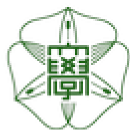

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Validation of insulin-like growth factor-I and its binding proteins as physiological growth indices in salmonid species

（サケ科魚類におけるインスリン様成長因子-I および
その結合蛋白の生理学的な成長指標としての有用性の検討）

Graduate School of Fisheries Sciences
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北海道大学大学院 水産科学院
海洋応用生命科学専攻

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2017
（平成 29 年）

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1. General introduction

Pacific salmon are anadromous species widely distributed throughout the north Pacific Rim. They belong to the genes *Oncorhynchus*, which consists of eight species as follows: pink salmon (*O. gorbuscha*), chum salmon (*O. keta*), coho salmon (*O. kisutch*), Chinook salmon (*O. tshawytscha*), sockeye salmon (*O. nerka*), masu salmon (*O. masou*), cutthroat trout (*O. clarki*) and rainbow trout (*O. mykiss*). These fish are targets of intensive capture fisheries which produce around one million tons in the north Pacific annually with chum and pink salmon being two dominant species captured (North Pacific Anadromous Fish Commission, 2016). Chum and pink salmon populations are supported by a large number of hatcheries, from which approximately 4.5 billion juveniles of chum and pink salmon are released every year (North Pacific Anadromous Fish Commission, 2016).

Pacific salmon share life-history patterns as anadromous fish but the timing of sea entry and the period of ocean life are characteristic of each species. All salmon hatch in freshwater and stay in the river or the lake for a certain period and migrate downstream to the ocean. Pink and chum salmon migrate downstream to the sea in their first spring with the body size of approximately one gram or less. On one hand, masu and coho salmon stay in freshwater for more than one year and migrate seaward in their second spring. Despite the difference in timing of downstream migration among species, all smolts go to the ocean to grow. In the ocean, juveniles spend a few months to several years feeding depending on species, genetic background and environment. After reaching final size, adult salmon go back to their natal rivers to spawn.

Most of juvenile salmon die during the early stage of their ocean life due to the size- and/or the growth-dependent mortality (Healey, 1982). Beamish and Mahnken (2001) proposed a critical size and period hypothesis to explain the high mortality during early marine life. Based on this hypothesis and others, there are two critical

periods; one occurs soon after sea entry and death occurs mainly due to maladaptation to new environments and/or high predation pressure, while the second critical period may be during fall to winter when shortage of energy reserve is lethal for young salmon (Healey, 1982; Beamish and Mahnken, 2001; Beamish et al., 2004; Farley et al., 2007; Kocik et al., 2009). Although the precise timing and mechanism of mortality still need to be elucidated, the importance of fish size and energy reserve for survival has been recognized. Therefore, measuring and evaluating growth status of salmon in the sea may have great value to assess the possibility of their survival.

There are several means to measure growth directly or indirectly. A direct measurement of growth by marking and recapturing the same individual is the most precise and reliable method and is an effective technique in controlled environments and in focused surveys. However, this method requires two sampling points for the same individual, which is labor-intensive, time-consuming and difficult when one needs to recover free-living fish in the sea. Instead, indirect measurements of growth using growth indices are widely used for monitoring growth of fish in the ocean. Such growth indices include the scales, otoliths and RNA/DNA ratio.

Scales reflect growth history of a fish. The scale is a hard tissue physically protecting the fish body. The scales have circuli which are added as the fish grows and the spacings of inter-circulus relate to growth; narrow spacings indicate slow growth and the wide spacings indicate fast growth (Friedland et al., 1993). The scale circuli are formed with 10-14 day durations (Fisher and Pearcy, 1990; Friedland et al., 1993; Wells et al., 2003). Thus, analyzing the increments of the radius of a scale allows the reconstruction of growth history.

Otoliths contain information on both living habitats and growth history (Campana and Thorrold, 2001). The otoliths are calcified structures located in the fish ear that consist mainly of calcium bicarbonate and also includes minerals such as strontium, manganese and zinc from the environments. The otolith forms daily rings and

its width is often correlated with growth status. Therefore, it is possible to back-calculate the age and daily growth patterns of a fish based upon patterns in the otolith (Fukuwaka, 1998; Courtney et al., 2000; Morita and Matsuishi, 2001).

Although the scale and otolith increments are very useful traits to evaluate past growth and living habitats, they may not be the best ones for evaluating current/recent growth status for salmon. The increment of the outermost layer of scales could be used to estimate recent growth for last 10-14 days (Wells et al., 2003), however, scale analysis is labor-intensive and in the case of salmon, scales are easily detached during trawl sampling. The otoliths may allow one to reconstruct daily growth patterns but otolith growth occurs even when fish are not growing (Maillet and Checkley, 1990; Campana and Thorrold, 2001), which would cause a decoupling between otolith size and growth status (Bradford and Geen, 1992).

Biochemical growth indices may be more reflective of recent growth status since they are directly involved in the growth process (Buckley, 1984; Couture et al., 1998; Chícharo and Chícharo, 2008). RNA/DNA ratio is one of the biochemical indices. Its utility as a growth index is based on the premise that DNA concentration in a cell is constant while ribosomal RNA concentration fluctuates depending on the degree of protein synthesis in the cell (Buckley, 1984; Chícharo and Chícharo, 2008). RNA/DNA ratio indeed has a strong correlation with growth rate in Atlantic salmon (*Salmo salar*) (MacLean et al., 2008; Caldarone et al., 2016). However, relationship of RNA/DNA ratio with growth varies among species and life-history stage (Johnson et al., 2002; Kawaguchi et al., 2013). Thus, either the validation of RNA/DNA ratio or the development of new growth indices may be needed for reliable assessment of recent/current growth status. A good growth index is preferably involved in the growth process (Couture et al., 1998).

The growth of animal is mainly regulated by the growth hormone (GH)-insulin-like growth factor-I (IGF-I) system (Daughaday and Retwein, 1989; Le

Roith et al., 2001; Ohlsson et al., 2009). In this system, GH from the pituitary gland promotes growth mainly through stimulating hepatic production of IGF-I. IGF-I is released into the bloodstream and mediates many of GH actions. The endocrine IGF-I in turn acts on the pituitary and hypothalamus to suppress the secretion of GH as a negative feedback loop. IGF-I circulates in the blood at relatively high levels as a hormone. This is due to the stabilization of IGF-I levels by IGF-binding proteins (IGFBPs). IGFBPs are important modulators of IGF-I activities by regulating its half-life and availability to the IGF-receptor on target tissues (Jones and Clemmons, 1995; Rajaram et al., 1997). In mammals, there are six types of IGFBPs in circulation and they regulate the activity of IGF-I differently. Some IGFBPs such as IGFBP-1 and -2 inhibit the activity of IGF-I by preventing it from interacting with the IGF-receptor. Other IGFBPs, such as IGFBP-3 and -5, can potentiate the activity of IGF-I by protecting it in the circulation and delivering it to the IGF receptor in the target tissues.

Recently, a new approach to assess recent/current growth status of fish using IGF-I has been proposed (Picha et al., 2008a; Beckman, 2011). Plasma IGF-I level varies in response to feeding status; when fish are fed at high rations, IGF-I levels are high and vice versa (Beckman et al., 2001, 2004a, b). In addition, a positive relationship between plasma IGF-I and individual growth rate over 2 weeks intervals in postsmolt coho salmon has been reported in the same species (Beckman et al., 2001, 2004a, b). Many laboratory studies using other salmon and fish species also reported that IGF-I level correlated to changes in nutritional status and circulating IGF-I has been shown to reflect recent growth rates of individuals. These findings support the notion that plasma/serum IGF-I is useful as a growth index (Picha et al., 2008a; Beckman, 2011).

Despite the utility of circulating IGF-I as an index of fish growth, care should be taken for situations where the IGF-I-growth relationship is disrupted. IGF-I-growth relationships were found to be disrupted by changes in environmental factors such as a rapid drop in water temperature and/or physiological conditions of individuals such as

precocious maturation (Beckman et al., 2004a, b, c). Thus, in order to make the assessment of growth status using IGF-I more reliable, developing new growth indices along with IGF-I is desirable.

IGFBPs are candidates of such growth indices to be used with IGF-I since they are important regulators of IGF-I in the circulation and respond well to environmental changes to adjust growth. In salmon, three major IGFBPs are consistently detected at 41-, 28- and 22-kDa. They have recently identified as IGFBP-2b, -1a and -1b, respectively (Shimizu et al., 2011a, b). The presence of paralogs of the each IGFBP type in fish is due to an extra-round of whole genome duplication in the teleost lineage. Circulating IGFBP-1a and -1b are induced under catabolic conditions, whereas IGFBP-2b is high under anabolic conditions as is the case for IGF-I (Shimizu et al., 2003a, 2006, 2011a). These circumstances suggest that circulating IGFBPs in salmon can be used as negative or positive indices.

Salmon IGFBP-1b has been proposed as a negative growth index (Shimizu et al., 2006, 2009; Kawaguchi et al., 2013; Fukuda et al., 2015). The studies on environmental regulation of circulating IGFBP-1b were facilitated by development of immunoassays (Shimizu et al., 2006; Fukuda et al., 2015). Quantification of circulating IGFBP-1b levels revealed that in coho salmon and masu salmon IGFBP-1b levels increased when fish were fed at low rations or fasted and there was a negative correlation with individual growth rate (Shimizu et al., 2006, 2009; Kawaguchi et al., 2013; Fukuda et al., 2015). These findings obtained under laboratory settings warrant examination of IGFBP-1b as a negative growth index in the field.

The usefulness of IGFBP-1a as a quantitative negative growth index has not been established. When both IGFBP-1 paralogs in the circulation were analyzed by ligand blotting using labeled IGF-I, they were responsive to catabolic condition such as fasting and stress, while IGFBP-1b might be more sensitive than IGFBP-1a to catabolic status in coho salmon (Shimizu et al., 2006, 2011a). Moreover, IGFBP-1a may have

different affinity for IGF-I and thus inhibit the IGF-I action for a different degree, suggesting that IGFBP-1a reflect severe catabolic conditions. Therefore, quantifying circulating IGFBP-1a and examining its relationship with growth along with IGFBP-1b is important. However, there is no immunoassay available for IGFBP-1a.

Given that IGF-I is being established as a positive growth index, the utility of IGFBP-1a and -1b as negative growth indices are worth examining. In this thesis, the terms "positive" or "negative" growth indices were used to reflect a plus or minus sign of the correlation coefficient with growth rate and a direction of metabolism (i.e. anabolism or catabolism). The first definition means if the slope of the regression line between a parameter and growth rate is positive, it is a "positive" growth index and vice versa. The second definition is based on its metabolic action. If a parameter, such as IGF-I, has an anabolic action and thus promotes growth, it is a positive growth index. If a parameter, such as IGFBP-1b, has a catabolic action and thus suppresses growth, it is a negative growth index.

The present study aims to validate candidates of biochemical and endocrine growth indices such as RNA/DNA ratio, IGF-I, IGFBP-1b, IGFBP-1a mainly for chum salmon, and utilize them at field survey to evaluate growth status of out-migrating juvenile fish. In Chapter 2, I validated usefulness of muscle RNA/DNA ratio and circulating IGF-I as positive growth indices for juvenile chum salmon and assessed growth status of juveniles in wild. In Chapter 3, I examined circulating IGFBP-1b as a negative growth index for juvenile chum salmon and monitored negative aspect of growth in wild. In Chapter 4, I attempted to evaluate growth retardation by using IGFBP-1b in coho salmon that had been migrating through the Strait of Georgia, British Columbia, Canada. In Chapter 5, I established a novel immunoassay for IGFBP-1a and examined its responses to changes in physiological conditions and relationship with growth rate in masu salmon.

2. Circulating IGF-I as a positive growth index of juvenile chum salmon

2.1. Introduction

Chum salmon is a target of intensive hatchery releases in north Japan because of its importance in fishery catches along the north Japanese coasts (Miyakoshi et al., 2013). Unlike other Pacific salmon except pink salmon, chum salmon migrate to the ocean in their first spring. Several studies based on marking-recapture experiment have revealed that during the initial stage of their marine life (i.e. soon after sea entry) mortality is high loosing at least 72% of released juveniles (Bax, 1983; Fukuwaka and Suzuki, 2002; Wertheimer and Thrower, 2007). This high mortality is likely size-selective (Healey, 1982). As most hatchery-reared chum salmon juveniles are released into the river, it is important to know how growth status is altered under changing feed availability, salinity and water temperature, and estimate their chance for survival during the critical periods at the estuary/coast and nearshore. However, few studies have linked physiological parameters with individual growth rate in this species.

As discussed earlier, biochemical or/and endocrine parameters involved in the growth process may be useful to evaluate recent/current growth status. These include RNA/DNA ratio and IGF-I. In a laboratory experiment using juvenile Atlantic salmon, RNA/DNA ratio in the muscle was positively graded by feeding rations (Arndt et al., 1996). In addition, MacLean et al. (2008) found that muscle RNA/DNA ratio was strongly correlated with individual growth rate in weight ($r^2 = 0.79$) in Atlantic salmon smolts. In contrast, muscle and liver RNA/DNA ratio in postsmolt masu salmon reared in freshwater were not correlated with individual growth rate (Kawaguchi et al., 2013). These findings suggest that the relationship of RNA/DNA ratio with growth rate varies among species, life-history stages and rearing environments.

Many studies reported that circulating IGF-I levels decreased by fasting and

well responded to changes in feeding ration in several fishes (Beckman et al., 2004b; Pierce et al., 2005; Shimizu et al., 2009; Picha et al., 2008b; Kawaguchi et al., 2013). In addition, circulating IGF-I has been shown to reflect recent growth rates of individuals since there is a strong, positive correlation with growth rates, making it as a candidate of growth index (Picha et al., 2008a; Beckman, 2011; Kawaguchi et al., 2013). However, it is not known whether RNA/DNA ratio and IGF-I can use as growth indices in juvenile chum salmon.

The present chapter first examined the validity of RNA/DNA ratio and circulating IGF-I as indices of growth rate using individually-tagged juvenile chum salmon under laboratory settings. I then measured these candidates of growth indices in juveniles during downstream and coastal migrations in order to monitor individual growth status at northeastern Hokkaido, Japan.

2.2. Materials and methods

2.2.1. Rearing experiment

Juvenile chum salmon were transferred from a local hatchery (Kamisato Hatchery) in Abashiri area, northeastern Hokkaido, to the rearing facility at Faculty of Fisheries Sciences, Hokkaido University and reared in 60 L freshwater glass aquariums (size 60 × 29.5 × 36 cm) in a temperature-controlled room (10°C). Each aquarium had a closed-circulation system installed with attached upper filtration throughout rearing. Fish were fed daily on a commercial diet (Marubeni Nisshin Feed Co. Ltd., Tokyo, Japan) to satiety until the beginning of the experiment. In May 2014, fish were acclimated to artificial seawater (Instant Ocean; Spectrum Brands Inc., Tokyo, Japan) by gradually increasing the salinity over one week. In June 2014, juveniles were lightly anesthetized in 3% 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan) and individually marked with PIT-tags (size φ1.4 mm × 8.4 mm, Biomark, Boise, ID, USA). They were

randomly placed into two 60 L seawater 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. Salinity was kept at full-strength seawater (31-34‰) and water temperature was maintained between 11.0-11.5°C throughout the experiment. The experiment was carried out in accordance with the guidelines of the Hokkaido University Animal Care and Use Committee.

Fork length (FL) and body weight (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)) × 100/(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$_2$, s_1 is length or weight on day$_1$ and $d_2 - d_1$ is the number of days among measurements. At the initial sampling, eight fish were sampled for blood and muscle. On day 10, fish from each treatment (Fed: n = 25, Fasted: n = 24) were sampled for blood and muscle. Blood was withdrawn using 10 or 20 μl plain glass tubes (Microcap; Drummond Scientific Company, Broomall, PA, USA) from the caudal vein, 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. A piece of muscle was excised from whole body, immediately frozen on dry ice and stored at -80°C until use.

2.2.2. Field survey

Field survey was carried out around the Abashiri coast, the Sea of Okhotsk side of Hokkaido Island, four times from mid May to mid June in 2013 and 2014, respectively (Fig.1). Fish were caught every 10 days by castnet at the Abashiri River, dragnet at the estuary and two-boat trawling at the port and coast. Trawling was conducted using a net (8-m-wide × 5-m-deep mouth, 18-m-long with wing nets 7-m-long and a central bag with 5-mm mesh) towed through the 1-2 m surface layer for 1-2 km at 4-6 km/h in the morning (6:00-8:00). Total catch at each site, trawling time and boat speed were

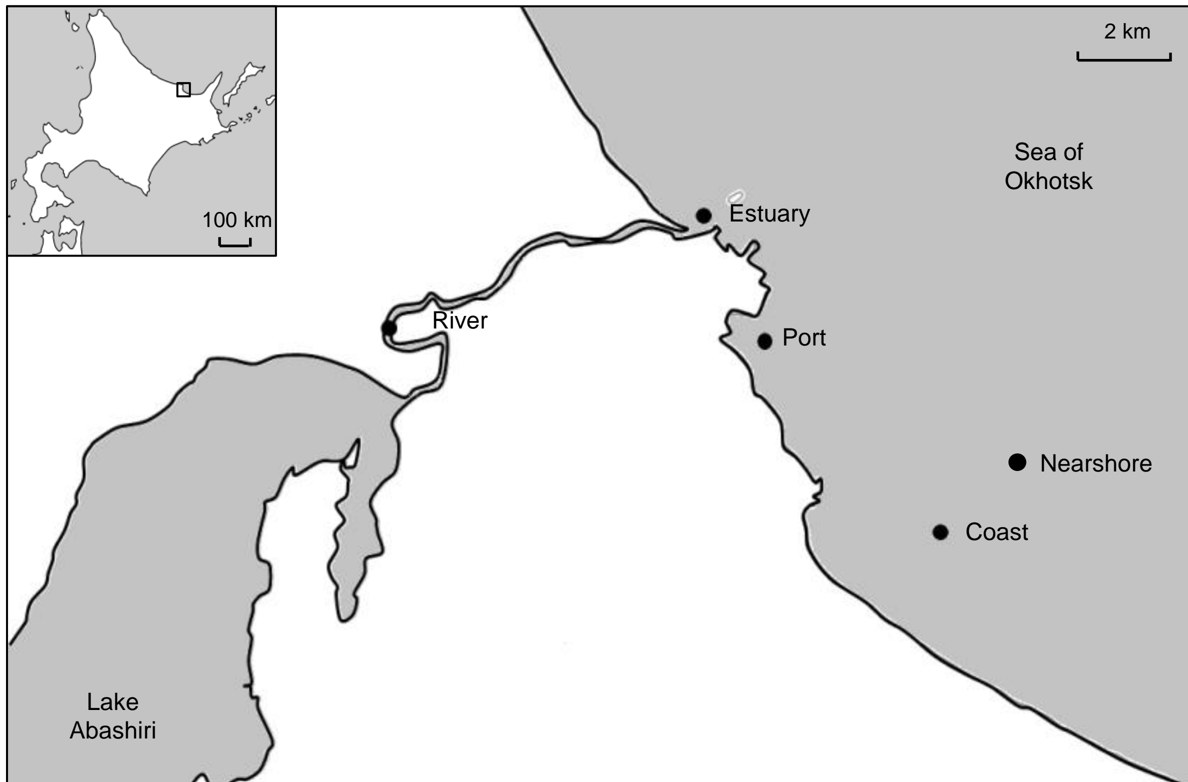

Fig. 1. Locations of the survey area and sampling sites. Black circles indicate the collecting sites for chum salmon juveniles. Fish were caught at the river, estuary, port , coast and nearshore.

recorded to calculate catch per unit effort (CPUE). Sea surface temperature (SST) and water temperature (WT) in the river were recorded at the beginning of catch at each site. Eight fish from each point were sampled for physiological analyses. After anesthetizing fish by 2-phenoxehanol, FL and BW were measured. Blood was withdrawn using 10 or 20 μl plain glass tubes from caudal vein, allowed to clot overnight at 4°C and centrifuged at 10,000 rpm for 15 min. Serum was collected and stored at -80°C. A piece of muscle was excised from whole body, which were mixture of white and red muscle but free of bone and other organs such as kidney, frozen on dry ice and store at -80°C until use. Gill arches were excised from the left side of the body, frozen on dry ice and stored at -80°C until use.

2.2.3. Sample analyses

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

For measuring IGF-I, serum was first extracted with an acid-ethanol as described in Shimizu et al. (2000). IGF-I was quantified by time-resolved fluoroimmunoassay (TR-FIA) based on the method described in Small and Peterson (2005) using recombinant salmon/trout IGF-I (GroPep Bioreagents Pty Ltd., Adelaide, SA, Australia) as a standard. Time-resolved fluorescence was measured using Wallac ARVO SX (PerkinElmer, Waltham, MA, USA).

Gill Na^+, K^+-ATPase (NKA) activity was measured by the method of Quabius et al. (1997) with minor modifications. This parameter was used to evaluate seawater adaptability since juvenile chum salmon experienced different salinities during their

downstream migration. This method is based on the ability of NKA hydrolyzing ATP to give ADP and inorganic phosphorus with or without presence of ouabain at 37°C. 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).

2.2.4. Statistical analyses

Results of the tank experiments were first analyzed by two-way ANOVA (date × treatment) using the JMP program (SAS Institute Inc., Cary, NC, USA). Simple regression analysis was also conducted using JMP program and the relations were considered to be significant at $P < 0.05$. Results of field survey were also analyzed by two-way ANOVA (date × site). When significant effects were found, differences were further identified by one-way ANOVA followed by Fisher's protected least significant difference (PLSD) test. Differences among groups were considered to be significant at $P < 0.05$.

2.3. Results

In the fasting experiment, BW and K significantly decreased after 10 days of fasting (Table 1). Fasting for 10 days resulted in little and negative SGRs in length and weight, respectively (Fig. 2). Muscle RNA/DNA ratio and circulating IGF-I levels in fasted fish were significantly lower than those in fed fish, although the magnitude of difference was greater for serum IGF-I (Fig. 3). Muscle RNA/DNA ratio was positively correlated with SGRs in length and weight with a higher regression coefficient for SGR in weight (Fig. 4). Serum IGF-I showed strong positive relations with SGRs in both length and weight (Fig. 5). Muscle RNA/DNA ratio also had a significant correlation with K, but no relationships with FL and BW were seen (Table 2). In contrast, serum IGF-I level

Table 1
Comparison of morphological parameters among treatments

		Day 0	Day 10
FL	Fed	5.8 ± 0.2	5.9 ± 0.1
	Fasted		5.7 ± 0.1
BW	Fed	1.37 ± 0.12 ^{ab}	1.43 ± 0.1 ^{a}
	Fasted		1.13 ± 0.07 ^{b}
K	Fed	0.69 ± 0.02 ^{a}	0.69 ± 0.01 ^{a}
	Fasted		0.58 ± 0.01 ^{b}

Values are expressed as mean ±SE (Day 0: n = 8, Day 10: n = 24-25). Symbols sharing the same letters are not significantly different from each other.

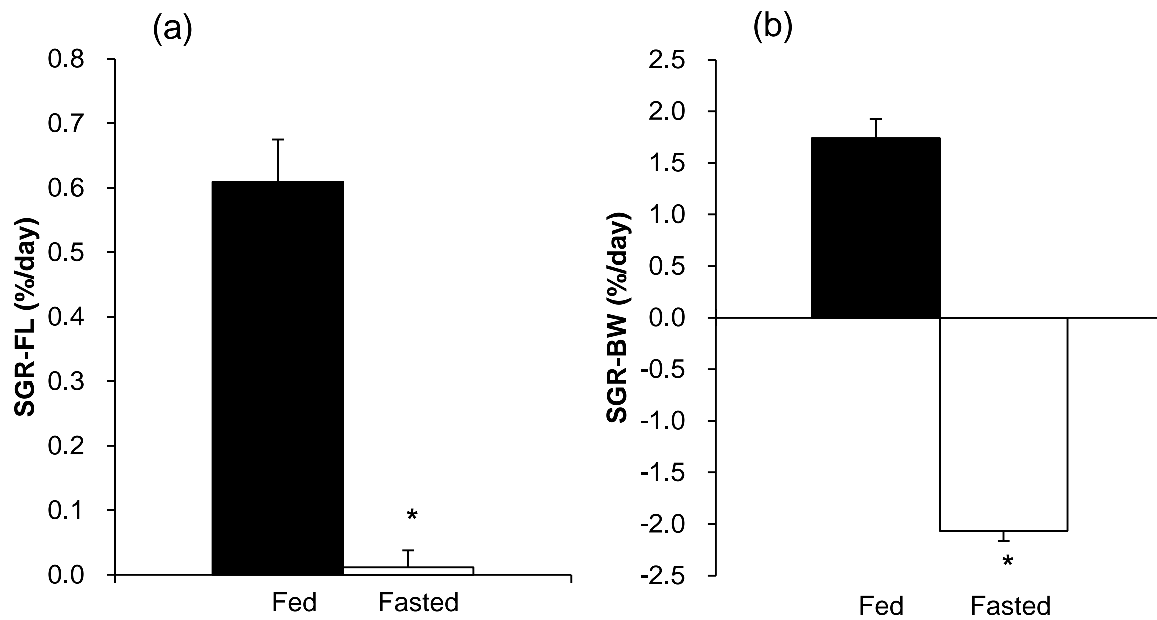

Fig. 2. Effect of fasting on SGR in length (a) and weight (b). Individually-tagged fish were fed or fasted for 10 days. Values are expressed as means ± SE (Fed: n = 25, Fasted: n = 24). Asterisks indicate significant differences between treatments.

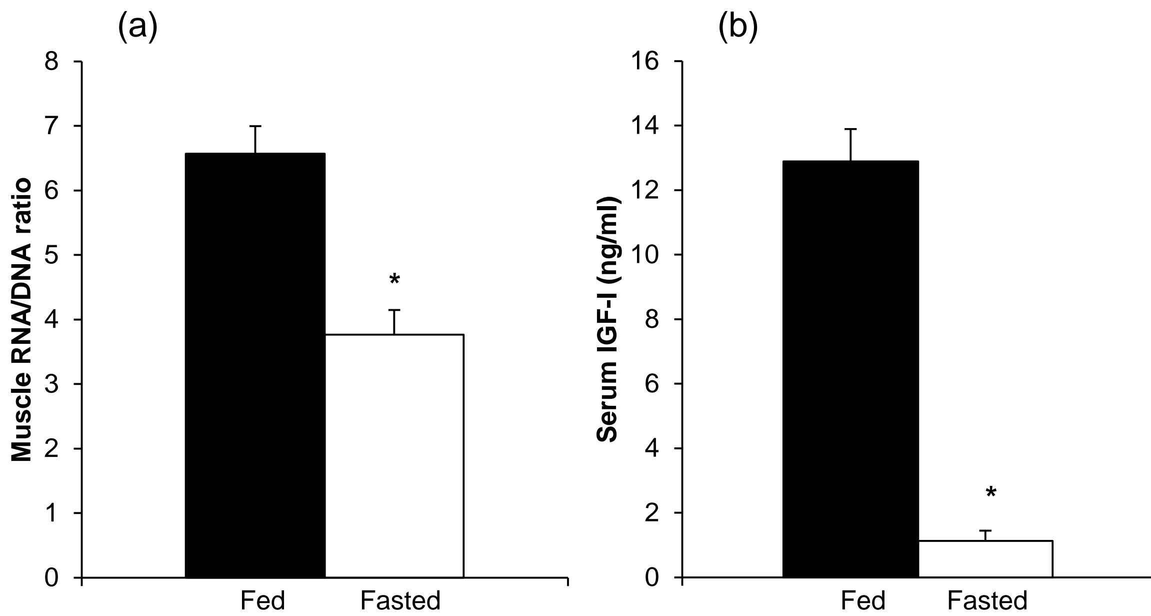

Fig. 3. Effect of fasting on muscle RNA/DNA ratio (a) and serum IGF-I level (b). Values are expressed as means ± SE (Fed: n = 25, Fasted: n = 24). Asterisks indicate significant differences between treatments.

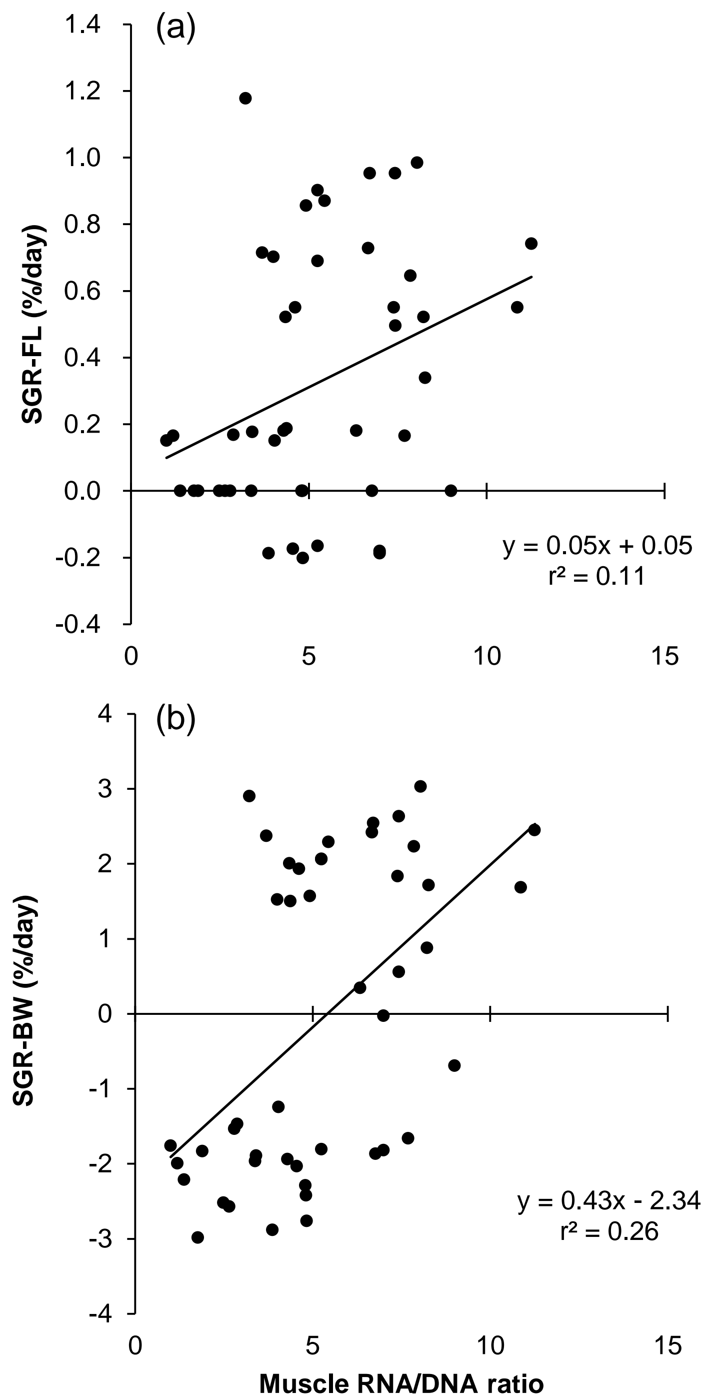

Fig. 4. Correlation between muscle RNA/DNA ratio and SGR in length (a) and weight (b). Dots are data from both fed and fasted fish ($n = 46$).

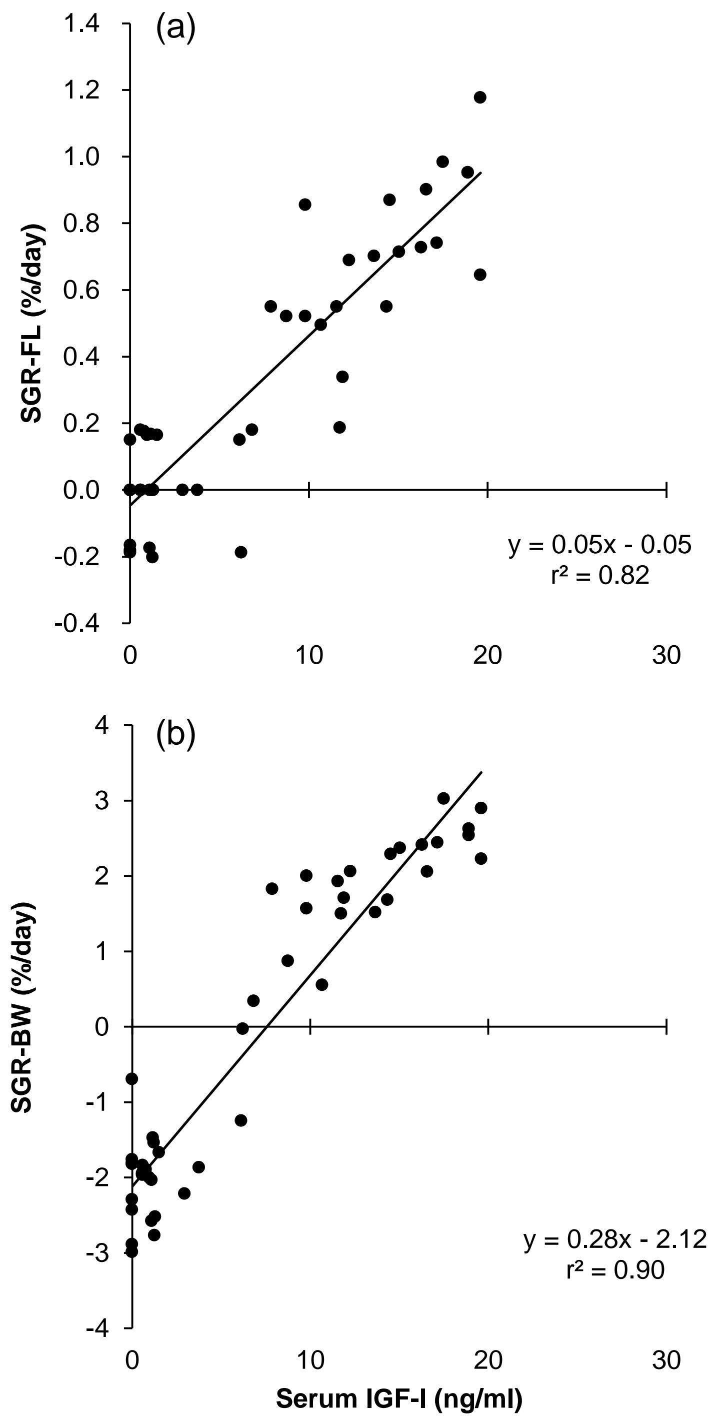

Fig. 5. Correlation between serum IGF-I level and SGR in length (a) and weight (b). Dots are data from both fed and fasted fish ($n = 46$).

Table 2
Regression coefficients (r^2) among physiological and biochemical parameters

	FL	BW	K	SGR-FL	SGR-BW	Muscle RNA/DNA
BW	0.87					
K	ns	0.33				
SGR-FL	0.23	0.44	0.45			
SGR-BW	0.15	0.42	0.71	0.82		
Muscle RNA/DNA	ns	ns	0.21	0.11	0.25	
Serum IGF-I	0.17	0.41	0.58	0.82	0.90	0.23

ns: not significant.

was significantly correlated with FL, BW and K (Table 2).

In the field survey, CPUE was highest at the estuary in May 2013, and the site of high CPUE shifted from the estuary to the port in June (Fig. 6). In May 2014, CPUE was high at the estuary as well as at the coast. The high CPUE at the coast was also observed in early June 2014. WT in mid May 2014 was higher than that in 2013 (Fig. 6). BW did not show clear trend in May in both years except fish caught at the coast in late May 2014, which were larger than those at other sites (Fig. 7). In June, BW of fish at the estuary tended to be small and that at the coast be large in both years (Fig. 7).

Activation of gill NKA after sea entry was observed in June 2013 (Fig. 8). In 2014, temporal increases in gill NKA activity in fish at the port were observed from late May to early June (Fig. 8). Muscle RNA/DNA ratio was generally similar among sampling sites in both years (Fig. 9). When the overall average values were compared among the sampling dates in each year, it was high in mid June in 2013 and early June in 2014, respectively. Serum IGF-I levels were low in fish at the estuary when compared to those at the coast from early June to mid June in both years (Fig. 10). The gradual increase of serum IGF-I levels in June was similar to the trend of BW.

2.4. Discussion

In the present study, I examined the relationship of muscle RNA/DNA ratio with individual growth rate in juvenile chum salmon in seawater. The muscle may be the best part to measure RNA/DNA ratio since the muscle constitutes the majority of fish body's mass. MacLean et al. (2008) found that muscle RNA/DNA ratio was strongly correlated with individual growth rate in weight in Atlantic salmon smolts. On the other hand, Johnson et al. (2002) examined an effect of variable rations on muscle RNA/DNA ratio in juvenile red drum (*Sciaenops ocellantus*) and found that fasting was effective to see a significant reduction of RNA/DNA ratio but no significant differences were observed

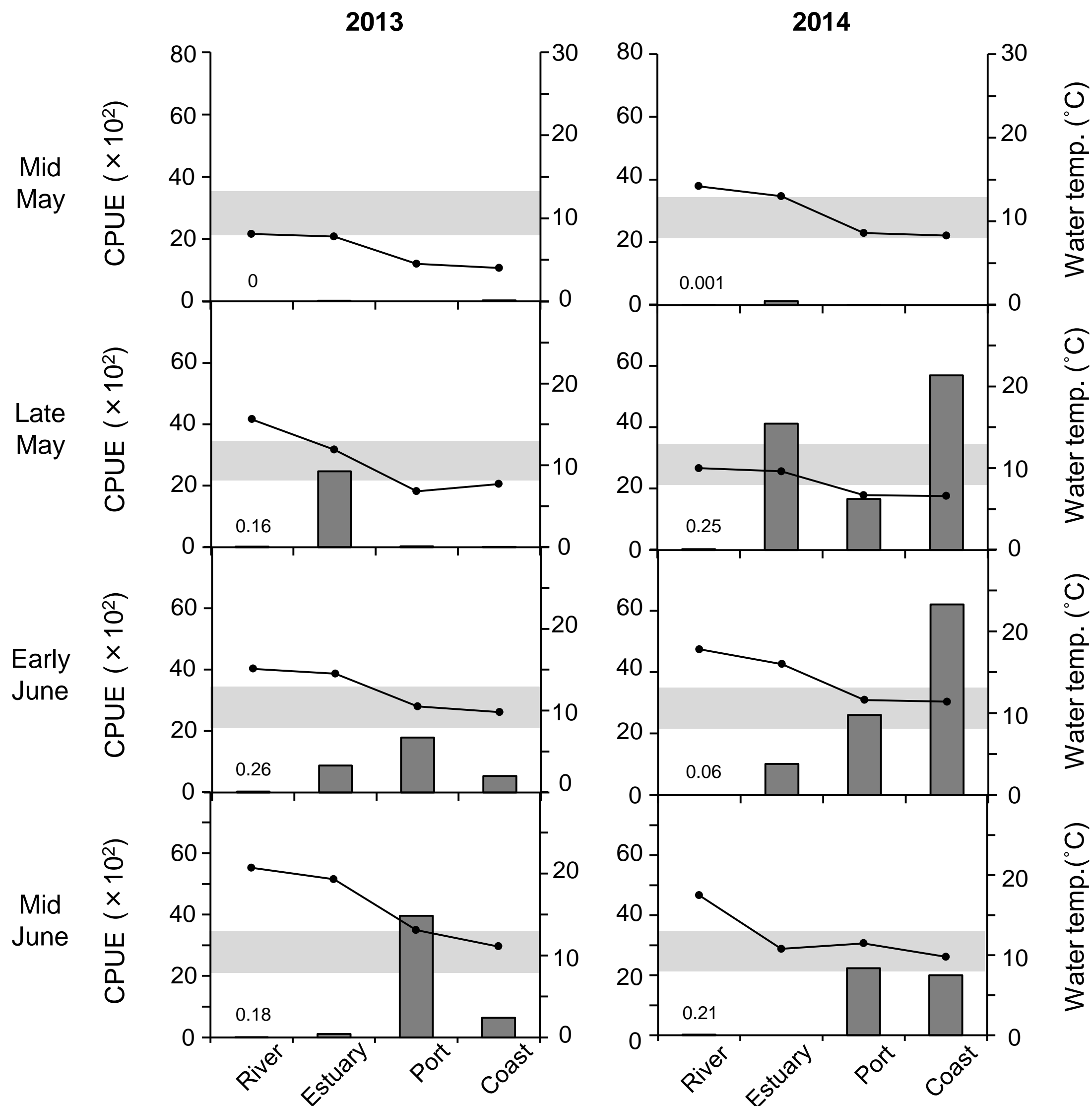

Fig. 6. CPUE for chum salmon juveniles (bar) and water temperature (line plot) at the river, estuary, port and coast from mid May to mid June in 2013 (left) and 2014 (right). CPUEs at the river is expressed as catch per casting and indicated by numbers, and that at estuary as catch per drag-netting. CPUEs at the port and coast are expressed as catch per 1-km trawling. Shaded areas indicate temperature range 8-13°C which has been suggested to be optimal for juvenile chum salmon in that area (Nagata et al. 2007).

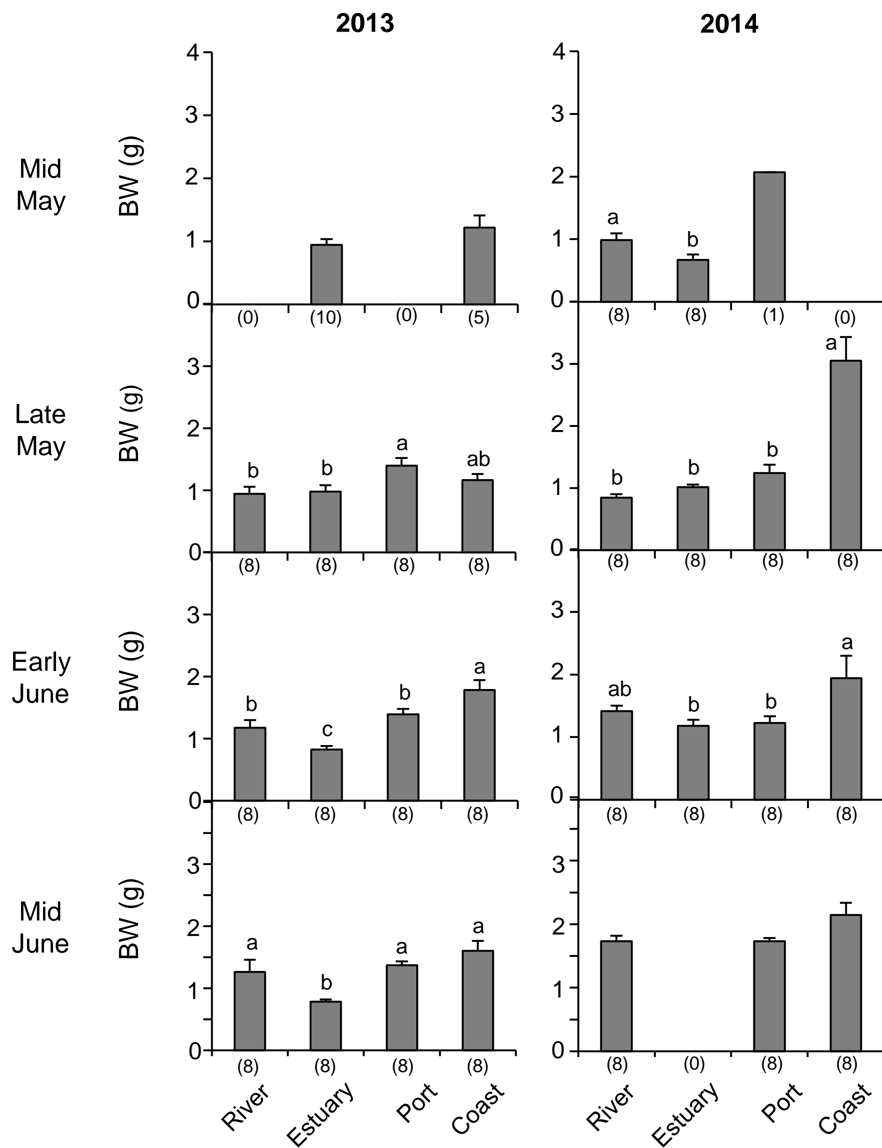

Fig. 7. BW of juvenile chum salmon at the river, estuary, port and coast from mid May to mid June in 2013 (left) and 2014 (right). Values are expressed as means ± SE. Arabic numeral under the x-axis indicates sample number. Symbols sharing the same letters are not significantly different from each other.

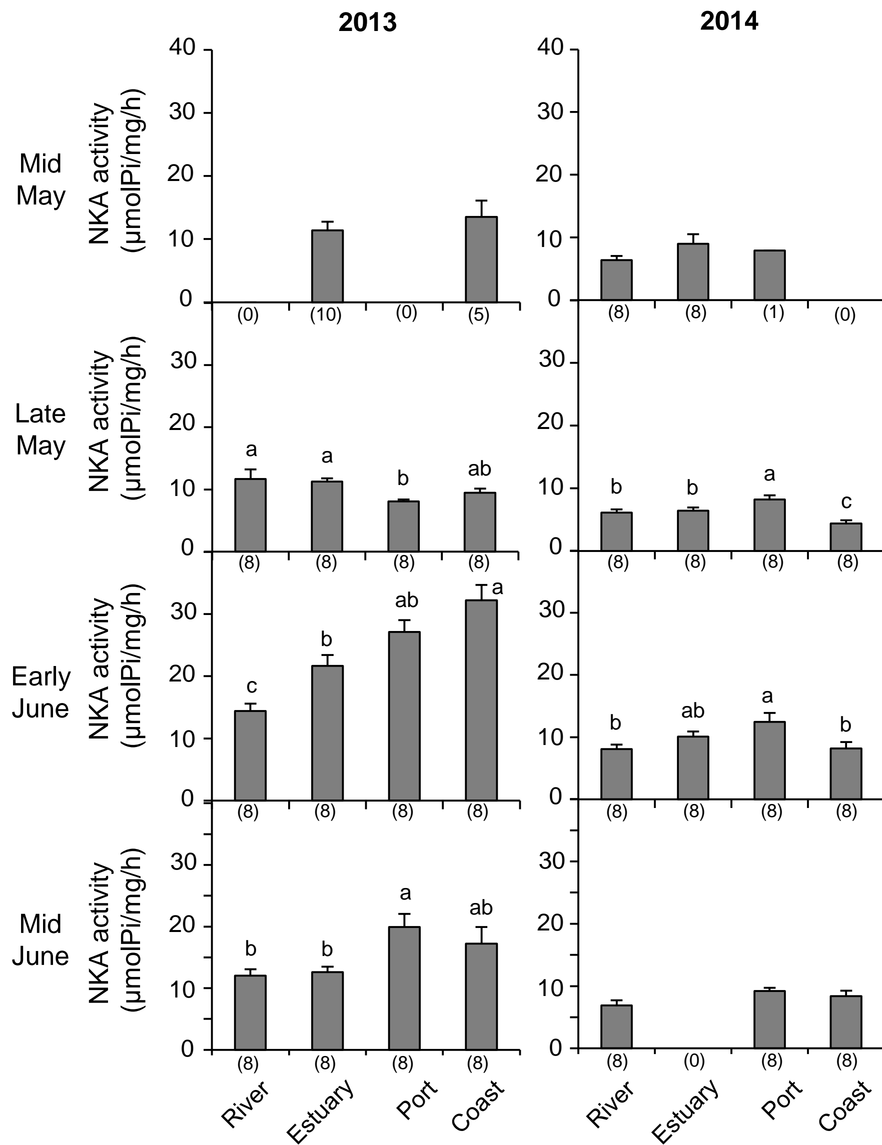

Fig. 8. Gill NKA activity of juvenile chum salmon at the river, estuary, port and coast from mid May to mid June in 2013 (left) and 2014 (right). Values are expressed as means ± SE. Arabic numeral under the x-axis indicates sample number. Symbols sharing the same letters are not significantly different from each other.

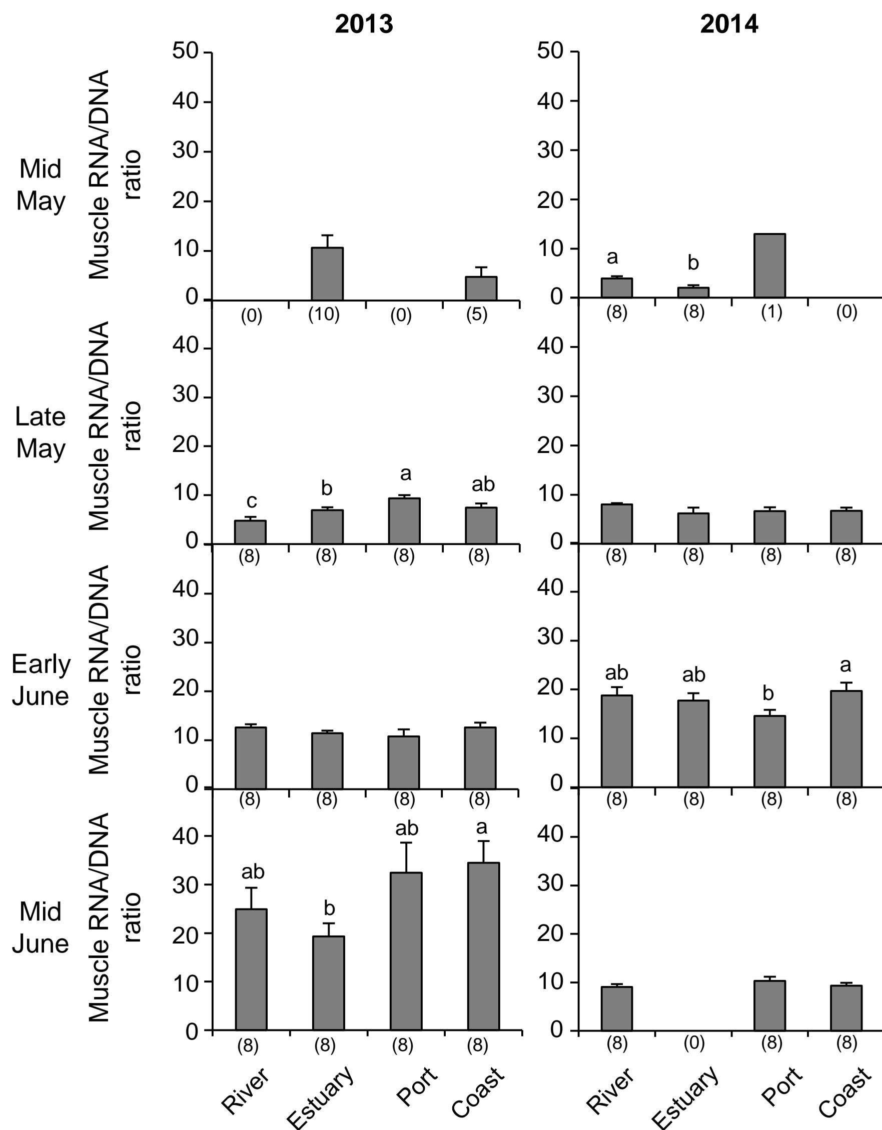

Fig. 9. Muscle RNA/DNA ratio of juvenile chum salmon at the river, estuary, port and coast from mid May to mid June in 2013 (left) and 2014 (right). Values are expressed as means ± SE. Arabic numeral under the x-axis indicates sample number. Symbols sharing the same letters are not significantly different from each other.

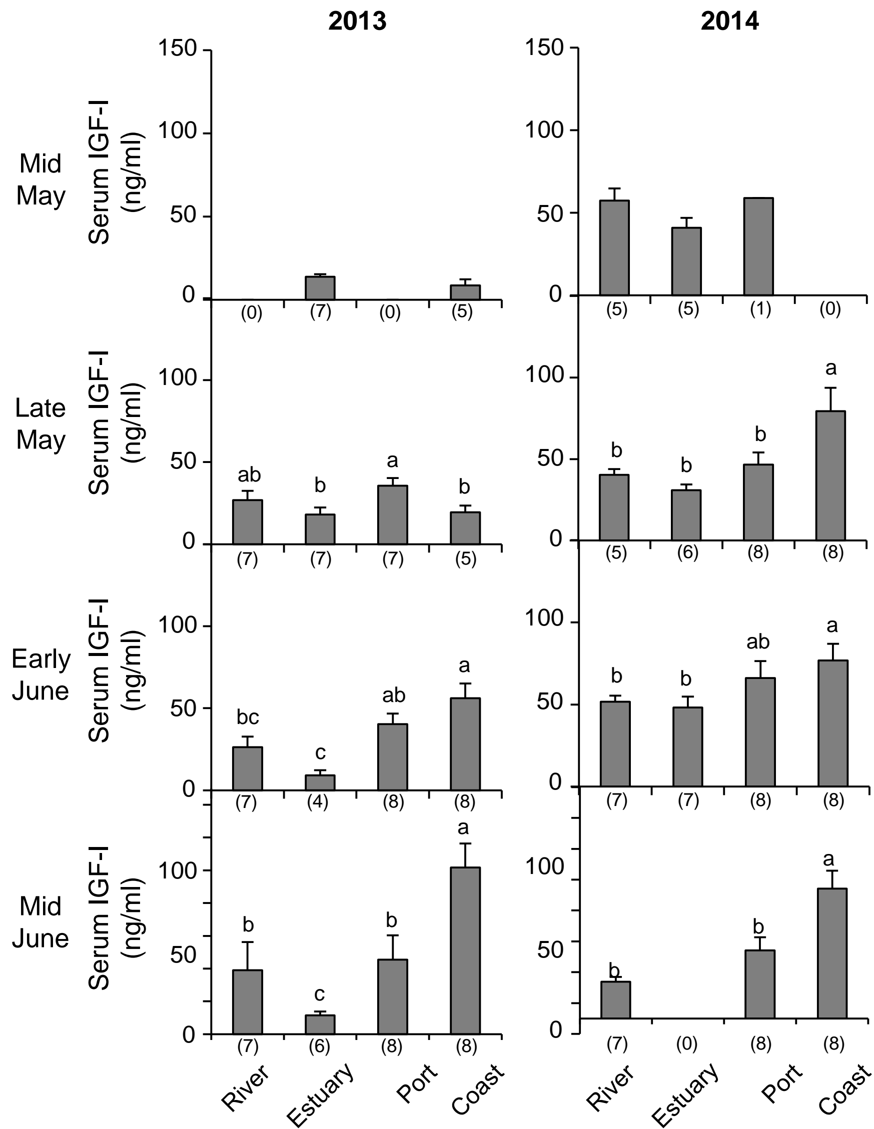

Fig. 10. Serum IGF-I level in juvenile chum salmon at the river, estuary, port and coast from mid May to mid June in 2013 (left) and 2014 (right). Values are expressed as means ± SE. Arabic numeral under the x-axis indicates sample number. Symbols sharing the same letters are not significantly different from each other.

among different feeding rations. Thus, the sensitivity of muscle RNA/DNA ratio as a growth index needs to be evaluated in each case. In the present study, muscle RNA/DNA ratio significantly decreased in fasting group and had a weak positive correlation with individual growth rate in both length and weight. I did not examine varying feeding rations in the present study but the correlation analysis suggests that muscle RNA/DNA ratio in juvenile chum salmon reflects nutritional status and recent body growth for some extent.

I also examined the response of serum IGF-I to fasting and its relationship with individual growth rate in juvenile chum salmon and the results were in accord with the previous findings. Circulating IGF-I in fish generally responds well to changing nutritional conditions; it is high when fish are fed and decreased when fish are restricted for feeding or deprived of feed (Beckman et al., 2004b; Picha et al., 2008b; Taniyama et al., 2016). In Chinook salmon, circulating IGF-I levels started to decline in 4 days during fasting (Pierce et al., 2005). In the present study, serum IGF-I levels were significantly decreased by fasting for 10 days. There were positive correlations between serum IGF-I level and individual growth rate in length and weight, and the IGF-I-growth relationship was stronger than that of muscle RNA/DNA ratio. Although positive relationships between circulating IGF-I level and individual growth rate have been reported in Atlantic salmon (Dyer et al., 2004), coho salmon (Beckman et al., 2004a, b, c) and masu salmon (Kawaguchi et al., 2013), this study is the first to clarify the relationship in juvenile chum salmon. The present results suggest that IGF-I is a better growth index than muscle RNA/DNA ratio in chum salmon, which is consistent with the findings in masu salmon (Kawaguchi et al., 2013). On the other hand, a time course of the IGF-I response may vary among experimental conditions such as temperature, season and fish status (Beckman et al., 1998, 2004c; Larsen et al., 2001; Beckman, 2011; Taniyama et al., 2016). Seasonal changes in the basal IGF-I levels may also affect the relationship (Beckman et al., 2004b; Beaudreau et al., 2011). I also

examined the IGF-I-growth relationship in August and found significant positive correlations in length and weight (data not shown). This result suggests that although the strength or slope of the relationship may vary, circulating IGF-I consistently reflects the growth rate at least during spring and summer in juvenile chum salmon.

Juvenile chum salmon were collected at the river, estuary, port, and coast by following their downstream and coastal migration route from May to June in Abashiri region, northeastern Hokkaido, Japan. Distribution of chum salmon juveniles along coastal waters in this region is largely influenced by SST (Nagata et al., 2007). When SST is low (i.e. <8°C), their distribution is limited to the estuaries and littoral zones. SST above 8°C allows juveniles to disperse to the coastal area but high SST (>14°C) drives them to leave for the offshore (Nagata et al., 2007). Thus, SST ranging 8-13°C is optimal for juvenile chum salmon to grow in this area. In the present study, a relatively high CPUE was observed at the estuary in May 2013 where SST was 8-12°C. Fish moved to the port when SST at the estuary became >14°C, which is in good agreement with the findings by Nagata et al. (2007). In 2014, high CPUEs were recorded at the estuary and coast in late May and their distribution shifted to the port and coast in June. The high CPUE at the coast in late May is somewhat peculiar since SST (7°C) was below the optimal range. Although the precise reason for this is not known, it may be related to the profile of SST at that area and fish conditions. SST in mid May 2014 at the port and coast were about 8°C which was favorable for juveniles. Fish caught at the coast were exceptionally large being 7-cm long and 3-g weight. These fish might be early migrants or fast-growing individuals that moved to the coast in late May just before SST slightly dropped.

In the present study, I monitored gill NKA activity since the sampling sites spanned over the different salinities and since successful adaptation to seawater is important to exhibit optimal growth in the ocean. NKA in the gills is the essential enzyme driving the extrusion of excess sodium, chloride and potassium ions from the

fish body (Mancera and McCormick, 2007; Hiroi and McCormick, 2012). Gill NKA activity was mediated by circulating IGF-I and actually its activation in masu salmon have a positive correlation with serum IGF-I level (Shimomura et al., 2012). The activity of NKA in several salmonid species that undergo parr-smolt transformation sharply elevates in freshwater to prepare for the future ocean life (McCormick, 2009; Shimomura et al., 2012). On the other hand, chum and pink salmon do not go through typical parr-smolt transformation and generally have high NKA activity soon after hatching in freshwater. However, gill NKA is often increased when fish enter seawater (Iwata et al., 2012). In the present study, gill NKA activity in fish at the port was generally higher than that at the river expect in late May 2013. Additionally, gill NKA activity showed no further increase or decrease when we compared fish at the port and coast. These results suggest that juvenile chum salmon increase gill NKA activity when entering seawater to fully hypoosmoregulate and the activity returns to the basal level to maintain the inside osmotic pressure. In early June 2013, there was a clear trend of increasing gill NKA activity from the river towards the coast and its absolute levels in the estuary, port and coast were highest during survey. How excess activation of gill NKA affects growth is not known at present.

Muscle RNA/DNA ratio showed no particular temporal or spatial pattern in this survey. Stefansson et al. (2012) monitored muscle RNA/DNA ratio in postsmolt Atlantic salmon migrating from the river through the fjord, coastal areas and the open ocean off the Norwegian coast. Muscle RNA/DNA ratio progressively increased as the fish migrated, suggesting activated muscle growth during this period. In the present study, muscle RNA/DNA ratio of juvenile chum salmon showed no such trend; it was relatively constant when different sampling sites were compared. However, when sampling dates were considered, the basal values were high in mid June 2013 and early June 2014. It might not be directly related to a change in growth status based on the relatively weak relation with growth rate in the laboratory experiment. Water

temperature or/and developmental stage should affect the basal levels, however, these need to be confirmed experimentally.

Although plasma/serum IGF-I levels in fish have been measured in many laboratory experiments, limited numbers of study have dealt with IGF-I in migrating salmon (Beckman et al., 2004a; Stefansson et al., 2012; McCormick et al., 2013; Ferris et al., 2014). Beckman and his colleagues validated IGF-I as an index of individual growth rate for immature coho salmon under laboratory conditions (Beckman et al., 2001, 2004b, c) and utilized it to evaluate growth status of postsmolt coho salmon caught at the Strait of Georgia, Canada, and Puget Sound, USA (Beckman et al., 2004a). This approach has been extended for sockeye, chum and Chinook salmon in British Columbian coastal waters (Ferriss et al., 2014). Variations in plasma IGF-I levels were found to reflect alterations of salmon growth status in response to local environments (Ferriss et al., 2014). A recent field study on coho salmon migrating through Strait of Georgia also suggests that plasma IGF-I levels was sensitive enough to detect variations of the productivity between local regions (Journey et al., 2017). These studies highlight the usefulness of IGF-I to monitor growth status of free-living salmon.

In the present study, serum IGF-I levels in juvenile chum salmon showed spatial and temporal variations during May to June. A general trend was that circulating IGF-I levels were lowest in fish at the estuary and high at the coast from late May through June. Although serum IGF-I level of the estuarine fish in mid June 2014 could not show because of no catch, fish caught in the subsequent survey in late June had lowest IGF-I in the estuary and highest IGF-I in the coast (data not shown). The increased serum IGF-I levels well corresponded to the increased fish size, supporting the result of the laboratory experiment that IGF-I could be used as a growth index in this species. Based on these results, chum salmon juveniles activated growth when they left the coast, which would be important to reach a critical size by the end of the summer in the Sea of Okhotsk. Our finding is also similar to that of Stefansson et al.

(2012) who reported increased IGF-I levels during downstream and coastal migration in Atlantic salmon smolts in Norwegian coastal waters. In contrast, plasma IGF-I levels in Atlantic salmon smolts migrating in the Gulf of Maine, USA, were lower than those in the Penobscot River (McCormick et al., 2013). This discrepancy in IGF-I profile may be due to a temporal increase in IGF-I during smoltification or/and difference in water temperature between the river and the ocean (McCormick, 2009; McCormick et al., 2013).

I found that small fish were consistently caught at the estuary throughout the sampling period and their serum IGF-I levels were low. These results suggest that fish in the estuary were under poor growth condition. Estuary is an important area for juvenile salmon especially for chum salmon (Salo, 1991; Hillgruber and Zimmerman, 2009) since it serves as a site for acclimation to seawater or a refuge from unfavorable temperature or feeding conditions. However, drawbacks are competition for food and delay of the migration timing. Indeed, fish in the estuary were often small and found to be at high density (Magnusson and Hilborn, 2003; Hillgruber and Zimmerman, 2009; Kocik et al., 2009). I observed the distribution of juveniles was similar to that reported in the previous studies above; larger fish were caught in the coast and smaller fish were caught in the estuary in June in both years. These results on IGF-I suggest that juveniles at the estuary had low growth rate and might suffer high mortality as indicated by the marking-recapture experiments (Bax, 1983; Fukuwaka and Suzuki, 2002; Wertheimer and Thrower, 2007). In order to test this hypothesis, more comprehensive sampling around the estuary and port is necessary in future survey.

In summary, the present study suggests that circulating IGF-I is a growth index in juvenile chum salmon in seawater. Monitoring growth status using IGF-I suggested that the growth of juvenile chum salmon in the Abashiri coastal waters was activated when they left the coast, while fish with low growth rate might stay in the estuary and experience size-selective mortality.

3. Circulating IGFBP-1b as a negative growth index of juvenile chum salmon

3.1. Introduction

Growth of animals is determined by their metabolic state which represents a balance between anabolism and catabolism. When the sum of the acquisition of energy by anabolism exceeds that of the consumption of energy by catabolism, there is surplus energy that can be used for growth. Chapter 2 described the utility of circulating IGF-I measures to evaluate positive growth status where anabolism is dominant over catabolism in juvenile chum salmon. Since circulating IGF-I levels in fasted fish were low, it could be also used to evaluate negative growth status where catabolism is dominant over anabolism. However, a shift from anabolic state to catabolic state sometimes occurs rapidly due to environmental changes such as acute stress. Stress responses would eventually slow down growth of an animal due to the catabolic cost to cope with stress. Circulating IGF-I may not be sensitive enough to an acute change of metabolic conditions to serve as a useful index in all conditions. For instance, in postsmolt coho salmon, when water temperature was rapidly dropped from 11°C to 7°C, the relationship between circulating IGF-I and growth rate was disrupted (Beckman et al., 2004c). The study suggests there are some situations where circulating IGF-I level is decoupled with growth status. Thus, it is preferable to have growth indices that reflect catabolic status of salmon along with IGF-I. Availability of such growth indices would stabilize and/or increase the accuracy and sensitivity of growth evaluation.

IGFBP-1 is a candidate for a negative growth index since in mammals it increases under catabolic conditions and inhibits the anabolic action of IGF-I (Jones and Clemmons, 1995; Rajaram et al., 1997; Firth and Baxer, 2002). In teleost, three types of IGFBPs are consistently detected at molecular ranges of 20-25, 28-32 and 40-45 kDa. Two low-molecular-weight IGFBPs have been assumed to be IGFBP-1 or -2 (Kelley et

al., 2001, 2002). Laboratory-based studies indicated that both forms were induced into circulation under acute but continuous stress such as handling for 1 h and confinement for 2 days and hormone treatments (Kelley et al., 2001, 2002, 2006). Kelley et al. (2001, 2002, 2006) proposed that the two low-molecular-weight IGFBPs are useful as markers of catabolic status in fish.

In salmon, two of three major circulating IGFBPs have been identified as IGFBP-1b and -1a (Shimizu et al., 2006, 2011a). A radioimmunoassay (RIA) for salmon IGFBP-1b have been developed and revealed that circulating IGFBP-1b were correlated to malnutritional status such as fasting and reduced feeding ration (Shimizu et al., 2006, 2009). In addition, a negative correlation between circulating IGFBP-1b and individual growth rate in postsmolt coho salmon has been reported (Shimizu et al., 2006). We have recently established a time-resolved fluoroimmunoassay (TR-FIA) for salmon IGFBP-1b using the same components to those for the RIA (Fukuda et al., 2015). TR-FIA has several advantages over RIA such as safety for operators, stability of the label and easiness. With this TR-FIA, we further revealed that IGFBP-1b showed a negative relationship with growth rate in yearling and underyearling masu salmon (Kawaguchi et al., 2013; Fukuda et al., 2015). Thus, it is worth examining whether or not circulating IGFBP-1b can be used as a negative growth index in juvenile chum salmon.

The present chapter examined the usefulness of circulating IGFBP-1b for juvenile chum salmon under laboratory conditions and evaluated their growth status during downstream and coastal migrations in the wild.

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3.2. Materials and methods

3.2.1. Rearing experiment

Juvenile chum salmon were transferred from a local hatchery (Kamisato Hatchery) in Abashiri area, northeastern Hokkaido, to a rearing facility at Faculty of Fisheries Sciences, Hokkaido University and reared in 60 L freshwater glass aquariums (size 60 × 29.5 × 36 cm) in a temperature-controlled room (10°C). Each aquarium had a closed-circulation system installed with attached upper filtration. Fish were fed daily on a commercial diet (Marubeni Nisshin Feed Co. Ltd., Tokyo, Japan) to satiety until the beginning of the experiment. In May and June 2015, fish were acclimated to artificial seawater (Instant Ocean; Spectrum Brands Inc., Tokyo, Japan) by gradually increasing the salinity to 31-34‰ seawater over one week. In May and June 2015, juveniles were lightly anesthetized in 3% 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan) and individually marked with PIT-tags (size φ1.4 mm × 8.4 mm, Biomark, Boise, ID, USA). Fish were randomly placed into three 60 L seawater tanks (15 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 second group was fasted throughout the experimental period. The third group was fasted for 5 days and refed for following 5 days with the same condition as fed group. Salinity was kept at full-strength seawater (31-34‰) and water temperature was maintained between 11.0-11.5°C throughout the experiment. The experiment was carried out in accordance with the guidelines of the Hokkaido University Animal Care and Use Committee.

FL and BW of all the fish were measured at the beginning of the experiment, 5 and 10 days after treatment. K was calculated as follows: (BW (g)) × 100/(FL (cm))^3. SGR was calculated as follows: SGR (%/day) = $\ln (s_2 - s_1) \times (d_2 - d_1)^{-1} \times 100$, where s_2 is length or weight on day_2, s_1 is length or weight on day_1 and $d_2 - d_1$ is the number of days among measurements. At the initial sampling, eight fish were sampled for blood. On day 10, fish from each treatment (Fed: n = 10-11, Fasted: n = 10, Refed: n = 10-11) were sampled for blood. Blood was withdrawn using 10 or 20 µl plain glass tubes (Microcap; Drummond Scientific Company, Broomall, PA, USA) from the caudal vein,

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.

3.2.2. Field survey

Field surveys were carried out around the Abashiri coast, the Sea of Okhotsk side of Hokkaido Island, five times from mid May to late June in 2015 (Fig.1). Fish were caught every 10 days by castnet in the Abashiri River, dragnet in the estuary and two-boat trawling within the port and along the coast and nearshore. Trawling was conducted using a net (8-m-wide × 5-m-deep mouth, 18-m-long with wing nets 7-m-long and a central bag with 5-mm mesh) towed through the surface layer (1-2 m) for 1-2 km at 4-6 km/h in the morning (6:00-8:00). Total catch, trawling time and boat speed were recorded at each site, and SST and WT in the river also were recorded at the beginning of catch at each site. Fish from each point were sampled for physiological analyses. After anesthetizing fish by 3% 2-phenoxehanol, FL and BW were measured. Blood was withdrawn using 10 or 20 μl plain glass tubes from the caudal vein, allowed to clot overnight at 4°C and centrifuged at 10,000 rpm for 15 min. Serum was collected and stored at -80°C.

3.2.3. Sample analyses

For measuring IGF-I, serum was first 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 Bioreagents Pty Ltd., Adelaide, SA, Australia) as a standard. Time-resolved fluorescence was measured using Wallac ARVO SX (PerkinElmer, Waltham, MA, USA). In the present study, serum IGF-I levels in juveniles caught in the wild related their size (serum IGF-I = 21.4 × FL - 78.2, $r^2 = 0.29$, $P < 0.0001$) when the whole samples were pooled and analyzed. In order to better understand growth profiles, I excluded size

effect on IGF-I levels by standardizing measured values to the mean length (Shimizu et al., 2009) using the following equation: standardized hormone $value_1$ = hormone $value_1$ – [($length_1$ – length mean) × slope], where hormone $value_1$ is the individual hormone level of a given fish, $length_1$ is the individual length of a given fish, length mean is the mean fish length juveniles caught in the field survey, and slope of hormone-length relation (Shimomura et al., 2012).

Serum IGFBP-1b levels were quantified by TR-FIA as described in Fukuda et al. (2015). Briefly, a competitive method was employed by following a procedure for DELFIA immunoassay (PerkinElmer). Plasma samples were first incubated with antiserum against purified salmon IGFBP-1b (Shimizu et al. 2006) overnight at 4°C in a 96-well microtiter plate coated with goat anti-rabbit IgG (PerkinElmer). A biotinylated salmon IGFBP-1b was added to each well and incubated overnight at 4°C. After washing, each well received europium-labeled streptavidin (PerkinElmer) followed by DELFIA Enhancement Solution (PerkinElmer). Time-resolved fluorescence was measured at 615 nm by using a Wallac ARVO X4 (PerkinElmer).

3.2.4. Statistical analyses

Results of the tank experiments were first analyzed by two-way ANOVA (date × treatment) using the JMP program (SAS Institute Inc., Cary, NC, USA). Simple regression analysis was also conducted using JMP program and the relations were considered to be significant at $P < 0.05$. When analyzing the regression, circulating IGFBP-1b levels were transformed into natural-log to obtain the normal distribution. The results of field survey were categorized by each month since the each physiological parameter tended similar within a month, and analyzed by two-way ANOVA (month × site). When significant effects were found, differences were further identified by one-way ANOVA followed by Fisher's protected least significant difference (PLSD) test. Differences among groups were considered to be significant at $P < 0.05$.

3.3. Results

In the laboratory experiment, FL, BW and K were significantly low in fish fasted for 10 days when compared with fed group in both months (Table 3). Refed group showed intermediate FL and BW between fish fed and fasted for 10 days. Fasting for 10 days also caused negative SGR in length and weight in both May and June (Fig. 11). Fish refed for 5 days showed an intermediate SGR between fed and fasted groups. Serum IGF-I levels in fasted fish in both months were low (Fig. 12a), while serum IGFBP-1b levels were high (Fig. 12b). Serum IGF-I and IGFBP-1b levels were the highest in June. Serum IGF-I and natural-log transformed IGFBP-1b levels had positive and negative correlations with SGR, respectively, in both months (Fig. 13). Although regression coefficients of IGF-I with SGR were stable between months (May: r^2 = 0.58, June: r^2 = 0.59), those of IGFBP-1b varied between months (May: r^2 = 0.62, June: r^2 = 0.41). Serum IGF-I also correlated with FL, BW and K, while serum IGFBP-1b had a strong correlation with only K in both months (Table 4). In addition, a negative correlation was found between serum IGF-I and IGFBP-1b levels but the correlation coefficient become weaker from May to June. Response of IGF-I/BP-1b ratio to fasting and refeeding were also analyzed (Fig. 14). The ratios in both months were lowest in fasted fish and showed no differences between fed and refed group.

In the field survey, larger fish were consistently in the nearshore in both months (Table 5). In May, small fish were caught in the estuary and/or port, while in June, FL and BW were low in fish caught at the river and estuary. Serum IGF-I levels in juveniles in May were high at the river and the port and slightly decreased at the nearshore (Fig. 15a). In June, serum IGF-I increased from the river to the port and dropped at the nearshore (Fig. 15b). Serum IGFBP-1b levels in May were consistently low from the river to the coast and sharply increased at the nearshore (Fig. 16a). In June, serum

Table 3
Comparison of morphological parameters among treatments in chum salmon reared on May and June

May		Day 0	Day 10
FL	Fed	5.6 ± 0.1^{c}	6.1 ± 0.1^{a}
	Fasted	5.7 ± 0.1^{bc}	5.8 ± 0.1^{bc}
	Refed	5.7 ± 0.1^{bc}	6.0 ± 0.1^{ab}
BW	Fed	1.36 ± 0.05^{c}	1.76 ± 0.11^{a}
	Fasted	1.52 ± 0.07^{bc}	1.32 ± 0.09^{c}
	Refed	1.47 ± 0.06^{bc}	1.58 ± 0.10^{ab}
K	Fed	0.77 ± 0.02^{bc}	0.75 ± 0.02^{bc}
	Fasted	0.81 ± 0.01^{a}	0.67 ± 0.02^{d}
	Refed	0.79 ± 0.01^{ab}	0.74 ± 0.01^{c}
June			
FL	Fed	6.4 ± 0.2^{bc}	7.4 ± 0.2^{a}
	Fasted	6.3 ± 0.1^{c}	6.4 ± 0.2^{c}
	Refed	6.3 ± 0.2^{c}	6.8 ± 0.2^{b}
BW	Fed	2.40 ± 0.18^{b}	3.29 ± 0.26^{a}
	Fasted	2.17 ± 0.15^{b}	1.72 ± 0.18^{b}
	Refed	2.18 ± 0.16^{b}	2.54 ± 0.24^{c}
K	Fed	0.89 ± 0.01^{a}	0.79 ± 0.02^{c}
	Fasted	0.86 ± 0.01^{ab}	0.63 ± 0.01^{d}
	Refed	0.85 ± 0.01^{b}	0.79 ± 0.02^{c}

Values are expressed as mean ± SE (Day 0: n = 16; Day 10: n = 10-11). Symbols sharing the same letters are not significantly different from each other.

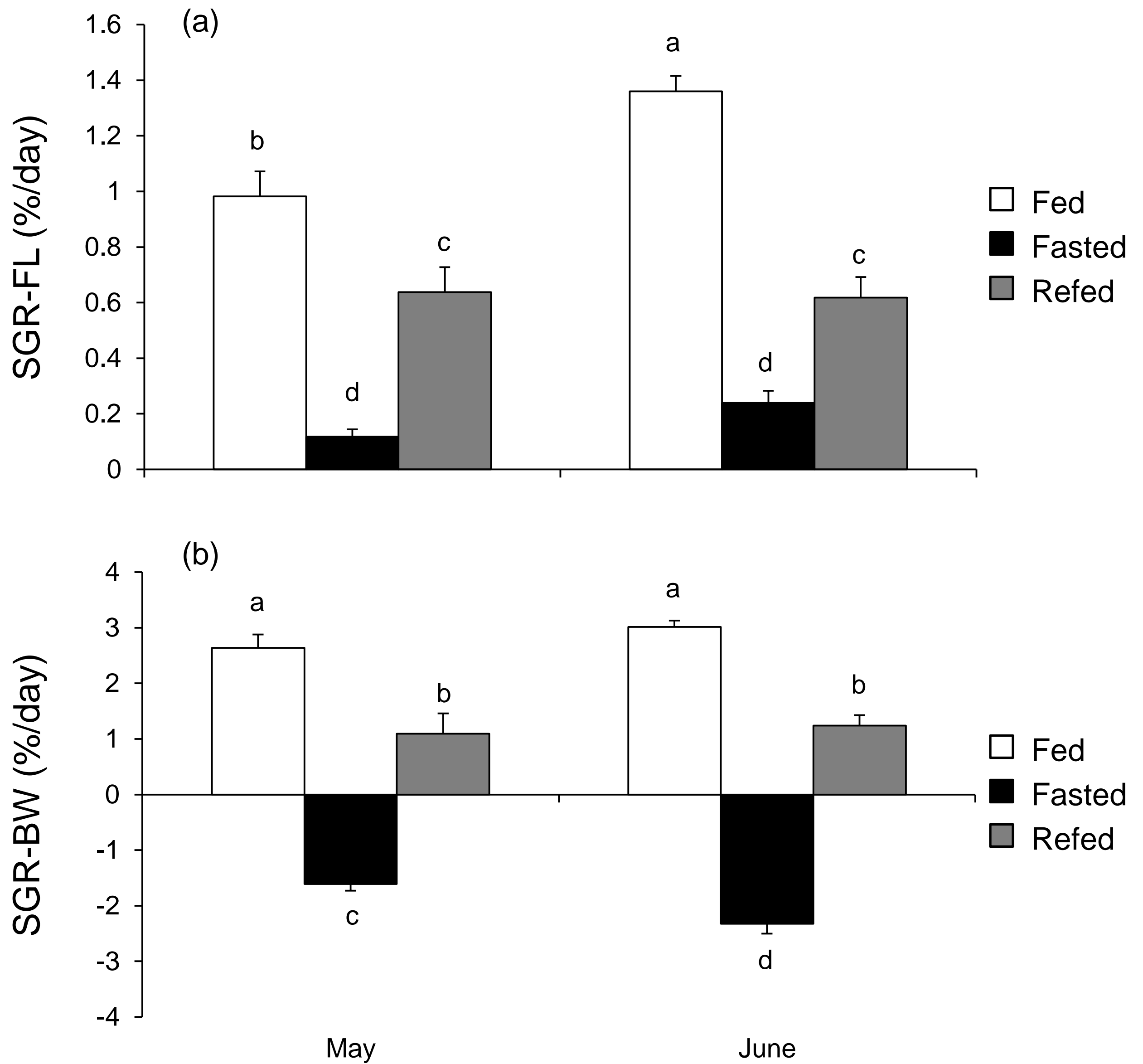

Fig. 11. Effects of fasting and refeeding on SGRs in length (a) and weight (b) on Day 10 in May and June. Individual-tagged fish were fed or fasted for 10 days, or fasted for first 5 days and then re-fed for the following 5 days. Values are expressed as mean ± SE (n = 10-11). Symbols sharing the same letters are not significantly different from each other.

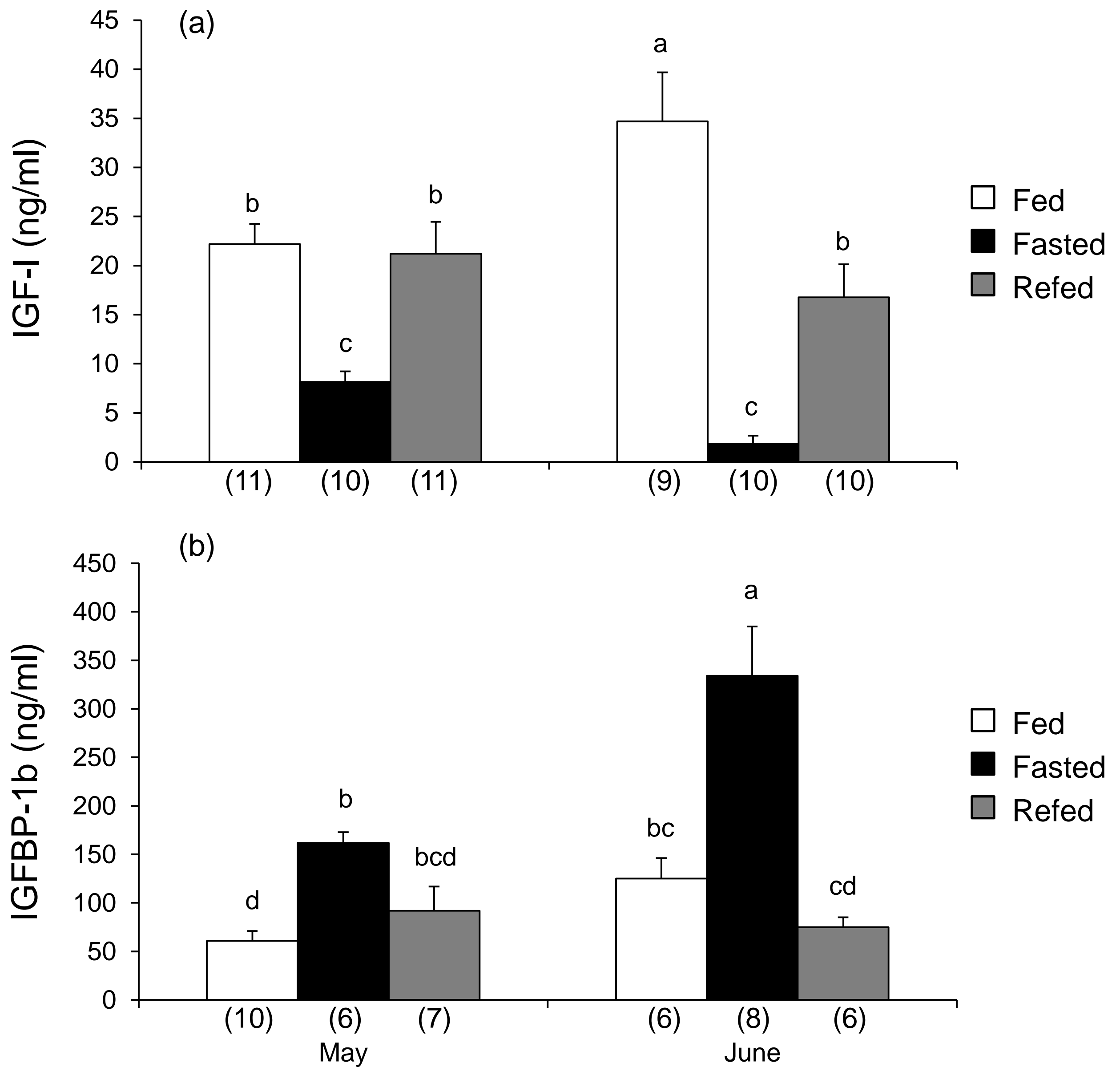

Fig. 12. Effects of fasting and refeeding on serum IGF-I (a) and IGFBP-1b levels (b) on Day 10 in May and June. Individual-tagged fish were fed or fasted for 10 days, or fasted for first 5 days and then re-fed for the following 5 days. Values are expressed as mean ± SE (*n* of each group is shown under corresponding bar). Symbols sharing the same letters are not significantly different from each other.

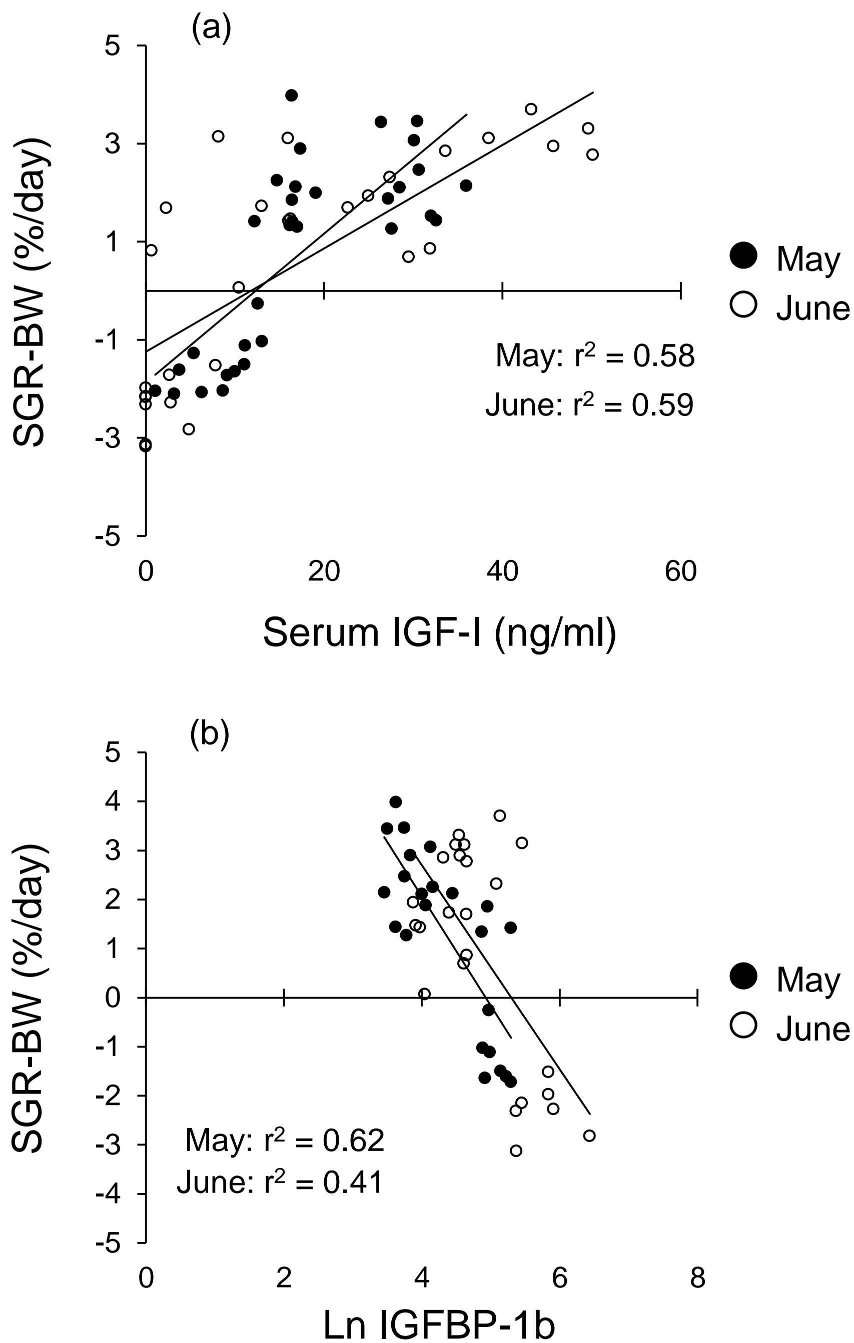

Fig. 13. Correlations of serum IGF-I (a) and natural-log transformed IGFBP-1b level (b) against SGR in weight of juvenile chum salmon in May (black circle) and June (while circle). Dots are data from fed, fasted and refed fish (IGF-I: *n* = 29-32, IGFBP-1b: *n* = 23-24)

Table 4
Correlation coefficients (r) between physiological and morphological parameters on Day 10 in May and June

May	FL	BW	K	SGR-FL	SGR-BW	IGF-I
BW	0.93					
K	Ns	0.52				
SGR-FL	0.47	0.62	0.59			
SGE-BW	0.52	0.68	0.64	0.95		
IGF-I	0.51	0.66	0.62	0.68	0.76	
IGFBP-1b	ns	ns	-0.67	-0.69	-0.76	-0.85
June						
BW	0.97					
K	0.58	0.72				
SGR-FL	0.60	0.62	0.57			
SGE-BW	0.62	0.71	0.85	0.86		
IGF-I	0.60	0.65	0.65	0.75	0.77	
IGFBP-1b	ns	ns	-0.73	ns	-0.64	-0.48

IGFBP-1b values are transformed to natural-log. ns: not significant.

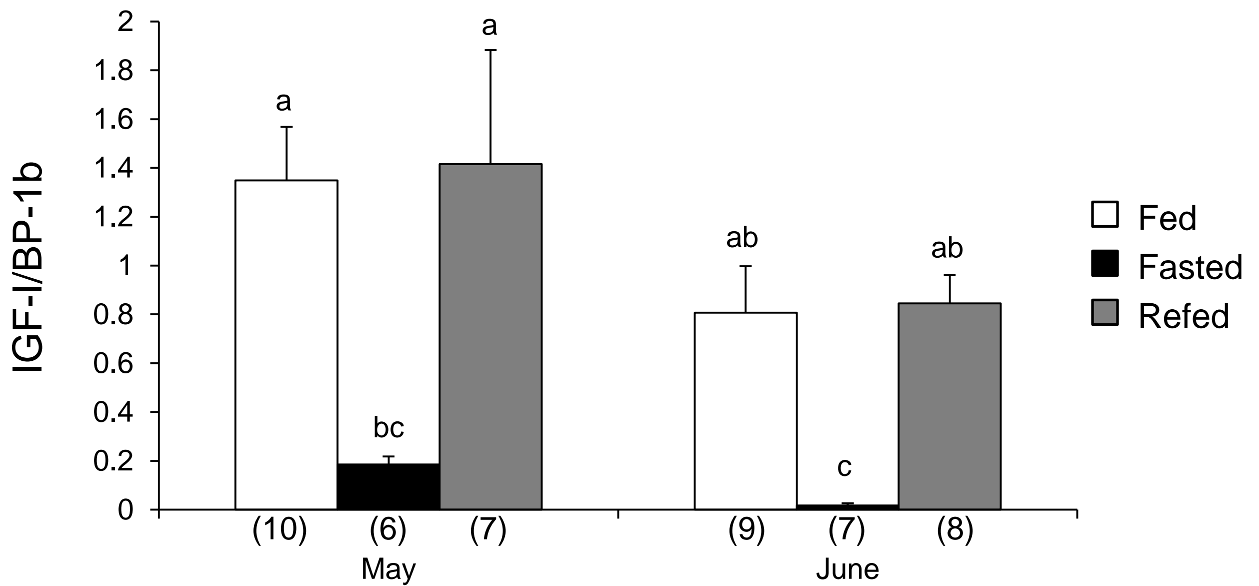

Fig. 14. Effects of fasting and re-feeding on the molar ratio of IGF-I to IGFBP-1b (IGF-I/BP-1b ratio) on Day 10 in May and June. Individual-tagged fish were fed or fasted for 10 days, or fasted for first 5 days and then refed for the following 5 days. Values are expressed as mean ± SE (*n* of each group is shown under corresponding bar). Symbols sharing the same letters are not significantly different from each other.

Table 5
Comparison of morphological parameters in juvenile chum salmon caught at the river, estuary, port, coast and nearshore in May and June

May	N	FL	BW	K
River	42	5.5 ± 0.1[a]	1.23 ± 0.04[b]	0.74 ± 0.01[c]
Estuary	2	5.2 ± 0.7[ab]	0.99 ± 0.36[bc]	0.70 ± 0.01[bc]
Port	21	5.0 ± 0.2[b]	1.08 ± 0.09[b]	0.81 ± 0.02[a]
Coast	34	5.7 ± 0.1[a]	1.50 ± 0.07[a]	0.80 ± 0.02[ab]
Nearshore	11	5.7 ± 0.1[a]	1.50 ± 0.08[ac]	0.79 ± 0.01[ab]
June				
River	48	5.8 ± 0.1[d]	1.42 ± 0.06[d]	0.71 ± 0.01[c]
Estuary	35	5.5 ± 0.1[e]	1.26 ± 0.05[d]	0.77 ± 0.01[b]
Port	101	6.5 ± 0.1[c]	2.14 ± 0.06[c]	0.76 ± 0.01[b]
Coast	58	6.8 ± 0.1[b]	2.64 ± 0.14[b]	0.80 ± 0.01[a]
Nearshore	36	7.2 ± 0.1[a]	3.11 ± 0.11[a]	0.83 ± 0.01[a]

Values are expressed as mean ± SE. Symbols sharing the same letters are not significantly different from each other.

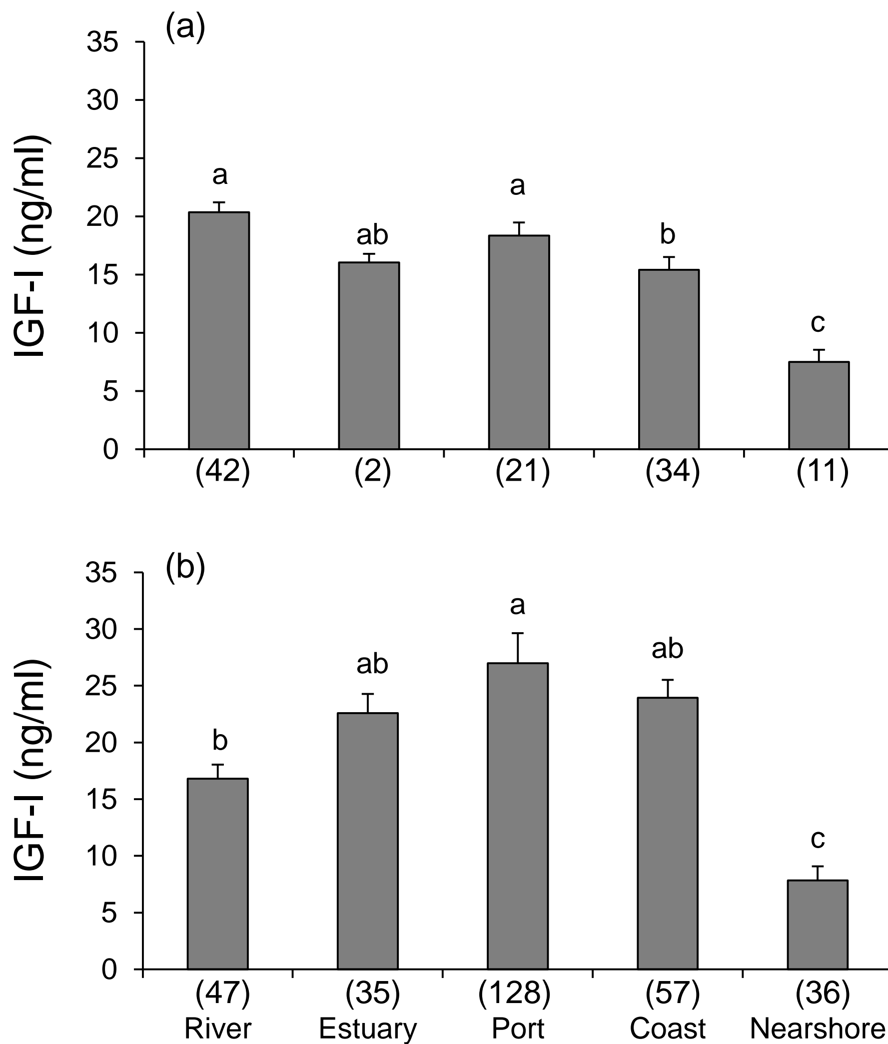

Fig. 15. Regional variations of size-standardized IGF-I in May (a) and June (b). Juvenile chum salmon were caught at the river, estuary, port, coast and nearshore in 2015. Values are expressed as mean ± SE (*n* of each group is shown under the corresponding bar). Symbols sharing the same letters are not significantly different from each other.

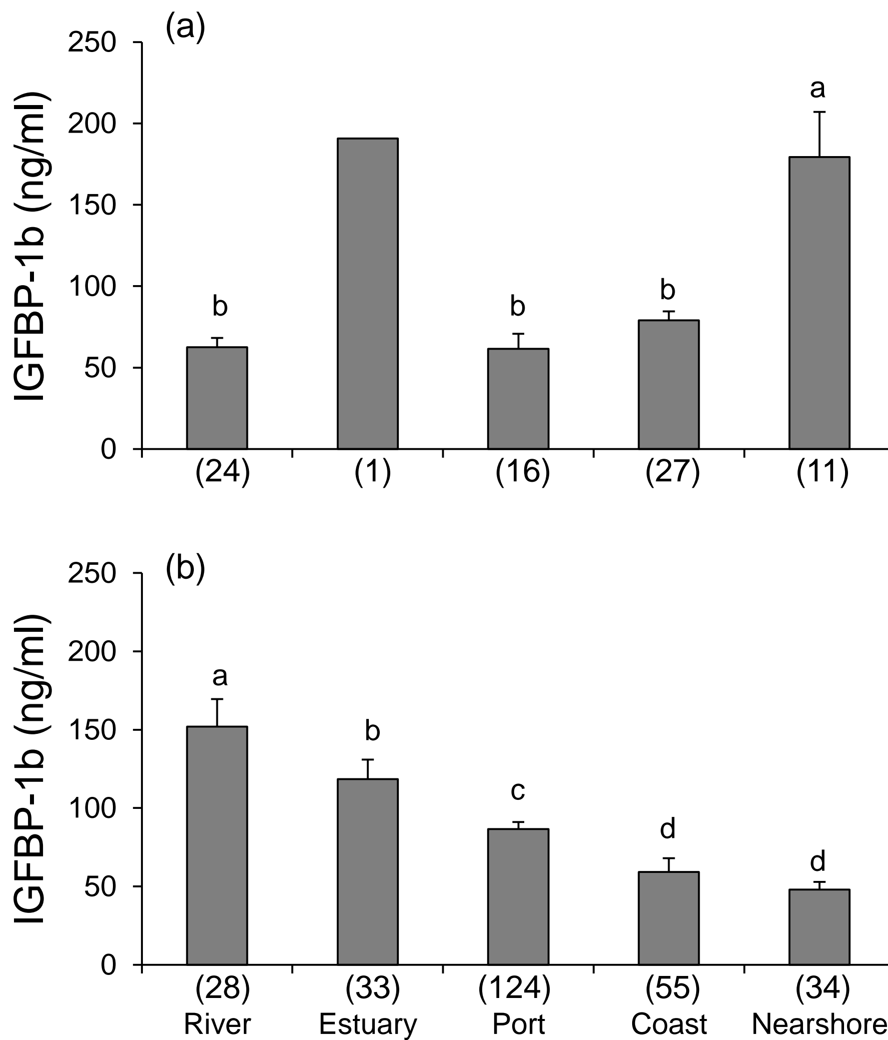

Fig. 16. Regional variations of serum IGFBP-1b levels in May (a) and June (b). Juvenile chum salmon were caught at the river, estuary, port, coast and nearshore in 2015. Values are expressed as mean ± SE (*n* of each group is shown under the corresponding bar). Symbols sharing the same letters are not significantly different from each other.

IGFBP-1b gradually decreased from the river to the nearshore (Fig. 16b). IGF-I/BP-1b ratio better distinguished regional differences (Fig. 17). The ratio in May decreased from the river to the nearshore, while it increased in June.

3.4. Discussion

I first examined the response of circulating IGFBP-1b to fasting and refeeding in juvenile chum salmon under laboratory conditions. Circulating IGFBP-1 in mammal and fish generally increases in response to fasting (Lee et al., 1993, 1997; Siharath et al., 1996; Kelley et al., 2001; Peterson and Small, 2004; Shimizu et al., 2006, 2009; Kawaguchi et al., 2013; Fukuda et al. 2015). In postsmolt coho salmon, plasma IGFBP-1b well correlated to the length of fasting and changes in feeding ration (Shimizu et al., 2006, 2009). In masu salmon, a significant increase of serum IGFBP-1b was seen in the fish fasted for 4 weeks and these values remained high throughout experimental period (Kawaguchi et al., 2013). In this study, serum IGFBP-1b levels increased when fish were fasted for 10 days and decreased to the basal levels after refeeding for 5 days in May and June. These responses were in good agreement with the previous studies on coho and masu salmon.

A negative correlation between IGFBP-1b levels and individual growth rates has been reported in postsmolt coho and masu salmon (Shimizu et al., 2006; Kawaguchi et al., 2013). However, the IGFBP-1b-growth relationships varied with different fish conditions such as age and season. Kawaguchi et al. (2013) found strong negative relationships in yearling masu salmon (r^2 = 0.71) between serum IGFBP-1b level and growth rate. In contrast, the correlation between these was weak in underyearling fish (r^2 = 0.25) (Fukuda et al. 2015), suggesting the relationship may be influenced by age. In addition, plasma IGFBP-1b in postsmolt coho salmon showed significant correlations with growth rate during June to September but regression coefficients varied among

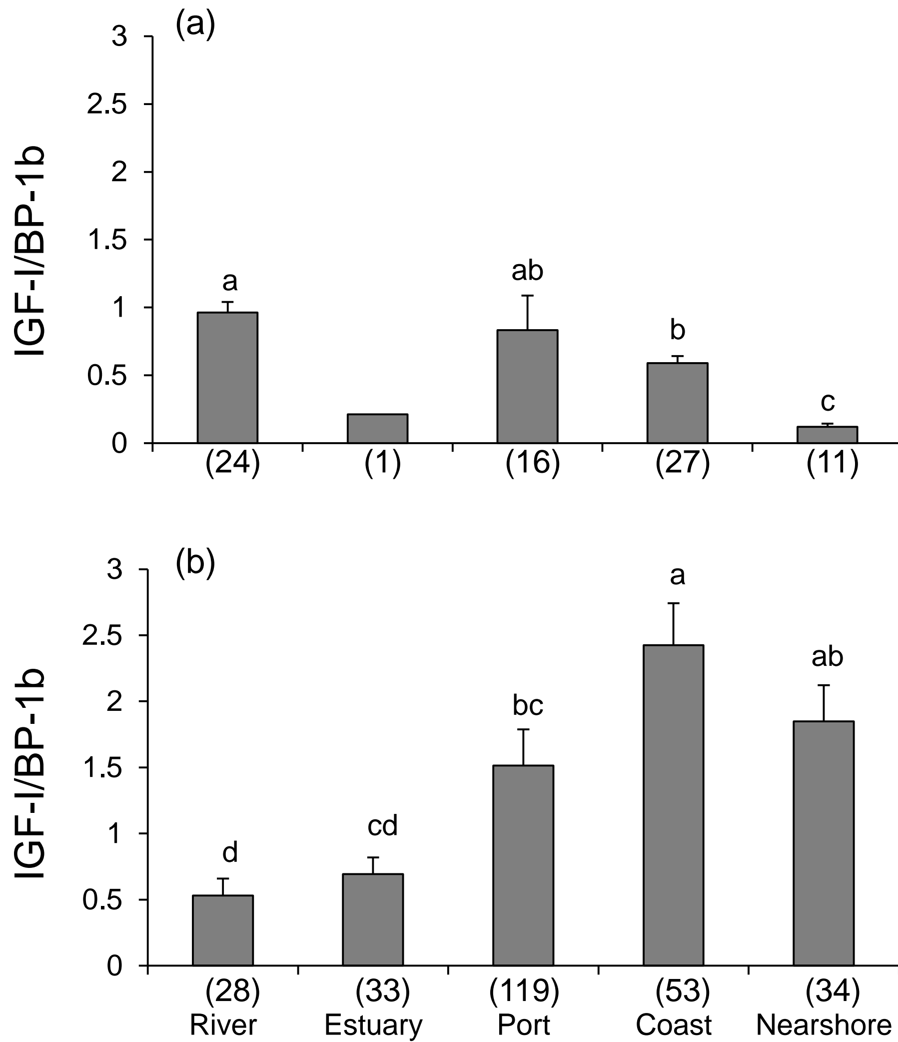

Fig. 17. The molar ratio of serum IGF-I to IGFBP-1b level (IGF-I/BP-1b) in May (a) and June (b). Juvenile chum salmon were caught at the river, estuary, port, coast and nearshore in 2015. Values are expressed as mean ± SE (*n* of each group is shown under the corresponding bar). Symbols sharing the same letters are not significantly different from each other.

sampling dates at 2-week intervals from July to September (r^2 = 0.24-0.52) (Shimizu et al., 2006). In the present study, circulating IGFBP-1b negatively correlated with growth rates in both May and June. The slopes of the regression lines between May and June were not different, while the regression coefficient value in June was weaker than that of in May. Thus, the seasonal variation of the IGFBP-1b-growth relationship is not severe in June which is the late period of downstream and coastal migrations of juvenile chum salmon.

In the laboratory experiment, there was a negative correlation between circulating IGFBP-1b and IGF-I in juvenile chum salmon. This is in accordance with the result from postsmolt masu salmon (Kawaguchi et al., 2013). This inverse relationship is not surprising since one of IGFBP-1b actions is to sequester IGF-I from the circulation (Lee et al., 1993, 1997). In contrast, in coho salmon plasma IGFBP-1b levels showed no correlation with IGF-I (Shimizu et al., 2006, 2009). This lack of the relationship may reflect differences in the sensitivity to nutritional input for IGF-I and IGFBP-1b. Shimizu et al. (2009) compared postprandial changes of plasma IGF-I and IGFBP-1b and suggested that IGFBP-1b was more sensitive to food intake, quickly responding within hours irrespective of fasting history. Thus, the balance between circulating IGF-I and IGFBP-1b can change within a short time scale after feeding, which may sometimes mask the relationship between IGFBP-1b and IGF-I.

Since salmon IGFBP-1b is potential inhibitor of IGF-I action, a ratio of IGF-I to IGFBP-1b may reflect a fraction of circulating IGF-I available for promoting growth. In order to test this hypothesis, I calculated the molar ratio and correlated it with SGR. However, the IGF-I/BP-1b ratio did not generate a higher regression coefficient (r^2 = 0.43) than found for IGF-I and growth. On the other hand, patterns of the average IGF-I/BP-1b ratio in response to fasting and refeeding differed to those of IGF-I and IGFBP-1b alone. Although the biological meaning of the ratio has not been elucidated, IGF-I/BP-1b ratios may inform us a balance between anabolism and catabolism.

Fish growth is affected by environmental factors such as feed availability, salinity, water temperature and stress. Effects of these environmental factors on IGFBP-1b need to be explored and taken into account when IGFBP-1b is used to evaluate growth status of fish in the wild since IGFBP-1b responses may be more rapid than changes in growth. As described earlier, acute changes of water temperature caused disruption to IGF-I-growth relationships (Beckman et al., 2004c), suggesting if changes in environmental factors are slow and lasted enough time for the endocrine system to respond, circulating IGFBP-1b should maintain its relationship with growth rate. However, in wild, these environmental factors sometimes change rapidly and may cause a decoupling between hormone levels and growth rates. I monitored growth status by measuring serum IGFBP-1b and IGF-I in juvenile chum salmon collected in the river, estuary, port, coast and nearshore. During their downstream and coastal migrations, juvenile chum experienced different salinity and water temperature.

Changes in salinity during downstream migration of juvenile chum salmon might have little effect on IGFBP-1b. When juvenile chum salmon were acclimated to full strength SW over 3 days, serum IGFBP-1 levels did not change in May and June (Nakamura et al., unpublished data). In juvenile Chinook salmon, a direct transfer to full strength SW caused an increase in plasma IGFBP-1b levels in 6 h, which was presumably owing to an osmotic stress (Shimizu et al., 2011a). A gradual acclimation to 66% SW of rainbow trout had no acute effect on plasma IGFBP-1b up to 3 days after transfer (Shepherd et al., 2005). Although an effect of a rapid change in salinity cannot be ruled out, juveniles could adapt themselves to adequate salinity by swimming vertically and horizontally. Thus, I assumed that the effect of salinity change is not significant in our survey on juvenile chum salmon.

Water temperature is an important parameter to consider when growth status is evaluated by IGFBP-1b. Shimizu et al. (2006) reported that a sudden drop in water temperature from 11°C to 7°C within a day changed plasma IGFBP-1b levels in

postsmolt coho salmon. In contrast, a gradual decrease in water temperature from 10°C to 5°C over 3 days had no influence on average serum IGFBP-1b levels although growth rate was not measured (Nakamura et al., unpublished data). Thus, care should be paid when comparing IGFBP-1b levels in fish between regions with very different water temperatures.

By taking account of the effect of water temperature, I attempted to evaluate growth status of juvenile chum salmon in wild by IGFBP-1b together with IGF-I. Serum IGFBP-1b levels were high at the nearshore in May, which was accompanied with low IGF-I levels. Water temperature at the nearshore in May (9.4 ± 0.6°C) was within the optimal range 8-13°C for growth (Nagata et al., 2007) and those at the port and the nearshore were not different. These data suggest that the high IGFBP-1b and low IGF-I levels in fish at the nearshore in May reflected poor growth conditions due to low nutritional conditions. On the other hand, in June serum IGFBP-1b levels were very low in fish at the nearshore while serum IGF-I levels were also low. The low IGF-I level suggests that juveniles were under poor growth conditions but might not be due simply to low nutritional conditions. Water temperature at the nearshore in June was within the optimal range, but it exceeded the optimal range at the port (13.3 ± 1.3°C). Thus, juveniles might have to move from the port to the nearshore due to the higher water temperature regardless of feed availability. It is not known water temperatures caused the unusual occurrence of both low IGFBP-1b and low IGF-I in these data. Unraveling the mechanism and consequence causing this situation will be important to allow the evaluation of growth status of fish by IGF-I and IGFBP-1b measures.

In view of the mechanism of growth regulation, the amount of IGF-I relative to the amount of IGFBP-1b may tell us a proportion of IGF-I used or being used for growth. If this is true, the IGF-I/BP-1b ratio is an index of growth potential. In Chapter 2, I hypothesized that small fish with low IGF-I level caught at the estuary were under poor growth conditions. In the 2015 survey, however, fish at the estuary did not exhibit

the lowest IGF-I levels. On the other hand, IGFBP-1b levels were relatively high at the estuary. I calculated the IGF-I/BP-1b ratio and found that it was low in fish in the estuary. This result supports my hypothesis that growth status of fish at the estuary is relatively poor in the Abashiri area. It is not known if the same trend existed in samples from 2013 and 2014 as IGFBP-1b was not measured. Sample analysis was not possible because of the limited amount of serum. Although the utility of IGF-I/BP-1b need to be fully explored by laboratory experiments, data from the estuary in 2015 illustrates one of advantages of having multiple growth indices.

In summary, the present study suggests circulating IGFBP-1b can be used as a negative growth index for juvenile chum salmon. Monitoring growth status by IGFBP-1b together with IGF-I suggested that juvenile chum salmon left the nearshore under poor growth conditions both in May and June as judged by the low IGF-I. However catabolic status was more severe in fish in June as suggested by the high IGFBP-1b level, which suggests a lower growth trajectory in these fish.

4. Regional variation of circulating IGFBP-1b in postsmolt coho salmon in the Strait of Georgia, British Columbia, Canada

4.1. Introduction

As discussed in Chapter 3, accumulating evidence suggests that circulating IGFBP-1b is useful as a negative growth index in several salmonid species including chum, coho and masu salmon (Beckman et al., 2004a, Kawaguchi et al., 2013). Measuring circulating IGFBP-1b level could provide important information on catabolic status of fish both under captivity and in wild, although its modulation by environmental factors other than feeding status needs to be explored more comprehensively especially when it is used in wild.

The Strait of Georgia is a marine fjord located between the Pacific Coast of Canada and Vancouver Island approximately 240 km long and 40 km wide. Water currents flow in from the Fraser River and flow out the both Strait of Juan de Fuca and Johnstone Strait, the southern and northern end of the strait, respectively. This area has high primary productivity and thus supports a large biomass of zooplankton and pelagic fish such as Pacific herring (*Clupea pallasii*) (Hourston and Haegele, 1980). Every year, hundreds of millions of juvenile Pacific Salmon including coho salmon migrate northward or southward from the Strait of Georgia to the Gulf of Alaska during spring and autumn (Tucker et al., 2009; Beamish et al., 2012; Beacham et al., 2014). The Strait of Georgia is an important coastal rearing area for juvenile Pacific salmon and growth conditions within the Strait of Georgia most likely influences year class strength (Beamish et al., 2008).

The Strait of Georgia can be divided into several regions with differing physical characteristics. Johnstone Strait is specific region connecting the Strait of Georgia to Queen Charlotte Strait and then the Pacific Ocean. The water column of

Johnstone Strait is highly mixed by tidal currents and it has been hypothesized that primary production is low in this area (Thomson, 1981). A recent retrospective analysis conducted by McKinnel et al. (2014) on a link between the abundance of sockeye salmon return and physical ocean conditions in Johnstone Strait led them to propose a “trophic gauntlet hypothesis”. The hypothesis states that within Johnstone Strait there is a nutritional hurdle that juvenile salmon have to go through every year and after passing Johnstone Strait they need to recover from the nutritional shortage in Queen Charlotte Strait and/or Queen Charlotte Sound otherwise they would be exposed to a higher risk of growth-dependent mortality.

Plasma IGF-I has been validated as a growth index in salmon under laboratory conditions (Beckman, 2011) and applied to assess growth status of coho salmon and other Pacific salmon in the Strait of Georgia, Puget Sound, British Columbian coastal waters and the northeastern Bering Sea (Beckman et al., 2004c; Ferriss et al., 2014; Wechter et al., 2017). Journey et al. (2017) investigated inter-annual growth status using IGF-I of coho salmon in Johnstone Strait and Queen Charlotte Strait, and revealed consistently low IGF-I levels in these areas when compared to that in the Northern Strait of Georgia. These regional characteristics might involve the influences of poor feeding availability caused by unsuitable environmental conditions in the region. Their study using IGF-I supported the trophic gauntlet hypothesis.

Based on these circumstances, I took an advantage of the unique feature of this region to examine the response of plasma IGFBP-1b in postsmolt coho salmon collected in the Strait of Georgia and Johnstone Strait. My hypothesis was that plasma IGFBP-1b levels in juvenile salmon would be highest in Johnstone Strait, among regions sampled in the Strait of Georgia due to the hypothesized poor feed availability in the region. I chose to analyze coho salmon since it is the best studied fish species with regard to the physiological and nutritional regulation of plasma IGF-I and IGFBP-1b and the relationships between IGF-I, IGFBP-1b and growth rate have been established under

laboratory conditions (Beckman et al., 2001, 2004a, b, c; Shimizu et al., 2006, 2009). In the present chapter, I analyzed plasma samples from postsmolt coho salmon caught during the trawl survey of the Strait of Georgia in 2014 by Fisheries and Oceans Canada, measured plasma IGFBP-1b and correlated it with seawater temperature and stomach fullness.

4.2. Materials and methods

4.2.1. Study location and fish sampling

Sampling sites of juvenile coho salmon were categorized into 11 regions (Fig. 18): Queen Charlotte Strait (QCST), Johnstone strait (JS), Northern Discovery Islands (Ndisc), Discovery Islands (disc), Desolation Sound (Des), Northern Strait of Georgia (NSOG), Malaspina Strait (Mala), Strait of Georgia (SOG), Southern Strait of Georgia (SSOG), Fraser River Plume (Plume) and Gulf Islands (Gulf) (Journey et al., 2017). Juvenile coho salmon were captured *via* fishing trawls aboard the C.C.G.S. W.E. Ricker and F/V Viking Storm in late June and early July of 2014. Specifics of survey design and complete methods are detailed in Beamish et al. (2008) and Journey et al. (2017). All fishing was conducted with a modified mid-water rope trawl (28-42 m wide × 12-18 m deep mouth at the surface (zero meter) or 15 meters deep) towed at average speed of 9.36 km/h (5 knots) for 30 min. Fish caught at each region were first measured for weight and fork length, and condition factor (K) was calculated as follows: $(BW (g)) \times 1000/(FL (mm))^3$. Blood samples were collected *via* heparinized syringe. Blood samples were immediately centrifuged, and plasma was collected. The plasma samples were kept frozen until use. Additionally, stomach samples were collected to calculate stomach fullness at some stations. Stomach fullness was calculated as both percent stomach fullness and volumetrically (cc). Seawater temperature was recorded with SBE 911plus CTD (Seabird Scientific, Bellevue, WA, USA).

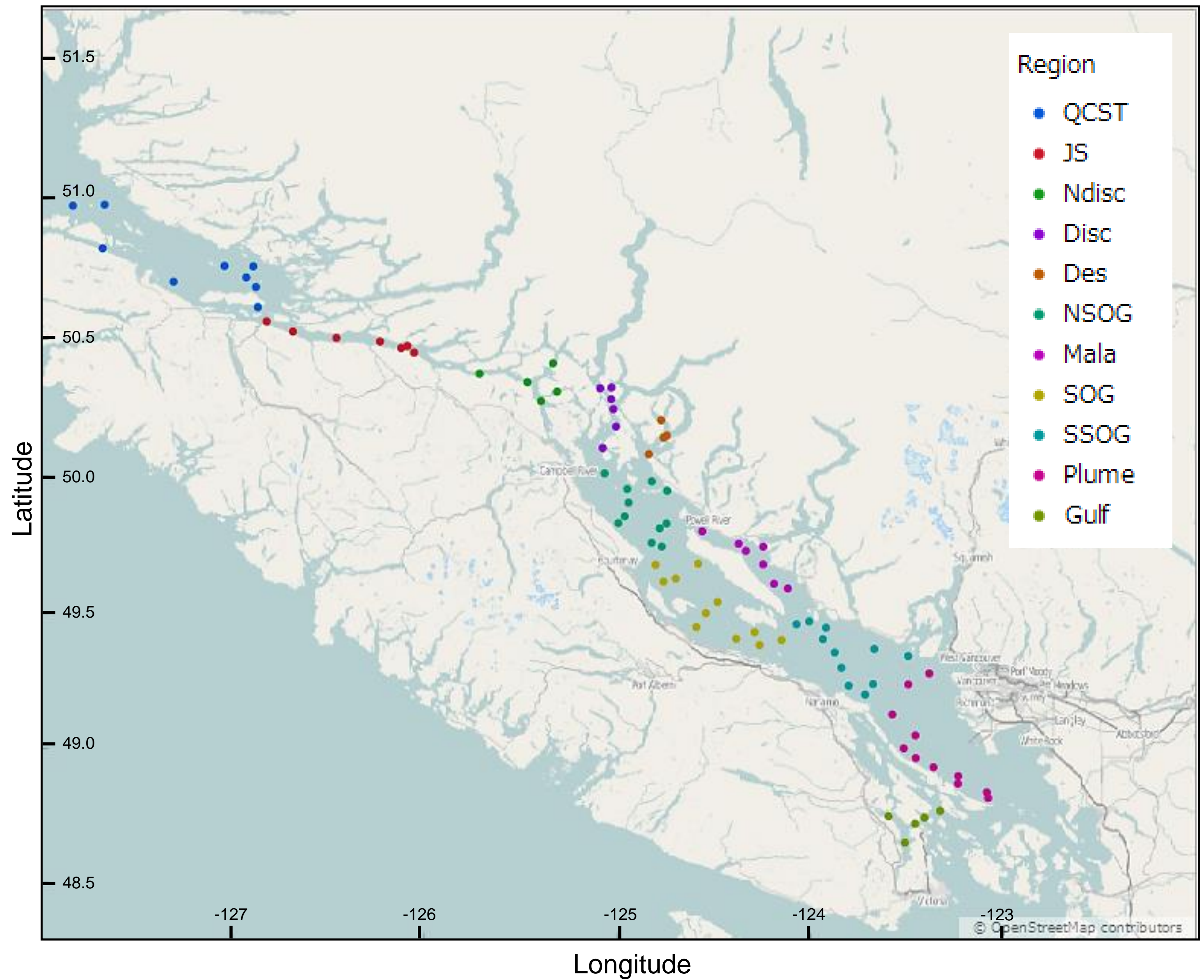

Fig. 18. Map of survey area in British Columbia, Canada and Strait of Georgia, USA. The tow location in 2014 are shown and each color coordinated by regions: Queen Charlotte Strait (QCST), Johnstone Strait (JS), Northern Discovery Islands (Ndisc), Discovery Islands (Disc), Desolation Sound (Des), Northern Strait of Georgia (NSOG), Malaspina Strait (Mala), Strait of Georgia (SOG), Southern Strait of Georgia (SSOG), Fraser River Plume (Plume) and Gulf Islands (Gulf). Sampling conducted in late June and early July according to Journey et al. (2017).

4.2.2. Sample analyses

Plasma IGF-I levels have been measured and reported in Journey et al. (2017). Plasma IGFBP-1b levels were quantified by TR-FIA as described in Fukuda et al. (2015). Briefly, a competitive method was employed to measure IGFBP-1b by following a procedure for DELFIA immunoassays (PerkinElmer). Plasma samples were first incubated with antiserum against purified salmon IGFBP-1b from Chinook salmon plasma overnight at 4°C in a 96-well microtiter plate coated with goat anti-rabbit IgG (PerkinElmer). Biotinylated salmon IGFBP-1b was added to each well and incubated overnight at 4°C. After washing with DELFIA Wash Buffer (PerkinElmer), each well received europium-labeled streptavidin (PerkinElmer) followed by DELFIA Enhancement Solution (PerkinElmer). Time-resolved fluorescence was measured using Wallac ARVO SX (PerkinElmer) at 615 nm. All samples were normalized using inter-assay pools of coho salmon plasma at three known IGFBP-1b concentrations (low, mid and high), corresponding to approximately 75, 50 and 25% binding in the plate.

4.2.3. Statistical analyses

Before statistical analysis, data from maturing coho salmon were excluded because their plasma IGF-I concentration is not exclusively indicative of relative growth (Beckman et al., 2004b, c). Results of the survey were first analyzed by one-way ANOVA (region) using the JMP program (SAS Institute Inc., Cary, NC, USA). When significant effects were found, differences were further identified by Fisher's protected least significant difference (PLSD) test. Differences among groups were considered to be significant at $P < 0.05$. Simple regression analysis was also conducted using JMP program, and relations were considered to be significant at $P < 0.05$. Regression coefficients were compared after calculating mean value of each tow.

4.3. Results

Slopes of the serial dilutions of plasma from coho salmon with different physiological conditions, such as immature or precociously maturing (jack), and low or high IGF-I and K were parallel with that of the standard (Fig. 19).

Significant regional differences in plasma IGFBP-1b and IGF-I/BP-1b ratio were found (one-way ANOVA, $P < 0.0001$, Fig. 20). Plasma IGFBP-1b levels tended to be high in fish captured in the Gulf Islands and was low for fish found between Fraser River Plume and the Northern Discovery Islands sites. Highest IGFBP-1b levels were found in Johnstone Strait and were also relatively high in Queen Charlotte Strait (Fig. 20a). In contrast, IGF-I/BP-1b ratios were the lowest in Johnstone Strait. Fish in Malaspina Strait and Gulf Islands also had a relatively low IGF-I/BP-1b ratio (Fig. 20b).

Plasma IGFBP-1b levels were plotted against fork length, weight and condition factor (K) in Johnstone Strait, Queen Charlotte Strait, Strait of Georgia and Gulf Islands (Fig. 21). K values were low in Johnstone Strait and Queen Charlotte Strait (Fig. 21c). In addition, in fish caught at Strait of Georgia there was a negative correlation between plasma IGFBP-1b and K. Seawater temperature was the lowest in Johnstone Strait and fish caught at that region had the lowest stomach fullness (Fig. 22a, c). However, plasma IGF-I level in Johnstone Strait was not the lowest (Fig. 22b). Plasma IGFBP-1b negatively correlated with temperature and plasma IGF-I in fish in Strait of Georgia, while plasma IGFBP-1b did not correlate with stomach fullness. When the data from all regions were pooled, IGFBP-1b negatively correlated with K and stomach fullness while positive relationships were seen between IGF-I/BP-1b ratio and these parameters (Table 6). Both IGFBP-1b and IGF-I/BP-1b ratio showed negative relationships with water temperature (Table 6).

4.4. Discussion

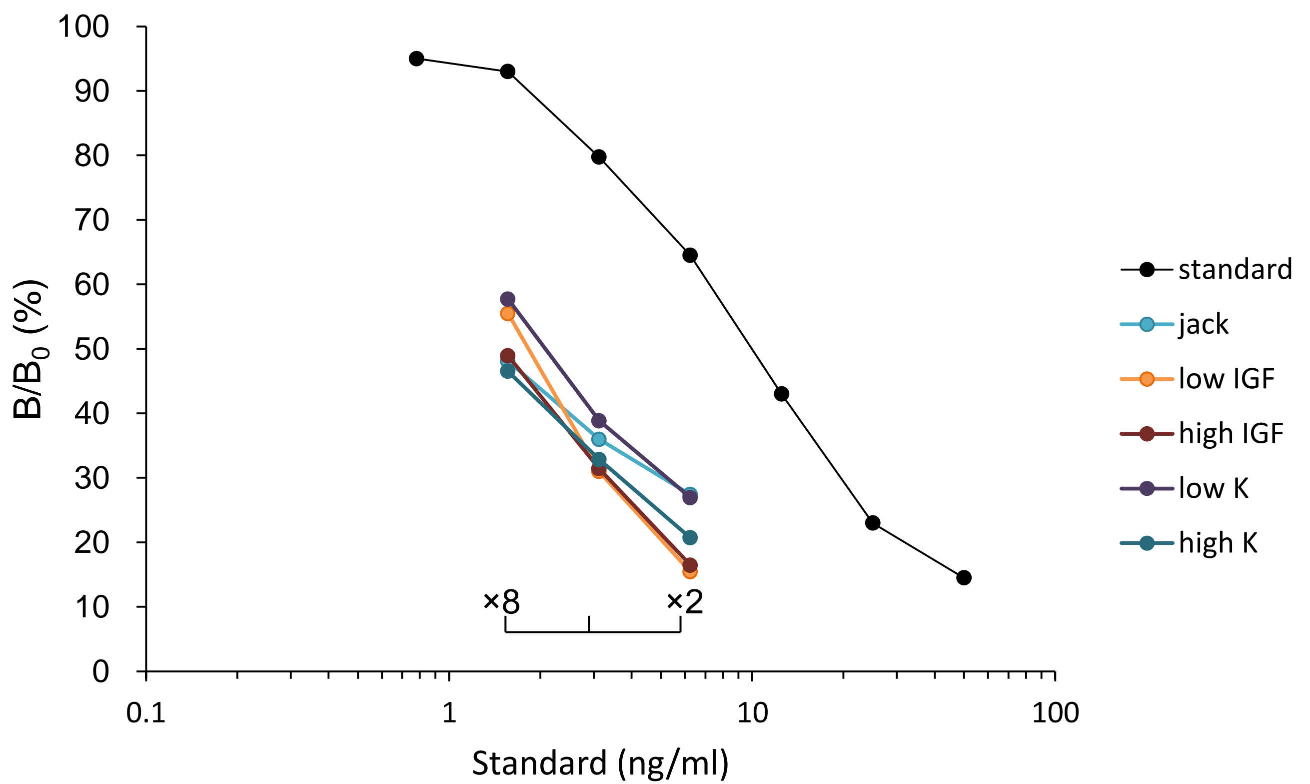

Fig. 19. Displacement curves of the biotinylated-salmon IGFBP-1b with purified IGFBP-1b and sera from coho salmon with different conditions. Jack: precocious maturing male, low/high IGF: sera showed low or high IGF-I concentrations (Journey et al., 2017), low/high K: sera showed low or high K values. Binding (B/B_0) is expressed as a percentage of specific binding.

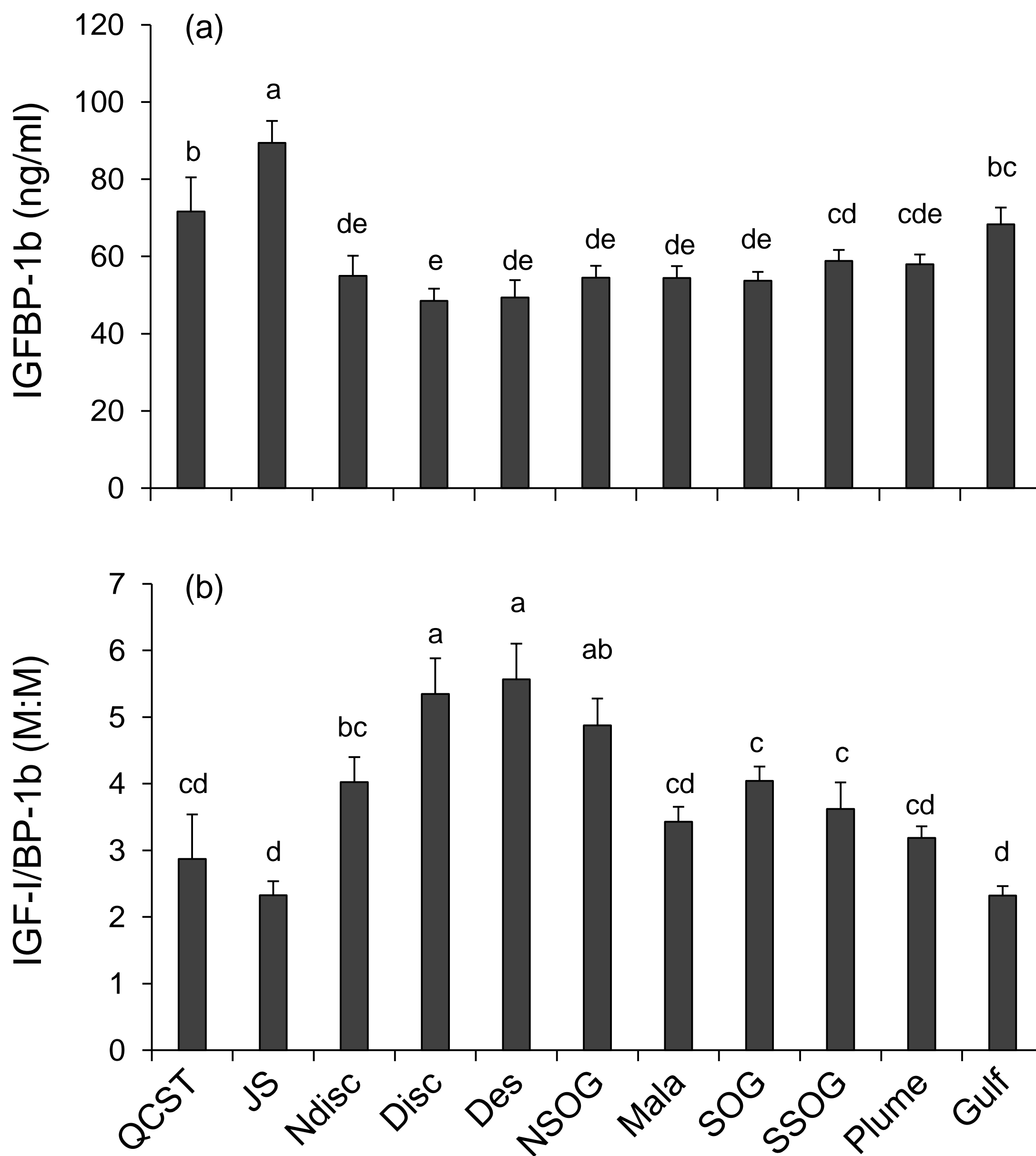

Fig. 20. Regional variations of plasma IGFBP-1b (a) and the molar ratio of IGF-I to IGFBP-1b (b). Values are expressed as mean ± SE. Symbols sharing the same letters are not significantly different from each other.

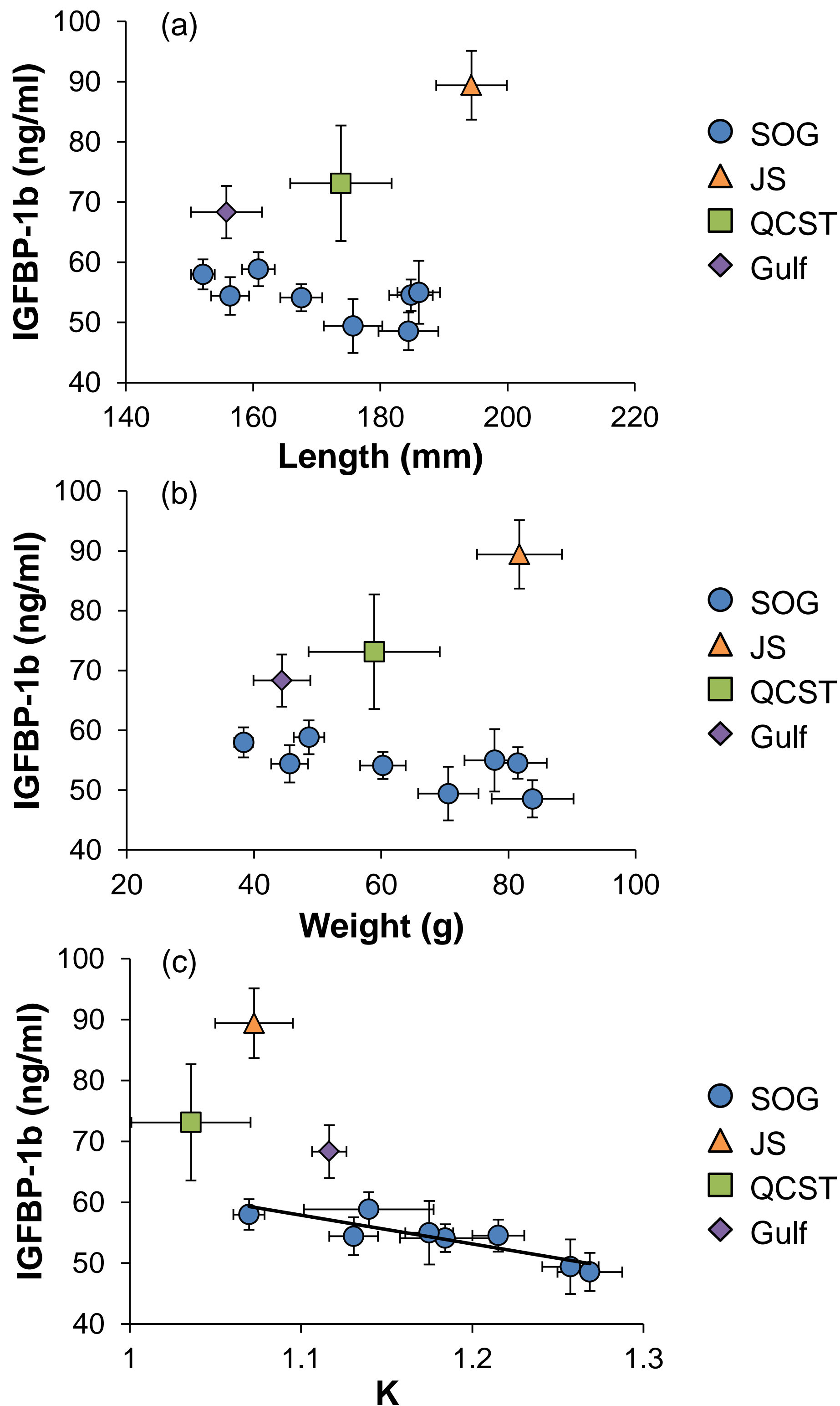

Fig. 21. Scatter plots of plasma IGFBP-1b levels against length (a), weight (b) and K (c). Regions were categorized oceanographically (Journey et al., 2017). Circles: eight regions in the Strait of Georgia, triangle: Johnstone Strait, diamond: Queen Charlotte Strait, and square: Gulf Islands. A correlation line was drawn against samples from Strait of Georgia.

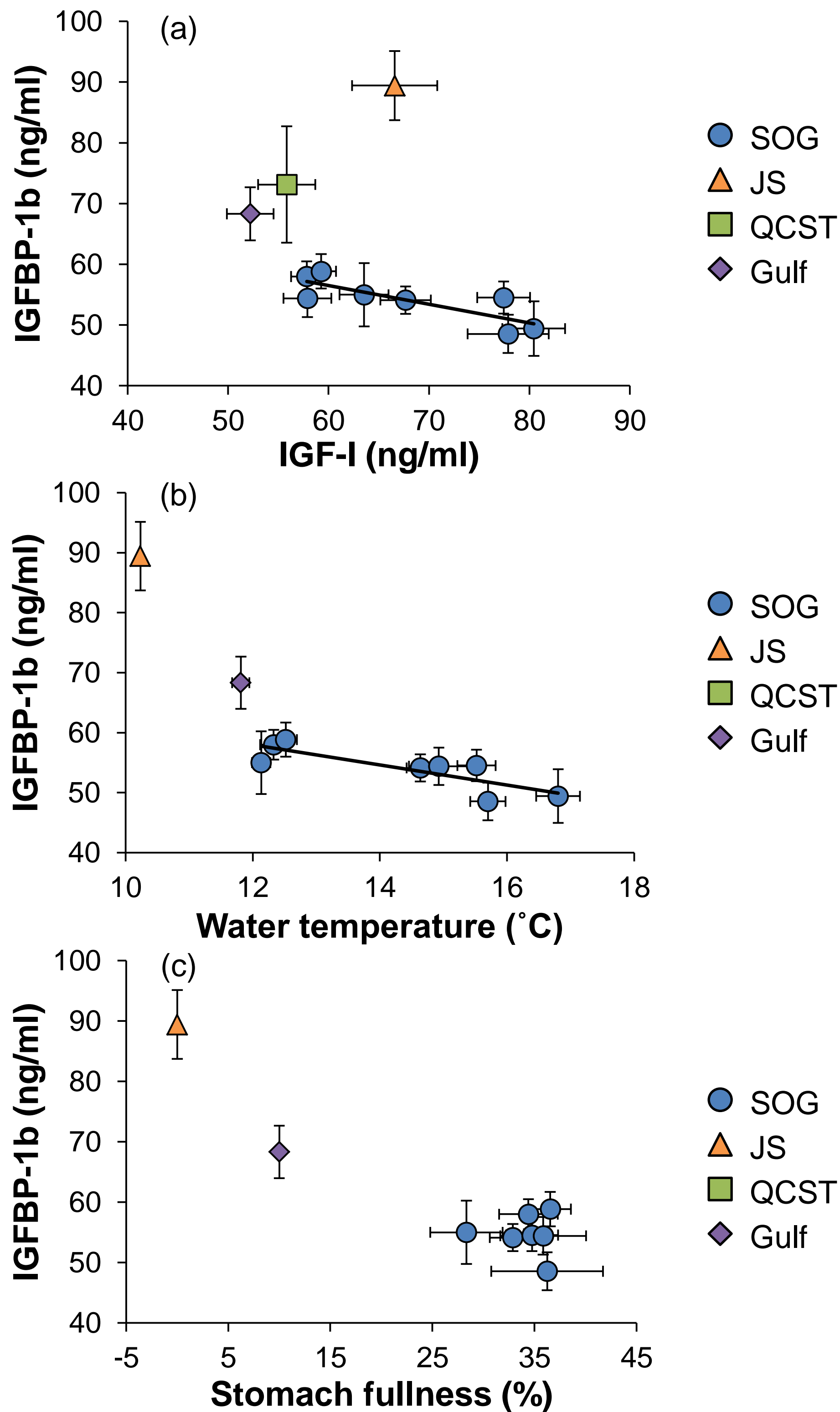

Fig. 22. Scatter plots of plasma IGFBP-1b levels against plasma IGF-I levels (a), water temperature (b) and stomach fullness (c). Regions were categorized oceanographically (Journey et al., 2017). Circles: eight regions in the Strait of Georgia, triangle: Johnstone Strait, diamond: Queen Charlotte Strait, and square: Gulf Islands. Correlation lines were drawn against samples from Strait of Georgia.

Table 6
Correlation coefficients (r) across whole regions

r	IGFBP-1b	IGF-I/BP-1b
Length	ns	ns
Weight	ns	0.36
K	-0.48	0.66
Water temperature	-0.49	-0.59
Stomach fullness	-0.58	0.33

IGFBP-1b values are translated to natural-log. ns: Not significant.

The present study attempted to further validate circulating IGFBP-1b levels as an index of negative growth and/or catabolic conditions in salmon in the ocean. I measured plasma IGFBP-1b levels in postsmolt coho salmon in the Strait of Georgia, where ocean conditions are relatively well defined and comprehensive trawl surveys for juvenile salmon are being conducted every year. In addition, data on plasma IGF-I levels in coho salmon and other Pacific salmon species in the strait were available (Journey et al., 2017). Thus, interpretations of the variations in plasma IGFBP-1b levels in relation to IGF-I and environmental factors were relatively easy. In particular, Johnstone Strait has been hypothesized to have poor primary productivity and thus to be an unfavorable area for juvenile salmon to grow (McKinnel et al., 2014). I thus assumed that plasma IGFBP-1b levels were high in fish in Johnstone Strait, and this hypothesis was proved to be correct by this study.

Evaluating plasma IGFBP-1b levels in fish in wild is challenging since environmental factors other than feeding/nutritional status can also affect plasma IGFBP-1b, these include stress and water temperature. The first concern was the possibility that an acute stress during trawl sampling (about 30 min) might affect plasma IGFBP-1b independent of growth/nutritional status. Plasma IGFBP-1b levels have been shown to be increased by stress in several fishes. An acute 15-min confinement stress on sunshine bass (a hybrid between female white bass, *Morone chrysops* and male striped bass *M. saxatilis*) increased plasma 24- and 28-kDa IGFBPs in 2 h (Davis and Peterson, 2006). In Jack mackerel (*Trachurus symmetricus*), a 30-kDa IGFBP was induced in response to a handling stress for 30-60 min (Kelley et al., 2006). However, Shepherd et al. (2011) assessed effects of acute stressor exposure (i.e. 5-min handling disturbance) on plasma IGFBPs in rainbow trout and found that the plasma levels of IGFBP-1a and -1b were unaffected. When juvenile chum salmon were sampled by trawling for 6 min and maintained in a bucket for 1 h post-capture, serum IGFBP-1b levels were not

affected (16.2 ± 2.3 ng/ml without holding vs 14.1 ± 1.5 ng/ml after holding in a bucket) (Kaneko, unpublished data). These findings suggest that although plasma IGFBP-1b may be induced by acute stress, capture by trawling may not have a significant impact. But, if the trawl sampling induced IGFBP-1b to some extent, I compared IGFBP-1b levels between sites with all fish having an increased baseline level of IGFBP-1b.

Water temperature has strong influence on metabolism and growth in fish. Temperature can affect either whole animal or specifically the IGF-I/IGFBP system (Gabillard et al., 2005). Shimizu et al. (2006) previously examined the effect of a drop in water temperature from 11 °C to 7 °C on plasma IGFBP-1b levels in postsmolt coho salmon. However, its effect was complex; A drop in water temperature decreased plasma IGFBP-1b during the first 2 weeks, while there were increased levels during the 4th-7th week and no effect was seen at 9th week (Shimizu et al., 2006). In the present study, there was a negative correlation between plasma IGFBP-1b and water temperature. This may suggest the higher IGFBP-1b levels for fish collected in Johnstone Strait might be due to the lower temperature in the region. But at the same time, these fish also had empty stomachs, which should also lead to increased IGFBP-1b levels. It is impossible to disentangle the effects of feed availability and water temperature in this survey. However, both feed availability and water temperature were low in Johnstone Strait and each had negative correlations with plasma IGFBP-1b, so that I could evaluate a combined effect of low feed availability and low water temperature on plasma IGFBP-1b. Although the exact causes of high IGFBP-1b levels in Johnstone Strait cannot not be specified, increased plasma IGFBP-1b in the region suggests growth retardation was occurring in the region.

As mentioned earlier, the reason for measuring plasma IGFBP-1b levels in postsmolt coho salmon at the Strait of Georgia in the present study was that this area was an important route for migrants and residences of juvenile salmon and set an unusual nutritional challenge at Johnstone Strait. Although most juvenile coho salmon

may exit the strait *via* the Strait of Juan de Fuca in autumn (Beamish et al., 2008; Chittenden et al., 2009), Johnstone Strait is the pathway for salmon out-migrating northward to Queen Charlotte Strait and characterized as a narrow, deep, cold and low production region. Low primary productivity in the region is mainly due to extensive water mixing by strong tides and sea breezes (McKinnell et al., 2014), which prevents water column from forming the thermal cline necessary for phytoplankton to photosynthesize. The low primary production results in the low abundance of zooplankton and small fishes such as Pacific herring. In the present study, fish caught at Johnstone Strait indeed had almost empty stomach demonstrating little feed was available in this region. Because IGFBP-1b is induced by reducing feeding ration, my assumption was IGFBP-1b levels were high in fish due to poor feeding conditions. As I expected, plasma IGFBP-1b levels in postsmolt coho salmon were the highest in Johnstone Strait.

Differences in water temperature between regions adjacent to Johnstone Strait might affect IGFBP-1b levels, however, there was only 2°C difference, which might not have a strong impact on IGFBP-1b. Moreover, there was a weak, negative correlation between plasma IGFBP-1b level and water temperature in fish under similar feeding conditions between Gulf Islands and Northern Discovery Islands. The slope of the IGFBP-1b *vs* temperature relationship was low so that 2°C difference in temperature between sites was related to an increase in IGFBP-1b of only 3.4 ng/ml. Thus, I consider the higher IGFBP-1b levels in Johnstone Strait are mainly due to low feed availability in that region. If fish were migrating from one region to another, one question may be that plasma IGFBP-1b in fish caught at Johnstone Strait did not reflect the poor feed availability in the region but that in other regions. Journey et al. (2017) estimated the residence time in Johnstone Strait of migrating juvenile salmon to be for a few days based on an optimal travel speed of salmon. This is enough time for IGFBP-1b to respond to food deprivation under laboratory conditions (Shimizu et al., 2009). Thus,

high plasma IGFBP-1b levels in fish in Johnstone Strait indicate that they were under catabolic conditions. This finding is in accord with the profiles of plasma IGF-I in juveniles of five Pacific salmon species migrating through the Strait of Georgia (Journey et al., 2017). The study by Journey et al. (2017) and the present study together support the trophic gauntlet hypothesis, which argues that fish passing Johnstone Strait should be under nutritional stress compared to fish in the Strait of Georgia since fewer prey are available in Johnstone Strait (McKinnel et al., 2014). Thus, monitoring growth/nutritional status by using IGF-I and IGFBP-1b is of great value to evaluate a probability of survival thereafter.

Circulating IGF-I has been proposed as a reliable growth index in fish (Picha et al., 2008a; Beckman, 2011). IGFBPs should also be useful as additional growth indices (Shimizu et al., 2003a, 2006). These endocrine growth indices have slightly different sensitivity to changes in feed availability and rearing environments (Shimizu et al., 2009, 2011a; Kawaguchi et al., 2013). In the present study, plasma IGFBP-1b levels were high in the Gulf Islands and low in the Discovery Islands, suggesting metabolic conditions of fish were varied among the regions. I calculated the molar ratio of IGF-I to IGFBP-1b to see if I could better evaluate growth status of salmon. In human, a few studies indicated a close inverse association between IGFBP-1 and free IGF-I (Frystyk et al., 2002), and IGF-I:IGFBP-1 ratio associated with metabolic parameters such as body mass index, insulin and glucose levels in circulation (Sandhu et al., 2004). IGFBP-1b in fish plays a critical role in limiting access of IGF-I to the receptor. In view of the mechanism of growth regulation, the amount of IGF-I relative to IGFBP-1b may tell us the proportion of IGF-I used or being used for growth. If this is true, the IGF-I/BP-1b ratio is an index of growth potential. Supporting this idea, the ratio had positive relationships with condition factor. In the present study, the IGF-I/BP-1b ratios were different among regions with Johnstone Strait being lowest and the regions were better distinguished by using the ratio compared to IGFBP-1b alone. In particular, an

intermediate ratio was found in the Northern Discovery Islands, suggesting growth was suppressed around the Johnstone Strait.

Fish in Malaspina Strait had relatively low IGF-I/BP-1b ratios despite an increasing trend of the ratio from Gulf Islands to Strait of Georgia, which also suggest growth retardation in that area. Therefore, juvenile coho salmon in Malaspina Strait might have had a lower feeding rate than these in the main basin, suggesting an unsuitable growth environment for juveniles (Journey, unpublished data). Although the biological meaning of the IGF-I/BP-1b ratio needs to be elucidated experimentally, the IGF-I/BP-1b ratio may reflect another aspect of growth performance in salmon in the ocean that cannot see by IGF-I or IGFBP-1b alone.

In summary, I measured plasma IGFBP-1b levels in postsmolt coho salmon in the Strait of Georgia. There were regional differences in plasma IGFBP-1b and the levels were highest at Johnstone Strait where low ocean productivity has been documented. These findings support the notion that plasma IGFBP-1b reflects catabolic status of fish and is useful as a negative growth index of salmon in wild.

5. Development of a TR-FIA for salmon IGFBP-1a and assessment of its utility as a negative growth index

5.1. Introduction

As discussed in Chapters 3 and 4, the availability of multiple growth indices should make growth evaluation more stable or/and sensitive. Three major IGFBPs at 41-, 28- and 22-kDa in salmon serum/plasma have been identified as IGFBP-2b, -1a and -1b, respectively (Shimizu et al., 2005, 2011a, b). An RIA for IGFBP-2b was developed and revealed that circulating IGFBP-2b was as good as IGF-I to evaluate positive growth status in coho salmon (Shimizu et al., 2003a; Beckman et al., 2004a, b). The third form IGFBP-1a in salmon corresponds to the 28-32 kDa form in other fishes. Analyses using ligand blotting revealed that the 28-32-kDa IGFBP of several fishes was up-regulated under catabolic conditions such as fasting, stress and cortisol treatment (Siharath et al., 1996, Park et al., 2000, Kelley et al., 2001, 2006, Kajimura et al., 2003, Peterson and Small, 2004, Hevrøy et al., 2011). These findings suggest that IGFBP-1a is also a good marker of catabolic states like IGFBP-1b.

Studies on fish IGFBP-1s suggest that they underwent subfunction partitioning (Kamei et al., 2008; Shimizu et al., 2011a). Subfunction partitioning is one of the fates of duplicated copies of a gene where ancestral regulatory and structural subfunctions are preserved by gene duplicates (Postlethwait et al., 2004). Kamei et al. (2008) identified two IGFBP-1s in zebrafish (*Danio rerio*) and compared the two subtypes for temporal/spatial expression patterns, responses to fasting and inhibitory actions on the IGF-induced cell proliferation. The authors found that the functions of the two IGFBP-1s overlapped but their IGF-binding characteristics were somewhat different, suggesting that the degree of their IGF-inhibitory action differs (Kamei et al., 2008). The physiological regulation of the two IGFBP-1s is also different. In underyearling

Chinook salmon, fasting for 6 weeks resulted in induction of IGFBP-1b but not IGFBP-1a (Shimizu et al., 2005). In contrast, in postsmolt masu salmon fasting for 4 weeks induced both subtypes, although IGFBP-1b was also detectable in fed fish (Kawaguchi et al., 2013). Based on these findings, I hypothesize that both IGFBP-1a and -1b are negative indices of growth, but their sensitivities to catabolic conditions and relationships with growth rate are different for some extent.

In order to test this hypothesis, quantification of IGFBP-1a and comparison with IGFBP-1b for its response to catabolic conditions are necessary. However, there is no immunoassay available for fish IGFBP-1a. This is due to lack of purified IGFBP-1a for antibody production since IGFBP-1a is present in the blood at a relatively low level compared to IGFBP-2b and IGFBP-1b (Shimizu et al., 2003a, b, 2005, 2011a). Purifying IGFBP-1a was possible from the medium of trout hepatoma cell culture (Bauchat et al., 2001), but the purification procedure involved many steps. Recently, Tanaka et al. (2017) produced and purified recombinant salmon (rs) IGFBP-1a using a bacterial expression system. Using this purified rsIGFBP-1a as antigen and assay standard, I developed a TR-FIA for salmon IGFBP-1a and quantified its levels in response to fasting and during parr-smolt transformation and correlated IGFBP-1a with growth rate.

5.2. Materials and methods

5.2.1. Assay components

Recombinant salmon (rs) IGFBP-1a was produced using a bacterial expression system as described in Tanaka et al., (2017). Briefly, a pET-32a(+) expression vector (Novagen, Madison, WI) carrying the open reading frame of masu salmon *igfbp-1a* cDNA was transformed into a strain of *Escherichia coli* (Rosetta-gami™ B(DE3)pLysS (Novagen)) and rsIGFBP-1a was expressed as a fusion protein with a histidine tag and thioredoxin.

The fusion protein was solubilized and isolated by Ni-affinity chromatography. The fusion partners were cleaved by entrokinase and rsIGFBP-1a was purified by reversed-phase high pressure liquid chromatography (HPLC) on a Vydac C-4 column (Separation Group, Hesperia, CA). Purified rsIGFBP-1a was aliquoted into low-absorption tubes (PGC Scientifics, Frederick, MD) and stored at -80°C until use. IGFBP-1b and -2b purified from Chinook salmon serum (Shimizu et al., 2003b, 2005) was used to examine the cross-reactivity.

Polyclonal antiserum against purified rsIGFBP-1a (anti-IGFBP-1a) was raised in a rabbit. Immunization of the rabbit was conducted in accordance with the guidelines of the Animal Care Committee of Hokkaido University. Purified rsIGFBP-1a in phosphate buffered saline (pH 7.0) was emulsified in an equal volume of TiterMax Gold Research Adjuvant (TiterMax USA, Inc., Norcross, GA). A rabbit was first immunized with 50 µg antigen by lymph node injection followed by subcutaneous boost injections with 100 and 150 µg antigen 2 and 3 weeks after the first injection, respectively. One week after the last boost, the blood was withdrawn from the ear vein and antiserum was collected after centrifugation. The antiserum was stored at -30°C until use.

5.2.2. Biotinylation of rsIGFBP-1a

Purified rsIGFBP-1a was labeled with a biotin (EZ-link Sulfo-NHS-Biotin, Thermo Scientific, Rockford, IL). Thirty-four micrograms of purified protein was reacted with 31 µl of 1 mM NHS-Biotin at a molar ratio of 1:25. A low-absorption 0.5 ml tube (PGC Scientific) containing the mixture was incubated on ice under dark with occasional flipping. Reaction was stopped by adding 45 µl 0.1 M Tris-HCl, pH 7.5 for 30 min and 59 µl 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5 were added to the tube. The biotinylated IGFBP-1a was dialyzed against 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5 using Slide-A-Lyzer 3.5 K dialysis cassette (Thermo Scientific). After dialysis, aliquots of the biotinylated IGFBP-1a were stored at -80°C until use.

5.2.3. TR-FIA for IGFBP-1a

A competitive method was employed in the assay. A 96-well strip assay plate coated with goat anti-rabbit IgG (DELFIA Strip Plate; PerkinElmer) was first washed with 200 µl DELFIA Wash Buffer (PerkinElmer) and each well received 80 µl DELFIA Assay Buffer (PerkinElmer), 20 µl anti-IGFBP-1a (1:8000) and 40 µl standard (purified rsIGFBP-1a) or serum diluted with Assay Buffer. The plate was sealed by BMPCR-SP Seal (BM Equipment Co., Ltd., Tokyo, Japan) and incubated at 4°C overnight with shaking at 600 rpm on a shaker. The plate was flash centrifuged and each well received 20 µl biotinylated rsIGFBP-1a (1:8000) and incubated at 4°C overnight at 600 rpm. After the plate was washed three times with DELFIA Wash Buffer (PerkinElmer), each well received 160 µl europium-labeled streptavidin (PerkinElmer) and incubated at room temperature for 1 h with shaking at 600 rpm. The plate was washed with 160 µl five times and 200 µl three times with DELFIA Wash Buffer (PerkinElmer). Room temperature-acclimated 200 µl DELFIA Enhancement Solution (PerkinElmer) was added to each plate. The plate was shook without sealing for 10 min at room temperature. Time-resolved fluorescence was measured by a fluorometer (ARVO X4; PerkinElmer) with emission and read wavelength at 340 nm and 615 nm, respectively.

5.2.4. Effect of fasting and refeeding

A captive broodstock of masu salmon from the Shiribetsu River held at Nanae Freshwater Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Japan was used for the experiment. In May 2012, yearling masu salmon were lightly anesthetized in water containing 3% 2-phenoxy ethanol (Kanto Chemical, Tokyo, Japan) and individually marked with PIT tags (Biomark, Boise, ID). They were randomly placed into three 300 L outdoor tanks, and allowed to recover and acclimate for 1 week with feeding. One week after tagging, their initial FL and BW were

measured. During the experiment, one group was fed daily on a commercial diet (Marubeni Nissin Feed Co. Ltd., Tokyo, Japan) to satiety for 6 weeks (Fed). The second group (Fasted) was fasted throughout the experimental period (6 weeks). The third group (Refed) was fasted for first 4 weeks and re-fed for following 2 weeks. They were reared using flow-through river water that ranged from 10.3-18.0°C during the experiment. The experiment was carried out in accordance with the guidelines of Hokkaido University Field Science Center Animal Care and Use Committee.

FL and BW of all fish were measured 4 and 6 weeks after the beginning of the experiment. K was calculated as follows: (BW (g)) × 100/(FL (cm))^3. 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_2, s_1 is length or weight on day_1 and d_2 - d_1 is the number of days between measurements. On 6 weeks, 18-21 fish per treatment were sampled for blood. Blood was withdrawn by a syringe from the caudal vein, allowed clotting overnight at 4°C and centrifuged at 10,000 rpm for 15 min. Serum was collected and stored at -80°C until use.

5.2.5. Changes during parr-smolt transformation

Yearling masu salmon reared in freshwater at the South Branch of Salmon and Freshwater Fisheries Institute, Hokkaido Research Organization (42'N, 140'E; Nikai-gun, Hokkaido, Japan) were sampled from January to June as described in Shimomura et al. (2012). In November 2012, underyearling masu salmon reared at Kumaishi hatchery were sorted by size (>11.5 cm as Large and 9.5-11.5 cm as Small) and only large group fish were used for the experiment. The large fish were further divided two treatments: large size in winter and high feeding ration in spring (Large-High) or large size in winter and low feeding ration in spring (Large-Low). Fish were reared separately in outdoor ponds (24.6 × 3.5 m) using river water. The Large-High group was fed twice (November-February) or four times (March-May) a

day on a commercial diet (Nippon Formula Feed Mfg, Kanagawa, Japan), with rations at 0.3-2.3%/BW/day. The Large-Low group received a restricted feeding ration at 0.2-1.9%. Feeding ration varied within the ranges in described above depending on growth status of fish since these were for stock enhancement and released to the river in May 2012. Some fish were kept in the same pond and reared until June in freshwater. Eight fish were sampled monthly. Fish were anesthetized in 3% 2-phenoxy ethanol and measured for FL and BW. K was calculated as described above. Gill arches were excised and a block of gill filaments was immediately frozen on dry ice and stored at -80°C until analyzed for Na^+, K^+-ATPase (NKA) activity. Blood was withdrawn by a syringe from the caudal vein, allowed clotting overnight at 4°C and centrifuged at 10,000 rpm for 15 min. Serum was collected and stored at -80°C until use. Gill and serum samples were used for the measurements of NKA activity to evaluate seawater adaptability and serum IGF-I level to compare with the responses of serum IGFBP-1a, respectively. Both analytical methods were described above.

5.2.6. Statistical analyses

Results of the fasting/refeeding experiment were first analyzed by two-way ANOVA (time × treatment) using the JMP program (SAS Institute Inc., Cary, NC). 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 $P < 0.05$. Simple liner regression analysis was used to assess the relationships between endocrine parameters and morphological/growth parameters.

For the seasonal samples, data from the June sample were not included in the analysis because fish held in freshwater in June were under quite different physiological state and disturbed the relationships (Shimomura et al. 2012). Data from January through May were analyzed by one-way ANOVA with time as a factor followed by

PLSD test as described above.

5.3. Results

Specific binding of the biotinylated IGFBP-1 was displaced by increasing amounts of cold IGFBP-1a. Serial dilutions of serum from masu salmon and rainbow trout were parallel with that of the standard (Fig. 23). The half-maximal displacement (ED_{50}) occurred at 61.5 ± 2.3 ng/ml (n = 8). The ED_{80} and ED_{20} were 18.1 ± 2.5 ng/ml (n = 8) and 226.9 ± 23.8 ng/ml (n = 8), respectively. The minimum detection limit of the assay, defined as the mean count of the zero standard minus two standard deviation, was 9.2 ng/ml (n = 6). The intra- and inter-assay coefficients of variation estimated using control sample were 5.3 % (n = 4) and 8.1 % (n = 4), respectively. The recovery of purified rsIGFBP-1a (50 ng/ml) added to rainbow trout serum was 96.3 % (n = 9).

Cross-reactivity of other salmon IGFBPs in the TR-FIA was examined (Fig. 24). Both IGFBP-2b (41-kDa form) and IGFBP-1b (22-kDa form) showed some displacement at higher concentrations and their cross-reactivity was calculated as 1.5 and 3.6 %, respectively. Adding salmon IGF-I at 1:1 or 1:10 molar ratio to rainbow trout serum did not considerably alter its displacement curve (Fig. 25).

Using the TR-FIA, responses of circulating IGFBP-1a to fasting/refeeding were examined in yearling masu salmon (Fig. 26). Serum IGFBP-1a levels did not show significant differences among treatments. Additionally, serum IGFBP-1a did not significantly correlate with SGRs in length and weight (Fig. 27).

Gill NKA activity, and circulating IGF-I and IGFBP-1a levels during smoltification in masu salmon reared under different growth regimen were measured (Fig. 28). Gill NKA activity gradually increased from February to April and showed a sharp increase in May in both groups (Fig. 28a). Serum IGF-I in Large-High fish consistently increased from February to April, while that in Large-Low was

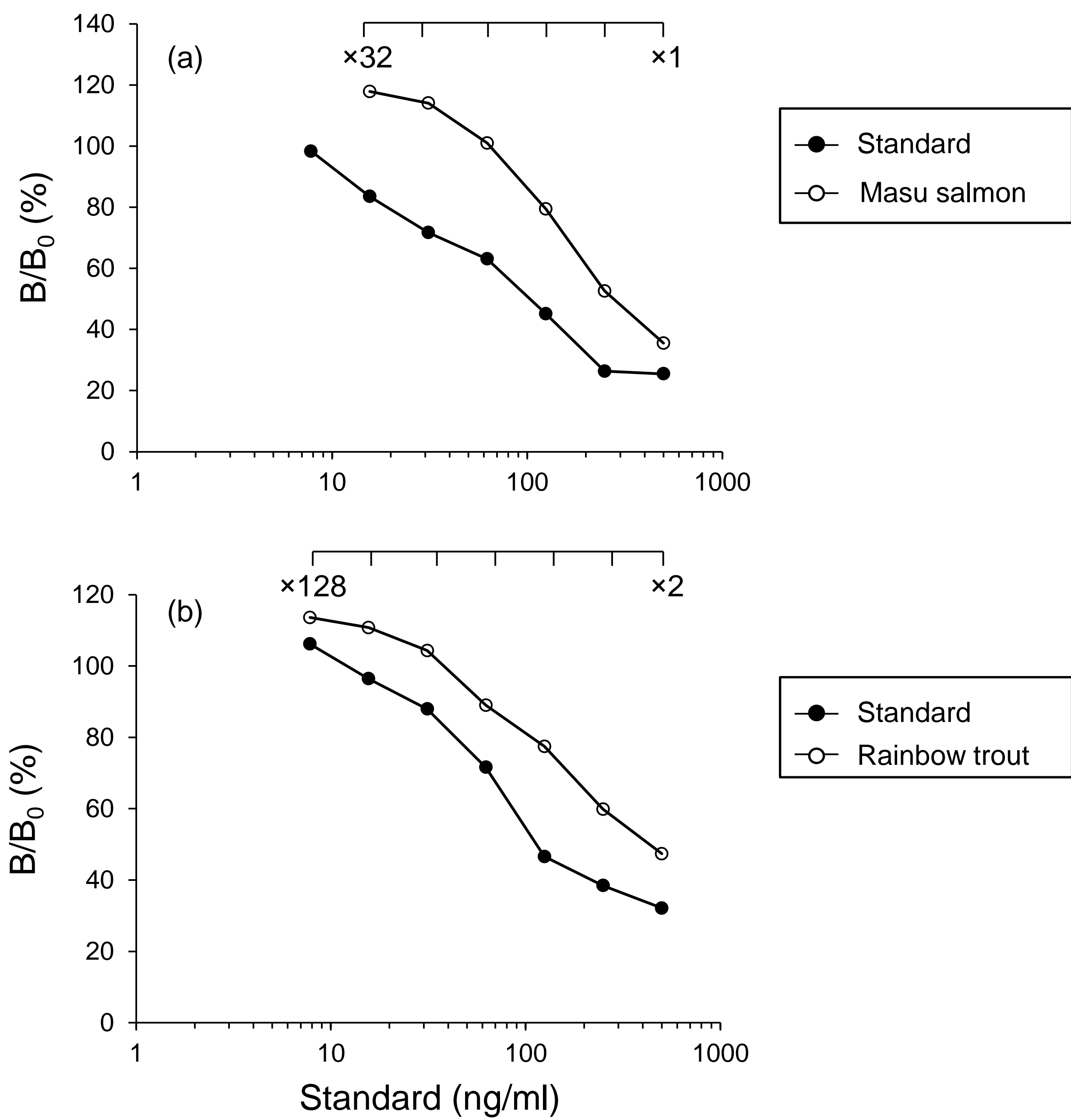

Fig. 23. Displacements of biotinylated salmon IGFBP-1a with purified IGFBP-1a and sera from masu salmon (a) and rainbow trout (b). Binding (B/B_0) is expressed as a percentage of specific binding.

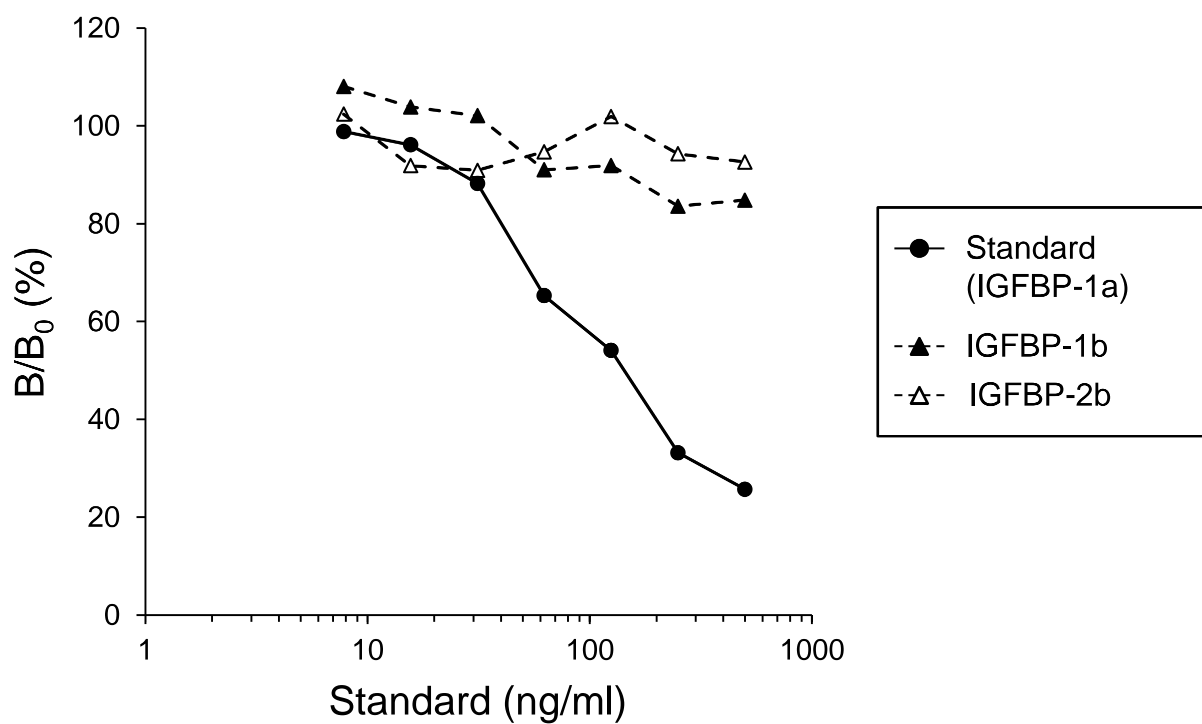

Fig. 24. Cross-reactivity of salmon IGFBPs in the TR-FIA. Displacement of the tracer was assessed by adding increasing amounts of purified salmon IGFBP-1a, -1b, -2b to the assay. Binding (B/B_0) is expressed as a percentage of specific binding.

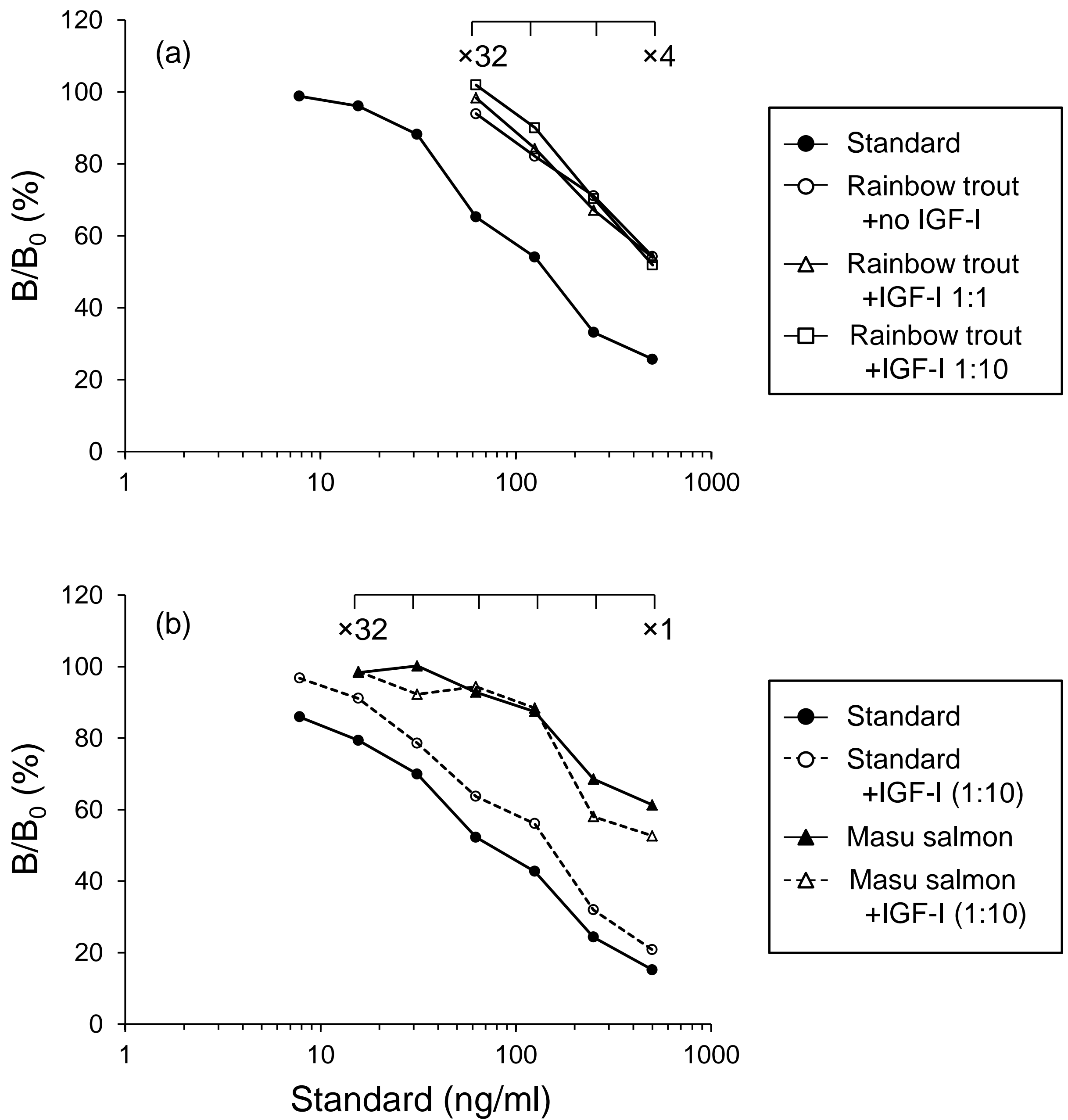

Fig. 25. Effects of exogenous salmon IGF-I on the displacement of rainbow trout serum (a), and standard and masu salmon serum (b) in TR-FIA. Salmon IGF-I was added to standard/serum at a molar ratio of 1:1 or 1:10. Binding (B/B_0) is expressed as a percentage of specific binding.

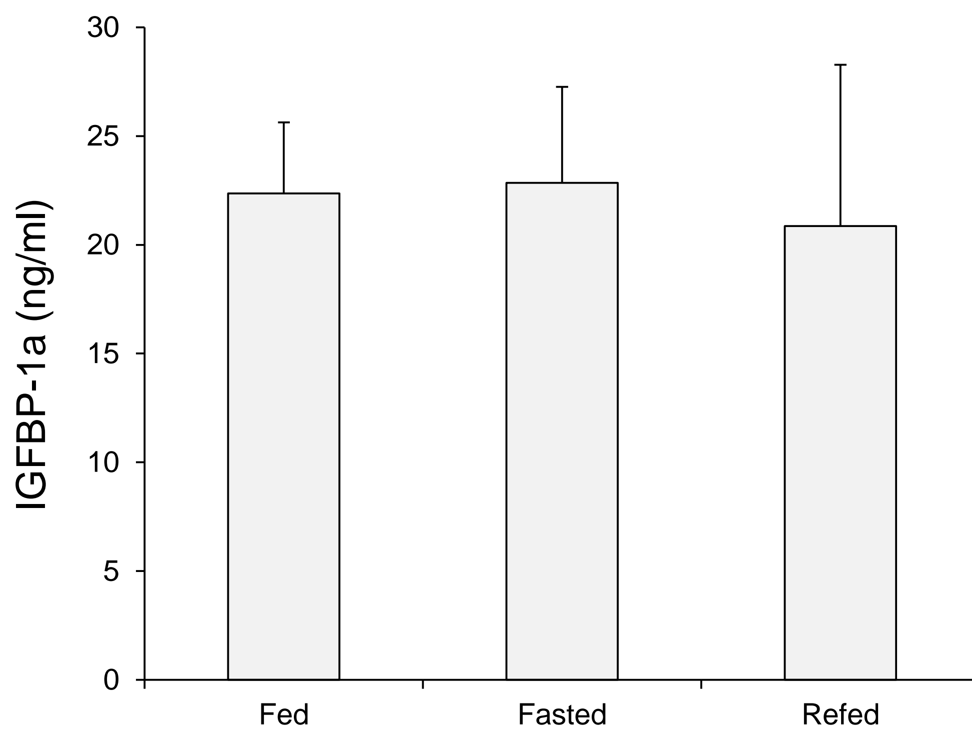

Fig. 26. Effects of fasting and refeeding on circulating IGFBP-1a on 6 weeks. Values are expressed as mean ± SE (n = 8-9).

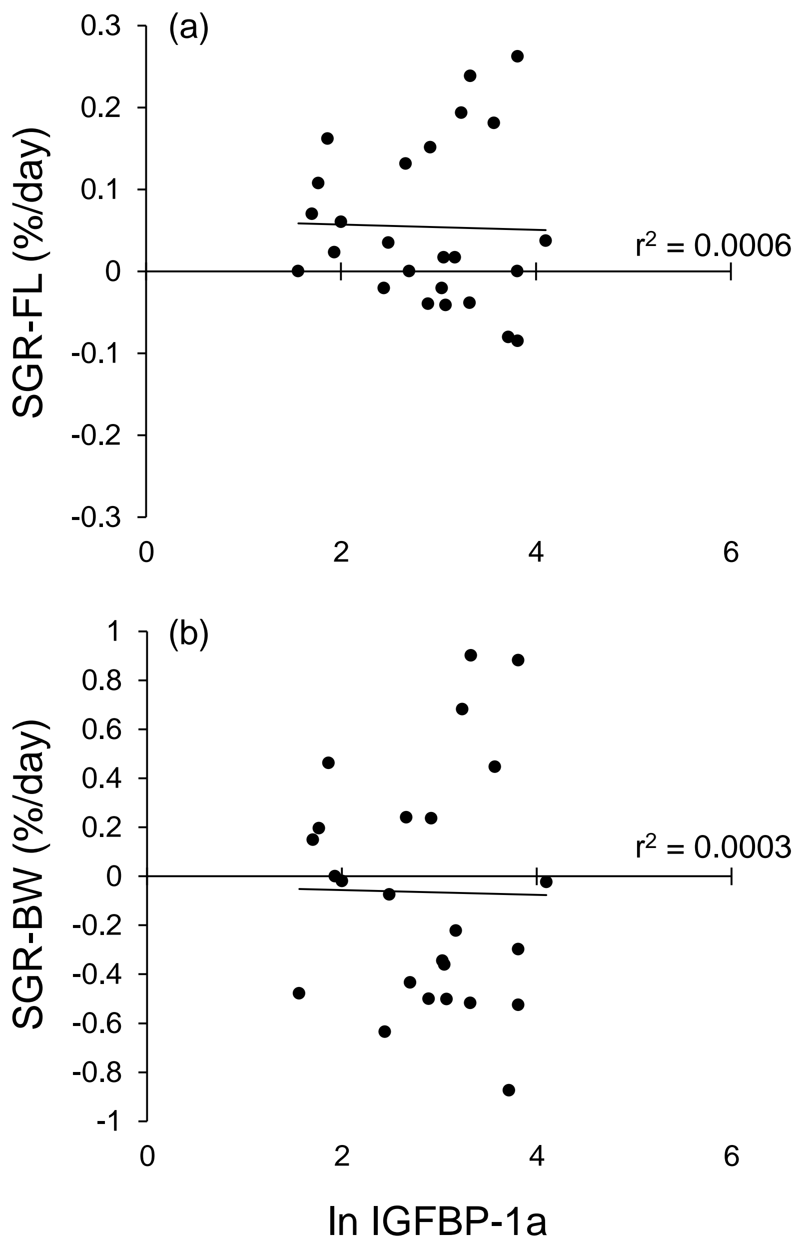

Fig. 27. Correlations between natural-log transformed IGFBP-1a level and SGR in standard length (a) and weight (b). Dots are data from fed, fasted and refed fish (n = 25)

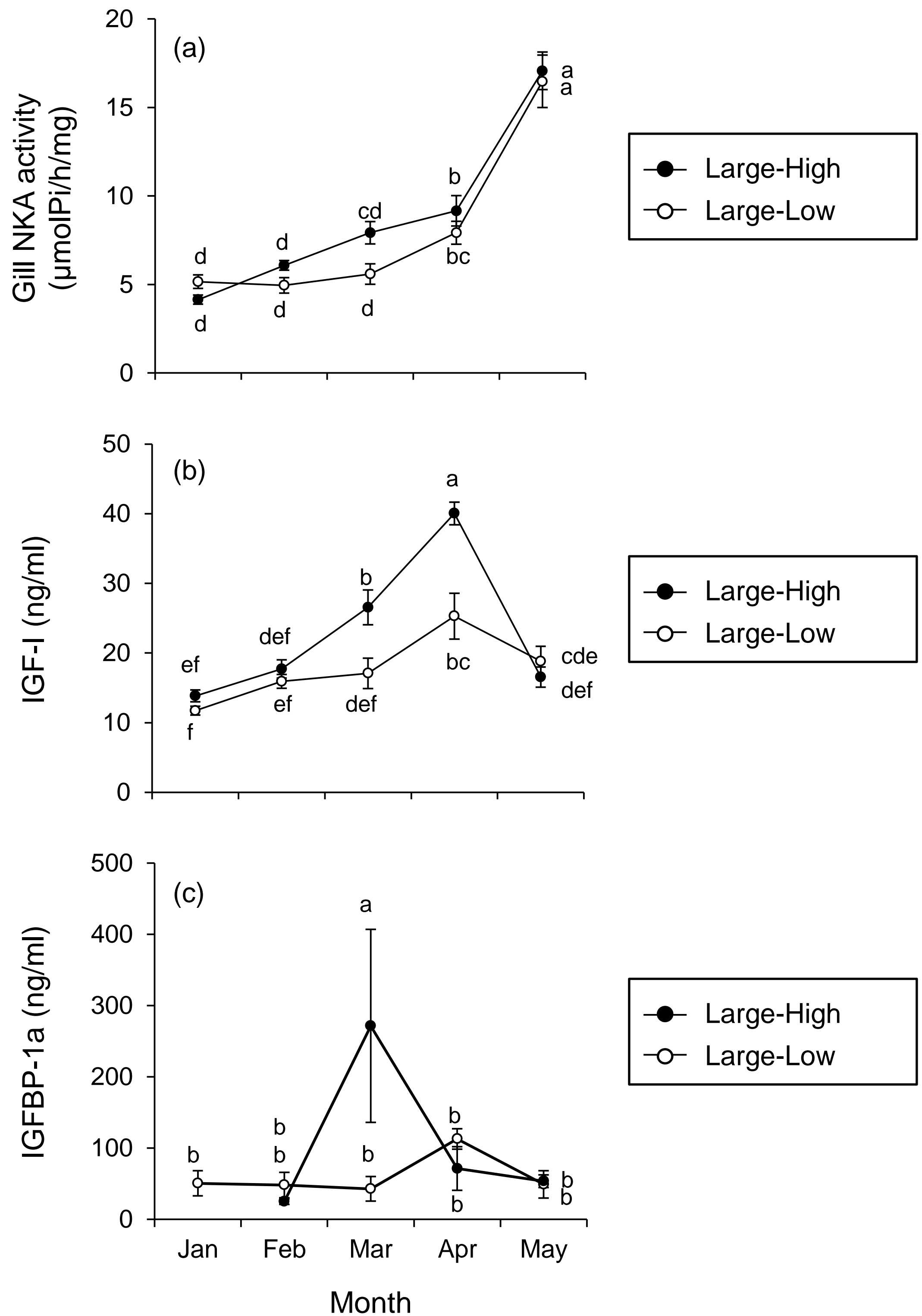

Fig. 28. Changes in gill Na^+, K^+-ATPase (NKA) activity (a), serum IGF-I (b) and IGFBP-1a levels (c) during smoltification of masu salmon. Values are expressed as mean ± SE (n = 4-8). Symbols sharing the same letters are not significantly different from each other. Most fish were released from the hatchery in May.

significantly lower than in the other group during March and April (Fig. 28b). Serum IGFBP-1a levels in the Large-Low group were relatively stable from January to March and significantly increased in April. While in the Large-High group, serum IGFBP-1a sharply increased in March and dropped in April (Fig. 28c).

5.4. Discussion

The present study is the first to report a development of a TR-FIA for salmon IGFBP-1a. Availability of immunoassay for quantifying fish IGFBPs is limited to salmon IGFBP-1b and -2b (Shimizu et al., 2003a, 2006; Fukuda et al., 2015). One of the challenges in establishing an immunoassay for a fish IGFBPs is to prepare enough purified IGFBP as antigen for immunization since circulating levels of IGFBPs are low being approximately 300 ng/ml or less (Shimizu et al., 2003a, 2006). In the case of the TR-FIA for salmon IGFBP-1b, antiserum was raised by immunizing purified protein from Chinook salmon serum. However, the yield from protein purification was very low (22 μg from 600 ml serum: Shimizu et al., 2005), making protein purification from serum not a practical method for antigen preparation. Recently, recombinant IGFBP-1a was produced in *E. coli* that had been transfected with an expression vector carrying cDNA of masu salmon IGFBP-1a (Tanaka et al., 2017). The present study used the rsIGFBP-1a for antiserum production, assay tracer and standard. The development of a TR-FIA for salmon IGFBP-1a should facilitate studies on its physiological regulation and relation to growth.

The assay was validated for its stability, specificity and cross-reactivity. Purified rsIGFBP-1a was biotinylated and used as tracer. This labeling appeared to have little effects on the binding to the antiserum and possible interaction with IGFs in the assay. Cross-reactivity of the TR-FIA with other IGFBPs particularly IGFBP-1b was a concern since IGFBP-1a and -1b shared 61% sequence homology (Shimizu et al.,

2011a). Indeed, antiserum against IGFBP-1b showed cross-reactivity with IGFBP-1a in the TR-FIA (Fukuda et al., 2015). In the present study, anti-IGFBP-1a also cross-reacted with IGFBP-1b but little with IGFBP-2b. Since the cross-reactivity with IGFBP-1b was low (3.6%), it should not severely affect the quantification of IGFBP-1a in the TR-FIA.

The assay components of the TR-FIA were prepared from recombinant masu salmon IGFBP-1a. Nevertheless, serial dilution of rainbow trout serum showed parallelism to the masu salmon standard, indicating its applicability to other salmonids. Although the serum from rainbow trout injected with cortisol showed a good parallel displacement curve, the parallelism was not as clear as in sera from other species. This is probably due to low IGFBP-1a levels in those sera. When this TR-FIA is used for other salmonid species such as Altantic salmon and coho salmon, the parallelism using serum that exhibits a strong IGFBP-1a band on ligand blotting should be confirmed.

As IGF and IGFBP are tightly associated, IGFBPs generally interfere an accurate measurement of IGFs in immunoassay. Therefore, IGF-IGFBP separation by acid-ethanol extraction is important (Shimizu et al., 2000). On the other hand, IGFs generally do not interfere performance of IGFBP assays, however in some RIAs the interference of IGF-I with IGFBPs binding were demonstrated (Baxter and Saunders, 1992; Shimizu et al. 2003a). I thus examined the effect of IGF-I on the serial dilution curve of rainbow trout serum by adding excess IGF-I. As results, all dilution curves with different ratios of exogenous IGF-I were parallel to the standard and the dilution curve of serum without IGF-I addition, indicating interference by IGF-I in the TR-FIA for salmon IGFBP-1a is minimum.

Unexpectedly, circulating IGFBP-1a measured by the TR-FIA did not respond to fasting or correlate with individual growth rate in yearling masu salmon. IGFBP-1 is believed to be an inhibitor of IGF-I actions in fish by increasing under catabolic conditions (Kajimura and Duan, 2007). There have been attempts to utilize IGFBP-1 circulating protein levels or hepatic mRNA levels as an index of negative growth and/or

stress (Kelley et al., 2001, 2006; Picha et al., 2008a; Kawaguchi et al., 2013). Kawaguchi et al. (2013) found that serum IGFBP-1a levels semi-quantified by ligand blotting were negatively correlated with SGR in weight in yearling masu salmon. In contrast, no such relationship was seen in the present study despite the fish used were from the same strain and same age as those used by Kawaguchi et al. (2013). This discrepancy may be attributed to the difference in detection sensitivities between ligand blotting and TR-FIA. Ligand blotting detects IGFBPs based on the ability to bind labeled-IGF, while TR-FIA measures immunoreactive components regardless of their IGF-binding ability. Thus, it is possible that IGFBP-1a in serum of fed fish is partly degraded by enzymes and that of fasted fish remains intact. Comparing patterns of the immunoreactive bands by immunoblotting using the antiserum against IGFBP-1a may address this question.

The results of the present study did not support my hypothesis that IGFBP-1a immunoreactivity measured by the TR-FIA was a quantitative, negative growth index. However, it does not mean that the TR-FIA established in the present study is useless. A conversion of the TR-FIA to a ligand immunofuctional assay (LIFA) as reported by Lassarre and Binoux (2001) may reveal a relationship between intact IGFBP-1a and growth rate. In a LIFA for human IGFBP-3, immunoreactive IGFBP-3 was first captured by specific antibody and their ability to bind a labeled IGF-I was quantified, which made an accurate and sensitive measurement of intact IGFBP-3 possible (Lassarre and Binoux, 2001). Establishing a LIFA for salmon IGFBP-1a is a future direction.

Circulating IGF-I and possibly IGFBPs are involved in parr-smolt transformation (smoltification). Smoltification is a series of changes pre-adaptive for salmon to the ocean life (Stefansson et al., 2008; McCormick, 2013). The acquisition of hypoosmoregulatory ability (seawater adaptability) through the activation of gill NKA is one of characteristic changes occurring during smoltification. A rapid lean growth,

generally reflected by reduction of K, is also seen during this period. Circulating IGF-I levels have been shown to change during smoltification in several salmonids species (Ágústsson et al., 2001; Aas-Hansen et al., 2003; Shimomura et al., 2012), which would improve seawater adaptability and/or promote body growth (McCormick, 2013). In masu salmon, IGF-I typically shows a peak or high values when gill NKA was activated, suggesting that circulating IGF-I is involved in the NKA activation (Shimomura et al., 2012). In addition, we previously reported changes in circulating IGFBP-1b during smoltification in masu salmon and found sharp increase from the end of March through late April (Fukuda et al., 2015). This finding suggests that circulating IGFBP-1b may have a positive effect on the IGF-I action by delivering it to the gills or protecting it from degradation. The finding of the present study suggests circulating IGFBP-1a also plays a role in smoltification. In this study, I measured seasonal changes in IGFBP-1a in fish with different growth pattern (low or high feeding rations in spring) and found that IGFBP-1a showed a peak in May or April. Especially, Large-High fish had the highest IGFBP-1a value prior to the peak month of circulating IGF-I and activation of NKA. It is possible that circulating IGFBP-1a regulates the availability of IGF-I to the gills and in turn the activity of NKA. It is of note that there were large individual differences in serum IGFBP-1a. Circulating IGFBP-1a may have diurnal rhythms as seen in IGF-I and IGFBP-1b and -2b (Shimizu et al., 2009). Assessing its variability within a day and functional significance are subject of future study. In addition, the availability of recombinant IGFBP-1a (Tanaka et al., unpublished data) should facilitate functional analysis of IGFBP-1a to reveal its role(s) in smoltification.

In summary, the present study established a TR-FIA for salmon IGFBP-1a. The IGFBP-1a immunoreactivity measured by the TR-FIA may not be a quantitative negative growth index since it did not respond to changes in nutritional status or correlate with growth rates in yearling masu salmon. However, a conversion from TR-FIA to LIFA may reveal a relationship between circulating intact IGFBP-1a and

growth rate. On the other hand, the sharp increase of circulating IGFBP-1a found at the same or prior to the peak of IGF-I during spring time suggests its involvement in smoltification in masu salmon.

6. General discussion

Forecasting stock recruitment and adult returns is one of the ultimate goals of surveys on salmon. Stock recruitment is determined by the number of juveniles that survived after periods of high and variable mortality. More than 90% of juvenile salmon die during the early phase of their marine life (Fukuwaka and Suzuki, 1998). Two critical periods have been proposed: the first, during a few months after sea entry and the second, during the first winter in the ocean (Beamish and Mahnken, 2001). Whether or not salmon juveniles survive during the critical periods depends on growth status of individuals. If juveniles cannot exceed a certain body size or energy store, they would be exposed to a higher risk to be eaten by a predator or die due to nutritional deficit (Beamish et al., 2004). Thus, assessing growth status of juveniles is important, and projecting their growth into the future should help forecast stock recruitment.

Chum salmon are the most important salmonid species as fisheries resources in Japan. Approximately 1.8 billion juveniles are released from the Hokkaido and Tohoku areas every year (Miyakoshi et al., 2013). However, adult returns fluctuate by a large extent both year-to-year and region-to-region (Miyakoshi et al., 2013). The regional variations in adult returns are likely influenced by differences in the coastal environments such as water temperature and feed availability that affect growth of juveniles. Intensive research has been conducted to evaluate the status of out-migrating juvenile chum salmon. However, there has been no attempt to utilize the endocrine growth indices for juvenile chum salmon. This thesis places a special emphasis on juvenile chum salmon growth.

There are several ways to measure growth of fish. Body size (weight and length) is the sum of growth from the first feeding to the present, and measuring body size gives information on past growth. In most fishes, pattern of past growth can be reconstructed by analyzing the circuli of the scales. Thus, the scales have provided vital

information for identifying a critical period for ocean conditions affecting their growth during the critical periods. However, analyzing the scales of returning adults is retrospective, and doing so of juveniles that will suffer growth-dependent mortality is possible, but labor-intensive and may be inaccurate since the outermost layer of the scale has no circulus line so that the time for the formation varies for a large extent. Therefore, tools that enable us to measure/assess proximate growth status of juveniles are valuable. In this thesis, I focused on the process and mechanism of growth and aimed to utilize the components involved in growth regulation as indices of recent/current growth status. These measures include RNA/DNA, IGF-I, IGFBP-1b and IGFBP-1a.

The findings of the present study suggested that the utility of muscle RNA/DNA as a positive growth index depends on species. RNA/DNA ratio is one of the biochemical indices widely used in studies on population dynamics in marine fishes and organisms (Chícharo and Chícharo, 2008). In juvenile Atlantic salmon, muscle RNA/DNA ratio responded to changes in feeding rations (Arndt et al., 1996) and Atlantic salmon smolts showed a positive correlation with growth rate (MacLean et al., 2008; Caldarone et al., 2016). In contrast, the RNA/DNA-growth relationship varies by species, life-history stage and tissue analyzed (Johnson et al., 2002; Kawaguchi et al., 2013). These findings indicate the necessity of validation in each target species to use RNA/DNA ratio as a growth index. In the present study, RNA/DNA ratio in juvenile chum salmon positively correlated with individual growth rate but the coefficient for the relationship between RNA/DNA and growth was weak, suggesting that RNA/DNA ratio is not the best index of growth at least for juvenile chum salmon. However, an advantage of using RNA/DNA is its versatility. Since chemical compositions of RNA and DNA are common in all organisms, assays of RNA/DNA ratio can be applied to any species if the nucleic acids are properly preserved and extracted. In addition, measuring RNA/DNA is relatively simple and relatively expensive. Thus, if RNA/DNA ratio

correlates well with growth rate in the target species, it is an option to estimate growth status.

The present study confirms that circulating IGF-I is an efficacious positive growth index in juvenile chum salmon. The utility of circulating IGF-I as a positive growth index has been proposed in salmonids and other fish species (Beckman et al., 2004a, b; Shimizu et al., 2006, 2009; Picha et al., 2008a; Beckman, 2011). In fact, there are few studies reporting a lack of relationship between circulating IGF-I and growth rate when IGF-I was measured after acid-ethanol extraction (Beckman et al., 2004a, b). As is the case in other studies, the laboratory experiments described in this thesis indicated that circulating IGF-I levels well reflected short-term fasting/refeeding and was strongly correlated with individual growth rate in juvenile chum salmon. In addition, the IGF-I-growth relationships were consistent between May and June. These findings suggested that circulating IGF-I is a reliable positive index of growth in this species. However, further validation of circulating IGF-I in juvenile chum salmon for its stability as a positive growth index is necessary. Criteria of a "stable" growth index include, no affect by sampling time, handling stress, season, temperature, developmental/maturational stages and growth history. However, plasma IGF-I is indeed influenced by these factors. For instance, a disruption of the IGF-I-growth relationship was observed when fish experienced an acute change in water temperature (Beckman et al., 2004a, b, c; Kawaguchi et al., 2013). Thus, a proper use of IGF-I as a growth index require knowing its variation and limitation through rearing experiments under different environmental settings. In this regard, the present study is incomplete as daily variation in IGF-I, the effect different feeding rations and effect of stress were not examined. In the present study, however, an attempt was made to stabilize or/and calibrate growth assessment by using IGF-I by adding other growth indices.

The present study suggested the utility of circulating IGFBP-1b as a negative growth index in juvenile chum and coho salmon. Circulating IGFBP-1b increased in

fasted fish and showed a strong, negative correlation with growth rates in juvenile chum salmon. In addition, a significant IGFBP-1b-growth relationship was found in both May and June. In addition, high plasma IGFBP-1b in coho salmon in the ocean likely reflected catabolic conditions owing to poor feed availability and low seawater temperature. Although more validation is needed, such as examining seasonal effects and diurnal changes, circulating IGFBP-1b is an index reflecting negative aspects of growth in juvenile salmon under catabolic or stressful conditions.

Although circulating IGF-I and IGFBP-1b are reliable growth indices in salmon, their mRNA levels in the liver or/and muscle may be also useful. IGF-I is produced in virtually all tissues and stimulates growth through endocrine and autocrine/paracrine actions. While, the major source of endocrine IGF-I is the liver (Daughaday and Rotwein, 1989; Le Roith et al., 2001; Ohlsson et al., 2009). Based on the assumption that the changes in *igf-1* mRNA level correspond to changes in protein level, some studies explored the relationship between hepatic *igf-1* and individual growth rate (Picha et al., 2008b; Kawaguchi et al., 2013). However, their regression coefficients were not as high as that of circulating IGF-I (Picha et al., 2008b; Kawaguchi et al., 2013). On the other hand, the usefulness of *igfbp-1b* and *-1a* as negative growth indices are not fully validated. We previously revealed hepatic *igfbp-1b* mRNA correlated with SGR but its coefficient was not higher than that of circulating level (hepatic *igfbp-1b*: r = -0.59, serum IGFBP-1b: r = -0.84) in yearling masu salmon (Kawaguchi et al., 2013). These findings suggest that circulating protein levels are better than mRNA levels as growth indices, but mRNA levels may be used as alternative growth indices when fish are too small to collect blood as is the case for chum salmon fry. Moreover, quantifying *igf-1* and/or *igfbps* mRNA level have an advantage in terms of the accumulation of the data on their environmental modulations such as effect of water temperature, salinity, hypoxia and stress in teleost (Shepherd et al., 2005; Kamei et al., 2008; Hevrøy et al., 2011; Taniyama et al., 2016). Since measurement of mRNA

level by quantitative real-time PCR is easy when compared with development of an immunoassay. Therefore, examining expression levels of *igf-1*/*igfbps* has a great value when immunoassays are not available.

The present study also revealed that IGF-I and IGFBP-1b may reflect their different specific roles in the growth process because there were different relations of IGF-I and IGFBP-1b with fish size and condition. It is worth considering differences among growth in length and weight, and condition factor (K). Changes in growth in length and weight are generally concordant but sometimes discordant. The body length of vertebrates is determined primarily by bone growth and theoretically cannot be negative. On the other hand, the body weight of vertebrates is determined mainly by the size of muscle, viscera and fat stores and weight growth can be negative when energy store are consumed by catabolic processes. Positive growth in juvenile fish therefore is accompanied with a balanced increase of both bone and muscle. Surplus energy would be stored as glycogen and fat. An index of energy stores can be generated by calculating the K values. The laboratory experiments in this study indicated that circulating IGF-I correlated with FL and BW, while IGFBP-1b related only with K. The former relationships are not surprising since IGF-I plays a crucial role in growth of cartilage and bone tissues and muscle (Ohlsson et al., 2009) and many studies using fish have reported relationships between circulating IGF-I and FL and BW (Beckman, 2011; Kawaguchi et al., 2013; Fukuda et al., 2015). The positive IGF-I-size relationships in fish support the notion that endocrine IGF-I is important for the size gain. On the other hand, IGFBP-1b was negatively correlated with K, which was in good agreement with the findings in coho and masu salmon (Shimizu et al., 2006, 2009; Kawaguchi et al., 2013; Fukuda et al., 2015). In mammals, IGFBP-1b is believed to be involved in catabolism especially glucose regulation (Lee et al., 1993, 1997). Although the function of circulating fish IGFBP-1 has not been revealed, its response to changes in nutritional status and stress suggests circulating IGFBP-1b plays crucial role in catabolism. These

functional differences between IGF-I and IGFBP-1b likely reflected disparate relationships with morphological growth/nutritional parameters.

The present study examined a new approach, using IGF-I/BP-1b ratio to evaluate growth potential. Mechanistically, when IGF-I is bound to IGFBP-1, it is removed from the circulation and does not promote growth. Indeed, in humans IGFBP-1 level is inversely related with biologically active IGF-I (Frystyk et al, 2002). Thus, the molar ratio of IGF-I to IGFBP-1b could tell us the fraction of IGF-I that can actually be delivered to target tissues to promote growth. My expectation was to see a higher correlation coefficient for IGF-I/BP-1b ratio with growth rate than that of IGF-I or IGFBP-1b to growth alone. However, the relationship between IGF/BP-1b ratio and growth did not result in an improved regression coefficient as compared to IGF-I, IGFBP-1b and growth (IGF-I: $r^2 = 0.59$, IGFBP-1b: $r^2 = 0.62$, IGF-I/BP-1b: $r^2 = 0.41$). Despite the lack of improvement in the regression coefficient, it is worth noting that the response of the average ratios to fasting and refeeding differed to those of IGF-I and IGFBP-1b alone. The biological meaning of IGF-I/BP-1b is not known at present. But the ratio provides us insight into a different aspect of the growth status of fish. More experiments need to be done to explore the relation of IGF-I/BP-1b to growth status under different experimental conditions.

The present study developed for the first time a TR-FIA for IGFBP-1a in fish and suggested that circulating IGFBP-1a might not be useful as a negative growth index. IGFBP-1a is a co-ortholog with IGFBP-1b in fish and increased under catabolic conditions similarly to IGFBP-1b. Semi-quantification of a IGFBP-1a band by ligand blotting in masu salmon found that there were weak but significant negative correlations between serum IGFBP-1a level and SGR in weight (Kawaguchi et al., 2013). As discussed earlier, TR-FIA measures both intact IGFBP-1a and IGFBP-1a fragments lacking the IGF-binding ability whereas ligand blotting detects only the IGF-binding ability. A selective measurement of intact IGFBP-1a will be a subject of future study.

Although circulating IGFBP-1a did not relate to growth status in the present study, it may be involved in smoltification. In any case, the newly developed TR-FIA for salmon IGFBP-1a provides us a tool of functional analysis and understanding of growth regulation in salmonid species. In particular, calculating IGF-I/BP-1a and BP-1b/BP-1a ratios are worth examining.

Assuming that endocrine IGF-I is important for growth in fish, measuring other endocrine components such as IGFBP-2b and IGF-II in the circulation and looking at relations with growth could be valuable in terms of analyzing the mechanism of growth regulation and developing new growth indices. IGFBP-2b is a major carrier protein of circulating IGF-I. Its plasma levels are influenced by GH treatment, fasting and seawater transfer (Shimizu et al., 2003a, 2007) and correlated with individual growth rate (Beckman et al., 2004b). In addition, circulating IGFBP-2b levels showed strong positive correlations with circulating IGF-I levels and growth rate (Shimizu et al., 2003a; Beckman et al., 2004b), suggesting it is another positive growth index. An RIA for salmon IGFBP-2b has been established but it is currently not being used. Thus, establishment of a new immunoassay is desired. Currently, production of recombinant salmon IGFBP-2b using a bacterial expression system is in progress, the recombinant IGFBP-2b can be used for assay components to develop a TR-FIA. A similar approach can be used for IGF-II, which is also in progress in our laboratory.

I propose that the new endocrine growth indices valuated in this thesis will be powerful tools for stock assessment when combined with traditional, comprehensive filed surveys. In this thesis, I monitored growth status of chum salmon in Hokkaido and coho salmon in Canada using circulating IGF-I and IGFBP-1b as indices of growth and/or metabolism. Although the both field surveys provided useful information on the growth status of chum and coho salmon, the data on coho salmon may be more relevant to stock assessment since more oceanographic and biological data are available from the survey in the Canadian coasts. Indeed, in addition to data collections for oceanographic

conditions, the survey in the Strait of Georgia consisted of a variety of biological analyses such as feed availability and stomach contents and of physiological analyses such as measurements of androgen and hepatic mRNA expression of GH/IGF axis. Although enormous labors and investments are needed to be devoted, scientific outcomes are more reliable and precise. This thesis emphasizes that the importance of systematic and intensive field survey in Hokkaido as well. Especially, chum salmon is an intensive target of capture fisheries in Hokkaido but numbers of adult return tended to decline in the last decade (Miyakoshi et al., 2013). Therefore, future direction of comprehensive survey in chum salmon is desired.

Evaluating growth status of juvenile salmon is valuable for assessing the risk of growth-dependent mortality, which would eventually determine year class strength. This thesis focused on the endocrine growth indices that enable us to evaluate positive and negative aspects of growth. This thesis also developed a new assay for a candidate of growth index. Circulating IGF-I is superior as a positive growth index in juvenile chum salmon whereas growth retardation or catabolic conditions can be evaluated by using IGFBP-1b as a negative growth index. Availability of multiple growth indices should make growth evaluation more stable, accurate and/or sensitive. Such robustness is important especially for surveys on juvenile salmon in the ocean, where many biotic and abiotic factors affect their growth directly or in combination with other factors.

7. Summary

Monitoring the growth of salmon during their early marine life is important for assessing the probability of growth-dependent mortality. However, the measurement of growth rate is challenging for free-living salmon in the ocean. Although there are several means to measure and evaluate individual growth directly and indirectly, I focused on biochemical and physiological indices such as RNA/DNA ratio, circulating insulin-like growth factor-I (IGF-I), IGF-binding protein (IGFBP)-1b and -1a since these are involved in growth process. RNA/DNA ratio reflects a degree of protein synthesis per cell and it correlates with growth rate in some fish species. IGF-I is an essential component of the growth hormone/IGF system that regulates animal growth. Circulating IGF-I responds well to changes in nutritional status and is strongly correlated with growth rate in fish. The level of IGF-I in the circulation is tightly regulated by multiple IGFBPs. In salmon blood, there are three IGFBPs. Among these, IGFBP-1b and -1a are believed to inhibit IGF-I actions. Circulating IGFBP-1b and -1a are induced into the blood by fasting and are negatively correlated with growth rate. Therefore, RNA/DNA, circulating IGF-I and IGFBP-1a/-1b are candidates for positive or negative growth indices. I validated and utilized RNA/DNA ratio and circulating IGF-I as positive growth indices in juvenile chum salmon. I also examined the usefulness of circulating IGFBP-1b as a negative index of growth and monitored potential negative growth in juvenile chum salmon and coho salmon in wild. Moreover, I established a time-resolved fluoroimmunoassay (TR-FIA) for salmon IGFBP-1a and evaluated its utility as a new negative index of growth in masu salmon.

Circulating IGF-I as a positive growth index of juvenile chum salmon (Chapter 2)

1) I validated the utility of muscle RNA/DNA ratio and serum IGF-I as positive

growth indices with a rearing experiment using individually-tagged juvenile chum salmon.

2) Muscle RNA/DNA ratio and serum IGF-I levels in fasted fish were significantly lower than those in fed fish, and the magnitude of difference was greater for serum IGF-I than RNA/DNA ratio.
3) Muscle RNA/DNA ratio and serum IGF-I levels had positive correlations with specific growth rates. The correlation coefficient of serum IGF-I was higher (r^2 = 0.90) than that of muscle RNA/DNA ratio (r^2 = 0.26).
4) The laboratory experiment suggested circulating IGF-I was a better than muscle RNA/DNA ratio as a growth index in juvenile chum salmon.
5) I monitored growth status of juvenile chum salmon caught in the river, estuary, port, coast sites that followed their downstream and coastal migration route in Abashiri area, eastern Hokkaido, Japan in 2013 and 2014.
6) Small fish were consistently observed in the estuary, while large fish were along the coast in both years.
7) Muscle RNA/DNA ratio was generally similar among sampling sites in both years.
8) Serum IGF-I levels were lower in fish at the estuary when compared to those along the coast in June in both years.
9) Field survey suggested that the growth of juvenile chum salmon in the Abashiri coastal waters was increased when fish left the coast, while fish with low growth rates might stay in the estuary and undergo size-selective mortality.

Circulating IGFBP-1b as a negative growth index of juvenile chum salmon (Chapter 3)

1) I examined the utility of circulating IGFBP-1b as a negative growth index with a fasting/refeeding experiment in juvenile chum salmon in May and June.

2) Serum IGF-I levels in fasted fish were low. Serum IGFBP-1b levels were increased by fasting and were reduced to the basal level by refeeding,
3) Serum IGF-I positively correlated with growth rate in both months (r^2 = 0.58-0.59), while serum IGFBP-1b was highly, negatively correlated with individual growth rate (r^2 = 0.41-0.62).
4) I calculated IGF-I/BP-1b ratio, which is a theoretical fraction of IGF-I available for binding with the IGF receptor. The ratios were lowest in fasted fish and showed no difference between fed and refed groups in either month.
5) The laboratory experiment suggested circulating IGFBP-1b was a good negative index in juvenile chum salmon.
6) I monitored growth status of juvenile chum salmon caught at the river, estuary, port, coast and nearshore sites that followed their downstream and coastal migration route in Abashiri area in May and June, 2015.
7) Serum IGF-I levels in May were high at the river site, while IGF-I levels were high at the port site in June. Fish caught at the nearshore site had consistently low IGF-I.
8) Serum IGFBP-1b levels in May were high at the nearshore site, while IGFBP-1b was consistently low at other sites. In June, serum IGFBP-1b gradually decreased from the river to the nearshore sites.
9) IGF-I/BP-1b ratio better distinguished regional difference in growth than IGF-I or IGFBP-1b alone; the ratio decreased from the river to the nearshore sites in May while the ratio increased across the same sites in June.
10) The field survey suggested juvenile chum salmon left the nearshore under poor growth conditions in both May and June as judged by the low IGF-I, however, catabolic status was severer in fish in nearshore sites in June as judged by the high IGFBP-1b.

Regional variation of circulating IGFBP-1b in postsmolt coho salmon in the Strait

of Georgia, British Columbia, Canada (Chapter 4)

1) I compared regional differences in plasma IGFBP-1b levels in postsmolt coho salmon in Strait of Georgia and Johnstone Strait, British Columbia, Canada.
2) Plasma IGFBP-1b levels were high in the Gulf Islands and low among the Discovery Islands.
3) Plasma IGFBP-1b levels were the highest in fish at the Johnstone Strait, where poor ocean conditions for salmon was assumed.
4) The IGF-I/BP-1b ratio discriminated differences between regions more precisely than IGFBP-1b alone.
5) I examined the relationships of plasma IGFBP-1b levels and IGF-I/BP-1b ratio with morphological parameters, stomach fullness and seawater temperature.
6) Plasma IGFBP-1b negatively correlated with K ($r = -0.48$), stomach fullness ($r = -0.49$) and water temperature ($r = -0.58$) when the data from all regions were pooled.
7) The IGF-I/BP-1b ratio positively correlated with K ($r = 0.66$) and stomach fullness ($r = 0.33$), and negatively correlated with water temperature ($r = -0.59$).
8) These findings suggest plasma IGFBP-1b reflects a catabolic status in postsmolt coho salmon and is a useful tool to monitor negative aspects of salmon growth in the ocean.

Development of a TR-FIA for salmon IGFBP-1a and assessment of its utility as a negative growth index (Chapter 5)

1) I developed a competitive TR-FIA for salmon IGFBP-1a by using recombinant IGFBP-1a as antigen for immunization and as an assay component.
2) The detection limit of the assay was calculated as 9.2 ng/ml, and ED_{80} and ED_{20} were 18.1 and 226.9 ng/ml, respectively.

3) The TR-FIA showed good parallelism with sera dilution from masu salmon and rainbow trout.
4) The TR-FIA showed 1.5 and 3.6% cross-reactivity with the 41-kDa form (IGFBP-2b) and 22-kDa form (IGFBP-1b), respectively. There is no interference by IGF-I in the assay.
5) I measured circulating IGFBP-1a in individually-tagged yearling masu salmon that had been fed or fasted for 6 weeks, or fasted for 4 weeks followed by refeeding for 2 weeks.
6) Serum IGFBP-1a did not respond to fasting for 6 weeks and refeeding for 2 weeks.
7) Serum IGFBP-1a did not correlate with individual growth rate.
8) I examined seasonal changes in circulating IGFBP-1a levels in hatchery-reared masu salmon during smoltification.
9) Serum IGFBP-1a showed a peak in April or May depending on growth history. These findings suggest the involvements of IGFBP-1a in smoltification in masu salmon.
10) These results suggest that circulating IGFBP-1a cannot be used as a negative growth index. A selective measurement of intact IGFBP-1a may better reveal its relationship with growth rate.

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