Title

Effects of seawater transfer and fasting on the endocrine and biochemical growth indices in juvenile chum salmon (*Oncorhynchus keta*)

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Abstract

Insulin-like growth factor (IGF)-I, IGF-binding protein (IGFBP)-1 and RNA/DNA ratio are endocrine and biochemical parameters used as growth indices in fish, however, they are subjected to environmental modulation. Chum salmon (*Oncorhynchus keta*) migrate from freshwater (FW) to seawater (SW) at fry/juvenile stage weighing around 1 g and suffer growth-dependent mortality during the early phase of their marine life. In order to reveal environmental modulation of the IGF/IGFBP system and establish a reliable growth index for juvenile chum salmon, we examined effects of SW transfer and fasting on IGF-I, IGFBP-1 and RNA/DNA ratio, and correlated them to individual growth rate. Among serum IGF-I, liver and muscle *igf-1*, *igfbp-1a*, *igfbp-1b* and RNA/DNA ratio examined, muscle RNA/DNA ratio and muscle *igfbp-1a* responded to SW transfer. Serum IGF-I, liver *igf-1* and liver RNA/DNA ratio were sensitive to nutritional change by being reduced in 1 week in both FW and SW while muscle *igf-1* was reduced 2 weeks after fasting. In contrast, *igfbp-1a* in both tissues was increased by 2 weeks of fasting and *igfbp-1b* in the liver of SW fish was increased in 1 week. These results suggest that the sensitivity of *igf-1* and *igfbp-1*s to fasting differs between tissues and subtypes, respectively. When chum salmon juveniles in SW were marked and subjected to feeding or fasting, serum IGF-I showed the highest correlation with individual growth rate. Liver *igfbp-1a* and *-1b*, and muscle *igf-1* exhibited moderate correlation coefficients with growth rate. These results show that serum IGF-I is superior to the other parameters as a growth index in juvenile chum salmon in term of its stability to salinity change, high sensitivity to fasting and strong relationship with growth rate. On the one hand, when collecting blood from chum salmon fry/juveniles is not practical, measuring liver *igfbp-1a* and *-1b*, or/and muscle *igf-1* is an alternative.

Keywords

insulin-like growth factor-I, insulin-like growth factor binding protein, salmon, salinity, fasting, growth index

1. Introduction

Insulin-like growth factor (IGF)-I is a 7.5-kDa polypeptide structurally similar to proinsulin and promotes growth of animals (Daughaday and Rotwein, 1989). IGF-I circulates in the blood at a relatively high level (~200 ng/ml) and acts as a classical hormone. Liver is the major site of circulating IGF-I producing 75% of endocrine IGF-I under stimulation by growth hormone (GH). IGF-I is also expressed in many tissues and exerts its actions through paracrine and autocrine manners (Daughaday and Rotwein, 1989; Le Roith et al., 2001). IGF-I is essential for normal postnatal growth, where local IGF-I is more important than endocrine IGF-I in mice (Le Roith et al., 2001; Ohlsson et al., 2009). Liver-derived endocrine IGF-I contributes to 30% of postnatal growth and plays a critical role in regulating GH secretion by the pituitary gland via a negative feedback loop (Le Roith et al., 2001; Ohlsson et al., 2009).

IGF-I is distinct from insulin in terms of the presence of high-affinity binding proteins. IGF-binding proteins (IGFBPs) regulate availability of IGF-I to target tissues and modulate its activity (Jones and Clemmons, 1995; Rajaram et al., 1997; Firth and Baxter, 2002). There are six IGFBPs in mammals and different types have different functions. IGFBP-1 is one of the major circulating IGFBPs and generally an inhibitor of IGF-I actions by preventing it from interacting with the receptor (Lee et al., 1993; Kajimura and Duan, 2007). IGFBP-1 is mainly produced by the liver but other tissues also express it. IGFBP-1 shows dynamic fluctuations in response to fasting and stress, and its increased level is often a sign of catabolic states.

In teleosts, the importance of IGF-I in growth, development, osmoregulation and maturation has been widely recognized (Wood et al., 2005; Reinecke 2010). Although the relative importance of endocrine and local IGF-I in fish growth has not been elucidated, circulating IGF-I generally reflects growth status (Picha et al., 2008a; Beckman, 2011). IGFBPs are also present in teleosts and their structure is well conserved (Wood et al., 2005; Kelley et al., 2006). However, due to an extra round of whole genome duplication in the teleost lineage, fish often have two copies of the each member of IGFBPs (Daza et al., 2011). Two *igfbp-1* subtypes were first identified in zebrafish (*Danio rerio*) (Kamei et al., 2008). Zebrafish IGFBP-1 co-orthologs were expressed predominantly in the liver and exhibited overlapping inhibitory actions on cell proliferation, although temporal expression patterns during embryonic development, response to hypoxia and affinity for IGF were different (Kamei et al., 2008). We identified two of three major IGFBPs of Chinook salmon (*Oncorhynchus tshawytscha*) as

IGFBP-1a and -1b and showed that both subtypes were induced under osmotic stress, suggesting that they have inhibitory actions on IGF-I (Shimizu et al., 2011). However, sites of their expression were different; *igfbp-1b* was exclusively expressed in the liver while *igfbp-1a* was detected in a variety of tissues (Shimizu et al., 2011). Recent detailed genomic analyses by Macqueen et al. (2013) revealed that salmonid species have 19 IGFBPs due to the tetraploidy origin of this group and the two IGFBP-1 subtypes of Chinook salmon corresponded to *igfbp-1a1* and *-1b1*, respectively. Tissue distributions of Atlantic salmon (*Salmo salar*) *igfbp-1a1* and *-1b1* were also different as is the case for Chinook salmon (Macqueen et al., 2013). These findings suggest that salmon IGFBP-1 subtypes partition their IGF-inhibitory actions, where the modes of IGFBP-1a and -1b actions are mainly local and systemic, respectively.

Analyses of the environmental modulation of IGF-I and IGFBPs in fish are important to understand how growth is adjusted to maximize performance and survival. Food availability, temperature, stress, salinity and water quality are major environmental factors affecting fish growth. Among these factors, the effect of feeding ration or/and fasting on circulating IGF-I in fish has been most intensively studied (Picha et al., 2008a; Beckman, 2011). In post-smolt coho salmon (*O. kisutch*), plasma IGF-I well reflected feeding status and growth rate (Beckman et al., 2004). In contrast, plasma IGFBP-1a and -1b levels were inversely related to feeding and growth status (Kawaguchi et al., 2013). Acute stress such as hypoxia, direct seawater transfer and handing increased tissue or circulating *igfbp-1*/IGFBP-1 in several fish species (Kelley et al., 2006; Kamei et al., 2008; Shimizu et al., 2011) and chronic stress reduces growth most likely through suppressing circulating IGF-I levels. Although the liver is the major site of production of circulating IGF-I and IGFBP-1, other tissues also express them and locally regulate growth. However, it is not well understood how local *igf-1* and *igfbp-1* respond to a change in food availability.

Adjusting growth under different salinities is crucial for euryhaline fishes and such modulation should be achieved by changes in IGF-I and IGFBPs. Salinity could affect IGF-I and IGFBPs directly at local tissues or indirectly through changes in GH and cortisol (Reinecke, 2010). Gill *igf-1* and in some case liver *igf-1* were shown to change when rainbow trout (*O. mykiss*) were transferred from freshwater (FW) to seawater (SW) (Sakamoto and Hirano, 1993; Poppinga et al., 2007). Plasma IGF-I in trout was also increased in a higher salinity (66% SW; Shepherd et al., 2005). Despite relatively a large number studies examined response of local or circulating IGF-I to salinity change in fish, there is no consensus on the direction of response; It

111 appears to vary depending on species, stages, salinity and duration (Reinecke et al., 2010;
112 Beckman, 2011). On one hand, only a few studies using salmonids examined effect of salinity
113 on plasma IGFBPs (Shepherd et al., 2006; Shimizu et al., 2007; 2011) and responses of *igfbp*
114 mRNAs to salinity change are scarce.
115 In addition to the mechanistic importance of IGF-I and IGFBPs in growth regulation,
116 they have drawn much attention from fish endocrinologists/biologists because of their potential
117 utility as growth indices (Picha et al., 2008a; Beckman, 2011). Estimating growth rate of fish is
118 important in aquaculture, and IGF-I and IGFBP-1 have been proposed as positive and negative
119 growth indices, respectively. Evaluating growth status of larvae, fry and juvenile fish is also
120 relevant to stock assessment since growth-dependent mortality is a key determinant of stock
121 recruitment (Beamish and Mahnken, 2001). Circulating IGF-I is so far the most reliable growth
122 index in several fishes (Picha et al., 2008a; Beckman, 2011). However, it is not clear whether
123 *igf-1* mRNA can be used as an alternative of plasma IGF-I. Moreover, utility of *igfbp-1a* and *-1b*
124 as negative growth indices are not fully validated.
125 Chum salmon (*O. keta*) is one of the eight Pacific salmon and an obligatory
126 anadromous species; They hatch in freshwater and all juveniles weighing around 1 g migrate to
127 SW in the first spring. This species is an important commercial fish in Japan and target of
128 intensive hatchery release. However, their returns fluctuate between years and local regions
129 (Miyakoshi et al., 2013). They are also believed to suffer growth-dependent mortality during the
130 early phase of their marine life (Bax, 1983; Fukuwaka and Suzuki, 2002; Wertheimer and
131 Thrower, 2007). Therefore, an accurate assessment of growth status of juvenile chum salmon at
132 the estuary and nearshore is important to assess their survival. We have recently shown that
133 plasma IGF-I is a good growth index in juvenile chum salmon in SW (Kaneko et al., 2015).
134 However, it is not known if salinity change affects basal IGF-I levels and its sensitivity to food
135 deprivation. Moreover, since chum salmon juveniles are sometimes so small that collecting
136 blood at field survey is not practical, availability of other growth indices that can be used as
137 alternative to serum IGF-I is valuable. The aims of the present study are twofold: 1) to analyze
138 responses of IGF-I/*igf-1* and *igfbp-1*s to salinity change and fasting, and 2) to examine their
139 utility as growth indices by comparing with a biochemical growth index, RNA/DNA ratio.
140
141 **2. Materials and methods**
142
143 *2.1. Fish and rearing experiments*

In May 2014, underyearling chum salmon (fork length (FL): 4.58 ± 0.08 cm; body weight (BW): 0.64 ± 0.03 g) were obtained from a local hatchery (Kamisato Hatchery) in Abashiri area, northeastern Hokkaido, Japan, transferred to the rearing facility at Faculty of Fisheries Sciences, Hokkaido University and reared in freshwater glass aquariums (60 × 29.5 × 36 cm^3; water volume: 60L) in a temperature-controlled room (10°C). Each aquarium was a closed-circulation system installed with a portable upper filter system. Fish were fed daily on a commercial diet (Marubeni Nisshin Feed Co. Ltd., Tokyo, Japan) to satiety. Three days after transfer, fish were divided into two groups: FW and SW groups. SW group was acclimated to full-strength (33 ppt) artificial SW (Marine Salt Pro; Spectrum Brands Inc., Tokyo, Japan) by increasing the salinity stepwisely over 5 days (6.6 ppt/day). Salnity was monitored by using a salinity meter (MotherTool, Nagano, Japan). They were transferred to four SW tanks and acclimated for 2 additional days. No mortality occurred during SW transfer. FW group was transferred to four FW tanks and acclimated for 2 days. Fish were fed during the acclimation/transfer period. Each group was further divided into Fed and Fasted groups to make four treatments (20 fish/treatment): FW-Fed, FW-Fasted, SW-Fed and SW-Fasted. Two tanks were used for each treatment (i.e. duplicated tanks/treatment). Fed groups were fed twice daily to satiety for 2 weeks while Fasted groups received no feed for the same period. Salinity was kept at full-strength seawater (31-34 ppt) and water temperature was maintained between 11.0-11.5°C during the experiment. Water was replaced one time at 1 week after fasting. Water quality (pH, NO_3^-, NO_2^-, HCO_3^-,Cl_2, and general and carbonate hardness) was monitored by using a test kit (Tetra test; Spectrum Brands, Tokyo, Japan). The experiment was carried out in accordance with the guideline of the Hokkaido University Animal Care and Use Committee. Eight fish from each treatment were sampled at 0, 1, 2 and 3 weeks after the beginning of SW acclimation. Fish were anesthetized using 3.3% 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan). After FL and BW were measured, the tail was cut and blood was withdrawn using 10 or 20 μL plain glass tubes (Microcap; Drummond Scientific Company, Broomall, PA, USA). Blood was allowed to clot overnight at 4°C and centrifuged at 10,000 rpm for 15 min. Serum was collected and stored at -80°C until use. Gills were collected and immediately frozen on dry ice and stored at -80°C. Two pieces of the liver were collected; One piece was immersed in RNAlater (Thermo Fisher Scientific, Waltham, MA), sit at 4°C overnight and stored at -30°C, and the other piece was frozen on dry ice and stored at -80°C. Muscle samples were collected similar to the liver.

In June 2014, juveniles (FL: 5.80 ± 0.17 cm; BW: 1.37 ± 0.12 g) that had been

acclimated to artificial SW were lightly anesthetized using 2-phenoxyethanol (approximately 1%) and individually marked with PIT-tags (size φ1.4 mm × 8.4 mm, Biomark, Boise, ID). They were randomly placed into two 60 L SW tanks (25 fish per tank) and one group was fed twice daily on the commercial diet with a ration at 3.0% body weight/day for 10 days. The other group was fasted throughout the experimental period. SW was not replaced during the experiment. The experiment was carried out in accordance with the guideline of the Hokkaido University Animal Care and Use Committee. FL and BW of all fish were measured at the beginning of the experiment and 10 days after treatment. Condition factor (K) was calculated as follows: (BW (g)) x 1000/(FL (cm))^3. Specific growth rate (SGR) was calculated as follows: SGR (%/day) = ln ($s_2 - s_1$) × ($d_2 - d_1$)$^{-1}$ × 100, where s_2 is length or weight on day$_1$ and $d_2 - d_1$ is the number of days among measurements. On day 10, fish from each treatment (Fed: n = 25, Fasted n = 24) were sampled for blood, liver and muscle as described above.

2.3. Na^+,K^+-ATPase activity assay

Gill NKA activity was measured according to Quabius et al. (1997) with minor modifications. This method is based on the ability of NKA to hydrolyze ATP to give ADP and inorganic phosphorus with or without presence of ouabain at 37°C for 10 min. Liberated inorganic phosphorus reacted with ammonium molybdate was quantified by measuring absorbance at 630 nm using a spectrophotometer (Corona Electronic, Ibaraki, Japan). Protein concentration was measured by using BCA (bicinchoninic acid) Protein Assay Kit (Thermo Scientific, IL). The activity was expressed as Pi (μmol) per protein (mg) per period (h).

2.4. Time-resolved fluoroimmunoassay (TR-FIA) for IGF-I

Prior to the assay, serum IGF-I was extracted with an acid-ethanol as described in Shimizu et al. (2000). IGF-I was quantified by TR-FIA based on the method described in Small and Peterson (2005) using recombinant salmon/trout IGF-I (GroPep, Adelaide, SA, Australia) for standard and labeling with europium, and anti-barramundi IGF-I (GroPep) as a primary antiserum.

2.5. Measurement of RNA/DNA ratio

RNA/DNA ratio was measured by a spectrofluorimetric method modified from Grémare and

Vétion (1994) as described in Kawaguchi et al. (2013). Briefly, amount of total nucleic acids (DNA + RNA) was measured using 4 μg/mL Thiazole orange (Sigma-Aldrich, St. Louis, MO) and that of DNA using 0.02 mg/mL Hoechst 33258 (Dojindo, Kumamoto, Japan). RNA/DNA ratio was calculated from these values.

2.6. RNA extraction and cDNA synthesis

Total RNA was extracted from livers and muscles using ISOGEN (Nippon gene; Tokyo, Japan) according to the manufacturer's instruction. One and half μg RNA was reverse-transcribed using SuperScript VILO cDNA Synthesis kit (Invitrogen, Carlsbad, CA, USA) in a 10-μl reaction according to the manufacturer's instruction. cDNA was stored at -30°C until use.

2.7. Real-time quantitative PCR (qPCR)

Sequences of primers for qPCR of *igf-1*, *igfbp-1a*, *igfbp-1b* and *ef-1α* were the same as described in Kawaguchi et al. (2013). Reverse transcribed-PCRs using these primers were performed to prepare assay standards for chum salmon. PCR products run on 1.5% agarose gel were excised and purified using QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA, USA). Copy numbers of the purified amplicon were calculated from the molecular weight of the amplion and concentration. The standard cDNA were serially diluted from 1×10^7 to 3×10^2 copies.

qPCR was set up using Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) in a reaction volume of 20 μl with primer concentration of 100 nM. qPCR was run on a 7300 Sequence Detector (Applied Biosystems) using the manufacturer's recommended cycling conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles at 95°C for 15 se and 60°C for 1 min. Measured values were normalized to those of *ef-1α*. Performance of qPCR was evaluated by confirming a single peak of the dissociation curve in each assay and calculating the amplification efficiencies of the standard curves, which were within the range of 97-100%. Coefficients of determination of the standard curves were also between 0.99-1.00.

2.8. Statistical analysis

Results of the experiments were analyzed first by three-way ANOVA (salinity x time x nutrition) using the JMP program (SAS Institute Inc., Cary, NC, USA). When significant effects were found, differences were further identified by one-way ANOVA followed by the Fisher's protected least significant difference (PLSD) test. Differences among groups were considered to be significant at $P < 0.05$. SGRs from the second experiment were biased to high and low values. In order to avoid anchor effects, the SGR data were first categorized into high, med and low, and eight samples were randomly selected from each category to analyze relationships between growth rate and the endocrine/biochemical parameters by simple linear regression.

3. Results

3.1. Effect of SW transfer

Average FL, BW and K of each treatment are shown in Table 1. There were no effects of SW transfer for 1 week on FL, BW and K. Gill NKA activity was lower in fish in FW (Fig. 1). There was no effect of SW transfer on serum IGF-I (Fig. 2). SW transfer had no effect on liver RNA/DNA ratio whereas muscle RNA/DNA was higher in fish in SW (Fig. 3a,b). Both liver and muscle *igf-1* were not affected by SW transfer (Fig. 3c,d). Liver *igfbp-1a* levels were not influenced by SW transfer but those in the muscle significantly increased 1 week after SW transfer (Fig. 4a,b). There was no effect of SW transfer on liver *igfbp-1b* (Fig4c).

3.2. Combined effects of salinity and fasting

FL, BW and K were reduced by fasting for 2 weeks in both salinities (Table 1). There were overall effects of salinity and fasting on gill NKA activity, and fasting for 2 weeks significantly reduced the activity in both salinities (Fig. 5). Serum IGF-I levels were reduced by fasting in both salinities and there was no interaction with salinity (Fig. 6). In addition, serum IGF-I levels in SW-Fed fish was higher than those in FW-Fed fish (Fig. 6). Liver RNA/DNA was reduced by fasting and prolonged fasting further reduced it in SW fish (Fig. 7a). In contrast, the effect of fasting on muscle RNA/DNA was found only in fish in SW after 2 weeks (Fig. 7b). There were overall effects of fasting to reduce liver and muscle *igf-1* (Fig. 7c,d). However, fasting effect on liver *igf-1* was seen in 1 week while it took 2 weeks of fasting to see the reduction of muscle *igf-1* (Fig. 7c,d). In addition, an overall effect of salinity on muscle *igf-1* was seen (Fig. 7d).

There were overall effects of fasting to increase both liver and muscle *igfbp-1a*, while no significant difference between Fed and Fasted groups 1 week after fasting was detected in both tissues (Fig. 8a,b). In muscle, effect of fasting on *igfbp-1a* was seen only in SW group at week 2 (Fig. 8b). Fasting also increased liver *igfbp-1b* in both salinities and the effect was first detected in 1 week in SW group (Fig. 8c).

3.3. Relationships of endocrine and biochemical parameters with growth

Data on FL, BW, K, SGR-FL and SGR-BW of juvenile chum salmon fed or fasted for 10 days in SW are provided in Supplemental Table 1. The relationships between the endocrine/ biochemical parameters and growth parameters were analyzed (Table 2). No endocrine/ biochemical parameters correlated with FL. Serum IGF-I showed a moderate positive relationship with BW and weak negative relationships were seen for liver *igfbp-1a* and *-1b*. Liver RNA/DNA and serum IGF-I showed moderate positive relationships with K while liver and muscle *igfbp-1a* and liver *igfbp-1b* were negatively correlated with K. Serum IGF-I showed the highest positive relationship with SGR-FL. Negative, weak relationships with SGR-FL were seen for liver *igfbp-1b* and muscle *igf-1*. Serum IGF-I also had the highest regression coefficient with SGR-BW. In addition, muscle *igf-1* had a moderate positive relationship with SGR-BW whereas negative relationships were seen for liver *igfbp-1a* and *-1b*. Scattered plots between the parameters and SGR-BW are shown in Figure 9.

4. Discussion

Chum salmon juveniles experience changes in salinity and food availability during their seaward migration. The general belief is that growth soon after sea entry is key for their survival (Bax, 1983; Fukuwaka and Suzuki, 2002; Wertheimer and Thrower, 2007). The present study examined effects of SW transfer and fasting on IGF-I/*igf-1* and *igfbp-1*s in order to analyze environmental modulation of the IGF/IGFBP system in chum salmon as well as assess their utility as growth indices.

Chum salmon is an obligatory anadromous species migrating to the sea in their first spring at fry/juvenile stage often weighing less than 1 g. This species is unique in terms of acquiring high hypo-osmoregulatory ability soon after yolk absorption and maintaining it for relatively long period of time in freshwater (Iwata et al., 1982; Hasegawa et al., 1986). Kojima

et al. (1993) reported a temporal decrease in hypo-osmoregulatory ability in chum salmon juveniles weighing 3-4 g but those fish were still able to restore increased plasma sodium ion concentrations in 24 hr after direct SW transfer. In the present study, chum salmon fry/juveniles (0.6 g) were acclimated stepwisely to full-strength SW (33 ppt) over 5 days. In addition to their high hypo-osmoregulatory ability before entering SW, the gradual acclimation employed in the present study should completely acclimated fish to SW without stress. Although we did not measure plasma osmolarity or ion concentrations, or plasma cortisol due to the limitation of serum volume, the high gill NKA activity before SW transfer and no further activation of the activity suggest their high hypo-osmoregulatory ability. In addition, fish were fed during acclimation. Thus, we considered changes in the parameters during the first week of SW transfer were responses to salinity change rather than osmotic stress or fasting.

There are a number of studies examined effect of salinity change on tissue *igf-1* or circulating IGF-I in fish. Sakamoto and Hirano (1993) reported transfer of rainbow trout to 26 ppt (80%) SW resulted in transient increases in gill and body kidney *igf-1* at 1 day and 8 days, respectively, but not in liver *igf-1*. In contrast, liver *igf-1* in the same species increased 12 hr after transfer to 20 ppt (61%) SW (Poppinga et al., 2007). Exposure of Caspian trout (*S. trutta caspius*) to brackish water (10.5 ppt) caused an increase in liver *igf-1* in 5 days (Sharif et al., 2015). Shephered et al. (2005) reported up-regulation of plasma IGF-I in rainbow trout 5 days after gradual transfer to 22 ppt (66%) SW. In the present study, serum IGF-I, liver *igf-1* and muscle *igf-1* were not altered by transfer to full SW (33 ppt) for up to 3 weeks. It is hard to compare these responses to other studies since species, salinity, feeding status and experimental settings are different. However, one study by Iwata et al. (2012) used chum salmon fry/juveniles similar to the present study. When chum salmon juveniles (fry) were transferred to SW, hepatic *igf-1* levels were similar, or even lower, to those in FW during first 2 weeks and became higher 4 weeks after transfer (Iwata et al., 2012). These results suggest that effect of SW transfer on hepatic *igf-1* in juvenile chum salmon is not acute and our finding supports the notion. The present study also reports no acute effect of SW transfer on muscle *igf-1* in chum salmon.

Effects of salinity on IGFBPs have been investigated in a few studies using trout/salmon. Shepherd et al. (2005) acclimated rainbow trout to 22 ppt SW and found increases in plasma levels of 42-50-kDa (most likely IGFBP-2b) and 21-kDa IGFBPs (most likely IGFBP-1b). A direct exposure to full-strength SW of underyearing Chinook salmon fall smolts had no effect on serum IGFBP-2b but induced both serum IGFBP-1a and -1b (Shimizu et al., 2011), suggesting the involvement of IGFBP-1s in osmoregulation or/and stress response. On

one hand, no studies examined effect of salinity on tissue *igfbp-1*. In the present study using juvenile chum salmon, both liver *igfbp-1a* and *-1b* did not respond to SW transfer, while muscle *igfbp-1a* levels increased by SW transfer but returned to the basal levels 2 weeks after transfer. These responses may be adjustment by the muscle to different salinity rather than stress response. Specific role of IGFBP-1a in the muscle during the early phase of SW adaptation is a subject of future research.

High activity of branchial NKA is essential for salmon to maintain the internal osmolarity and ion balance in SW. As in its name, NKA consumes ATP to extrude sodium ions in exchange with potassium ions. Under the shortage of nutritional input, decreased gill NKA activity and increased Cl^- ions were observed in rainbow trout and Atlantic salmon (Jürss et al., 1987; Stefansson et al., 2009). Consistent with the previous studies, a negative effect of fasting on gill NKA activity was observed in juvenile chum salmon. A decrease in gill NKA activity during fasting may be adaptive in terms of saving energy, but long-term disturbance of ion balance under such condition should increase risk of mortality of juvenile salmon in wild.

Circulating IGF-I has been shown to be sensitive to changes in nutritional input. Beckman et al. (2004) reported plasma IGF-I levels in post-smolt coho salmon responded well to varying feeding ration, being higher in fish with high feeding ration. Fasting reduced plasma IGF-I levels in Chinook salmon as fast as 4 days (Pierce et al., 2005). In the present study, fasting for 1 week reduced serum IGF-I levels in fish in both FW and SW and prolonged fasting for another week tended to further reduce it. Thus, the response of serum IGF-I levels in juvenile chum salmon to fasting is consistent with the previous studies. It is worth noting that IGF-I levels in SW-Fed fish were higher than those in FW-Fed fish 3 weeks after transfer, suggesting that activation of the somatotropic axis in SW.

The liver is the major site of production of circulating IGF-I while virtually all tissues express *igf-1*. Responses of *igf-1* in the liver and muscle to fasting have been compared in several fish species. In rainbow trout and channel catfish (*Ictalurus punctatus*), fasting for 30 days reduced both liver and muscle *igf-1* and refeeding for 15 days was enough to restore its levels (Gabillard et al., 2006; Peterson and Waldbieser, 2009). On the other hand, a higher sensitivity of muscle *igf-1* to fasting than liver *igf-1* has been reported in Mozambique tilapia (*Oreochromis mossambicus*), yellowtail (*Seriola quinqueradiata*) and a cichlid fish (*Cichlasoma dimerus*) (Fox et al., 2010; Fukada et al., 2012; Delgadin et al., 2015), suggesting local regulation of *igf-1* is important to adjust tissue growth under fasting condition. It should be emphasized that the results of these studies do not necessarily deny the importance of hepatic

375 *igf-1* in growth regulation since the mRNA level is a reflection of the balance between
376 translation and degradation. In the present study, the basal mRNA levels of *igf-1* were higher in
377 the liver than in the muscle, and hepatic *igf-1* responded to fasting for 1 week while muscle
378 *igf-1* levels were not reduced by fasting for 1 week but 2 weeks. These results suggest that in
379 juvenile chum salmon, liver *igf-1* is more sensitive to fasting. Thus, the sensitivity of tissue *igf-1*
380 to fasting appears to vary depending on species, stage and conditions. Salinity doesn't seem to
381 have a strong impact on tissue (liver and muscle) *igf-1* although there was a small but
382 significant difference in muscle *igf-1* levels between FW-Fed and SW-Fed groups.
383 The present study compared the responses of liver and muscle *igfbp-1a* to fasting as
384 well as those between *igfbp-1a* and *-1b* in the liver. Fasting fish usually causes an increase in
385 hepatic *igfbp-1a/b* (Kamei et al., 2008; Pedroso et al., 2009; Peterson and Waldbieser, 2009;
386 Kawaguchi et al,, 2013; Breves et al., 2014), which is adaptive to save energy under nutritional
387 deficiency (Kajimura and Duan, 2007). Selection for large size resulted in lower muscle
388 expression of *igfbp-1a* and *-1b* in zebrafish (Amaral and Johnston, 2012), suggesting IGFBP-1
389 negatively regulate growth. However, there is no study comparing their responses to fasting
390 between different tissues. In the present study, *igfbp-1a* was expressed both in the liver and
391 muscle at the similar levels while *igfbp-1b* was detectable only in the liver. The exclusive
392 hepatic expression of *igfbp-1b* has been also reported in tilapia (Breves et al., 2014). Hepatic
393 and muscle *igfbp-1a* were increased by fasting in FW as well as in SW, suggesting the
394 importance of muscle IGFBP-1a under fasting conditions. Our finding is in good agreement
395 with those in zebrafish (Amaral and Johnston, 2011, 2012) where muscle *igfbp-1a* responded to
396 fasting and refeeding. In zebrafish, *igfbp-1b* was also expressed in the muscle and showed the
397 similar responses to fasting and refeeding although the levels were much lower than *igfbp-1a*
398 (Amaral and Johnston, 2011). Liver *igfbp-1b* was increased by fasting in both FW and SW as is
399 the case for liver *igfbp-1a*, but a significant increase was first seen in SW fish in 1 week. In
400 addition, a larger response of muscle *igfbp-1a* to fasting was found in SW fish. These results
401 suggest that the sensitivity of *igfbp-1*s is higher in SW although how salinity influences the
402 fasting response of *igfbp-1*s is not known at present. IGFBP-1s produced in the liver under
403 fasting condition should be released into the bloodstream and act on circulating IGF-I to adjust
404 growth rate.
405 One of the objectives of the present study was to validate the endocrine and
406 biochemical parameters as growth indices for juvenile chum salmon. There are considerable
407 interests in assessing growth status of fish using growth indices in aquaculture industry and

stock assessment. The latter is based on the hypothesis that high growth-dependent mortality soon after SW entry occurs in juvenile chum salmon (Bax, 1980; Fukwaka and Suzuki, 2002; Wertheimer and Thrower, 2007). However, since chum salmon juveniles experience large changes in environment such as salinity and food availability during downstream and coastal migration, good candidates of growth indices for this species should be stable during salinity change, sensitive to nutritional change and well correlated with growth rate. We have recently reported that serum IGF-I in juvenile chum salmon acclimated in SW was strongly, positively correlated with growth rate (Kaneko et al., 2015). The present study revealed that IGF-I was stable after SW transfer and there was no interaction between fasting response and salinity. The results of the present study strengthen the utility of serum IGF-I as a growth index in juvenile chum salmon. In addition, the present study compared IGF-I with the other candidates of growth index in terms of correlation with growth rate. As a result, serum IGF-I still showed the strongest relationship with growth rate. This result confirms that serum IGF-I is a good growth index for juvenile chum salmon.

The reliability of circulating IGF-I as growth index has been examined and discussed (Picha et al., 2008a; Beckman, 2011). Although the basal levels of circulating IGF-I fluctuates in response to season, sudden water temperature change, developmental stage and maturation status, it is so far the most reliable growth index in fish (Picha et al., 2008a; Beckman, 2011). However, collecting blood from small fish is sometimes not possible/practical. Chum salmon start migrating to the ocean even they are below 1 g. In such case, measuring mRNA levels of growth indices is an alternative. The present study assessed the utility of *igf-1* and *igfbp-1*s as alternative growth indices for juvenile chum salmon. Liver *igf-1* was sensitive to fasting but not correlated with grow rate. On the other hand, muscle *igf-1* was less sensitive to fasting but showed a moderate, positive correlation with growth rate. This is consistent with the finding in a hybrid striped bass (*Morone chrysops* x *M. saxatilis*) where muscle *igf-1* was positively correlated with growth rate ($r^2 = 0.23$) (Picha et al., 2014). However, it should be noted that the same authors reported that liver *igf-1* also correlated with growth rate ($r^2 = 0.48$) in the hybrid striped bass (Picha et al., 2008b). Although there is species difference in relationships of liver and muscle *igf-1* with growth rate, our finding shows that muscle *igf-1* is a better indicator of growth rate than liver *igf-1* in juvenile chum salmon in SW.

In addition to muscle *igf-1*, *igfbp-1a* and *-1b* may also be alternatives of serum IGF-I. We reported that circulating protein and hepatic mRNA levels of IGFBP-1a and -1b were negatively correlated with growth rate in post-smolt masu salmon in FW (Kawaguchi et al.,

2013). In accord with the previous study, hepatic *igfbp-1a* and *-1b* had negative relationships with growth rate in juvenile chum salmon. Their coefficients of determination were moderate being not as strong as that of serum IGF-I. Small serum volume prevented us to measure serum levels of IGFBP-1a and -1b in the present study. Although their protein and hepatic mRNA levels are expected to be similar as is the case in masu salmon (Kawaguchi et al., 2013), it needs to be confirmed in future research. Muscle *igfbp-1a* responded to fasting but showed no significant relationship with growth rate, indicating that the liver is the tissue to be analyzed when *igfbp-1a* is used as a growth index.

RNA/DNA ratio has been used as a biochemical index of growth in fish and marine organisms (Chícharo and Chícharo, 2008). The ratio is believed to reflect the degree of protein synthesis (RNA) per unit cell (DNA) and thus growth. Muscle RNA/DNA has been shown to be well correlated with growth rate in Atlantic salmon smolts (Maclean and Caldarone, 2008). The present study analyzed liver and muscle RNA/DNA ratio to compare its utility with tissue *igf-1* and *igfbp-1*s as an alternative of plasma IGF-I. As results, in juvenile chum salmon liver and muscle RNA/DNA ratio showed low or no correlation with growth rate, making it difficult to use as an alternative of serum IGF-I in this species.

In conclusion, our findings suggest that in chum salmon IGF-I and IGFBP-1s adjust growth under fasting condition by responding systemically as well as locally. The present study also shows that serum IGF-I is superior as a growth index for juvenile chum salmon. However, when juveniles are too small to collect blood, liver *igfbp-1a* and *-1b* and muscle *igf-1* are alternatives of IGF-I.

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637

Table 1
Fork length (FL), body weight (BW) and condition factor (K) of juvenile chum salmon used for seawater transfer and fasting experiments in May

		Time after SW transfer		Time after fasting	
Parameter	Treatment	0 week	1 week	1 week (2 wk)	2 week (3 wk)
FL	FW-Fed	4.58±0.08^{B}	4.91±0.14^{A}	5.21±0.12^{b}	5.70±0.16^{a}
	FW-Fasted			4.95±0.10^{bc}	4.95±0.06^{bc}
	SW-Fed		4.78±0.04^{AB}	4.96±0.08^{bc}	5.71±0.05^{a}
	SW-Fasted			4.88±0.07^{c}	4.85±0.10^{c}
BW	FW-Fed	0.64±0.03^{B}	0.73±0.04^{A}	0.95±0.05^{b}	1.45±0.15^{a}
	FW-Fasted			0.74±0.04^{cd}	0.75±0.04^{cd}
	SW-Fed		0.68±0.02^{AB}	0.88±0.04^{bc}	1.14±0.06^{a}
	SW-Fasted			0.70±0.03^{cd}	0.65±0.04^{d}
K	FW-Fed	0.67±0.04^{A}	0.62±0.03^{A}	0.67±0.03^{bc}	0.77±0.02^{a}
	FW-Fasted			0.60±0.01^{d}	0.62±0.03^{cd}
	SW-Fed		0.62±0.01^{A}	0.72±0.02^{ab}	0.76±0.03^{a}
	SW-Fasted			0.60±0.01^{d}	0.57±0.02^{d}

Values are expressed as mean ±SE (n＝8). Numbers in parences are weeks after seawater transfer. Within a parameter, groups sharing the same letter are not significantly different. Effects of SW transfer, and SW transfer x fasting were analyzed separately as shown by upper and lower case letters.

Table 2
Correlation coefficients (r) between endocrine/biochemical parameters and growth parameters

	FL	BW	K	SGR-FL	SGR-BW
Liver R/D	-	-	0.65	-	0.50
Muscle R/D	-	-	-	-	-
Serum IGF-I	-	0.65	0.46	0.88	0.92
Liver *igf-1*	-	-	-	-	-
Muscle *igf-1*	-	-	0.56	0.44	0.65
Liver *igfbp-1a*	-	-0.42	-0.71	-	-0.63
Muscle *igfbp-1a*	-	-	-0.53	-	-
Liver *igfbp-1b*	-	-0.54	-0.60	-0.48	-0.59

FL: fork length; BW: body weight; K: condition factor; SGR: specific growth rate; (-): not significant.

638 **Figure legends**

639

640 Fig. 1. Effects of SW transfer on gill NKA activity in juvenile chum salmon. Values are
641 expressed as means ± SE (n=8). Groups sharing the same letter are not significantly different.

642

643 Fig. 2. Effects of SW transfer on serum IGF-I levels in juvenile chum salmon. Values are
644 expressed as means ± SE (n=6-8).

645

646 Fig. 3. Effects of SW transfer on liver (a,c) and muscle (b,d) RNA/DNA ratio (a,b) and *igf-1*
647 mRNA levels(c,d) in juvenile chum salmon. Values are expressed as means ± SE (n=6-8).
648 Groups sharing the same letter or those without letter are not significantly different.

649

650 Fig. 4. Effects of SW transfer on liver (a,c) and muscle (b) *igfbp-1a* (a,b) and *igfbp-1b* (c)
651 mRNA levels in juvenile chum salmon. Values are expressed as means ± SE (n=6-8). Groups
652 sharing the same letter or those without letter are not significantly different.

653

654 Fig. 5. Effects of salinity and fasting on gill NKA activity in juvenile chum salmon. Values are
655 expressed as means ± SE (n=8). Asterisks indicate overall effects. Groups sharing the same
656 letter are not significantly different.

657

658 Fig. 6. Effects of salinity and fasting on serum IGF-I levels in juvenile chum salmon. Values are
659 expressed as means ± SE (n=7-8). An asterisk indicates an overall effect. Groups sharing the
660 same letter are not significantly different.

661

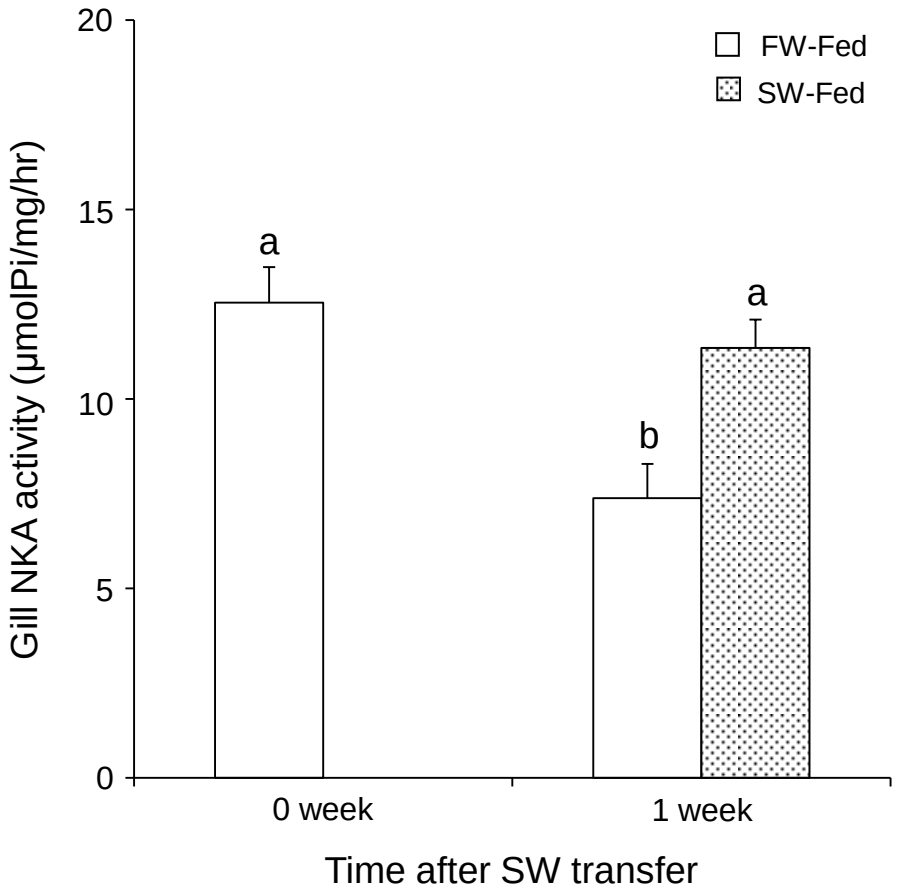
662 Fig. 7. Effects of salinity and fasting on liver (a,c) and muscle (b,d) RNA/DNA ratio (a,b) and
663 *igf-1* mRNA levels (c,d) in juvenile chum salmon. Values are expressed as means ± SE (n=6-8).
664 Asterisks and crosses indicate overall effects and interactions, respectively. Groups sharing the
665 same letter are not significantly different.

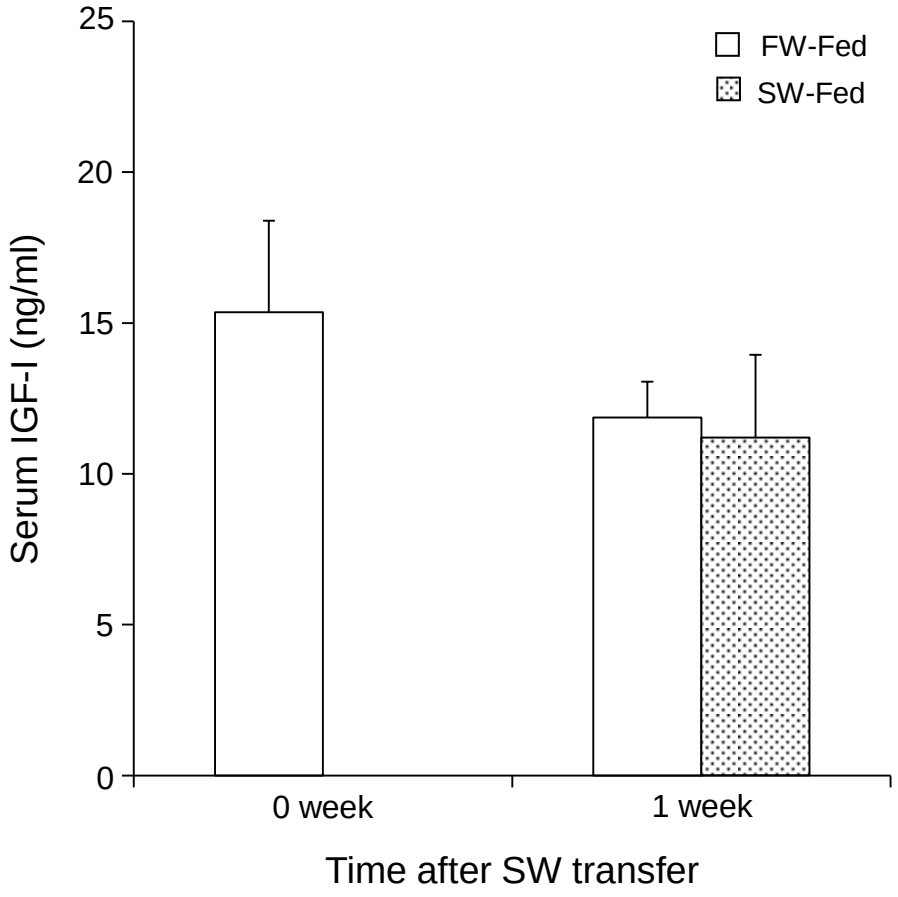
666

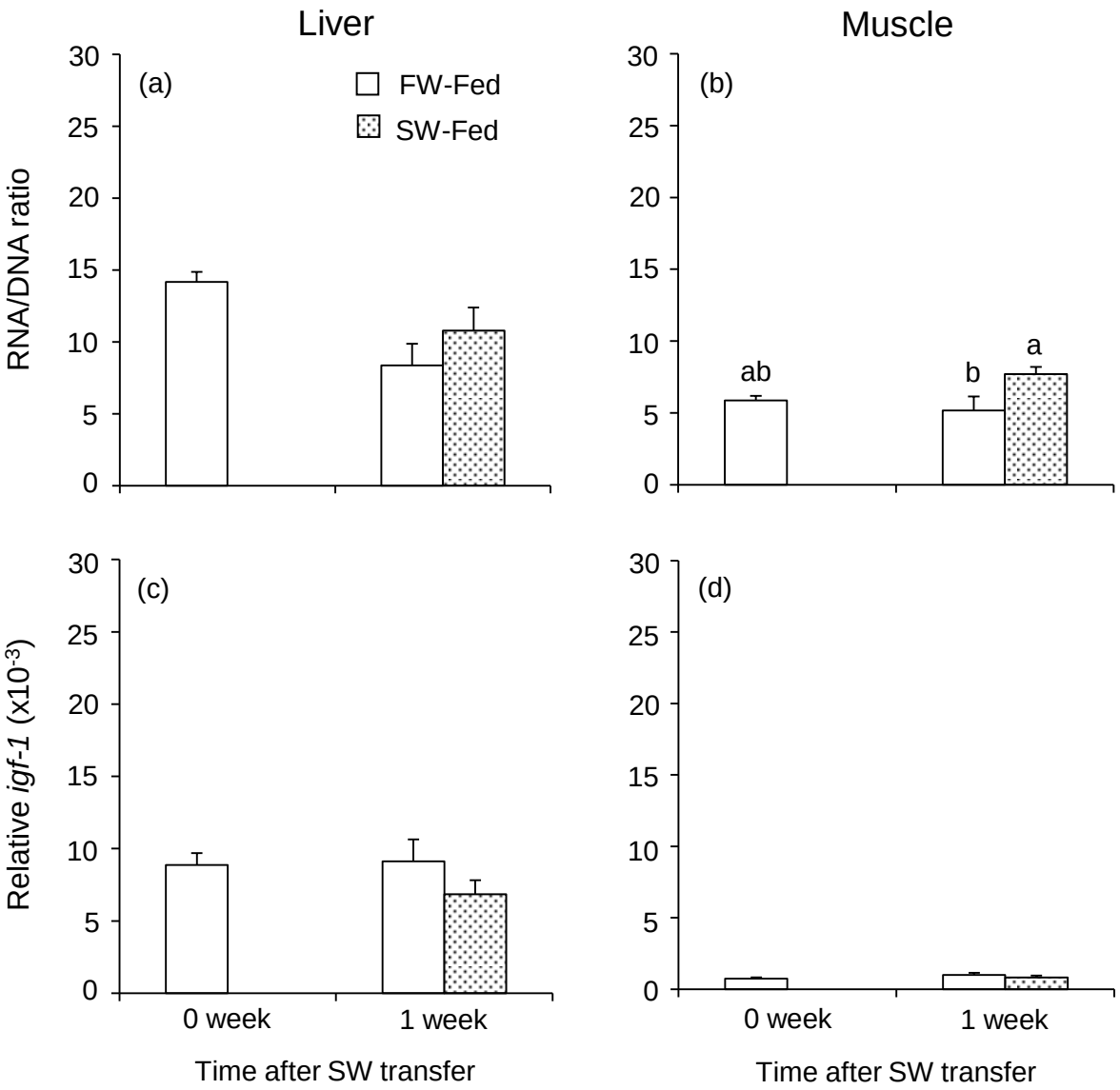
667 Fig. 8. Effects of salinity and fasting on liver (a,c) and muscle (b) *igfbp-1a* (a,b) and *igfbp-1b*
668 (c) mRNA levels in juvenile chum salmon. Values are expressed as means ± SE (n=7-8).
669 Asterisks and crosses indicate overall effects and interactions, respectively. Groups sharing the
670 same letter are not significantly different.

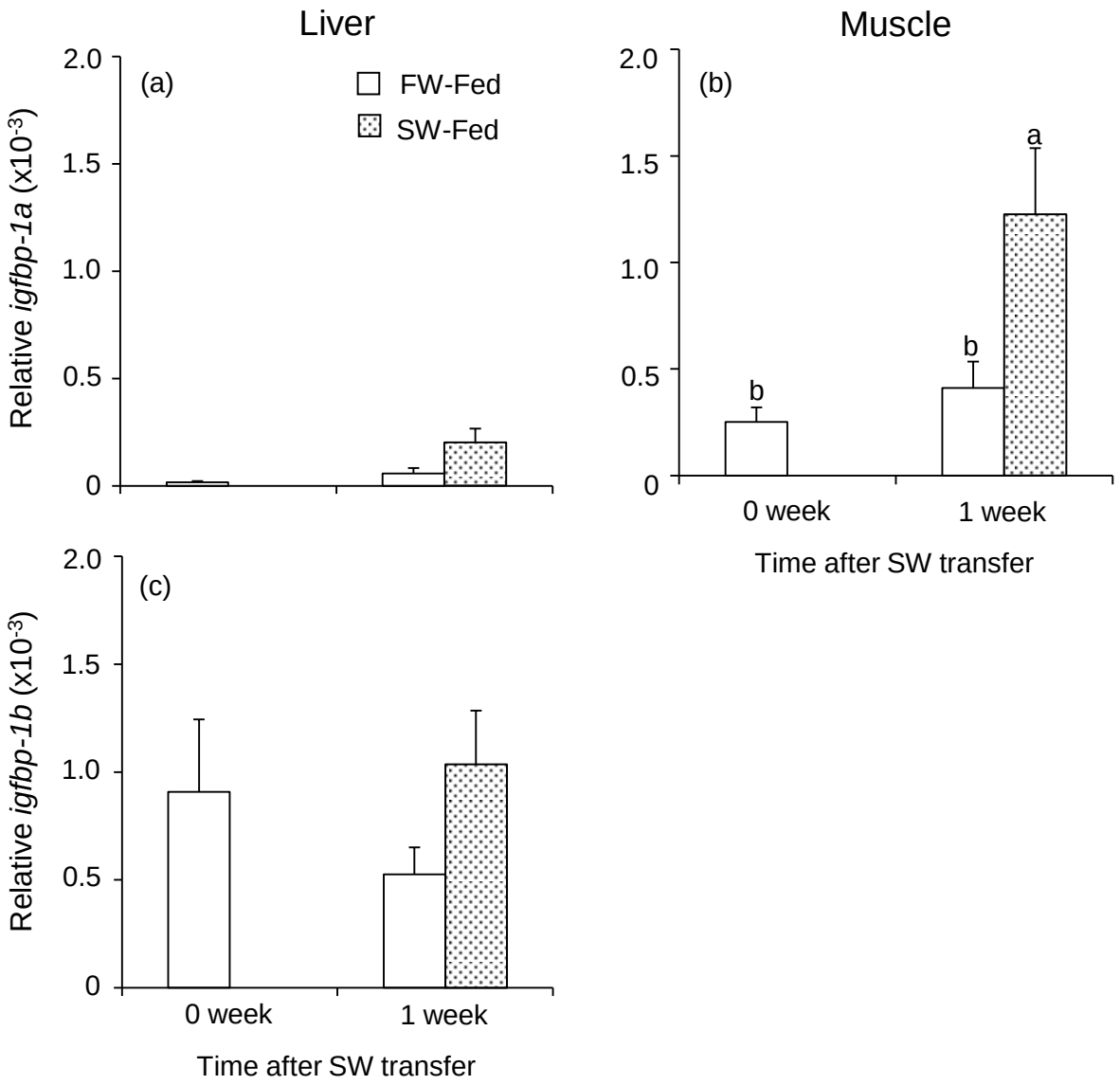
671

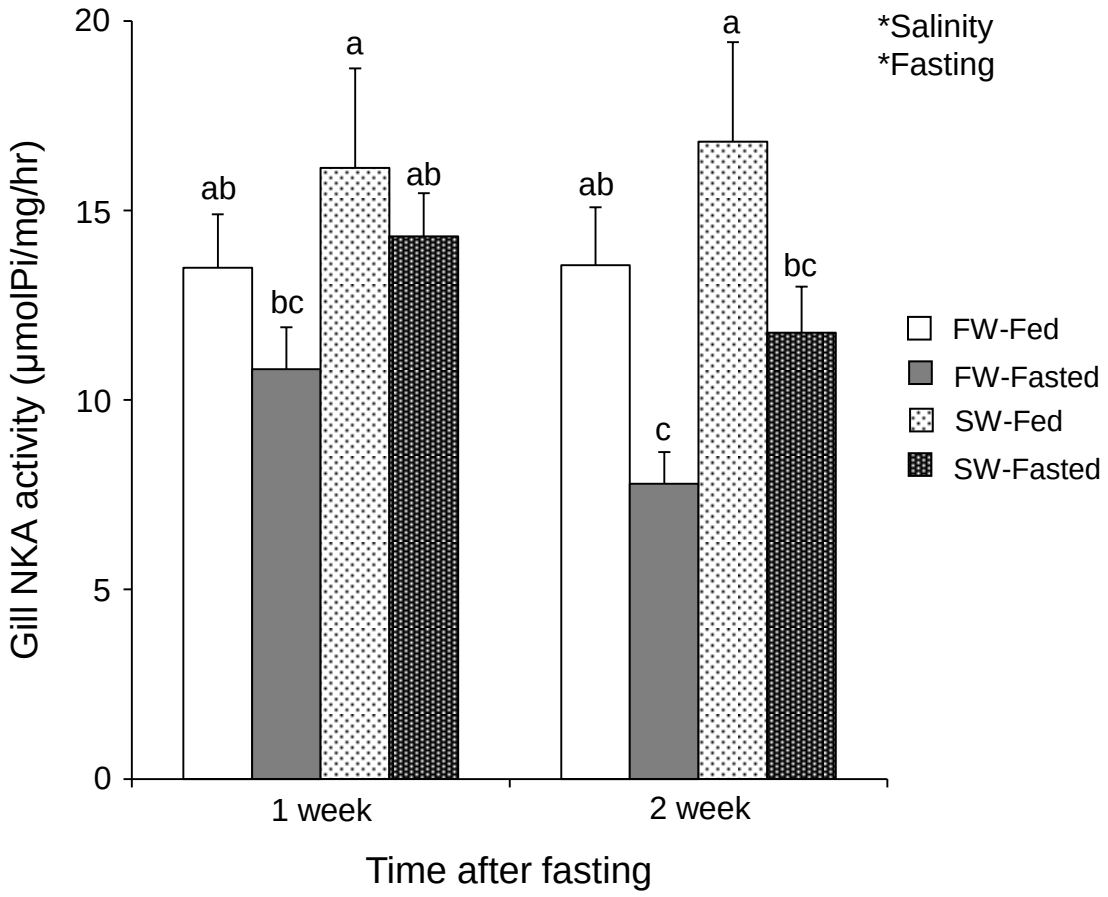
672 Fig. 9. Relationships with SGR-BW of biochemical and endocrine parameters. (a, d-f): liver, (b,
673 e,g): muscle, (c) serum. (a,b) RNA/DNA ratio, (c) IGF-I, (d,e) *igf-1*, (f,g): *igfbp-1a*, (h) *igfbp-1b*.
674 Regression lines were drawn when the relationships were significant. r^2: coefficient of
675 determination. Data on muscle RNA/DNA ratio and serum IGF-I were from Kaneko et al.
676 (2015).

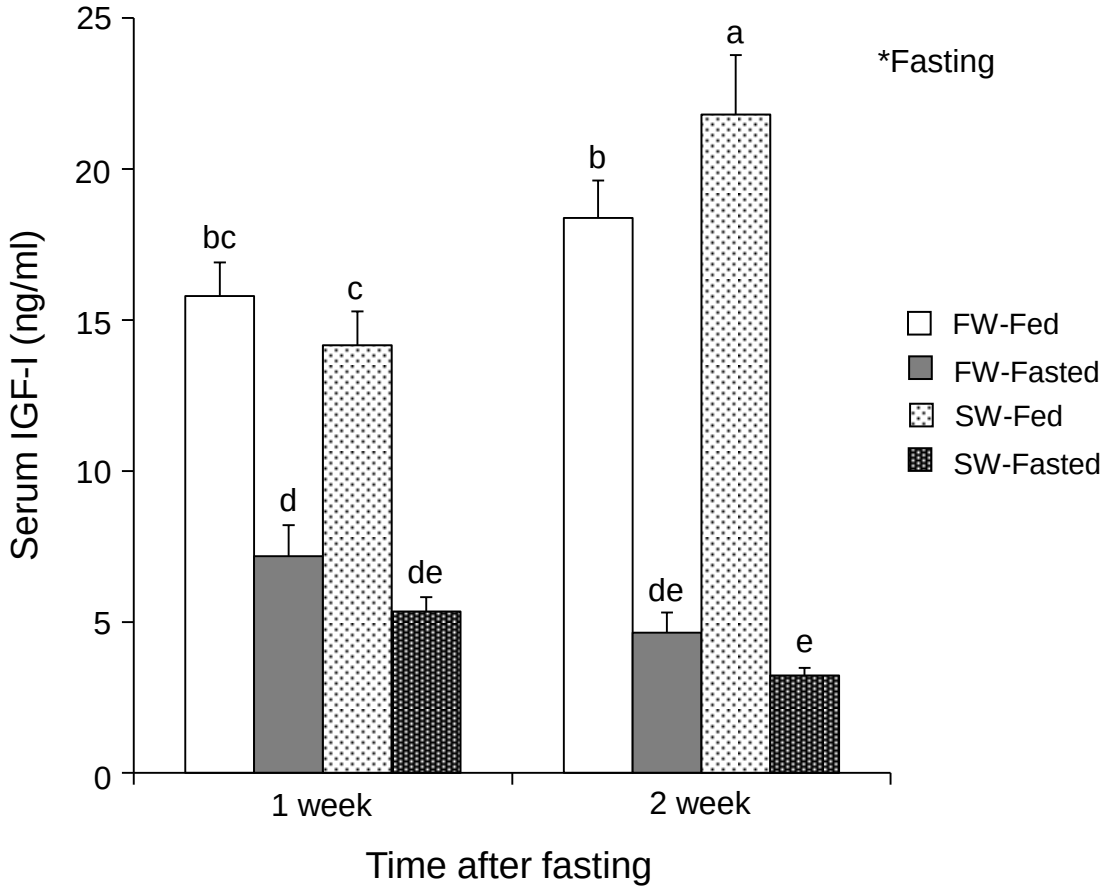

20
15
10
5
0
Gill NKA activity (μmolPi/mg/hr)
FW-Fed
SW-Fed
a
a
b
0 week
1 week
Time after SW transfer

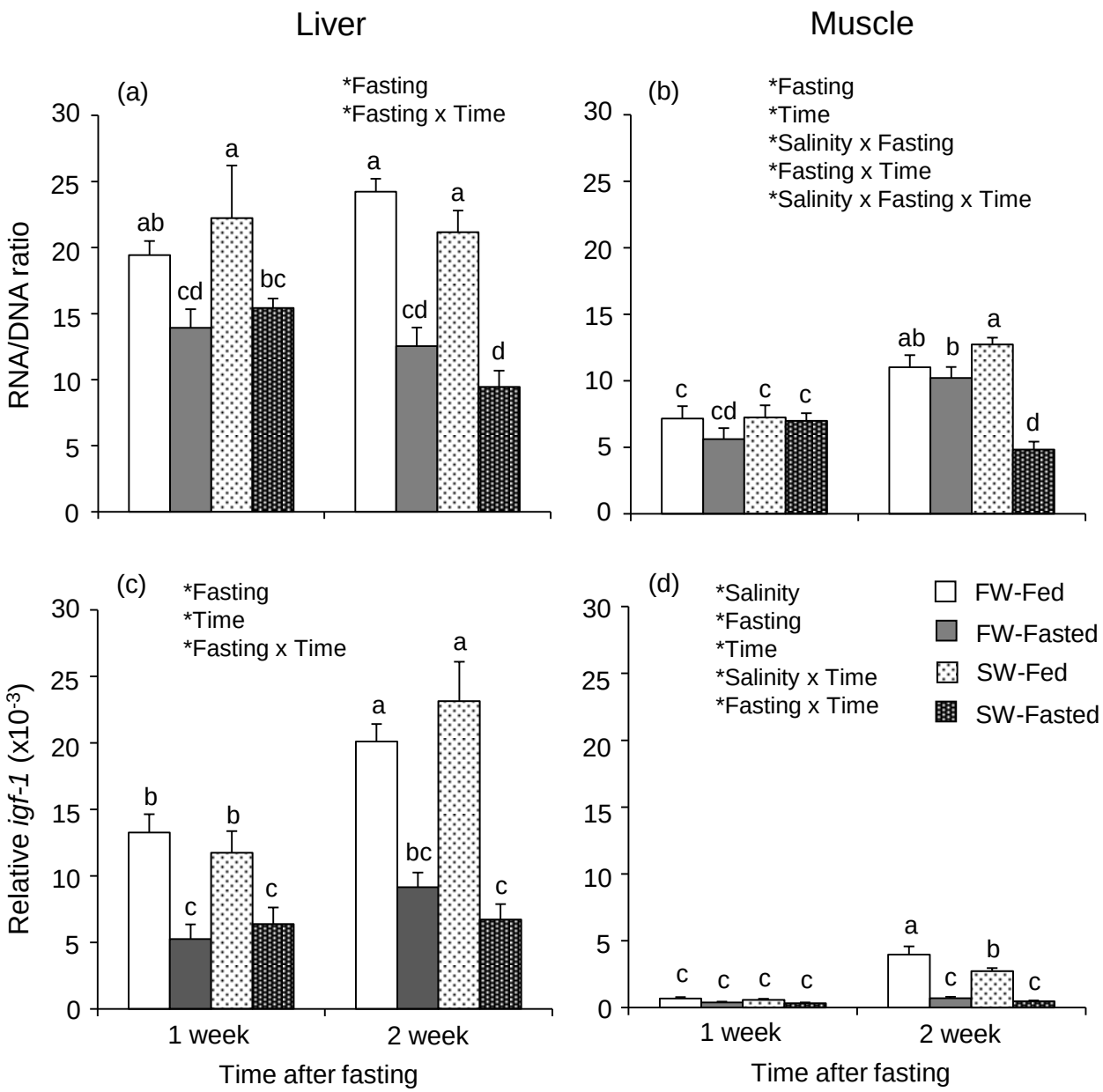

25
20
15
10
5
0
Serum IGF-I (ng/ml)
FW-Fed
SW-Fed
0 week
1 week
Time after SW transfer

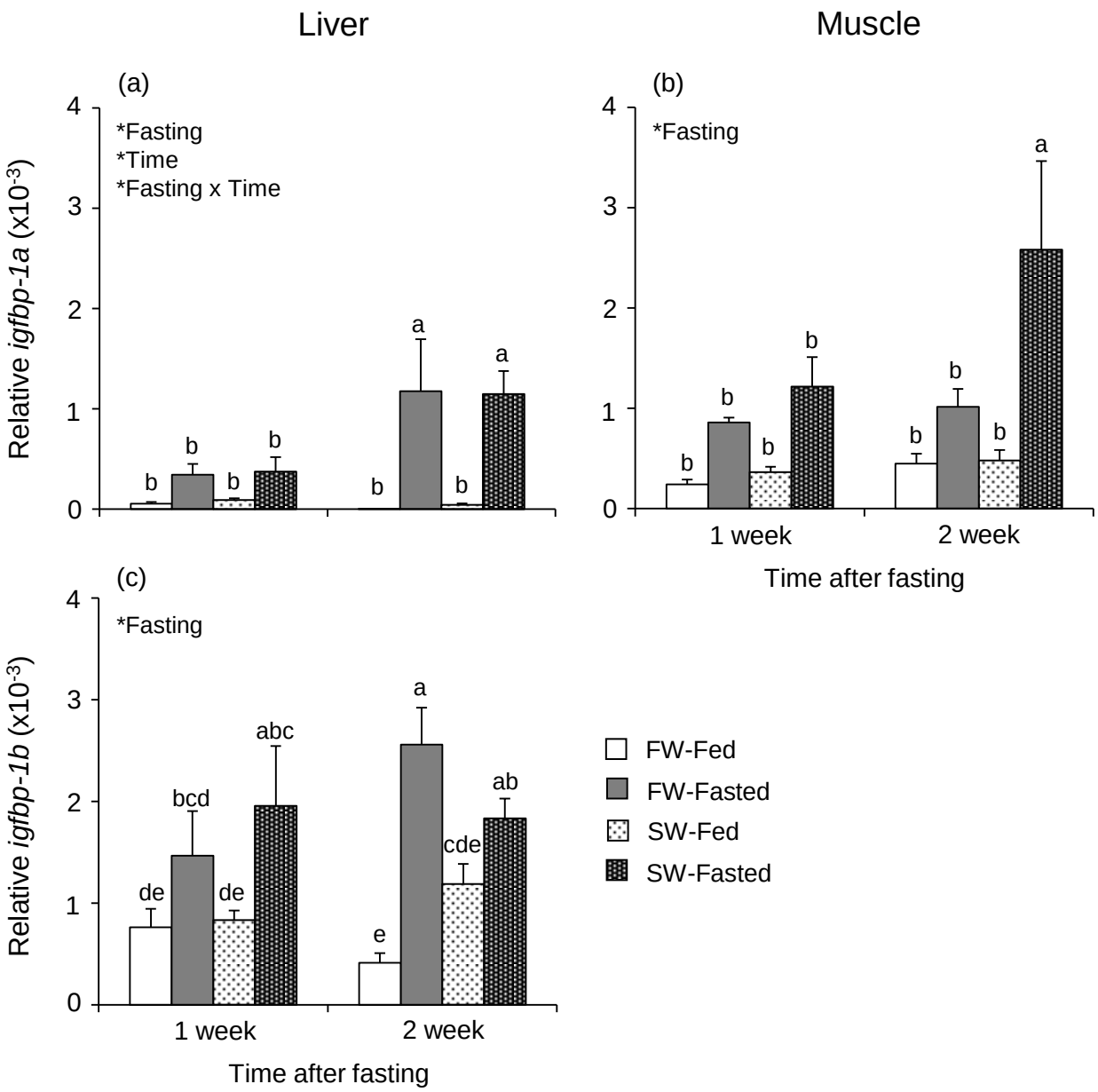

Liver
Muscle
(a)
(b)
(c)
(d)
FW-Fed
SW-Fed
RNA/DNA ratio
Relative igf-1 (x10-3)
ab
b
a
0 week
1 week
Time after SW transfer

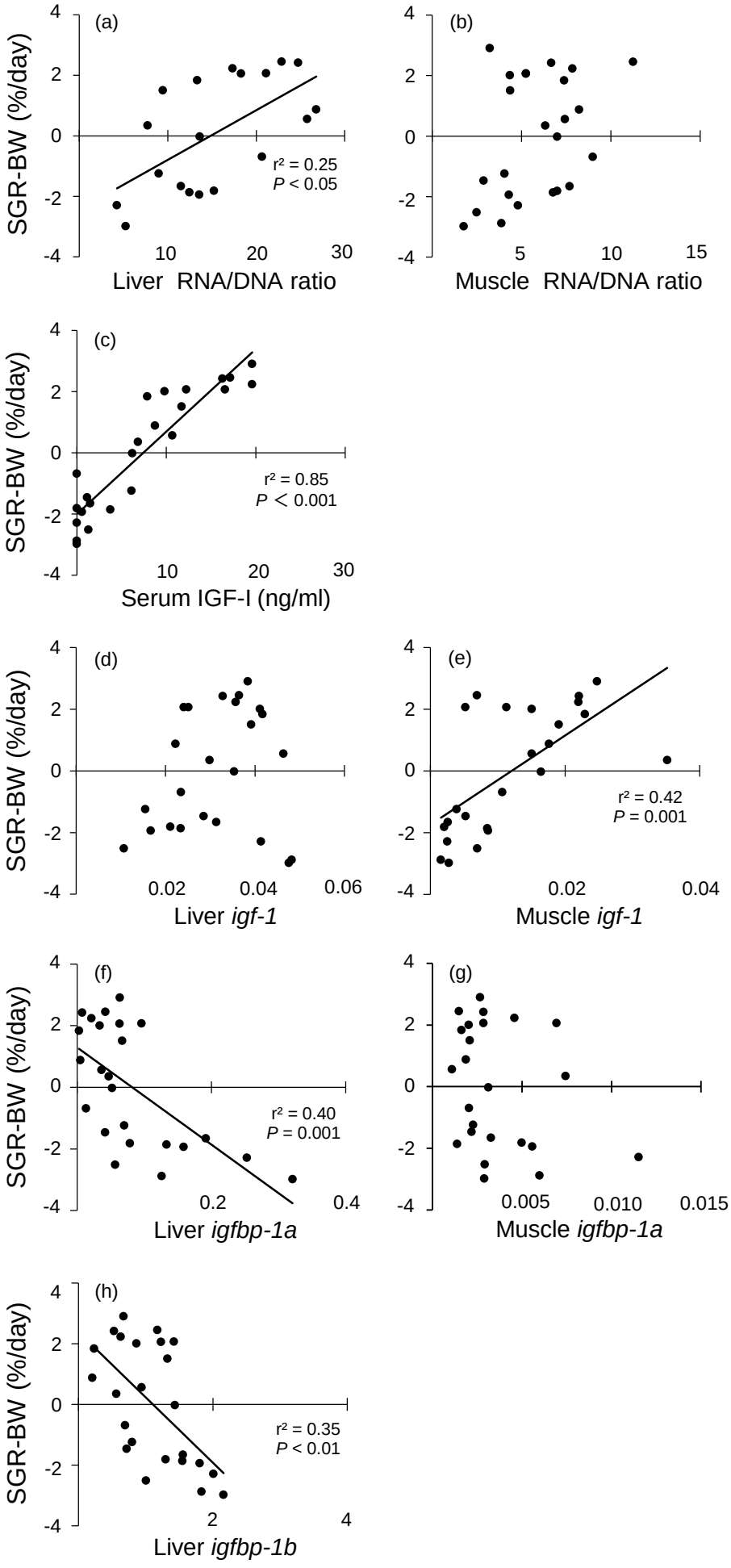

Liver
Muscle
(a)
(b)
(c)
FW-Fed
SW-Fed
Relative igfbp-1a (x10-3)
Relative igfbp-1b (x10-3)
2.0
1.5
1.0
0.5
0
b
b
a
0 week
1 week
Time after SW transfer


20
15
10
5
0
Gill NKA activity (μmolPi/mg/hr)
ab
bc
a
ab
ab
c
a
bc
1 week
2 week
Time after fasting
*Salinity
*Fasting
FW-Fed
FW-Fasted
SW-Fed
SW-Fasted


25
20
15
10
5
0
Serum IGF-I (ng/ml)
bc
d
c
de
b
de
a
e
*Fasting
FW-Fed
FW-Fasted
SW-Fed
SW-Fasted
1 week
2 week
Time after fasting


Liver
Muscle
(a)
*Fasting
*Fasting x Time
(b)
*Fasting
*Time
*Salinity x Fasting
*Fasting x Time
*Salinity x Fasting x Time
RNA/DNA ratio
(c)
*Fasting
*Time
*Fasting x Time
(d)
*Salinity
*Fasting
*Time
*Salinity x Time
*Fasting x Time
FW-Fed
FW-Fasted
SW-Fed
SW-Fasted
Relative igf-1 (x10-3)
1 week
2 week
Time after fasting


Liver
Muscle
(a)
*Fasting
*Time
*Fasting x Time
Relative igfbp-1a (x10-3)
(b)
*Fasting
1 week
2 week
Time after fasting
(c)
*Fasting
Relative igfbp-1b (x10-3)
1 week
2 week
Time after fasting
FW-Fed
FW-Fasted
SW-Fed
SW-Fasted


(a)
SGR-BW (%/day)
r² = 0.25
P < 0.05
Liver RNA/DNA ratio
(b)
Muscle RNA/DNA ratio
(c)
r² = 0.85
P < 0.001
Serum IGF-I (ng/ml)
(d)
Liver igf-1
(e)
r² = 0.42
P = 0.001
Muscle igf-1
(f)
r² = 0.40
P = 0.001
Liver igfbp-1a
(g)
Muscle igfbp-1a
(h)
r² = 0.35
P < 0.01
Liver igfbp-1b

Supplemental Table 1
Growth parameters of juvenile chum salmon from fasting experiment in SW

Parameter	Treatment	Day 0	Day 10
FL	Fed	5.80±0.17	5.80±0.09
	Fasted		5.80±0.14
BW	Fed	1.37±0.12	1.37±0.06
	Fasted		1.16±0.10
K	Fed	0.69±0.02[a]	0.70±0.01[a]
	Fasted		0.59±0.02[b]
SGR-FL	Fed	-	0.51±0.10[a]
	Fasted		0.03±0.04[b]
SGR-BW	Fed	-	1.46±0.29[a]
	Fasted		-2.07±0.18[b]

FL: fork length; BW: body weight; K: condition factor. Values are expressed as mean ±SE (initial n=8, Fed n=14, Fasted n=10). (-): not significant. For each parameter, groups sharing the same letter are not significantly different.