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Title:

Evaluation of binding capacity of circulating insulin-like growth factor binding protein-1b in salmonids using a ligand immunofunctional assay

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Abstract

Insulin-like growth factor-binding proteins (IGFBPs) regulate the activity of insulin-like growth factor (IGF)-1. Among the three major circulating IGFBPs in salmonids, IGFBP-1b is an inhibitor of IGF activity induced under catabolic conditions. IGFBP-1b is considered to quickly sequester IGF-1 from the circulation. However, the level of circulating IGFBP-1b present in its unoccupied free form is unknown. Here, we aimed to develop a non-equilibrium ligand immunofunctional assay (LIFA) to evaluate IGF-binding capacity of circulating intact IGFBP-1b. Purified Chinook salmon IGFBP-1b, its antiserum, and europium-labeled salmon IGF-1 were used as the assay components. In the LIFA, IGFBP-1b was first captured by the antiserum, allowed to bind to the labeled IGF-1 for 22 h at 4°C, and quantified its IGF-binding capacity. Serial dilutions of the standard and serum were prepared simultaneously within a certain concentration range (1.1–12.5 ng/ml). In underyearling masu salmon, IGF-binding capacity of intact IGFBP-1b was higher in fasted fish than in fed fish. Transferring Chinook salmon parr to seawater also increased IGF-binding capacity of IGFBP-1b, most likely due to osmotic stress. In addition, there was a strong relationship between total IGFBP-1b levels and its IGF-binding capacity. These results suggest that IGFBP-1b expressed under stress is mostly present in the free form. On the contrary, during smoltification of masu salmon, IGF-binding capacity of IGFBP-1b in the serum was relatively low and less related to the total IGFBP-1b level, suggesting its functional difference under certain physiological conditions. These results indicate that estimating both total IGFBP-1b level and its IGF-binding capacity is useful for evaluating the catabolic status and unraveling the regulation of IGF-1 activity by IGFBP-1b.

Keywords

binding capacity, immunoassay, insulin-like growth factor, marker, salmon, smolt, stress

1. Introduction

Insulin-like growth factor (IGF)-1 is a member of the insulin family and has strong mitogenic activity. It circulates and mediates the growth-promoting effect of growth hormones by binding to the IGF-1 receptor (Daughaday and Rotwein, 1989; Jones and Clemmons, 1995). Unlike insulin, circulating IGF-1 mostly binds to one of the six IGF-binding proteins (IGFBPs) (Rajaram et al., 1997). IGFBPs are not structurally related to the insulin/IGF-1 receptor family members, but their affinity for IGF-1 is comparable to that of the IGF-1 receptor (Rechler, 1993). IGFBPs can inactivate circulating IGF-1 and release it to bind to the receptor when they are degraded by specific enzymes or associated with the cell surface (Zapf, 1995; Rajaram et al., 1997; Bunn and Fowlkes, 2003; Bach, 2018; LeRoith et al., 2021). Thus, IGFBPs regulate IGF-1 activity and somatic growth.

In mammalian circulation, IGFBP-3 and IGFBP-1 play pivotal roles in stabilizing and sequestering IGF-1, respectively (Baxter, 1994; Rajaram et al., 1997). IGFBP-3 carries approximately 80% of IGF-1 by forming a ternary complex with an acid-labile subunit, and the half-life of circulating IGF-1 is prolonged from 10 min to 12 h (Baxter, 1994; Zapf, 1995; LeRoith et al., 2021); this complex is likely a source of free IGF-1. On the contrary, IGFBP-1 generally inhibits IGF-1 activity by sequestering free IGF-1 (Lee et al., 1993, 1997). Circulating levels of IGFBP-1 show large variations within a day; fasting increases the IGFBP-1 level, whereas food intake reduces it through the dominant suppressive effect of insulin and the counter-stimulatory activity of cortisol (Powell et al., 1991; Katz et al., 1998). In mammals, circulating IGFBP-1 is unoccupied with IGFs (Lee et al., 1989) and is therefore readily available to bind and inactivate free IGF-1 (Lee et al., 1993, 1997).

Teleosts have two subtypes of IGFBP-1, namely, IGFBP-1a and IGFBP-1b, because of an additional round of whole-genome duplication (Ocampo Daza et al., 2011; Macqeen et al., 2013). In salmonids, IGFBP-1a and -1b are two of the three major subtypes of IGFBP-1 (Shimizu and Dickhoff, 2017). They are similar to mammalian IGFBP-1 in terms of their response to fasting/meal intake and stress (Shimizu and Dickhoff, 2017). In masu salmon (*Oncorhynchus masou*), prolonged fasting induces both the subtypes, but IGFBP-1b responds earlier than IGFBP-1a (Kawaguchi et al., 2013). Transferring juvenile Chinook salmon (*O. tshawytscha*) from freshwater to seawater results in an osmotic stress, which in turn induces IGFBP-1b and -1a expression within 6 and 24 h, respectively (Shimizu et al., 2011). In contrast, IGFBP-1a is more effective in inhibiting IGF-1 activity in the pituitary cells of masu salmon (Hasegawa et al., 2020).

In our previous study, we established a radioimmunoassay (RIA) and time-resolved fluoroimmunoassay (TR-FIA) for measuring total immunoreactive IGFBP-1b (Shimizu et al., 2006; Fukuda et al., 2015). Quantification of total IGFBP-1b in the circulation revealed that its level is generally inversely correlated with the growth rate in coho salmon (*O. kisutch*) and masu

salmon (Shimizu et al., 2006; Fukuda et al., 2015). Therefore, circulating IGFBP-1b level is a useful inverse growth index. In contrast, circulating IGF-1 is regarded as a positive growth index in salmonids and other fishes (Picha et al., 2008; Beckman, 2011). However, under certain conditions such as a rapid water temperature change, the relationship between IGF-1 and growth rate is disrupted (Beckman et al., 2004). These findings suggest that combining these two growth indices, namely, IGFBP-1b and IGF-1, provides an accurate estimation of the growth status because they reflect the degree of anabolism and catabolism, respectively (Kaneko et al., 2019).

A TR-FIA has also been established for measuring salmon IGFBP-1a (Kaneko et al., 2020). However, the measurement of immunoreactive IGFBP-1a does not provide physiologically relevant profiles, such as an increase in response to fasting in masu salmon. However, when serum IGFBP-1a was detected and semi-quantified using ligand blotting with labeled IGF-1 in the same species, the band intensity of IGFBP-1a was higher in fasted fish samples than in fed fish samples (Kawaguchi et al., 2013). This difference in band intensity may be due to the partial degradation of circulating IGFBP-1a. Based on this assumption, a ligand immunofunctional assay (LIFA) was established to semi-quantify the fraction of circulating IGFBP-1a capable of binding to IGF (Kaneko et al., 2020). This fraction corresponds to free (unoccupied with IGFs) and intact (not degraded) IGFBP-1a, whereas the fraction measured using TR-FIA is the total immunoreactive IGFBP-1a, regardless of its IGF-binding ability or occupancy by endogenous IGFs. As a result, not total, but free intact IGFBP-1a showed an inverse relationship with growth rate in masu salmon. These results suggest that the measurement of the free intact form may provide a more accurate estimation of the degree of growth retardation. However, there has been no assessment of free intact IGFBP-1b in any fish species. In the present study, we aimed to establish a LIFA to evaluate IGF-binding capacity of circulating intact IGFBP-1b in salmonids and compare its responses and levels with those of total IGFBP-1b.

2. Material and methods

2.1. Assay components

Purified IGFBP-1b from the serum of spawning Chinook salmon (Shimizu et al., 2005), an antiserum against the purified IGFBP-1b (Shimizu et al., 2006), and europium (Eu)-labeled IGF-1 for TR-FIA (Shimomura et al., 2012) were used for the LIFA.

2.2. Immunoprecipitation, electrophoresis, and ligand blotting

A mixture of 50 µl of spawning Chinook salmon serum and 10 µl solution of 20 mM Tris, 150 mM NaCl containing 0.5% bovine serum albumin, 1% deoxycholic acid sodium salt (DOC), and 1% Triton X in a 1-ml tube was reacted with 10 µl of antiserum against IGFBP-1b at 4°C for 5 h followed by incubation with 50 µl of goat anti-rabbit immunoglobulin G at 4°C overnight. The

incubate was laid on 0.5 ml of SDT-PBS (0.9 M sucrose, 1% DOC, and 1% Triton X in phosphate-buffered saline) and centrifuged at 5000 rpm for 15 min. The supernatant was discarded, and the precipitate was washed three times with Tris-NaCl and solubilized in 10 µl of PBS.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 3% stacking and 12.5% separating gels. The precipitated sample was prepared as described above and Chinook salmon serum was treated with an equal volume of sample and buffer containing 2% SDS and 10% glycerol at 85°C for 5 min. The stacking and separating gels were placed in a solution of 50 mM Tris, 400 mM glycine, and 0.1% SDS at 8 and 12 mA, respectively, until the bromophenol blue dye front reached the bottom of the gel.

Ligand blotting with digoxigenin-labeled human IGF-1 (DIG-hIGF-1) was performed as previously described (Shimizu et al. 2000). Nitrocellulose membranes were incubated overnight with DIG-hIGF-1 and then with antibodies against DIG-conjugated horseradish peroxidase (Roche, Indianapolis, IN, USA) at a dilution of 1:2500 for 1.5 h at room temperature (20°C–25°C). IGFBP was visualized using enhanced chemiluminescence (ECL) western blotting reagents (Amersham Life Science, Arlington Heights, IL, USA).

2.2. LIFA for intact IGFBP-1b

A LIFA for intact IGFBP-1b was established according to the method to quantify salmon IGFBP-1a (Kaneko et al., 2019). A 96-well assay plate coated with goat anti-rabbit IgG (DELFIA Yellow Plate; Perkin Elmer, Waltham, MA, USA) was first washed with 200 µl of DELFIA Wash Buffer (PerkinElmer). To each well, 40 µl of DELFIA Assay Buffer (PerkinElmer), 40 µl of IGFBP-1b antiserum (1:400), and 40 µl of standard (purified IGFBP-1b) or serum diluted with the Assay Buffer were added. This step immobilizes IGFBP-1b captured by the antiserum to the plate. The plate was sealed and incubated at 4°C overnight on a shaker at 600 rpm. Subsequently, 40 µl of Eu-IGF-1 (6.3 ng/ml) was added to each well to allow binding to IGFBP-1b and the plate was incubated at 4°C overnight on a shaker at 600 rpm. The plate was then washed six times with 200 µl of Wash Buffer, and 200 µl of DELFIA Enhancement Solution (PerkinElmer) thawed to room temperature (20°C–25°C) was added. The plate was shaken without sealing for 10 min at 25°C. Time-resolved fluorescence was measured using a Wallac ARVO X4 multilabel counter (PerkinElmer). Measured values were expressed as a binding capacity unit (BCU), IGF-1-binding ability relative to that of purified IGFBP-1b. In the present study, 1 BCU was defined as the IGF-1-binding ability of 1 ng/ml purified IGFBP-1b used as the standard.

2.3. Fasting experiment

Underyearling masu salmon maintained at the Nanae Freshwater Laboratory, Field Science Center for Northern Biosphere, Hokkaido University (Kameda-gun, Hokkaido, Japan), were used.

They were randomly placed in two 16-L tanks supplied with flow-through 10℃ well-water. During the experiment, one group (Fed) was fed daily to satiety with a commercial diet (Marubeni Nisshin Feed, Tokyo, Japan) for 10 days. The other group (Fasted) was fasted throughout the experimental period of 10 days. The experiments were performed in accordance with the guidelines of the Hokkaido University Field Science Center Animal Care and Use Committee (Approval No. 30-3). After 10 days of treatment, fork length and body weight were measured, and blood samples were collected from 10 fish per treatment. Blood was collected from the caudal vein into heparinized 75-μl glass tubes (Hirschmann Laborgeräte, Eberstadt, Germany) after cutting the tail. It was centrifuged at 3000 × *g* for 5 min. Plasma was collected and stored at −80°C until use.

2.4. Seawater transfer experiment

Plasma samples from the seawater transfer experiment conducted by Shimizu et al. (2011) were used to measure intact IGFBP-1b level. Briefly, underyearling Chinook salmon (*O. tshawytscha*) parr, juvenile fish that had not yet been prepared for marine life, were transferred to either seawater or freshwater tanks. Plasma samples collected 0, 6, 12, 24, 72 (3 days), and 168 h (7 days) after transfer and stored at −80°C were subjected to intact IGFBP-1b analysis.

Serum IGFBP-1b was visualized via ligand blotting and semi-quantified using ImageJ version 1.440 (Schneider et al., 2012). Its intensity was normalized to human IGFBP-4 band intensity and expressed as an arbitrary density unit (ADU).

2.5. Photoperiod manipulation experiment

Plasma from fish in the photoperiod manipulation experiment conducted by Suzuki et al. (2020) was used in this study. Underyearling masu salmon were reared under an advanced photoperiod in which LD15:9 was interrupted by LD9:15 from June for 4 months to induce smoltification. Plasma samples were obtained monthly during October and February and stored at −80°C until analysis.

2.5. Statistical analysis

The results were analyzed using Student's *t*-test and a one-way ANOVA for pairwise and multiple comparisons, respectively, using JMP Pro 16 (SAS Institute Inc., Cary, NC, USA). Three intact IGFBP-1b values that were outliers in the quantile range were excluded from the analysis (tail quantile = 0.1; Q = 3). When significant effects were found using the one-way ANOVA, differences were further identified using Tukey's honest significant difference (HSD) test, with differences considered significant at $P < 0.05$. A linear correlation analysis was also performed to determine the relationship between parameters.

3. Results

3.1. Specificity of antiserum

Ligand blotting of Chinook salmon samples using DIG-IGF-1 revealed three bands for IGFBP-2b, -1a, and -1b; however, the IGFBP-2b band was weak (Fig. 1). The serum sample that was precipitated with an antiserum against IGFBP-1b exhibited an IGFBP-1b band.

3.2. Establishment of the LIFA

Specific binding of Eu-IGF-1 increased with increasing amounts of IGFBP-1b standard in the LIFA (Fig. 2). The serial dilution curve of Chinook salmon serum was parallel to the standard curve within a certain dilution range (i.e., 1:128–1:8); however, the curve plateaued at higher concentrations (Fig. 2). When the standard was mixed with 20-time volume of rainbow trout serum, binding to Eu-IGF-1 was reduced at higher concentrations; the dilution curve constructed was parallel to the serial dilution curve of Chinook salmon serum (Fig. 3), suggesting interference of serum components. Based on these results, a certain concentration range of standard (i.e., 0.8–12.5 ng/ml) was selected and plotted against serial dilutions of Chinook salmon serum/plasma (Fig. 4). The curves were parallel when the concentration of the standard was equal or less than 12.5 ng/ml, which was set as the upper limit of the LIFA. Total binding of Eu-IGF-1 in the assay was dependent of the incubation time and did not reach an equilibrium state up to 32 h (Fig. 5). The incubation time was set for 22 h at 4°C for reproducibility. The minimum detection rate of LIFA, defined as the mean of zero standard plus two standard deviations, was 1.1 ng/ml (n = 4). Thus, the assay range for the LIFA was 1.1–12.5 ng/ml. The intra-assay and inter-assay coefficients of variation were 6.4% (n = 4) and 11.8% (n = 5), respectively. The recovery rate of IGFBP-1b added to the sera of Chinook salmon parr that was transferred to seawater was 85.6% (n = 7).

3.3. Measurement of IGF-binding capacity of IGFBP-1b using the LIFA

Fasting underyearling masu salmon for 10 days increased IGF-binding capacity of IGFBP-1b in serum (Fig. 6). When Chinook salmon parr individuals were transferred to seawater, the IGF-binding capacity increased (Tukey's HSD test, $P < 0.0001$) and became higher than that in control fish in freshwater (Fig. 7a). This response was similar to that observed for the total IGFBP-1b level (Fig. 7b). The IGF-binding capacity strongly correlated with the total IGFBP-1b level (Fig. 8). In addition, there was a positive correlation between the IGF-binding capacity and IGFBP-1b level semi-quantified using ligand blotting although the coefficient of determination (r^2) was 0.76 ($P < 0.0001$).

During photoperiod-induced smoltification in masu salmon, the binding capacity of

IGFBP-1b gradually increased from November to February (Fig. 9a). In contrast, the total IGFBP-1b level sharply increased from November to December and remained relatively high thereafter (Fig. 9b). There was a positive correlation between the binding capacity and total IGFBP-1b levels, although the r^2 value was relatively low (Fig. 10).

4. Discussion

In this study, we developed a LIFA for salmonid IGFBP-1b using components used for TR-FIAs of total IGFBP-1b and IGF-1, purified Chinook salmon IGFBP-1b, its antiserum, and Eu-labeled salmon IGF-1 (Shimomura et al., 2012; Fukuda et al., 2015). This LIFA was specific enough to detect free intact IGFBP-1b, because the antiserum immunoprecipitated exclusively IGFBP-1b from the serum, and its cross-reactivity with IGFBP-1a and -2b in TR-FIA was 3.1% and undetectable, respectively (Fukuda et al., 2015). Moreover, purified IGFBP-1b captured using this antiserum showed a dose-dependent increase in IGF-1 binding. These results validate the usefulness of the LIFA for detecting intact IGFBP-1b. However, care should be taken when setting the assay range because the standard curve and the serial dilution curve of salmon serum were not parallel at higher concentrations. Such a difference in the binding property between the standard and salmon serum is presumably due to interference by component(s) in the serum, because incubation of purified IGFBP-1b with rainbow trout serum inhibited IGF-1 binding at higher concentrations. The identity of the components or factors are currently unknown. We set an assay range (i.e., 1.1–12.5 ng/ml) where the serial dilution curves of the standard and serum were parallel. Given this limitation, quantification of IGF-binding capacity of IGFBP-1b in the sera of salmonids is possible.

It should be pointed that the LIFA established in the present study is useful to evaluate IGF-binding capacity of IGFBP-1b but may not adequately quantify absolute amount of free intact IGFBP-1 in samples. This is partly because this assay does not remove endogenous IGFs; Varying concentrations of endogenous IGFs in samples likely affect the binding of IGFBP-1b to Eu-IGF-1. In addition, the current LIFA uses a non-equilibrium condition under which the fluorescent count depends on incubation time with Eu-IGF-1. We therefore set the incubation time for 22 h at 4°C. By taking account of these limitations, measured values were expressed as IGF-binding capacity unit (BCU).

Measurement of IGF-binding capacity of IGFBP-1b in serum using the LIFA revealed that its response to fasting was similar to that of total IGFBP-1b. Fasting increases the total IGFBP-1b level in the bloodstream of salmonids (Shimizu et al., 1999, 2006, 2009; Kawaguchi et al., 2013). In addition, the IGF-1-binding capacity assessed using a binding assay with ^{125}I-IGF-1 in fasted fish was higher than that in fed fish (Shimizu et al., 1999), suggesting that IGFBP-1b is responsible for the increase in binding capacity. The results of the present study are in

accordance with the findings on IGFBP-1 in humans (Lee et al., 1989) and support the assumption that IGFBP-1b in salmon is secreted and present in an unoccupied free form to block the anabolic activity of IGF-1 under fasting conditions.

Osmotic stress also increased IGF-binding capacity of IGFBP-1b, similar to the total IGFBP-1b level. In our previous study, we quantified total IGFBP-1b using RIA in Chinook salmon parr and smolts transferred to seawater (Shimizu et al., 2011). In this experiment, smolts were capable of hypo-osmoregulation and exhibited only a transient increase in circulating IGFBP-1b level, whereas parr suffered from osmotic stress and showed a large increase in IGFBP-1b level (Shimizu et al., 2011). In the present study, we measured IGF-binding capacity of IGFBP-1b in the same plasma samples of parr and found that its response and level were similar to those of total IGFBP-1b. In addition, the binding capacity of IGFBP-1b correlated with the intact IGFBP-1b level semi-quantified using ligand blotting, which represents the sum of occupied and unoccupied free IGFBP-1b. These results provide further evidence that IGFBP-1b induced under catabolic conditions mostly circulates in the free form.

However, it should be noted that under stress conditions, IGF-binding capacity of IGFBP-1b sometimes exceeded the total level, which is theoretically contradictory. This difference is likely due to IGFBP-1b phosphorylation. Phosphorylation is a major post-translational modification of IGFBP-1 in mammals and is known to increase the IGF-1-binding ability of human IGFBP-1 by six times (Jones et al., 1991), whereas the binding affinity of rat IGFBP-1 does not change with phosphorylation (Peterkofsky et al., 1998). The PEST (Pro-Glu-Ser-Thr) domain in the mid region is responsible for the phosphorylation of mammalian IGFBP-1 (Julkunen et al, 1988; Lee et al., 1993, 1997). Although the phosphorylation of salmon IGFBP-1b is yet to be demonstrated, it possesses a PEST domain (Shimizu et al., 2005). Considering these findings, it is possible that the degree of phosphorylation of intact IGFBP-1b induced under catabolic conditions was higher than that of purified Chinook salmon IGFBP-1b, resulting in higher IGF-1-binding ability of the former. If this was true, IGF-binding capacity of IGFBP-1b determined using the LIFA would reflect both its quantity and IGF-binding affinity. Therefore, care should be taken to evaluate BCU. Although not a rigid quantification of free intact IGFBP-1b level, BCU is still useful for evaluating the ability of IGFBP-1b to bind to IGF-1 and inhibit the activity of circulating IGF-1.

The profiles of IGF-binding capacity of IGFBP-1b during smoltification differed from those under catabolic conditions. Smoltification involves a series of pre-adaptive changes to ocean life (Stefansson et al., 2008; McCormick et al., 2013). The acquisition of hypo-osmoregulatory ability through the activation of gill Na^+/K^+-ATPase (NKA) activity is a characteristic change that occurs during smoltification. We previously reported that the total IGFBP-1b level increases during smoltification in coho and masu salmon (Shimizu et al., 2006; Fukuda et al., 2015; Suzuki

et al., 2020). Although the binding capacity of IGFBP-1b levels increased during smoltification, its increase and levels were not as high as those of total IGFBP-1b. Moreover, the correlation between the binding capacity of IGFBP-1b and its total levels was relatively weak (r^2 = 0.28) compared with that under stress conditions (r^2 = 0.85). These results suggest that a portion of the circulating IGFBP-1b binds to endogenous IGF-1. In smolting masu salmon, a correlation between circulating total IGFBP-1b level and gill NKA activity has been reported (Fukuda et al., 2015; Suzuki et al., 2020). In contrast, IGF-binding capacity of IGFBP-1b did not show a significant correlation in the present study (data not shown). We hypothesized that IGFBP-1b during smoltification represents a fraction of circulating IGF-1 in the gills. In addition, our results indicate that IGFBP-1b might plays roles other than inhibiting IGF-1 activity in certain developmental stages or under physiological conditions in salmon. These hypotheses warrant further investigation.

There are only a few reports on the establishment of LIFA for IGFBPs (Lassarre and Binoux, 2001; Kaneko et al., 2020). The LIFA was originally established for human IGFBP-3 to assess the degree of limited proteolysis of IGFBP-3, and a comparison between the total and intact forms provided useful information for evaluating the availability of IGF-1 in target tissues (Lassarree and Binoux, 2001). Similarly, we previously developed an LIFA for salmonid IGFBP-1a and found that a part of the circulating IGFBP-1a was possibly fragmented, and not the total but intact form, showed an inverse correlation with the growth rate (Kaneko et al., 2020). These studies highlight the importance of limited proteolysis of IGFBP in regulating its function. However, the results of the present study suggest that such proteolysis does not occur with IGFBP-1b in salmonids under catabolic conditions. However, the distinction between free (intact) and total IGFBP-1b remains useful for elucidating the mechanism by which IGFBP-1b regulates IGF-1 activity, particularly partitioning of circulating IGF-1 into different tissues during smoltification.

In summary, we developed an LIFA for estimating IGF-binding capacity of IGFBP-1b. The responses of the binding capacity of IGFBP-1b to catabolic conditions were similar to those of total IGFBP-1b, and level of IGFBP-1b was as high as that of the total IGFBP level, suggesting that IGFBP-1b is mostly present in its free form to quickly sequester IGF-1 from the circulation. On the contrary, during smoltification, a portion of circulating IGFBP-1b binds to IGF-1 and may be delivered to target tissues, such as the gills. Measurement of both IGF-binding capacity of IGFBP-1b and its total levels is useful for assessing catabolic conditions and further unraveling the role of circulating IGFBP-1b in salmonids.

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Figure legends

Fig. 1. Ligand blotting of Chinook salmon serum before and after immunoprecipitation using the antiserum against salmon IGFBP-1b. For immunoprecipitation, Chinook salmon serum was first reacted with the antiserum and incubated with goat anti-rabbit IgG. After centrifugation, the precipitate was electrophoresed and subjected to ligand blotting using digoxigenin-labeled IGF-1. NHS: normal human serum, Chinook: Chinook salmon serum. Arrows indicate the migration positions of human (left) and salmon (right) IGFBP bands.

Fig. 2. Binding of europium (Eu) labeled IGF-1 by serially diluted purified Chinook salmon IGFBP-1b (open circles) and Chinook salmon serum (closed squares) in the ligand immunofunctional assay.

Fig. 3. Effect of addition of rainbow trout serum to standard on IGF-1 binding in the ligand immunofunctional assay. Purified Chinook salmon IGFBP-1b was mixed with 20-time volume of rainbow trout and serially diluted serum samples. Binding of europium-labeled IGF-1 was compared between standards with and without the addition trout serum.

Fig. 4. Standard curve of ligand immunofunctional assay for measurement of binding capacity of IGFBP-1b in the sera of salmonids. Concentrations of the standard between 0.8 and 12.5 ng/ml were selected to ensure that the standard curve was parallel to serial dilution curves of Chinook salmon sera.

Fig. 5. Effect of incubation time with europium (Eu)-labeled IGF-1 on binding count in ligand immunofunctional assay. Assay standard (6.3 ng/ml) and Chinook salmon serum diluted at 1:8 were incubated with Eu-IGF-1 with varying time. Arrow indicates incubation time for 22 h used for routine measurements.

Fig. 6. Effect of fasting on plasma intact IGFBP-1b level in underyearling masu salmon. Fish were subjected to either 10 days of feeding or fasting. Measured intact IGFBP-1b level is expressed as mean ± SE (n = 10) of binding capacity unit (BCU) relative to purified salmon IGFBP-1b. Asterisk indicates a significant difference between treatments (Student's t-test, $P < 0.05$).

Fig. 7. Effects of direct seawater transfer of underyearling Chinook salmon parr on plasma intact (a) and total (b) IGFBP-1b levels. Measured intact IGFBP-1b level is expressed as mean ± SE (n = 5-7) of binding capacity unit (BCU) relative to purified salmon IGFBP-1b. Data of total IGFBP-

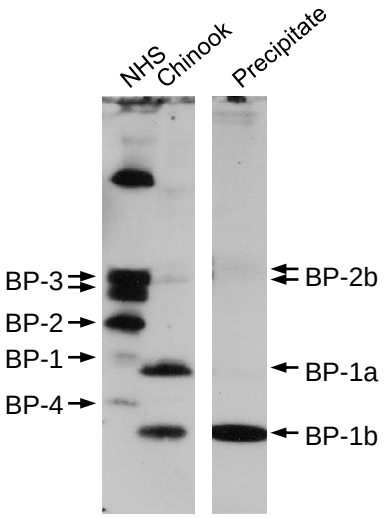
1b level measured using radioimmunoassay were from Shimizu et al. (2006), used with permission. Asterisk indicates a significant difference between treatments at a given time point (Student's *t*-test, $P < 0.05$).

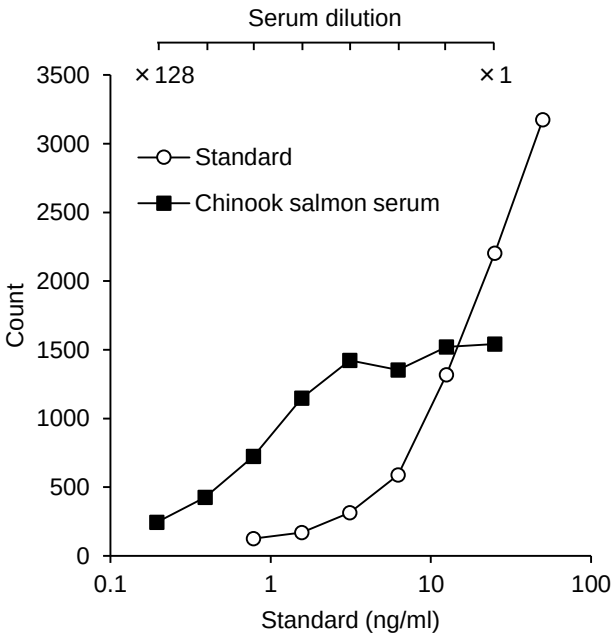
Fig. 8. Relationship between plasma intact and total IGFBP-1b levels in the seawater transfer experiment. Data ($n = 65$) of total IGFBP-1b level measured using radioimmunoassay were from Shimizu et al. (2006), used with permission.

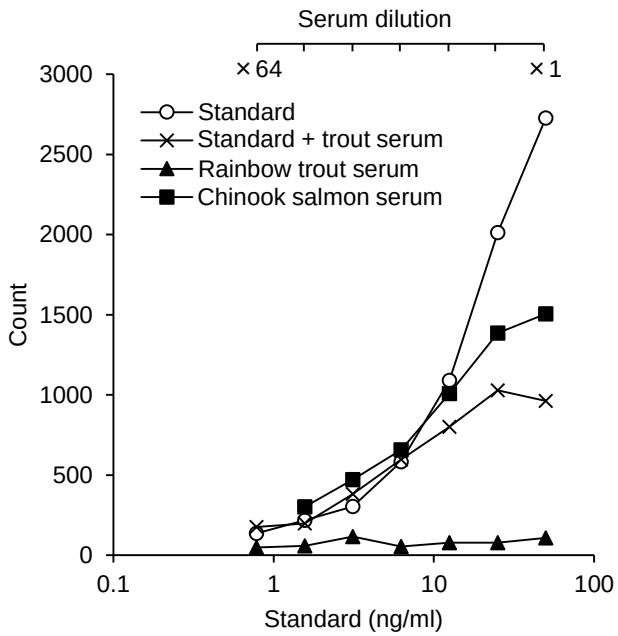
Fig. 9. Changes in serum intact (a) and total (b) IGFBP-1b levels in juvenile masu salmon during smoltification. Smoltification was induced by an advance photoperiod regime in which light:dark (LD)15:9 was interrupted by LD9:15 from June for 4 months. Measured intact IGFBP-1b level is expressed as mean ± SE (n = 9–14) of binding capacity unit (BCU) relative to purified salmon IGFBP-1b. Data of total IGFBP-1b level measured using time-resolved fluoroimmunoassay were from Suzuki et al. (2020), used with permission. Values are expressed as mean ± SE (n = 9–14). Symbols sharing the same letters are not significantly different from each other (Tukey's HSD, $P < 0.05$).

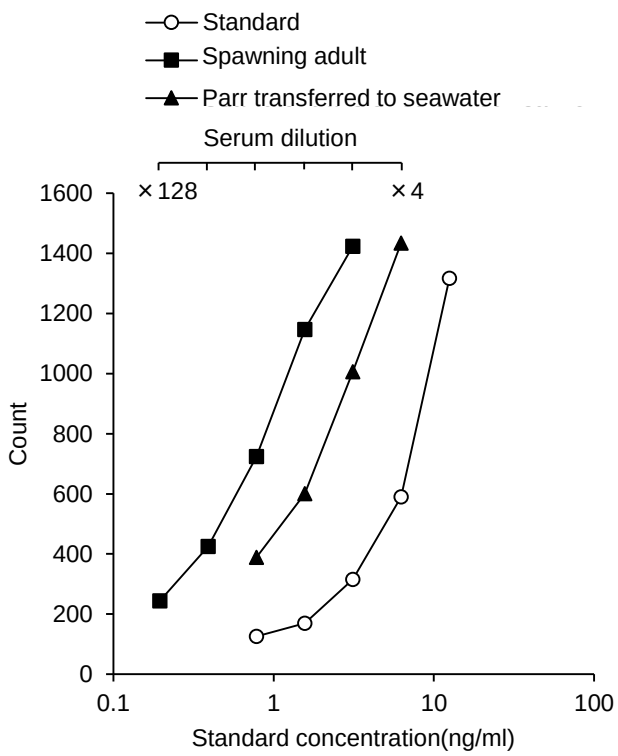
Fig. 10. Relationship between serum intact and total IGFBP-1b levels in smolting masu salmon. Data ($n = 61$) of total IGFBP-1b level measured using the time-resolved fluoroimmunoassay were from Suzuki et al. (2020), used with permission.

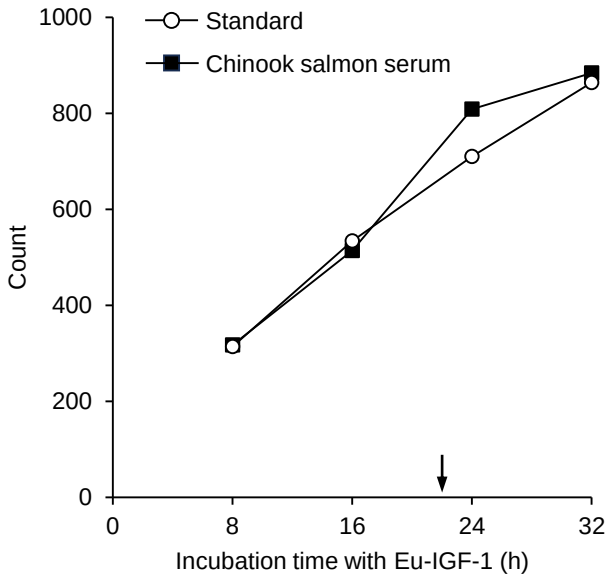
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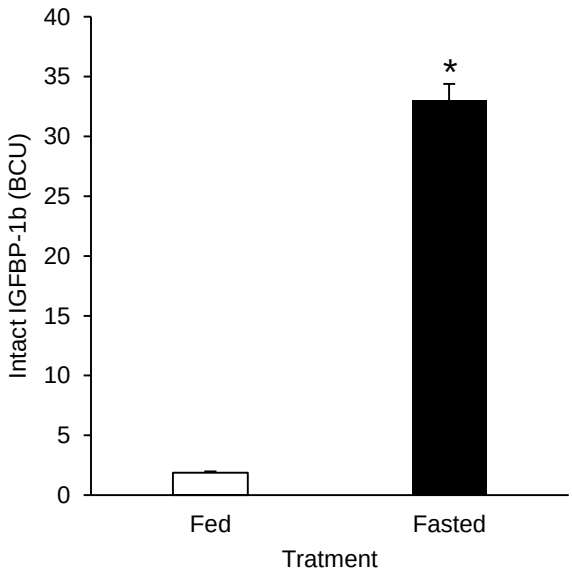


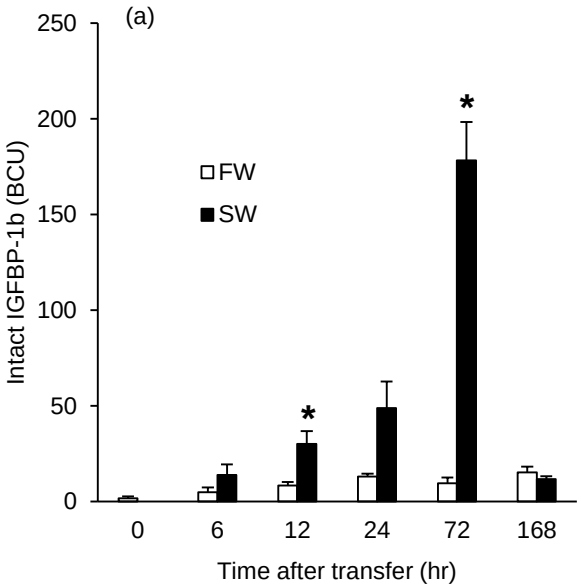

Serum dilution
×128
×1
3500
3000
2500
2000
1500
1000
500
0
Count
Standard
Chinook salmon serum
0.1
1
10
100
Standard (ng/ml)

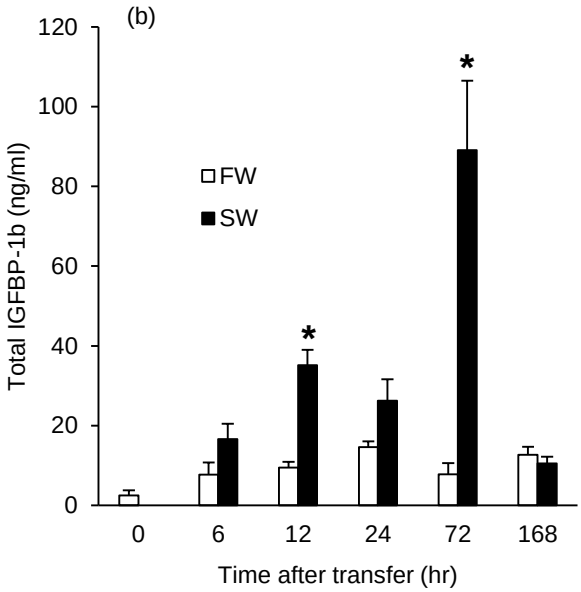

Serum dilution
× 64
× 1
3000
2500
2000
1500
1000
500
0
Count
Standard
Standard + trout serum
Rainbow trout serum
Chinook salmon serum
0.1
1
10
100
Standard (ng/ml)

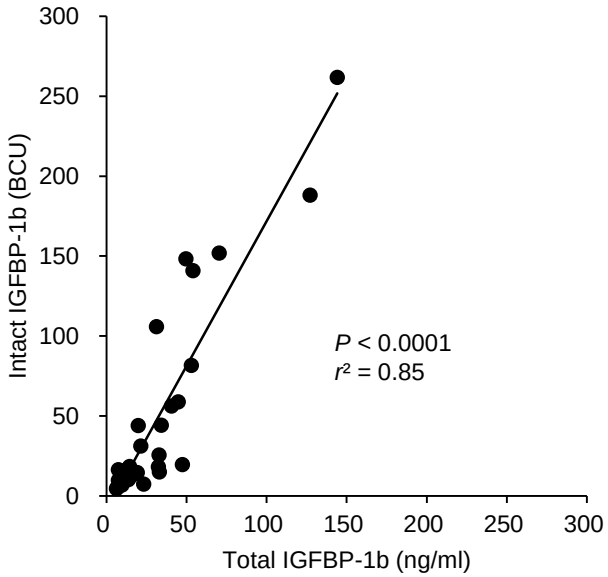

Standard
Spawning adult
Parr transferred to seawater
Serum dilution
×128
×4
1600
1400
1200
1000
800
600
400
200
0
Count
0.1
1
10
100
Standard concentration(ng/ml)

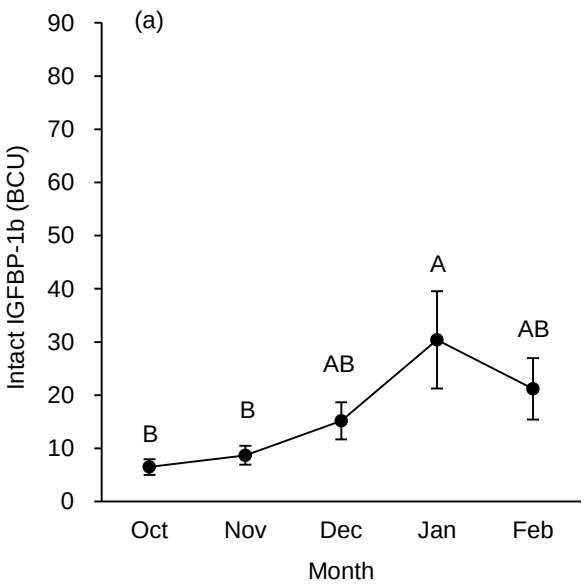

Standard
Chinook salmon serum
Count
1000
800
600
400
200
0
0
8
16
24
32
Incubation time with Eu-IGF-1 (h)

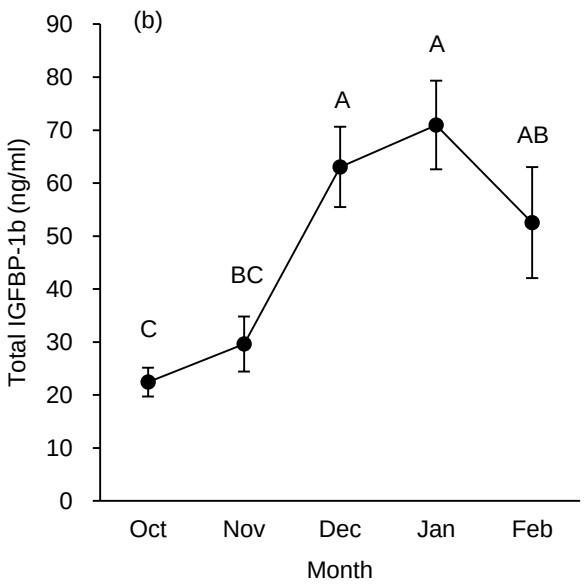

40
35
30
25
20
15
10
5
0
Intact IGFBP-1b (BCU)
*
Fed
Fasted
Tratment

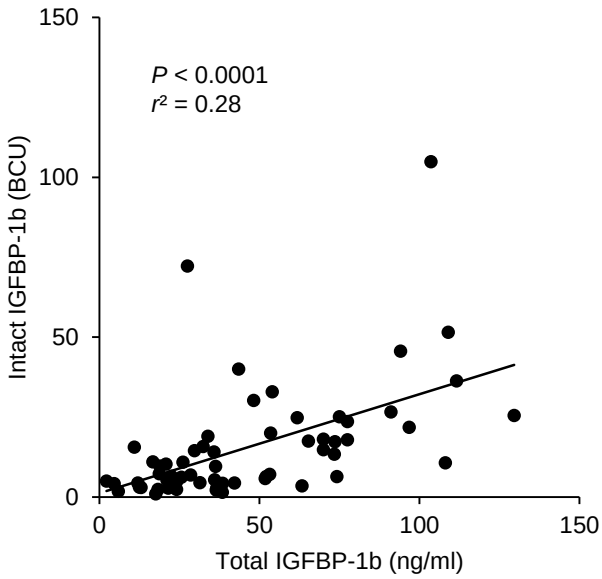

(a)
Intact IGFBP-1b (BCU)
250
200
150
100
50
0
FW
SW
*
*
0
6
12
24
72
168
Time after transfer (hr)


(b)
Total IGFBP-1b (ng/ml)
120
100
80
60
40
20
0
FW
SW
*
*
0
6
12
24
72
168
Time after transfer (hr)


300
250
200
150
100
50
0
Intact IGFBP-1b (BCU)
0
50
100
150
200
250
300
Total IGFBP-1b (ng/ml)
P < 0.0001
r² = 0.85


(a)
Intact IGFBP-1b (BCU)
90
80
70
60
50
40
30
20
10
0
B
B
AB
A
AB
Oct
Nov
Dec
Jan
Feb
Month


(b)
Total IGFBP-1b (ng/ml)
90
80
70
60
50
40
30
20
10
0
C
BC
A
A
AB
Oct
Nov
Dec
Jan
Feb
Month


150
100
50
0
0
50
100
150
P < 0.0001
r² = 0.28
Intact IGFBP-1b (BCU)
Total IGFBP-1b (ng/ml)