Title:

Measurement of circulating salmon insulin-like growth factor binding protein-1: assay development, response to feeding ration and temperature, and relation to growth parameters

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Salmon IGFBP-1 RIA

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Abstract

Fish plasma/serum contains multiple insulin-like growth factor binding proteins (IGFBPs), although their identity and physiological regulation are poorly understood. In salmon plasma, at least three IGFBPs with molecular masses of 22, 28 and 41 kDa are detected by Western ligand blotting. The 22-kDa IGFBP has recently been identified as a homolog of mammalian IGFBP-1. In the present study, a radioimmunoassay (RIA) for salmon IGFBP-1 was established and regulation of IGFBP-1 by food intake and temperature, and changes in IGFBP-1 during smoltification were examined. Purified IGFBP-1 from serum was used for standard, tracer preparation and antiserum production. Crosslinking ^{125}I-IGFBP-1 with salmon IGF-I eliminated interference by IGFs. The RIA had little cross-reactivity with salmon 28- and 41-kDa IGFBPs (< 0.5%) and measured IGFBP-1 as low as 0.1 ng/ml. Fasted fish had significantly higher IGFBP-1 levels than fed fish (21.6 ± 4.6 ng/ml vs 3.0 ± 2.2 ng/ml). Plasma IGFBP-1 was measured in individually tagged one-year old coho salmon reared for 10 weeks under four different feeding regimes as follows: high constant (2% body weight/day), medium constant (1% body weight/day), high variable (2% body weight/day - 0.5% body weight/day) and medium variable (1% body weight/day - 0.5% body weight/day). Fish fed with high ration had lower IGFBP-1 than those with medium ration. Circulating IGFBP-1 increased following a drop in feeding ration to 0.5% and returned to the basal levels when feeding ration was increased. Another group of coho salmon were reared for nine weeks under different water temperatures (11°C or 7°C) and feeding rations (1.75, 1 or 0.5% body weight/day). Circulating IGFBP-1 levels were separated by temperature during the first four weeks; a combined effect of temperature and feeding ration was seen in week seven; only feeding ration influenced IGFBP-1 level thereafter. These results indicate that IGFBP-1 is responsive to moderate nutritional and temperature changes. There was a clear trend that circulating IGFBP-1 levels were negatively correlated with body weight, condition factor (body weight/body length^3 x 100), growth rates and circulating 41-kDa IGFBP levels but not IGF-I levels. During parr-smolt transformation of coho salmon, IGFBP-1 levels showed a transient peak in late April,

which was opposite to changes in condition factor. Together, these findings suggest that salmon IGFBP-1 is inhibitory to IGF-action. In addition, IGFBP-1 responds to moderate changes in dietary ration and temperature, and shows a significant negative relationship to condition factor.

Introduction

Insulin-like growth factor (IGF)-I is a potent mitogen that is essential to postnatal growth of animals. IGF-I exerts its actions through endocrine, paracrine and autocrine manners by binding to IGF-receptors. Regardless of the mode of action, availability of IGF-I to bind receptors is regulated by a family of high-affinity IGF-binding proteins (IGFBPs). Six IGFBPs have been identified and characterized in mammals (Shimasaki & Ling, 1991; Rajaram *et al*., 1997). Depending on the type of IGFBP and cellular environment, IGFBPs can either inhibit or potentiate the biological action of IGF-I. In addition, different tissues produce different IGFBPs. These features add to the complexity of IGF-I actions. Besides regulating IGF-I availability, IGFBPs have IGF-independent actions on cell growth (Ferry *et al*., 1999).

IGF-I and IGFBPs are widely found among vertebrates including teleosts, and they appear to have co-evolved throughout the vertebrate lineage (Reinecke & Collet, 1998). Evidence for at least five IGFBP sequences (IGFBP-1 to -5) can be found in zebrafish (*Danio rerio*) and fugu (*Fugu rubripes*) genome databases and their sequences show 40-60% homology to mammalian counterparts (Wood *et al*., 2005a) . This conserved nature of IGFBP structure supports the concept that IGFBPs play a crucial role in regulating IGF-I action in vertebrates. In zebrafish, a series of studies based on IGFBP knockdown has shown that IGFBP-1, -2 and -3 regulate developmental rate under hypoxia, formation of the cardiovascular system, and formation of the pharyngeal skeleton and inner ear (Kajimura *et al*., 2005; Li *et al*., 2005; Wood *et al*., 2005b). These studies argue for the importance of IGFBPs during development as well as postnatal growth.

Endocrine IGF-I forms a large pool bound to IGFBPs in the blood. Although an essential role of endocrine IGF-I in the regulation of postnatal growth of mice has been questioned (Le Roith *et al*., 2001), its contribution to growth and metabolism is a subject of active investigation. Among six IGFBPs present in the circulation of mammals, IGFBP-1 may be one of the most critical factors regulating availability of circulating IGF-I to peripheral tissues (Lee *et al*., 1997). IGFBP-1 generally acts as an inhibitor of IGF-I action presumably through sequestering free IGF-I levels.

Unlike other IGFBPs, circulating IGFBP-1 shows a diurnal change in response to food intake (Busby *et al*., 1988; Cotterill *et al*., 1988). IGFBP-1 levels increase during fasting, and return to basal levels after a meal. This rapid change in IGFBP-1 is primarily due to the suppressive effect of insulin (Snyder & Clemmons, 1990). However, amino acids also influence the synthesis of IGFBP-1 in rats (Straus *et al*., 1993). The increase in IGFBP-1 may be a mechanism by which the action of IGF-I is blocked to redirect energy during malnutrition. Circulating IGFBP-1 is also increased under other catabolic states such as prolonged exercise, stress, hypoxia and critical illness (Lee *et al*., 1997). These responses of IGFBP-1 may be mediated, at least partly, by glucocorticoids such as cortisol. Glucocorticoid stimulates IGFBP-1 production, but its stimulatory effect is secondary to the suppressive effect of insulin in mammals (Unterman *et al*., 1991).

Candidates for fish IGFBP-1 have been detected in the circulation of several teleosts (Siharath *et al*., 1996; Kelley *et al*., 2001, 2002; Park *et al*., 2000; Kajimura *et al*., 2003). Western ligand blotting of fish plasma/serum typically reveals three IGFBP bands at 20-25, 25-30 and 40-50 kDa. The two smaller forms may be fish IGFBP-1 and/or -2 based on their size and response to fasting and stress (Kelley *et al*., 2001). This is further supported by hormone treatments with insulin and cortisol (Kelley *et al*., 2001; Kajimura *et al*., 2003). However, because the exact identity of lower molecular weight IGFBPs is obscure; it is not known if their physiological response is due to a conserved nature of the same type of IGFBP, or a similar regulation of different types of IGFBPs. In addition, there is no specific assay for fish IGFBP-1 available at present, which makes a detailed quantitative analysis difficult. We have recently purified a 22-kDa IGFBP from Chinook salmon serum, cloned its cDNA and identified it as a homolog of mammalian IGFBP-1 (Shimizu *et al*., 2005). Salmon IGFBP-1 lacks a PEST (Pro, Glu, Ser, Thr)-rich domain involved in rapid turnover of protein and an RGD (Agr-Gly-Asp) integrin recognition sequence (Shimizu et al., 2005), which might influence kinetics and function of circulating salmon IGFBP-1. Thus, the physiological role of IGFBP-1 in fish is unclear. The present study describes development of a radioimmunoassay (RIA) for salmon IGFBP-1. With this RIA, we measured plasma IGFBP-1 in

response to feeding ration and water temperature, and during the parr-smolt transformation, and compared it to growth, condition factor, and plasma IGF-I and 41-kDa IGFBP.

Materials and Methods

Fish, sampling procedure and rearing experiments

Rearing conditions: Yearling Chinook salmon (*Oncorhynchus tshawytscha*) and coho salmon (*O. kisutch*) were reared in fresh water at the Northwest Fisheries Science Center in Seattle, WA, USA. They were maintained in recirculated fresh water in circular fiberglass tanks under natural photoperiod; flow rate was 25 L/min; temperature ranged from 10.5°C to 13.0°C. Before fish were used for experiments, they were fed standard rations (0.6-1.0% body weight/day) of a commercial diet (Biodiet Grower; Bioproducts Inc., Warrenton, OR, USA). The experiments were conducted under guidelines of the University of Washington Institutional Animal Care and Use Committee.

Blood collection: Fish were anesthetized in 0.05% tricane methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA, USA). Blood was withdrawn by cutting the caudal peduncle and letting blood flow into a heparinized glass tube. Plasma was collected after centrifugation at 700*g* for 15 min and stored at -80°C until use.

Effect of fasting: Yearling Chinook salmon were fed at 52% of the maximum ration or fasted for six weeks (October to December). Blood was collected six weeks after treatment as described above.

Effect of feeding ration: Detailed experimental design was described in Beckman *et al*. (2004a). Briefly, yearling postsmolt coho salmon were individually tagged by passive integrated transponder (PIT)-tags (Destron). From June to September, four groups of fish were fed at 2% (HiFeed) or 1% (MedFeed) body weight/day, and feeding rates were maintained (Constant) through the experiment or decreased to 0.5% body weight/day for four weeks and returned to their original feeding rates (Variable). These combinations created four feeding groups: HiFeedConstant, HiFeedVaribale, MedFeedConstant and MedFeedVariable. Fish were sampled at two week

intervals. Instantaneous growth rate was calculated as: Growth (%/day) = (ln s_2 - ln s_1) x (d_2 - d_1)$^{-1}$ x 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 the number of days between measurements. Condition factor was calculated as: body weight (g)/body length (cm)^3 x 100. Five out of 147 fish were found to be precociously maturing males. IGFBP-1 levels in those fish were not included in the analysis since a disturbance in the relationship among IGF-I, 41-kDa IGFBP and growth rate has been reported in maturing males (Beckman *et al*., 2004a).

Effects of temperature and feeding ration: Detailed experimental design was described in Beckman et al (2004b). Briefly, individually tagged one-year-old coho salmon were reared at 11°C (Warm) or 7°C (Cool), and fed at 1.75% (HiFeed), 1.0% (MedFeed) or 0.5% (LowFeed) for nine weeks (from June to August). These combinations created four treatment groups: WarmHiFeed, WarmMedFeed, CoolMedFeed and CoolLowFeed. Fish were sampled at two or three week intervals.

Parr-smolt transformation: One-year-old coho salmon undergoing the parr-smolt transformation were sampled for blood every two weeks from March to July as described previously (Shimizu *et al*., 2003).

Sample analyses

Plasma IGF-I levels were measured by RIA as described in Shimizu *et al*. (2000). Briefly, IGF-I was first extracted from plasma by acid-ethanol and quantified by the RIA using recombinant salmon IGF-I as standard and tracer, and anti-recombinant barramundi IGF-I as primary antibody (GroPep Pty Ltd, Adelaide, Australia). Plasma 41-kDa IGFBP was quantified by a homologous salmon RIA as described in Shimizu *et al*. (2003). In this RIA, tracer was prepared from cross-linking purified 41-kDa IGFBP with ^{125}I-IGF-I.

Purification of salmon IGFBP-1

IGFBP-1 was purified from serum of spawning male Chinook salmon (Shimizu *et al*., 2005).

Briefly, salmon serum was first fractionated by ammonium sulfate precipitation and loaded onto an IGF-affinity column. IGFBP-1 was eluted from the column with 0.5M acetic acid and further purified by reversed-phase high pressure liquid chromatography (HPLC) on a Vydac C-4 column (0.46 x 5 cm; Separation Group, Hesperia, CA). Purified IGFBP-1 was quantified with the BCA Protein Assay Kit (Pierce Chemical, Rockford, IL), aliquoted into prelubricated microcentrifuge tubes (PGC Scientifcs, Frederick, MD) and stored at -80°C until use.

Preparation of antiserum

Polyclonal antiserum against purified IGFBP-1 (anti-IGFBP-1) was raised in a rabbit. Immunization of the rabbit was conducted in accordance with the guidelines of the Animal Care Committee of Hokkaido University, Japan. A total of 52 μg purified protein in 1 ml were emulsified in an equal volume of Freund's complete adjuvant (Iatoron; Tokyo, Japan). The rabbit was first immunized with 24 μg antigen by lymph node injection and boosted subcutaneously with 28 μg antigen in the back three weeks after the first injection. Two weeks after the boost, blood was withdrawn from the ear vein and antiserum was collected after centrifugation. The antiserum was stored at -30°C until use.

Preparation of tracers

Purified IGFBP-1 was iodinated with 0.5 mCi $Na^{125}I$ (Amersham Life Sicence Inc.) by the chloramine-T method. Five micrograms of IGFBP-1 in 31 μl were mixed with 41 μl 0.5 M phosphate buffer, pH 7.4. The mixture was reacted with 20 μl of 0.4 mg/ml chloramine-T (Sigma; St. Louis, MO, USA) for 90 sec and 20 μl of 0.6 mg/ml metabisulfite was added to stop the reaction. Iodinated IGFBP-1 (^{125}I-IGFBP-1) was separated from free $Na^{125}I$ using Biogel P-6 (1 x 18 cm; Bio-Rad). An aliquot of ^{125}I-IGFBP-1 (1.3 μg) was incubated with 4.3 μg salmon IGF-I (GroPep Pty Ltd, Adelaide, Australia) for 2 hr and they were cross-linked by disuccinimidyl suberate (Pierce; Rockford, IL, USA) according to manufacturer's instruction. The ^{125}I-IGFBP-1

cross-linked with salmon IGF-I (^{125}I-IGFBP-1/IGF-I) was separated from non-reacted IGF-I by gel filtration using Sephadex G-50 (1 x 18 cm, superfine; Pharmacia, Uppsala, Sweden). Specific activity of the tracer estimated by the self-displacement assay was 69.9 μCi/μg.

RIA for salmon IGFBP-1

RIA was carried out in 12 x 75 mm polystyrene test tubes. Purified IGFBP-1 was used for the standard. One hundred microliters of standard or plasma (10-20 μl) diluted in 20 mM phosphate, 150 mM NaCl, pH 7.4 containing 1.0% bovine serum albumin and 0.05% Triton X-100 were incubated with 100 μl anti-IGFBP-1 at a dilution of 1:2500 overnight at 4°C. Approximately 7000 cpm of tracer in 100 μl was added to the tubes and incubated overnight at 4°C. Free and antibody-bound tracers were separated by the addition of 0.5% Pansorbin (Calbiochem-Novabiochem Corp., La Jolla, CA, USA). After incubating overnight at 4°C, tubes were centrifuged at 1350*g* for 30 min and the supernatant was aspirated. Radioactivity in the pellets was measured by a gamma counter (Packard, Meriden, CT, USA). Standard and plasma samples were run in triplicate and duplicate, respectively, unless otherwise indicated.

Statistical analyses

Values of IGFBP-1, IGF-I and body weight were natural-log transformed to improve normality of distribution. Results of the experiments were analyzed by paired *t*-test, unpaired *t*-test or one-way analysis of variance (ANOVA) followed by the Fisher's protected least-significant difference (PLSD) test using the Statview 512+ program (Abacus Concepts, Inc., Berkeley, CA, USA). Simple regression was used to assess the relationship of IGFBP-1 to growth and other parameters. Differences between groups were considered to be significant at $P < 0.05$.

Results

Specific binding of ^{125}I-IGFBP-1/IGF-I was displaced by increasing amount of cold IGFBP-1 and

the displacement with the serial dilution of salmon serum was parallel with the standard (Fig. 1). Adding an excess amount of salmon IGF-I (1:100 molar ratio) to the standard did not alter the curve. The same lack of effect was seen with human IGF-I and IGF-II at various ratios (1:1, 1:10 and 1:100; data not shown). Cross-reactivity of other salmon IGFBPs in the RIA using ^{125}I-IGFBP-1/IGF-I was examined (Fig. 2). The 41-kDa IGFBP had no effect on displacing the binding. The 28-kDa IGFBP showed some displacement at higher concentrations, but its cross-reactivity was less than 0.5%, showing that the RIA is specific to IGFBP-1.

The specific and non-specific binding to the antiserum (1:2500 dilution) under the assay conditions were 24.3 ± 2.0% (mean ± S.E.M; n = 9) and 0.70 ± 0.04%, respectively. The half-maximal displacement (ED_{50}) occurred at 2.28 ± 0.13 ng/ml (n = 9). The ED_{80} and ED_{20} were 0.37 ± 0.03 ng/ml and 25.98 ± 1.54 ng/ml, respectively. The minimal detection limits of the assay, defined as the mean count of the zero standard minus two standard deviations, was 0.11 ± 0.03 ng/ml. The precision profile of the standard curve indicates that the functional sensitivity, defined as the concentration at which the inter-assay coefficients of variation is < 20%, was 0.05 ng/ml (n = 9). The intra- and inter-assay coefficients of variation estimated using a control serum were 5.3% (n = 8) and 4.6% (n = 9), respectively.

Effect of IGFs on measured IGFBP-1 was assessed by adding varying concentrations of IGFs to plasma (Table 1). Salmon IGF-I, human IGF-I and human IGF-II had no effect on IGFBP-1 levels up to 100 ng/ml, whereas 1000 ng/ml human IGFs significantly altered measured IGFBP-1 levels (paired t-test, $P < 0.05$). However, because circulating IGF levels in salmon rarely exceed 100 ng/ml and because the rank of IGFBP-1 levels in individuals was not altered, the IGF effect is practically not a problem in the assay. Recoveries of purified IGFBP-1 added to plasma with and without salmon IGF-I were 90.3-94.9% and 94.3-103.3%, respectively. These data show that IGFs do not interfere with the RIA. The RIA was also biologically validated as fasted fish had higher IGFBP-1 levels than fed fish (21.6 ± 4.6 ng/ml vs 3.0 ± 2.2 ng/ml, n = 11-14), which is in agreement with Western blotting analysis (Shimizu *et al*., 2005).

Using the validated RIA, the response of the circulating IGFBP-1 to feeding ration was examined in postmolt coho salmon (Fig. 3). When fish were reared under two different feeding rations (HiFeed and MedFeed), IGFBP-1 levels were higher in MedFeed groups (Fig. 3b). In HiFeedVariable group, a decrease in feeding ration caused an increase in IGFBP-1 within two weeks. A subsequent increase in feeding to the original amount caused a decrease in IGFBP-1 to the original level. In MedFeedVariable group, the same trend was seen in response to a decrease in feeding ration except that the elevation in IGFBP-1 took four weeks to be significant.

The results of simple regression of IGFBP-1 with growth and morphological parameters are shown in Table 2. For most dates, IGFBP-1 was negatively correlated with body weight, condition factor, growth rates and 41-kDa IGFBP. No significant relation was found between IGFBP-1 and IGF-I on any given date (Table 2, Fig. 4b), whereas a consistent positive relation was evident between 41-kDa IGFBP and IGF-I (Fig. 4a). IGFBP-1 appears to show the strongest negative relationship to condition factor (Table 2, Fig. 4c).

Effects of temperature and feeding ration were also examined in postsmolt coho salmon (Fig. 5). Although IGFBP-1 levels fluctuated during the first two sampling dates, they were separated by temperature (Fig. 5b). On the third sampling date, feeding ration became a major factor separating IGFBP-1 levels. Temperature might still have some effect as the differences in IGFBP-1 levels were close to being significant between WarmMedFeed and CoolMedFeed (P = 0.0521). At the end of the experiment, IGFBP-1 levels were ranked by feeding ration only.

Changes in IGF-I, 41-kDa IGFBP, IGFBP-1 and condition factor were assessed during the parr-smolt transformation of coho salmon. IGFBP-1 showed a transient peak in late April, which corresponds to the second peak in IGF-I (Fig. 6a,c). The change in IGFBP-1 was opposite to the change in condition factor (Fig. 6c,d). The inverse relationship between IGFBP-1 and condition factor was best represented by a exponential curve fitting ($r^2 = 0.416$, $P < 0.0001$).

Discussion

We developed a specific RIA for salmon IGFBP-1, which is the first RIA for a non-mammalian IGFBP-1. This assay should facilitate quantitative studies of the role of IGFBP-1. Most previous studies of fish IGFBPs have used Western ligand blots for semi-quantifying IGFBP levels. RIAs have a number of advantages over the Western ligand blot analyses, including greater precision and capacity for measuring large numbers of samples. There are additional challenges in developing an RIA for a binding protein in that the ligand (IGF) in samples may interfere with assay performance. For example, we found during development of the RIA for salmon 41-kDa IGFBP that IGF interfered with the assay when the binding protein was directly labeled with ^{125}I (Shimizu *et al.* 2003). Interference was avoided by using labeled IGF cross-linked to the 41-kDa IGFBP. Similarly for the salmon IGFBP-1 RIA, IGF interference was avoided by using labeled IGFBP-1 cross-linked to IGF. Labeled IGF cross-linked to unlabeled IGFBP-1 resulted in a less sensitive assay, probably due to lower specific activity (data not shown). Eliminating interference by IGF allows direct assay of plasma samples without extraction. Although purified Chinook salmon IGFBP-1 was used for standard and label, the coho salmon samples showed parallel displacement, suggesting that the assay could be used for other salmonids.

Elevation of circulating IGFBP-1 during fasting is a well-known response in a wide range of vertebrates including fish (Busby *et al*., 1988; Shiharath *et al*., 1996). In most experiments, the two extreme nutritional conditions of fasting and feeding *ad libitum* were compared. On the other hand, much less is known about the response of IGFBP-1 to moderate nutritional change. In humans, caloric restriction of 50% for six days resulted in an increase in plasma IGFBP-1 in adults but not in children (Smith *et al*., 1995). Dietary energy restriction of 42.5% and 56 % for two weeks had no effect on IGFBP-1 in dogs (Mawell *et al*., 1998). Plasma IGFBP-1 levels decreased in guinea pigs fed rations of 70% of *ad libitum* levels for 80 days (Sohlström *et al*., 1998). These results indicate that changes in IGFBP-1 in response to moderate feed restriction differ depending on stage and species. In the present study, the effect of ration on circulating IGFBP-1 was assessed in growing coho salmon. When ration was reduced from 2% to 0.5% body weight per

day, IGFBP-1 increased at two weeks, whereas reducing ration from 1% to 0.5% increased IGFBP-1 at four weeks. The later response with the more moderate reduction in ration suggests that the relative change in food intake is an important cue for inducing IGFBP-1. Alternatively, the fish fed on the higher ration (2%) may be more sensitive to reduction in food intake. The response of IGF-I and 41-kDa IGFBP to ration reduction was generally opposite to that of IGFBP-1; they declined by two weeks at the 2% to 0.5% ration change (Beckman *et al*., 2004a). However, IGF-I did not decline in response to the 1% to 0.5% ration change, suggesting that IGFBP-1 may be more sensitive than IGF-I to ration change. Overall, the results indicate that IGFBP-1 is quite responsive to moderate ration change in salmon.

Environmental temperature is a crucial factor affecting metabolic rate of poikilotherm and the change in the metabolic setting by temperature, in turn, may alter the endocrine system. In Atlantic salmon (*Salmo salar*), hormonal changes associated with parr-smolt transformation were limited by lowering temperature (McCormick *et al*., 2000). A relatively short-term effect of temperature change (one week) on insulin and IGF-I has been reported in coho salmon; a drop of temperature increased insulin and decreased IGF-I, respectively (Larsen *et al*., 2001). Temperature also affects IGFBP in catfish (*Ictalurus punctatus*) (Johnson *et al*., 2003). Increasing temperature from 21°C to 26 °C resulted in an induction of a 19-kDa IGFBP whereas other IGFBPs remained unchanged. We have previously shown that salmon 41-kDa IGFBP as well as IGF-I was temporally affected by temperature (Beckman *et al*., 2004b). In the present study, temperature change appeared to disrupt IGFBP-1 levels for at least six weeks as feeding level had little relation to plasma IGFBP-1 level until the seventh week of the experiment. After this acclimation period feeding level again appeared to be the primary determinant of IGFBP-1 as fish receiving less feed had higher levels and temperature had no effect on IGFBP-1 at the end of the experiment (nine weeks). These results suggest that it took nine weeks for fish to adjust IGFBP-1 levels to the different temperature. The response of IGF-I, 41-kDa IGFBP and IGFBP-1 to temperature change appears to differ. Lowering temperature resulted in a decrease in IGF-I and an increase in 41-kDa

IGFBP (Beckman *et al*., 2004b), whereas IGFBP-1 response was in both directions. Gabillard *et al*. (2003) found that higher environmental temperature increased plasma GH levels in rainbow trout (*Oncorhynchus mykiss*). These findings suggest that temperature influences the somatotropic axis not simply through changing metabolic rate, which would result in all components of the axis changing similarly, but through specific responses for each component.

We also studied changes in circulating IGFBP-1 levels during smoltification, which is a pre-adaptation to ocean life accompanied by many hormonal changes including IGF-I (Dickhoff *et al*., 1997). Shimizu *et al*. (2003) reported that IGF-I showed two peaks during the smolting process; one in late March and one in late April. In the present study, plasma IGFBP-1 levels in the same samples used in Shimizu *et al*. (2003) showed a peak in late April, which corresponds to the second peak in IGF-I. However, IGFBP-1 and IGF-I levels were not correlated (data not shown) similar to the result in the feeding experiment. The increase in IGFBP-1 may be driven by an increase in cortisol, which becomes elevated during smoltification. In contrast, a peak of 41-kDa IGFBP corresponded to the first peak in IGF-I, and their levels were positively correlated. These findings indicate that the IGF-I/IGFBP system changes during smoltification and suggest different roles of IGFBP-1 and 41-kDa IGFBP in this process. The significance of the change in IGF binding proteins during smoltification is unknown.

We analyzed the relationship of IGFBP-1 to growth, fish size, condition factor, IGF-I and 41-kDa IGFBP in individually tagged fish from the feeding experiment. Simple regression analysis revealed a clear trend that circulating IGFBP-1 is negatively correlated with body weight, condition factor, growth rates and 41-kDa IGFBP. These results support the hypothesis that IGFBP-1 is generally inhibitory to growth. Our findings are in agreement with studies in humans showing that IGFBP-1 is inversely related to anthropometric and endocrine factors (Travers *et al*., 1998; Voskuil *et al*., 2001; Wolk *et al*., 2004). Among the growth and morphometric factors tested in the present study, the strongest and most consistent negative relation was with condition factor (body weight/body length^3). The negative relation between IGFBP-1 and condition factor was

present in both sets of data analyzed; one from the ration manipulation and the other from the smoltification study. In the experiment where some fish received reduced ration, the decline in condition factor was due to a greater loss in weight relative to growth in length. Growth in length ceased but did not become negative, and some individual fish lost weight due to dietary restriction. The group that went from 2% to 0.5% ration had a significant weight loss by two weeks. In the study of smoltification, it is well established that the decline in condition factor is due to a more rapid growth in length relative to growth in weight (Winans & Nishioka, 1987). Thus, the inverse relationship between IGFBP-1 and condition factor is present during nutritional restriction and development of growing fish. In humans a strong relationship was observed between IGFBP-1 and body mass index (BMI: body weight/height^2), which is similar to condition factor of fish. The inverse relationship in humans held for early pubertal children (Travers *et al*., 1998), premenopausal women (Voskuil *et al*., 2001), and middle-aged and elderly men (Wolk *et al*., 2004). The underlying mechanisms for the relationship of lean body index and high IGFBP-1 in humans and salmon is not known, but invites additional study in other species.

Findings from the present study support different roles of salmon 41-kDa IGFBP and IGFBP-1 in regulating IGF-I activity. The 41-kDa IGFBP is the main carrier of circulating IGF-I as its levels are generally highly correlated with IGF-I levels (Shimizu *et al*., 2003; Beckman *et al*., 2004a,b). On the other hand, salmon IGFBP-1 can not be a main carrier of IGF-I based on 1) the lack of correlation with IGF-I levels, and 2) the molar concentrations of IGFBP-1 in blood is an order of magnitude lower than those of total IGF-I and 41-kDa IGFBP. In mammals, IGFBP-1 is postulated to be an important regulator of free IGF-I levels, which are biologically active and available to bind with IGF receptor (Frystyk *et al*., 1994). This hypothesis has recently been tested by *in vivo* infusion of human IGFBP-1 into catheterized rats. Infused human IGFBP-1 did not significantly alter the plasma concentration of total IGF-I, but decreased circulating free IGF-I levels (Lang *et al*., 2003). Although free IGF-I levels were not measured in the present study, a possible role of salmon IGFBP-1 in the regulation of free IGF-I may explain why IGFBP-1 shows

no correlation with total IGF-I despite negative correlations with growth rates.

In conclusion, we developed a specific RIA for salmon IGFBP-1 for the first time and analyzed its regulation by food intake and temperature, and during smoltification. A range of moderate nutritional and temperature manipulations indicate that they are critical factors controlling circulating salmon IGFBP-1. Regression analysis revealed that plasma IGFBP-1 is negatively correlated with growth and condition factor, among others. These findings suggest that growth-inhibitory action of IGFBP-1 is conserved in salmon.

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Figure legends

Fig. 1 Displacement of ^{125}I-IGFBP-1/IGF-I by purified IGFBP-1 with or without IGF-I, and Chinook salmon serum. Increasing amounts of purified IGFBP-1, IGFBP-1 incubated with excess salmon IGF-I (1:100 molar ratio) and serum from spawning male Chinook salmon were added to the assay. Each point is mean of duplicate.

Fig. 2 Cross-reactivity of salmon IGFBPs in the RIA. Displacement of tracer was assessed by adding increasing amounts of purified salmon IGFBP-1, 28- and 41-kDa IGFBPs to the assay. Each point is mean of duplicate.

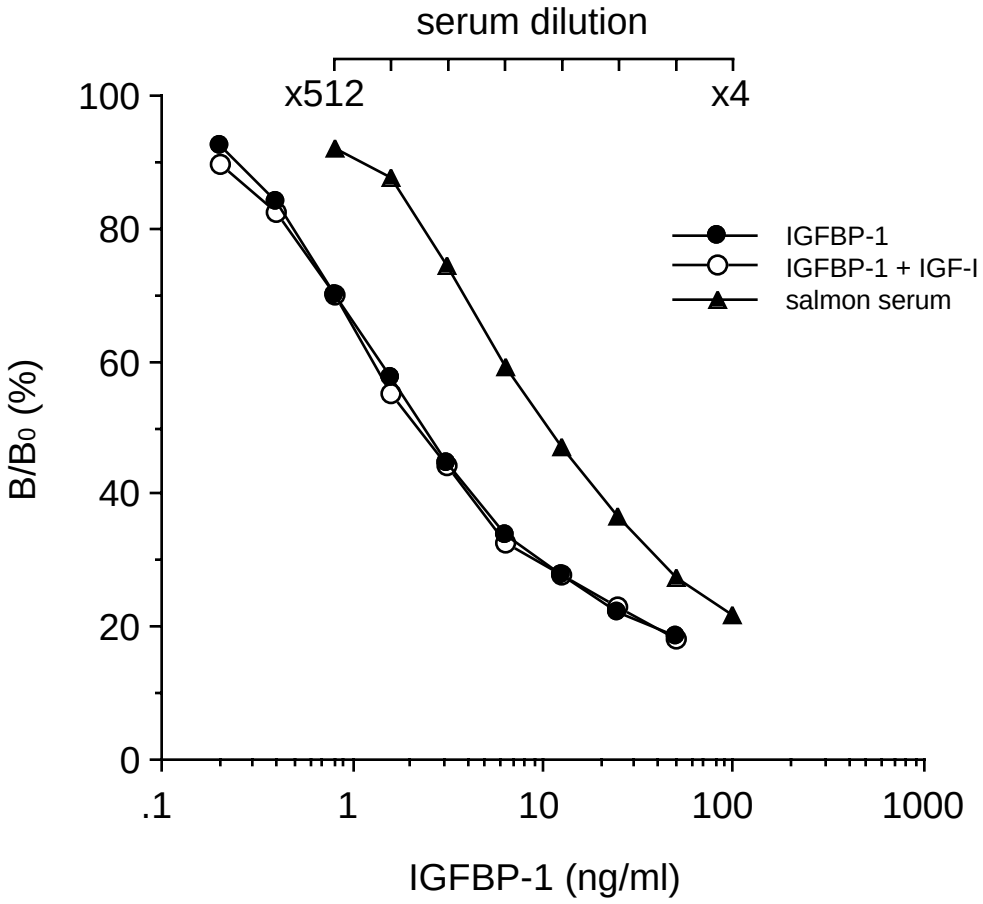
Fig. 3 Effect of feeding ration and its change on plasma IGFBP-1 levels. (a) Experimental design. Postsmolt coho salmon were reared under high (2% body weight/day) or medium (1%) ration (HiFeed or MedFeed, respectively). Feeding rations were maintained or changed during the experiment (Constant or Variable, respectively). (b) Profiles of plasma IGFBP-1 levels measured by RIA. One-way ANOVA followed by Fisher's PLSD test was performed after natural-log transforming IGFBP-1 values. Symbols sharing the same letters are not significantly different from each other for a given time point (Fisher's PLSD, $P < 0.05$). n = 6-10 per point.

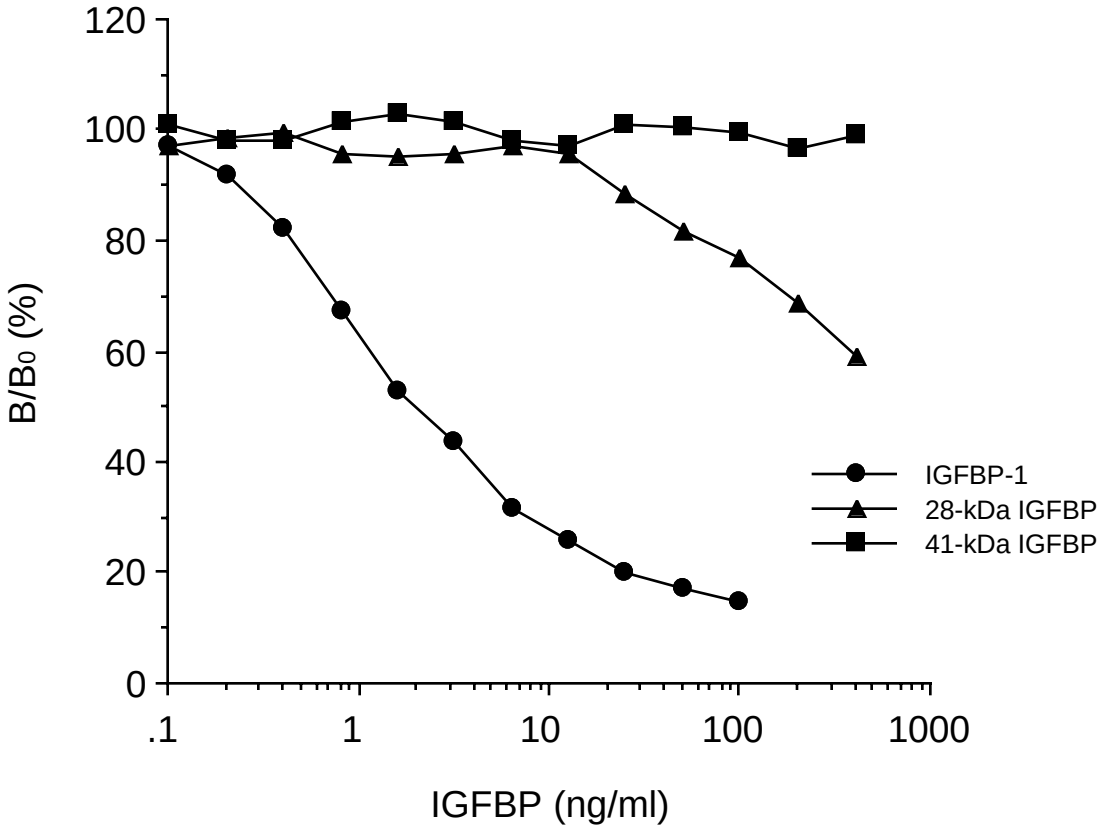
Fig. 4 Representative correlations between IGF-I and 41-kDa IGFBP (a), IGF-I and IGFBP-1 (b), and condition factor and IGFBP-1 (c) on July 26 (week 2). Values of IGF-I and IGFBP-1 were natural-log transformed (ln). Data on IGF-I and 41-kDa IGFBP are from Beckman *et al.* (2004a).

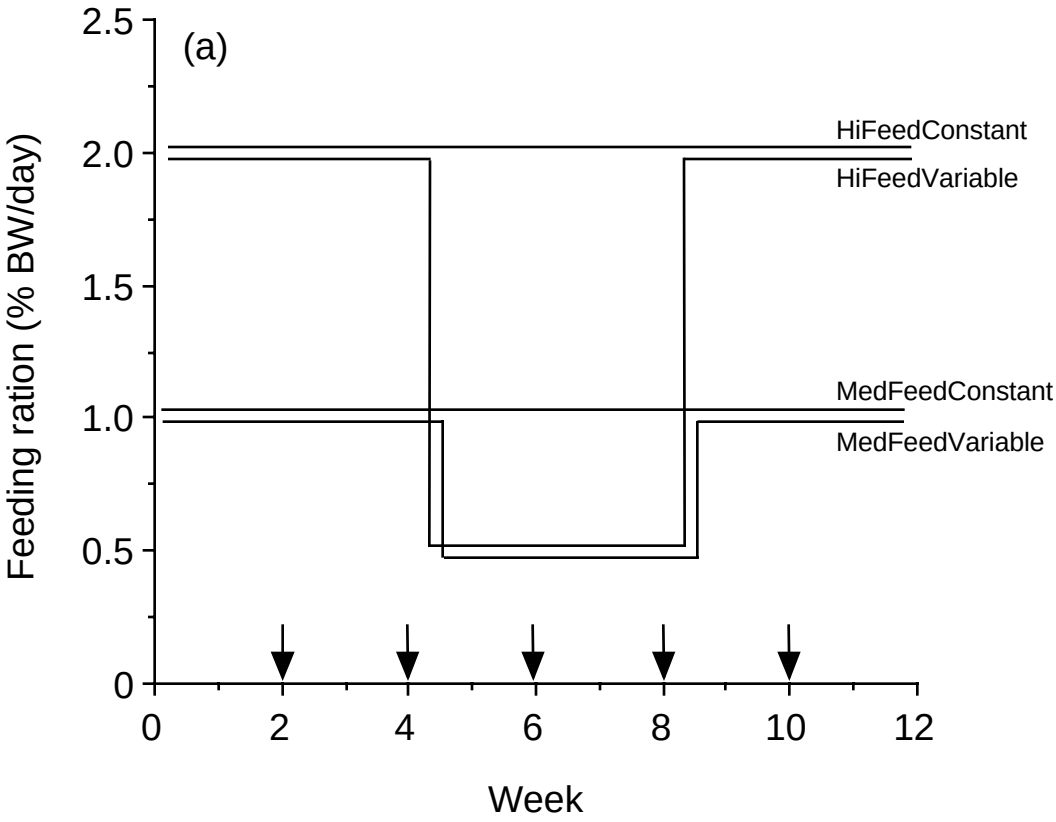
Fig. 5 Effects of temperature and feeding ration on plasma IGFBP-1 levels. (a) Experimental design. Postsmolt coho salmon were reared under warm (11°C) or cool (7°C) water temperature. Fish were fed at high (1.75% body weight/day), medium (1%) or low (0.5%) ration (HiFeed, MedFeed or LowFeed, respectively). (b) Profiles of plasma IGFBP-1 levels measured by RIA.

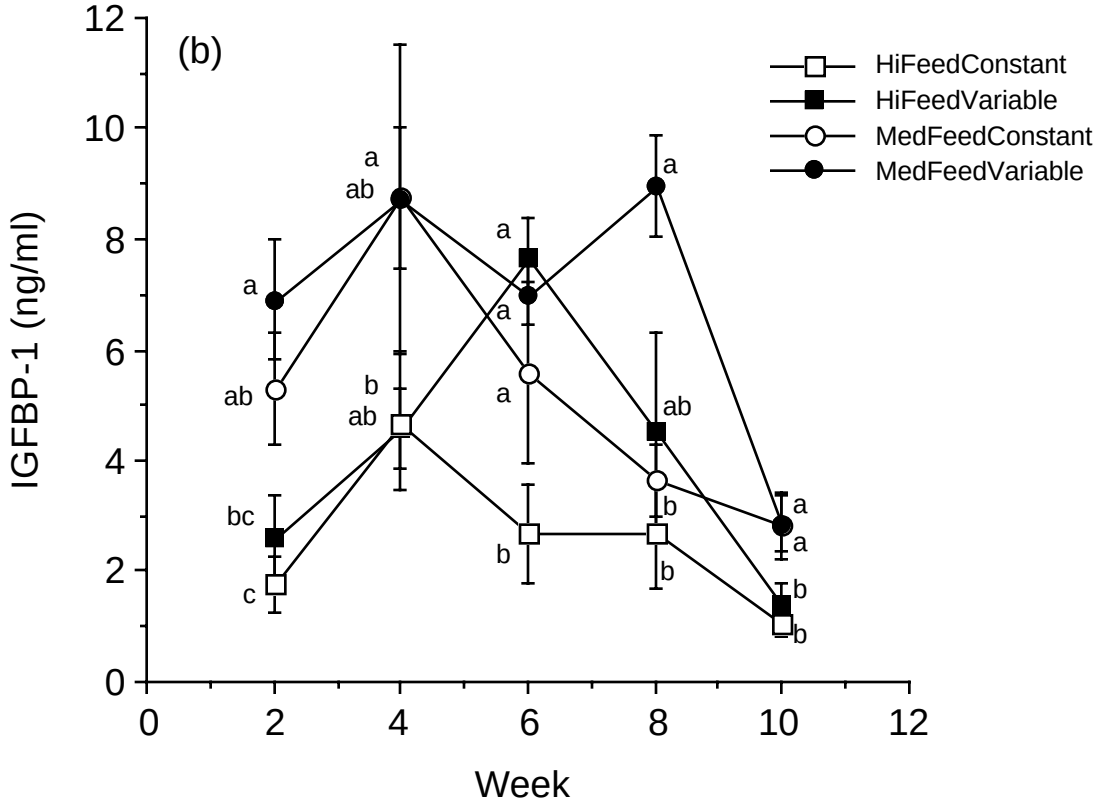
One-way ANOVA followed by Fisher's PLSD test was performed after natural-log transforming IGFBP-1 values. Symbols sharing the same letters are not significantly different from each other for a given time point (Fisher's PLSD, $P < 0.05$). n = 6-11 per point.

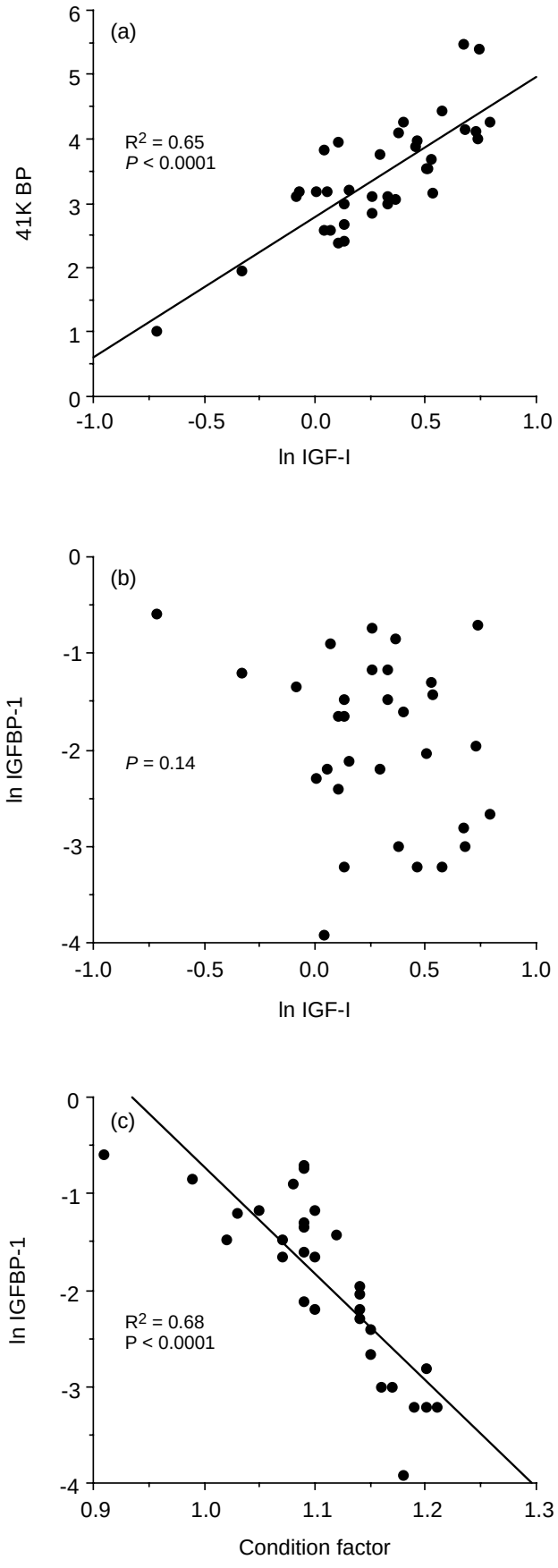
Fig. 6 Changes in plasma IGF-I (a), 41-kDa IGFBP (b), IGFBP-1 (c) levels and condition factor (d) during parr-smolt transformation. Blood samples were collected from one-year-old coho salmon during parr-smolt transformation. Condition factor was calculated as body weight (g)/body length $(cm)^3$ x 100. n = 11-12 per point. Graphs on IGF-I and 41-kDa IGFBP were reproduced from Shimizu *et al*. (2003) with permission. For statistical analyses, values of IGF-I, 41-kDa IGFBP and IGFBP-1 were natural-log transformed. Symbols sharing the same letters are not significantly different from each other for a given parameter (one-way ANOVA followed by Fisher's PLSD, $P < 0.05$).

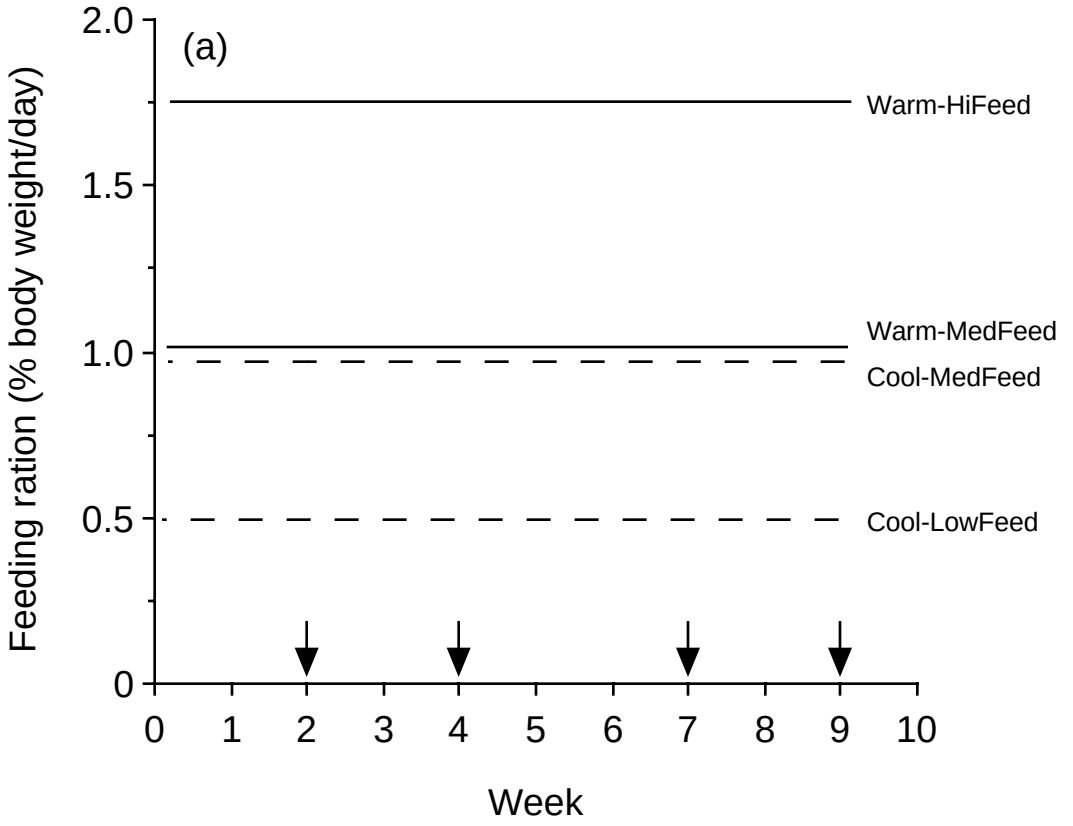

serum dilution
x512
x4
100
80
60
40
20
0
B/B0 (%)
.1
1
10
100
1000
IGFBP-1 (ng/ml)
IGFBP-1
IGFBP-1 + IGF-I
salmon serum

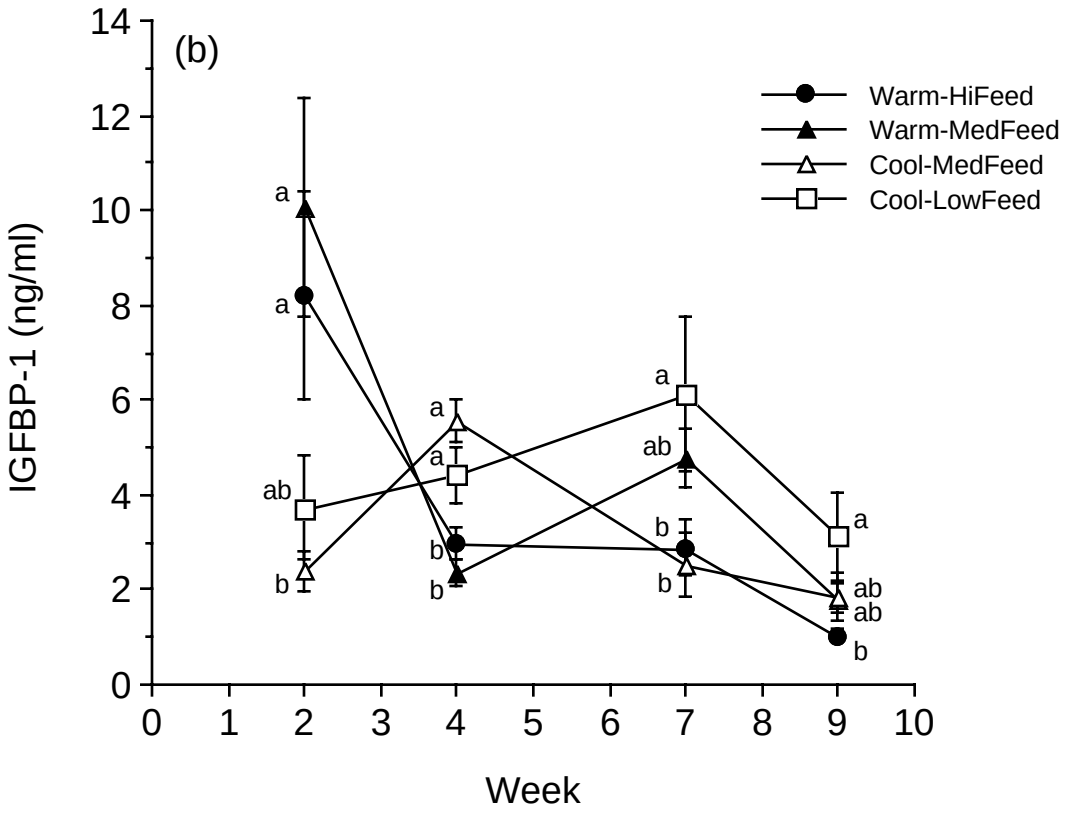

120
100
80
60
40
20
0
B/B0 (%)
.1
1
10
100
1000
IGFBP (ng/ml)
IGFBP-1
28-kDa IGFBP
41-kDa IGFBP


(a)
Feeding ration (% BW/day)
2.5
2.0
1.5
1.0
0.5
0
HiFeedConstant
HiFeedVariable
MedFeedConstant
MedFeedVariable
0
2
4
6
8
10
12
Week


(b)
IGFBP-1 (ng/ml)
12
10
8
6
4
2
0
HiFeedConstant
HiFeedVariable
MedFeedConstant
MedFeedVariable
a
ab
a
ab
b
ab
a
a
a
ab
a
ab
bc
c
b
b
b
b
a
a
b
b
0
2
4
6
8
10
12
Week


(a)
R2 = 0.65
P < 0.0001
41K BP
ln IGF-I
(b)
P = 0.14
ln IGFBP-1
ln IGF-I
(c)
R2 = 0.68
P < 0.0001
ln IGFBP-1
Condition factor


(a)
Feeding ration (% body weight/day)
2.0
1.5
1.0
0.5
0
Warm-HiFeed
Warm-MedFeed
Cool-MedFeed
Cool-LowFeed
0 1 2 3 4 5 6 7 8 9 10
Week


(b)
IGFBP-1 (ng/ml)
14
12
10
8
6
4
2
0
Warm-HiFeed
Warm-MedFeed
Cool-MedFeed
Cool-LowFeed
a
a
ab
b
a
a
b
b
a
ab
b
b
a
ab
ab
b
0 1 2 3 4 5 6 7 8 9 10
Week

Shimizu et al., Fig. 6

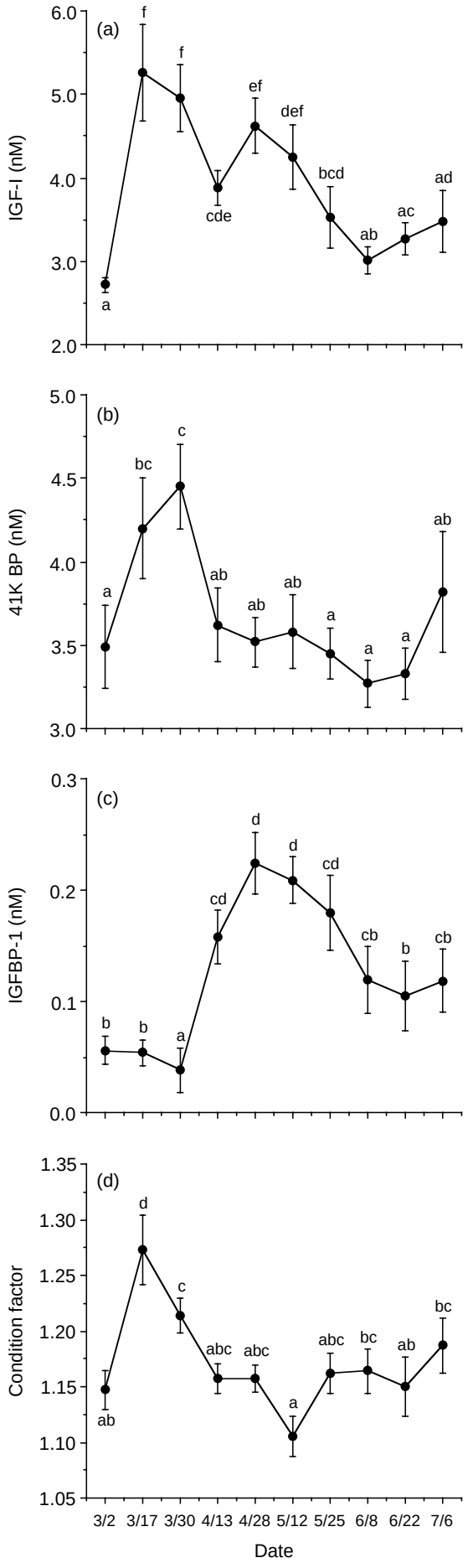


Table 1. Effect of IGFs on measured IGFBP-1 levels

Treatment	Dose (ng/ml)	IGFBP-1 (ng/ml)
plasma only		23.39 ± 5.46
salmon IGF-I	10	24.42 ± 6.35
	100	24.85 ± 5.51
	1000	27.18 ± 7.23
human IGF-I	10	23.31 ± 2.30
	100	24.44 ± 5.95
	1000	25.98 ± 6.19 *
human IGF-II	10	24.03 ± 5.69
	100	25.96 ± 6.74
	1000	28.56 ± 7.30 *

Values are mean ± S.E. (n = 5).
Asterisks (*) indicate values are significantly different from control (paired t-test, $P < 0.05$).

Table 2. Negative relations of ln IGFBP-1 to growth and metabolic parameters

Week	Date	P	r2	Week	Date	P	r2
vs length				*vs IG weight*			
2	26-Jul	0.1294	ns	2	26-Jul	0.0002	0.38
4	10-Aug	0.1543	ns	4	10-Aug	0.1536	ns
6	24-Aug	0.0216	0.23	6	24-Aug	0.0001	0.52
8	8-Sep	0.2068	ns	8	8-Sep	0.0181	0.29
10	27-Sep	0.0416	0.11	10	27-Sep	0.0021	0.24
vs ln weight				*vs ln IGF-I*			
2	26-Jul	0.0081	0.22	2	26-Jul	0.1390	ns
4	10-Aug	0.0278	0.16	4	10-Aug	0.3001	ns
6	24-Aug	0.0008	0.42	6	24-Aug	0.1632	ns
8	8-Sep	0.0185	0.29	8	8-Sep	0.2105	ns
10	27-Sep	0.0069	0.19	10	27-Sep	0.0911	ns
vs Condition factor				*vs 41-kDa IGFBP*			
2	26-Jul	< 0.0001	0.68	2	26-Jul	0.0071	0.22
4	10-Aug	0.0004	0.35	4	10-Aug	0.0033	0.26
6	24-Aug	< 0.0001	0.59	6	24-Aug	0.0013	0.40
8	8-Sep	0.0021	0.44	8	8-Sep	0.1246	ns
10	27-Sep	< 0.0001	0.38	10	27-Sep	0.0007	0.27
vs IG length							
2	26-Jul	0.0523	ns				
4	10-Aug	0.0596	ns				
6	24-Aug	0.0005	0.44				
8	8-Sep	0.0149	0.32				
10	27-Sep	0.0006	0.28				

Data on instantaneous growth (IG), IGF-I and 41-kDa IGFBP levels are from Beckman et al. (2004a)
Slopes of regression lines are all negative.
ln: natural log-trasformed
ns: not significant