx.His.rtIGFBP-2b was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 3% stacking gel and 12.5% separating gel. The sample was treated with an equal volume of the sample buffer containing 2% SDS and 10% glycerol with or without 5% 2-mercaptoethanol at 85 °C for 5 min. Gels were placed in a solution of 50 mM Tris, 400 mM glycine, and 0.1% SDS at 8 mA for the stacking gel and 12 mA for the separating gel until the bromophenol blue dye front reached the bottom of the gel, and were stained with 0.1% Coomassie Brilliant Blue (CBB) R250 (Bio-Rad, Hercules, CA). Molecular mass was estimated with Precision Marker (Bio-Rad).

IGF-binding ability of Trx.His.rtIGFBP-2b was assessed by ligand blotting with digoxigenin-labeled human IGF-1 (DIG-hIGF-1) according to a previously described protocol (Shimizu et al., 2000). Four hundred nanograms of the recombinant protein was separated by SDS-PAGE and electroblotted onto a nitrocellulose membrane. The membrane was incubated overnight with DIG-hIGF-1 (50 ng/ml) and then incubated with antibodies against DIG conjugated horseradish peroxidase (HRP; Roche, Indianapolis, IN, USA) at a dilution of 2,000 for 1.5 h at room temperature (20–25 °C). IGFBP bands were visualized using enhanced Chemiluminescence (ECL) Prime western blotting reagents (Amersham Life Science, Arlington Heights, IL, USA) and ImageQuant LAS 500 (GE Healthcare, Uppsala, Sweden).

For immunoblotting, an electroblotted nitrocellulose memberane was incubated with antiserum against purified Chinook salmon IGFBP-2b (Shimizu et al., 2003) at a dilution of 1:2,500 for 2 h at room temperature (20–25 °C). The membrane was then probed with goat anti-

rabbit IgG-HRP conjugate (Bio-Rad) at a dilution of 1:10,000 for 1 h at room temperature (20–25 °C). Immunoreactive bands were visualized using the ECL reagents as described above.

2.3. Preparation of tracer

His.rtIGFBP-2b and Trx.His.rtIGFBP-2b were labeled with Europium (Eu)-Labeling Reagent (6 mM) using a DELFIA Eu-N1 ITC chelate labeling kit (PerkinElmer, Waltham, MA, USA) according to the manufacturer's protocol, with optimization. Briefly, approximately 70 μg recombinant protein in 20 mM Tris-HCl (pH 8.0) containing 0.5M NaCl was dialyzed against 50 mM sodium bicarbonate/sodium carbonate (pH 9.3) at 4 ℃. Trx.His.rtIGFBP-2b1 (70 μg in 80 μl) was mixed with 14 μl of 4 mg/ml Eu-N1-ITC chelate (1:60 molar ratio) and reacted overnight at 4 ℃ under dark conditions. Eu-labeled His.rtIGFBP-2b1 and Trx.His.rtIGFBP-2b1 were separated from the unreacted Eu-chelate by gel filtration using a Sephadex G-25 (Superfine; Pharmacia Fine Chemicals, Uppsala, Sweden) column (10 × 80 mm). Samples were eluted by applying 50 mM Tris-HCl (pH 7.8) containing 0.9% NaCl to the top of the gel, and 0.5 ml fractions were collected manually. Time-resolved fluorescence and protein concentrations in each fraction were measured using a Wallac ARVO X4 (PerkinElmer) and Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), respectively.

2.4. TR-FIA for IGFBP-2b

A competitive method was employed in this study. A 96-well assay plate coated with goat anti-rabbit IgG (DELFIA Yellow Plate; Perkin Elmer) was first washed with 200 μl DELFIA Wash Buffer (Perkin Elmer), and 20 μl anti-Chinook salmon IGFBP-2b (Shimizu et al., 2003; 1:125) and 120 μl standard (Trx.His.rtIGFBP-2b1) or serum was added to each well. The plate was sealed and incubated overnight at 4 °C with shaking at 600 rpm on a shaker. On the next day, the plate was flash centrifuged and 20 μl Eu-Trx.His.rtIGFBP-2b1 (1:5,500) was added to each well and the plate was incubated overnight at 4 °C with shaking at 600 rpm. On the last day, the plate was washed six times with 200 μl wash buffer, and 200 μl DELFIA Enhancement Solution (PerkinElmer), which had been acclimated to room temperature before use, was added to each well. The plate was shaken without sealing for 10 minutes at room temperature. Time-resolved fluorescence was measured using a Wallac ARVO X4 multi-label counter (PerkinElmer).

2.5. Biological validation

IGFBP-2b levels in sera from fed, fasted, and refed immature rainbow trout were measured using

TR-FIA and LIFA for biological validation. Details of the rearing experiment have been described by Izutsu et al. (2022). Briefly, three groups of underyearling rainbow trout (body weight: 265.4 ± 7.8 g) individually tagged using passive integrated transponder (PIT) tags (12.5 × 2.1 mm, Biomark, Boise, ID, USA) were fed or fasted for 33 days or refed for 11 days after 22 days of fasting. Sera collected on day 33 were used for the analyses. As stated in Izutsu et al. (2022), Rearing and handling fish were carried out in accordance with the guidelines of the Hokkaido University Animal Care and Use Committee (#30-3).

2.6. Statistical analysis

The results were analyzed by one-way ANOVA using JMP Pro 16 (SAS Institute Inc., Cary, NC, USA). When significant effects were found, differences were further identified using Tukey's honestly significant difference (HSD) test, with differences considered significant at $P < 0.05$. Linear correlation analysis was also performed to determine the relationship between serum IGFBP-2b levels and the growth rate or serum IGF-1 concentrations of individual trout.

3. Results

3.1. Production of recombinant IGFBP-2b used for Eu-labeling

Affinity-purified Trx.His.rsIGFBP-2b exhibited two bands around 46 kDa on SDS-PAGE (Fig. 1). One of the two bands had IGF-binding ability on ligand botting while the other did not. On the other hand, both bands were immunostained with antiserum against IGFBP-2b on immunoblotting (Fig. 1). Affinity-purifed His.rsIGFBP showed patterns similar to those of Trx.His.rsIGFBP-2b (data not shown).

3.2. Development of TR-FIA

When the Eu-labeled proteins were separated from the unreacted Eu, Eu-Trx.His.rtIGFBP-2b showed a clear peak in the pass-through fraction of gel filtration, whereas Eu-His. rtIGFBP-2b showed no peak (Data not shown). Eu-Trx.His.rtIGFBP bound to anti-IGFBP-2b and showed time-resolved fluorescence (data not shown). Thus, Eu-Trx.His.rtIGFBP-2b was used as a tracer for further development of TR-FIA.

The utility of unlabeled Trx.His.rtIGFBP-2b and His.rtIGFBP-2b as assay standards were examined in TR-FIA using Eu-Trx.His.rtIGFBP-2b (Fig. 2). Tracer binding was displaced by competition with Trx.His.rtIGFBP-2b in a dose-dependent manner, which was parallel to the serial dilution of rainbow trout serum. In contrast, His.rtIGFBP-2b was ineffective in displacing

tracer binding. Based on these results, Trx.His.rtIGFBP-2b was selected as the assay standard.

The addition of varying amounts of salmon IGF-1 (equivalent to 0.3 to 80 ng/ml) did not affect the binding of the standard (equivalent to 209 ng/ml) and serum (equivalent to 386 ng/ml) (Fig. 3). The assay range (ED_{80}–ED_{20}) of the TR-FIA was 60.4 ± 3.3 to 251.3 ± 18.6 ng/ml, and its minimum detection limit, defined as the mean of the zero standard minus two standard deviations, was 21 ng/ml (n = 4). No displacement was observed in recombinant Atlantic salmon IGFBP-1b with or without fusion partners, thioredoxin, or a histidine-tag (Maruoka et al., unpublished data) (Fig. 4a). The intra- and interassay coefficients of variation were 5.68% (n = 8) and 5.65% (n = 4), respectively. Recovery of Trx.His.rtIGFBP-2b added to rainbow trout serum was 98.7% (n = 6). Serial dilutions of the sera from spawning Chinook salmon and adult chum salmon were parallel to the standard curve (Fig. 4b).

3.3. Biological validation

Serum IGFBP-2b levels in fed, fasted, and refed rainbow trout were measured using TR-FIA and compared with those semi-quantified by ligand blotting using labeled hIGF-1 as reported by Izutsu et al. (2022). Serum IGFBP-2b levels measured using TR-FIA decreased after 33 days of fasting and remained low after 22 days of fasting, followed by 10 days of refeeding (Fig. 5a). These responses were generally consistent with those semi-quantified by ligand blotting, whereas the latter appeared to be more sensitive in terms of the magnitude of the response (Fig. 5b). In addition, there was a positive correlation between IGFBP-2b levels measured using TR-FIA and ligand blotting (Fig. 5c).

Serum IGFBP-2b levels on day 33, as measured using TR-FIA, were positively correlated with SGRs in length during both days 0–33 and 22–33 (Fig. 6a,b). There was also a correlation between the serum IGFBP-2b and IGF-1 levels on day 33 (Fig. 6c). Serum IGFBP-2b levels on day 33 correlated with SGR in weight, whereas the coefficient of correlation appeared to be higher than that measured using ligand blotting (Table 1).

4. Discussion

To establish a competitive immunoassay, tracer preparation is critical. Although intact protein purified from the blood is ideal, the amount of purified salmon IGFBP-2b is limited, and purifying it from serum is not practical due to low recovery (Shimizu et al., 2003). The present study utilized recombinant trout (rt) IGFBP-2b to prepare tracers. Two types of rtIGFBP-2b with different fusion partners were prepared for this purpose (Trx.His.rtIGFBP-2b and His.rtIGFBP-2b) and

243 labeled with Eu. However, the recovery of the Eu-His.rtIGFBP-2b from gel filtration was very
244 low, possibly because of adsorption onto the column matrix (data not shown). Thus, only Eu-
245 Trx.His.rtIGFBP-2b was used as the tracer. Although intact salmon IGFBP-2b is *N*-glycosylated
246 while Trx.His.rtIGFBP-2b produced by bacterial cells is not, Eu-labeled Trx.His.rtIGFBP-2b was
247 bound to the antiserum raised against purified Chinook salmon IGFBP-2b (Shimizu et al., 2003),
248 confirming that the lack of glycosylation or addition of a fusion partner did not diminish
249 antigenicity.

250 Trx.His.rtIGFBP-2b was also useful as a standard because its serial dilutions showed
251 a good displacement curve for the tracer in TR-FIA. In contrast, His.rtIGFBP-2b showed little
252 displacement. Such a difference in TR-FIA performance between Trx.His.rtIGFBP-2b and
253 His.rtIGFBP-2b might be due to the degree of protein folding. Generally, linkage to thioredoxin
254 increases the solubility of proteins synthesized in the *E. coli* cytoplasm (LaVallie et al., 1993). In
255 addition, it helps proteins fold correctly (Berndt et al., 2008). Although His.rtIGFBP-2b possessed
256 an IGF-binding ability in ligand blotting (data not shown), its protein folding might have affected
257 its cross-reactivity with antiserum against purified Chinook salmon IGFBP-2b. Based on these
258 results, Trx.His.rtIGFBP-2b was selected as both the tracer and standard.

259 IGF-1 interference in the RIA of salmon IGFBP-2b has been reported previously
260 (Shimizu et al., 2003). This is likely due to a conformational change upon binding to IGF-1 (Arai
261 et al., 1996), which may alter the antigenicity of IGFBPs. This study assessed the possible
262 influence of IGF-1 by adding various amounts of IGF-1 to the serum or standard. Neither the
263 slope of the standard curve nor the measured circulating IGFBP-2b level was affected by the
264 addition of IGF-1. Thus, the interference by IGF-1 in TR-FIA appears to be minimal.

265 TR-FIA is specific enough to measure IGFBP-2b in salmonid blood, in which subtypes
266 of IGFBP-1 are also present. When recombinant masu salmon IGFBP-1b was examined for cross-
267 reactivity with TR-FIA, no displacement was observed. In RIA, the antiserum used in the present
268 study showed only 3% of cross-reactivity with purified Chinook salmon IGFBP-1a and -1b
269 (Shimizu et al., 2003). These findings confirmed the specificity of the antiserum. Given that low
270 cross-reactivity with Chinook salmon IGFBP-1b, we expect that this new TR-FIA will be useful
271 for measurement of IGFBP-2b across salmonids species. The recombinant IGFBP-2b used in the
272 assay was produced based on the sequence from the rainbow trout. Nevertheless, serial dilutions
273 of serum from Chinook salmon and chum salmon showed parallelism with the standard,
274 indicating that TR-FIA is applicable to salmonids other than rainbow trout, as is the case with
275 RIA. However, the potential cross-reactivity with IGFBP-2a should be taken into account.

Although IGFBP-2a has not been detected in the circulation of salmonid fishes, it is expressed in the liver and has a 56% deduced amino acid sequence homology with IGFBP-2b (Shimizu et al., 2011b). In rainbow trout and masu salmon sera, the presence of a forth IGFBP band at 32 kDa has been reported (Cleveland et al., 2018; Hayashi et al., 2021; Izustu et al., 2022). If this form is identified as IGFBP-2a, its cross-reactivity in the TR-FIA needs to be tested in future study.

Measurement of circulating IGFBP-2b levels using TR-FIA revealed that fasting decreased its levels in immature rainbow trout, which is in good agreement with previous studies (Shimizu et al., 2003, 2009; Cleveland et al., 2020; Izutsu et al., 2022). However, the decreased levels of circulating IGFBP-2b after 22 days of fasting were not restored, even after 11 days of refeeding. As discussed by Izutsu et al. (2022), domesticated rainbow trout selected under intensive feeding might become less adaptive to recovery from fasting conditions.

A positive relationship between circulating IGFBP-2b levels measured using TR-FIA and circulating IGF-1 levels was also confirmed. IGFBP-2b in salmonids is thought to be a major carrier of circulating IGF-1 (Shimizu et al., 2003, 2011b, 2017) and there is generally a positive correlation between circulating IGF-1 and IGBP-2b (Beckman et al., 2004a,b). The results of the present study suggest that IGFBP-2b measured using TR-FIA could be a tractable approach for exploring relationships between individual variation in circulating IGFBP-2b levels and growth rate in rainbow trout and other salmonid fishes, with the potential to provide a new physiological growth rate index. Individual variation in IGF-1 is already being used as a growth index in some fishes (Picha et al., 2008; Beckman, 2011) and, based on the patterns observed here (see Fig. 6), measurments of IGFBP-2b may be useful as an additional growth rate marker in salmonid fishes.

Notably, the response to fasting and the relationship with the growth rate of IGFBP-2b levels measured using TR-FIA were less sensitive and weaker, respectively, than those measured using ligand blotting. For instance, when rainbow trout were subjected to 22 days of fasting, serum IGFBP-2b levels were 54% lower than those of fed controls in ligand blotting, while only a 17% reduction was observed in TR-FIA. These differences can be attributed to the different principles of these two methods. Ligand blotting detects intact IGFBP with IGF-binding ability after treatment with sodium dodecyl sulfate before electrophoresis, whereas TR-FIA measures both intact and fragmented IGFBP without IGF-binding ability as long as the fragmented IGFBP retains antigenicity (Shimizu et al., 2000; Kaneko et al., 2020). A possible explanation for this discrepancy is that certain parts of the circulating IGFBP-2b are present as fragments and do not respond to fasting. In pregnant women, a considerable portion of circulating IGFBP-3, the major carrier of IGFs, is partly fragmented, which increases the availability of IGFs to the target tissues

(Lassarre and Binoux, 2001; Gibson et al., 2001). However, the significance of IGFBP-2b fragmentation in salmon is not known at present and is a subject for future study.

In summary, the present study established a TR-FIA for IGFBP-2b using a recombinant protein as the assay component. Although intact IGFBP-2b semi-quantified by ligand blotting may be more sensitive to changes in nutritional and growth status, IGFBP-2b quantified using TR-FIA is also sensitive enough as a growth index and allows the processing of a larger number of samples, making it useful to evaluate the role of IGFBP-2b in modulating growth.

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Figure legends

Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), ligand blotting (LB) and immunoblotting (IB) of recombinant trout IGFBP-2b with fusion partners thioredoxin and histidine-tag (Trx.His.rtIGFBP-2b). Four microgams of affinity-purified recombinant protein was separated by 12.5% SDS-PAGE under non-reducing conditions and stained with Coomassie Brilliant Blue (CBB; left panel). Four hundred nanograms of the recombinant protein was electrophored, blotted onto nitrocellulose membranes and subjected to LB using digoxigenin-labeled human IGF-1 (50 ng/ml; middle panel) or IB using antiserum against purified Chinook salmon IGFBP-2b (1:2,500; right panel). Arrows indicate migration positions of the molecular markers (left) and Trx.His.rtIGFBP-2b (right).

Fig. 2. Displacement of europium-labeled recombinant trout IGFBP-2b with fusion partners thioredoxin and histidine-tag (Trx.His.rtIGFBP-2b) with unlabeled Trx.His.rtIGFBP-2b, His.rtIGFBP-2b, and rainbow trout serum. Binding (B/B_0) is expressed as a percentage of specific binding. All values are means of duplicate determinations.

Fig. 3. Effects of adding increasing amounts of salmon IGF-1 (0.3 to 80 ng/ml) on the specific binding (B/B_0) of the standard (equivalent to 209 ng/ml) and rainbow trout serum diluted at 1:4. All values are single determinations.

Fig. 4. Cross-reactivity of recombinant salmon IGFBP-1b (a) and salmon sera (b) in time-resolved fluoroimmunoassay (TR-FIA). Displacement of the tracer was assessed by adding increasing amounts of recombinant Atlantic salmon IGFBP-1b with/without fusion partners Trx and His (31.3 to 500 ng/ml) or serial dilutions of Chinook and chum salmon sera to the assay. All values are means of duplicate determinations.

Fig. 5. Effects of fasting and refeeding on serum IGFBP-2b levels, measured using time-resolved fluoroimmunoassay (TR-FIA; a) and ligand blotting (b), and their correlation (c). Intensities of the IGFBP-2b bands on ligand blotting were semi-quantified and expressed as arbitrary density units (ADU). Data on ligand blotting were provided by Izutsu et al. (2020). Values are expressed as means ± SE (day 33: $n = 12–14$). Symbols sharing the same letters are not significantly different from each other (Tukey's HSD test, $P < 0.05$). Data from fed (black circules), fasted (white

467 circules) and refed (grey circles) fish were pooled for correlation analysis.

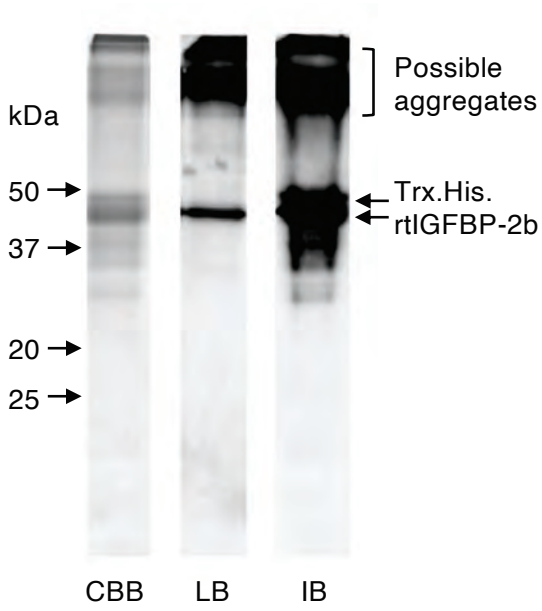
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469 Fig. 6. Correlations of serum IGFBP-2b level with specific growth rate in length (SGRL) during
470 0–33 days (a) and 22–33 days (b), and with IGF-1 levels (c) in rainbow trout. Data on SGRL were
471 from Izutsu et al. (2022) with permission. Data from fed (black circules), fasted (white circules)
472 and refed (grey circles) fish were pooled for correlation analyses.

Figure 1

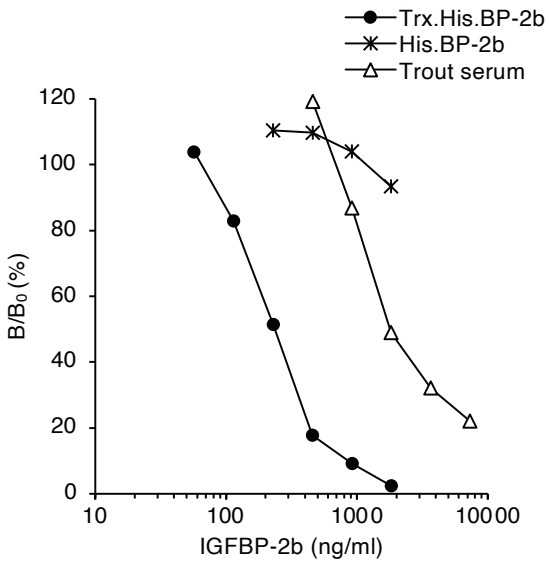
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Izutsu et al., Fig. 1



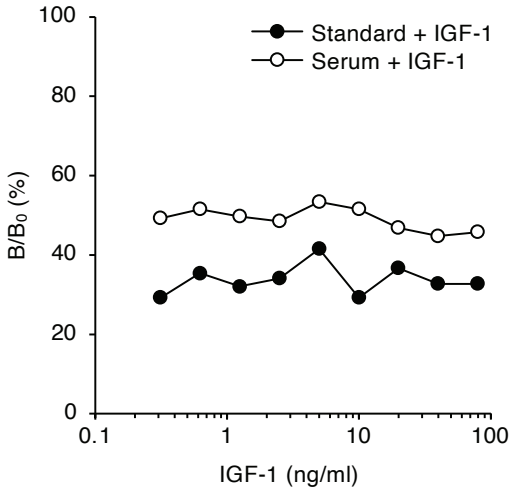
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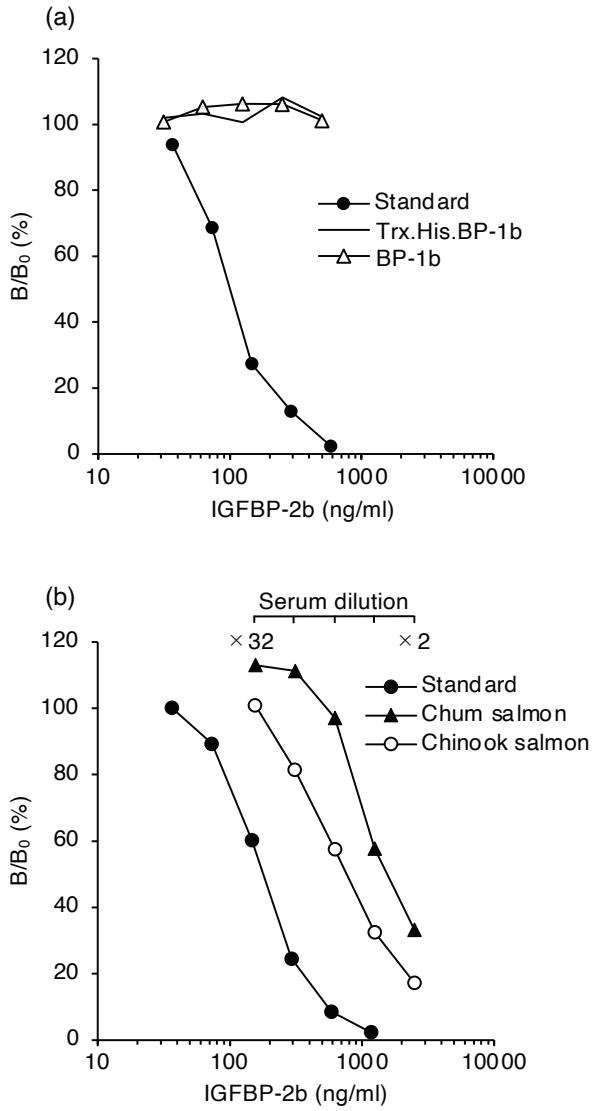


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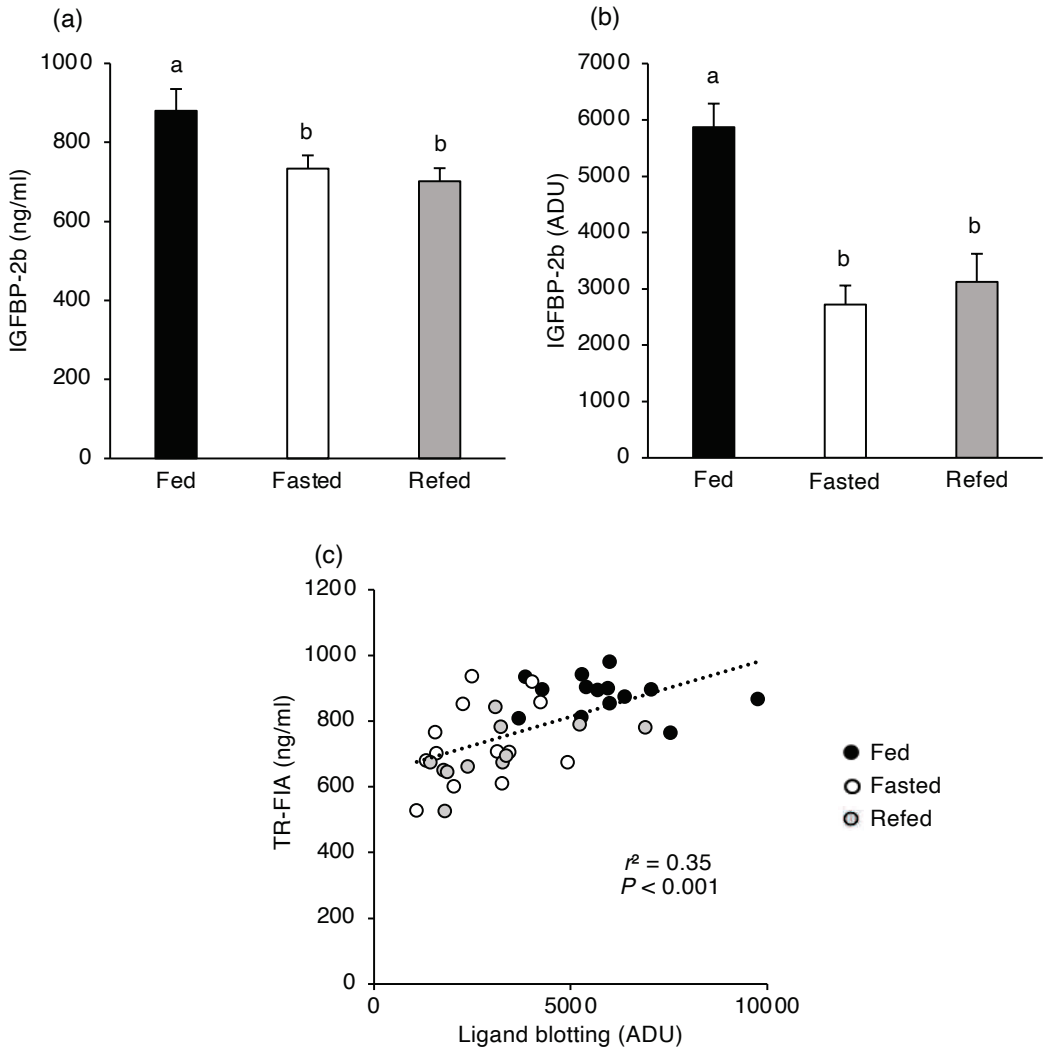
Izutsu et al., Fig. 3



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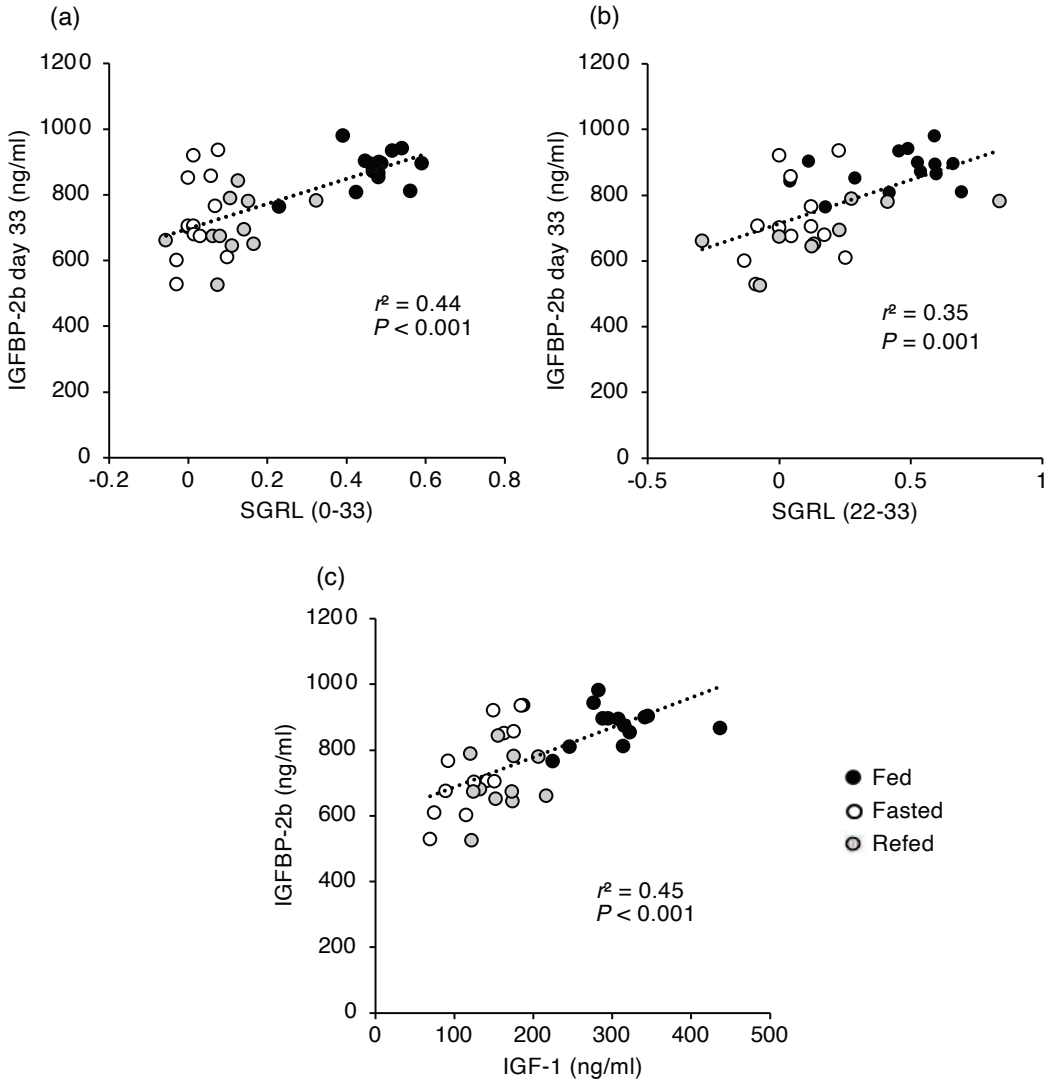


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Table 1. Correlations of serum IGFBP-2b levels measured by TR-FIA and ligand blotting with IGF-1 level and specific growth rates in length and weight (SGRL and SGRW).

		IGF-1	SGRL		SGRW	
	Day	33	0-33	22-33	0-33	22-33
TR-FIA	r^2	0.45	0.44	0.35	0.37	0.10
	P	< 0.001	< 0.001	0.001	< 0.001	0.051
Ligand blotting	r^2	0.58	0.46	0.34	0.49	0.24
	P	< 0.001	< 0.001	< 0.001	< 0.001	0.0021

Data on serum IGF-1 levels and SGRs are from Izutsu et al. (2022) with permission.

Authors' contributions. A.I.: conceptualization, data curation, methodology, formal analysis, investigation, validation, visualization, writing – original draft. S.H.: methodology, investigation. N.K.: conceptualization, methodology, investigation. D.T.: investigation, resources. A.H.: methodology, investigation. M.S.: conceptualization, data curation, funding acquisition, investigation, project administration, supervision, validation, visualization, writing –original draft, writing – review & editing.

Declaration of interests. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.