**Title:**

Relationships between gill $Na^+$,$K^+$-ATPase activity and endocrine and local insulin-like growth factor-I levels during smoltification of masu salmon (*Oncorhynchus masou*)

**Authors:**

Takahiro Shimomura[1], Takuro Nakajima[1], Moeri Horikoshi[1], Anai Iijima[2], Hirokazu Urabe[2], Shinya Mizuno[2], Naoshi Hiramatsu[1], Akihiko Hara[1], and Munetaka Shimizu[1]*

**Affiliations:**

[1]Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate, Hokkaido 041-8611, [2]Salmon and Freshwater Fisheries Research Institute, Hokkaido Research Organization, Kitakashiwagi 3-373, Eniwa, Hokkaido 061-1433, Japan

*Corresponding author: Tel&Fax: +81-138-40-8897

E-mail address: mune@fish.hokudai.ac.jp

**Abstract**

We established profiles of insulin-like growth factor (IGF)-I mRNA in the liver, gill and white muscle and circulating IGF-I during smoltification of hatchery-reared masu salmon, and compared with that of gill $Na^+$,$K^+$-ATPase (NKA) activity. Gill NKA activity peaked in May, and dropped in June. Liver *igf1*mRNA was high in March and decreased to low levels thereafter. Gill *igf1* increased from March, maintained its high levels during April and May and decreased in June. Muscle *igf1* mRNA levels were relatively high during January and April when water temperature was low. Serum IGF-I continuously increased from March through June. Serum IGF-I during March and May showed a positive correlation with NKA activity, although both were also related to fish size. These parameters were standardized with fork length and re-analyzed. As a result, serum IGF-I and gill *igf1* were correlated with NKA activity. On the other hand, samples from desmoltification period (June) that had high serum IGF-I levels and low NKA activity disrupted the relationship. Expression of two IGF-I receptor (*igf1r*) subtypes in the gill decreased in June, which could account for the disruption by preventing circulating IGF-I from acting on the gill and retaining it in the blood. The present study suggests that the increase in gill NKA activity in the course of smoltification of masu salmon was supported by both endocrine and local IGF-I, and the decrease during desmoltification in freshwater was due at least in part to the down-regulation of gill IGF-I receptors.

**Keywords**

insulin-like growth factor-I; salmon; smoltification; gill; $Na^+$,$K^+$-ATPase; serum

## 1. Introduction

All anadromous salmonids are hatched in freshwater, stay in the river/lake for certain period and migrate to the ocean. With a few exceptions, juvenile salmon are intolerant to full seawater and need to acquire seawater adaptability as well as changes adaptive to ocean life prior to the downstream migration. Such transition is called smoltification (parr-smolt transformation) that involves development of seawater adaptability, body silvering, darkening of fin margins, decrease in condition factor, change in rheotaxis and formation of school [19,55]. These changes are sometimes independent one another but occur in spring through synchronization by photoperiod [8,19,55,56]. Several endocrine systems are involved in smoltification and often act synergistically to induce a change. For instance, the acquisition of seawater adaptability is under control by cortisol and the growth hormone (GH)-insulin-like growth factor (IGF)-I system [26,27]. On the other hand, some changes may be coordinated by a single endocrine system. The GH-IGF-I system controls animal growth and also plays a crucial role in development of seawater adaptability in salmonids [11,26,42]. The GH-IGF-I system promotes growth via multiple pathways [10,22,34]. GH acts on target tissues directly or indirectly through IGF-I, which is primarily produced by the liver in stimulation with GH, secreted into bloodstream and mediates GH actions [10,34]. IGF-I is also expressed in virtually all types of tissues and exerts autocrine/paracrine actions [22]. Understanding how these hormones improve seawater adaptability is particularly important for hatchery programs of several salmonid species since degree of seawater adaptation directly affects initial survival of released fish in seawater, growth in following summer and survive as adults [3,7,12].

The Gill, along with the kidney and intestine, is a major organ responsible for maintaining ion concentrations of the body. Improvement of seawater adaptability at the gill level is largely achieved by proliferation, differentiation/transformation and specific localization of the chloride cells with enhanced activity of $Na^+,K^+$-ATPase (NKA) and other ion transporters/channels [17,37,46]. NKA is located in the basolateral membrane of chloride cells and essential for extrusion of sodium ions from the cells. Cortisol and GH are known to enhance NKA activity by acting on its mRNA and/or protein and by inducing the changes of the chloride cells [23,25-27,40,43]. As is the case for growth regulation, some of the GH actions on NKA activity may be mediated by IGF-I, and local IGF-I (i.e. gill IGF-I) should also play a role [24,29]. However, what source of IGF-I is important is a matter of debate [42]. Accumulating evidence emphasizes importance of gill IGF-I in osmoregulation [42,44,57]. On the other hand, assessing involvement of endocrine IGF-I in activating NKA has been encountered by the fact that during

smoltification, a rapid lean growth also occurs in response to increasing day length, water temperature and food availability. Circulating IGF-I typically shows an increase during smoltification and may be important for both promoting growth and NKA activity [4,11]. However, what percentages of circulating IGF-I are partitioned to promote growth and osmoregulation, respectively, is not known. In order to analyze the IGF-I roles in the regulation of osmoregulation, a comprehensive data set on circulating IGF-I levels and tissue *igf1* mRNA during smoltification is necessary, which is somewhat incomplete to date. Indeed, there is no study measuring circulating IGF-I and liver *igf1* mRNA levels simultaneously during smoltification. The first goal of this study is to establish profiles of circulating IGF-I and *igf1* mRNA in tissues responsible for growth and osmoregulation (i.e. liver, gill and white muscle) in masu salmon (*Oncorhynchus masou*). We then performed correlation analyses to assess involvement of endocrine and local IGF-I in increasing gill NKA activity.

## 2. Materials and methods

### *2.1. Fish*

Under-yearling and yearling masu salmon were reared in freshwater at the South Branch of Salmon and Freshwater Fisheries Institute, Hokkaido Research Organization (42°N, 140°E) (Nikai-gun, Hokkaido, Japan). Under-yearling masu salmon were sorted by size (> 10.5 cm) and visual inspection in November 2009 to remove precociously maturing males and potential non-smolting fish in the following spring. Fish were maintained in the river water in outdoor ponds (24.6 x 3.5 m) and fed twice (November-February) or three times (March-June) a day on a commercial diet (Nippon Formula Feed Mfg, Kanagawa, Japan) with standard rations at 0.4-1.9%/body weight. These fish were for stock enhancement and released to the river in May 2010. Some fish were kept in the same pond and reared until June. From November 2009 to June 2010, seven fish were sampled monthly. Fish were anesthetized by 3.3% 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan) and measured for fork length and body weight. Condition factor was calculated as follows: (body weight) x 1000/(fork length)$^3$. Blood was withdrawn by a syringe from the caudal vein, allowed to clot overnight at 4°C and centrifuged at 8,050*g* for 10 min. Serum was collected and stored at -30°C until use.

### *2.2 Cloning of partial cDNAs for IGF-I and elongation factor-1a (EF-1α)*

Liver cDNA was prepared from yearling masu salmon reared at Nanae Freshwater Experimental Station, Hokkaido University (Kameda-gun, Hokkaido, Japan) as described in Shimizu et al. [51].

105 Primer sets designed for Atlantic salmon (*Salmon salar*) IGF-I and EF-1α (Genbank ID:
106 EF432852 and BG933853 [9]) (Table 1) were applied to masu salmon. Reverse transcriptase
107 (RT)-PCR was performed with a Veriti Thermal Cycler (Applied Biosystems, Carlsbad, CA) and
108 AmpliTaq Gold® 360 Mater Mix (Applied Biosystems, Foster City, CA). PCR cycles consisted
109 of 1 cycle of 95°C for 10 min; 36 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 1 min 30
110 sec; 1 cycle of 72°C for 7 min. PCR products were cloned into the pGEM-T Easy Vector Systems
111 (Promega, Madison, WI) and positive clones were sequenced as described in Shimizu et al. [51].
112
113 *2.3. RNA extraction and cDNA synthesis*
114 Total RNA was extracted from the tissues as described in Shimizu et al. [51]. One and half μg
115 RNA was reverse-transcribed using SuperScript VILO cDNA Synthesis kit (Invitrogen, Carlsbad,
116 CA) in a 10 μl reaction according to the manufacturer's instruction. cDNA was stored at -30°C
117 until use.
118
119 *2.4. Real-time quantitative PCR (qPCR)*
120 Primer sets for qPCR of IGF-I and EF-1α were designed based on the cloned masu salmon cDNA
121 sequences using MacVector Ver 9 (MacVector Inc., Cary, NC). One primer in each assay was
122 placed across an exon/exon boundary predicted from the gene structure of zebrafish from
123 Ensembl data base (http://asia.ensembl.org/index.html). The primers for IGF-I target the signal
124 peptide region.
125 RT-PCRs using these primers were performed to prepare assay standards. PCR
126 products run on 1.5% agarose gel were excised and purified using QIAEX II Gel Extraction Kit
127 (Qiagen, Valencia, CA). Copy numbers of the purified amplicon were calculated from the
128 molecular weight of the amplion and concentration. The standard cDNA were serially diluted
129 from $1 \times 10^7$ to $3 \times 10^2$ copies.
130 qPCR was set up using Power SYBR Green PCR Master Mix (Applied Biosystems) in
131 a reaction volume of 20 μl with primer concentration of 100 nM. The reaction mixture contained
132 0.1-2 μl cDNA template. qPCR was run on a 7300 Sequence Detector (Applied Biosystems)
133 using the manufacturer's recommended cycling conditions: 50°C for 2 min, 95°C for 10 min
134 followed by 40 cycles at 95°C for 15 se and 60°C for 1 min. Measured values were normalized by
135 those of *ef1a* and expressed as relative values.
136 Primers specific to type I IGF receptor subtypes (IGF-IRa and IGF-IRb) were
137 designed based on the sequences of rainbow trout (*Oncorhynchus mykiss*) (Genbank ID:

AF062499 and AF062500 [16]) (Table 1). qPCR was performed as described above and measured values were normalized by those of *ef1α*.

*2.5. Time-resolved fluoroimuunoassay (TR-FIA) for IGF-I*

Prior to the assay, serum IGF-I was extracted with an acid-ethanol as described in Shimizu et al. [52]. IGF-I was quantified by TR-FIA based on the method described in Small and Peterson [53] using recombinant salmon/trout IGF-I (GroPep) as a standard.

*2.6. NKA activity assay*

Gill NKA activity was measured according to Quabius et al. [41] with minor modifictions. This method is based on the ability of NKA hydrolyzing ATP to give ADP and inorganic phoshporus with or without presence of ouabain at 37°C for 10 min. 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).

*2.7. Standardization of data*

Previous work has shown that plasma IGF-I levels may be related to fish size [5], and this was the case in the present study (Serum IGF-I = 4.82 x fork length - 42.9, $r^2 = 0.58$) when the March to May samples were pooled and analyzed. In order to better understand a relationship between gill NKA activity and IGF-I levels, we excluded size effect on both parameters by standardizing measured values to the mean length [50] using the following equation: standardized hormone $value_1$ = hormone $value_1$ - [($length_1$ - length mean) x 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 in a treatment, and slope is the slope of hormone-length relation. NKA activity (NKA = 1.25 x fork length - 12.3, $r^2 = 0.51$) and some other parameters were also correlated with fish size and thus standardized as described above.

*2.8. Statistical analysis*

Values from precociously maturing males were not included in the analysis since those disturb the IGF-I-growth relationship [6]. Results of the experiments were analyzed by one-way ANOVA. When significant effects were found, differences were identified by the Fisher's protected least significant difference (PLSD) test using the JMP program (SAS Institute Inc., Cary, NC). Differences among groups were considered to be significant at $P < 0.05$.

## 3. Results

Water temperature and morphological changes during the experimental period (November to June) were shown in Figure 1. Water temperature ranged from 2°C (January) to 15°C (June) (Fig. 1a). Fork length and body weight of masu salmon stayed relatively constant from November to April and increased in May through June (Fig. 1b,c). There was no significant change in condition factor (Fig. 1d).

Gill NKA activity was measured from March to June (Fig. 2). It increased in the course of smoltification, showed a peak in May and significantly dropped in June.

Liver *igf1* mRNA levels were relatively high during November to March (Fig. 3a). The level significantly decreased in April and stayed low thereafter. Gill *igf1* was low during November to February, started to increase in March, maintained high values during April and May, and decreased in June (Fig. 3b). Muscle *igf* gradually increased from November to April and decreased thereafter (Fig. 3c). Serum IGF-I levels were low during November to March and continuously increased from April through June (Fig. 3d).

Since the fish sampled in June were held in freshwater beyond their migration period (April to May), they were most likely undergoing desmoltification that accompanies many physiological changes to re-adapt to freshwater [19]. We thus excluded the data from June for correlation analyses. Serum IGF-I was strongly correlated with gill NKA activity in the course of smoltification (March to May) ($r^2 = 0.74$, $P < 0.0001$; Table 2). However, both serum IGF-I and gill NKA were also correlated with body size ($r^2 = 0.58$, $P = 0.0002$ for IGF-I and $r^2 = 0.51$, $P = 0.0006$ for NKA; Table 2). In order to further analyze a possible involvement of endocrine and local IGF-I in the development of gill NKA activity, serum IGF-I and gill NKA values were standardized by body length using the slopes of the correlation lines (Fig. S1, Fig. S2). As a result, both serum IGF-I and gill *igf1* levels positively correlated with gill NKA activity (Table 3, Fig. 4). On the other hand, inclusion of the June data disrupted the relationship between serum IGF-I and gill NKA activity (data not shown).

Profiles of *igf1ra* and *igf1rb* transcripts in the liver, gill and white muscle were analyzed by qPCR (Fig. 5). *igf1ra* and *igf1rb* were expressed in the liver but at low levels (Fig. 5a,b). Gill *igf1ra* mRNA tended to increase from March to May and significantly dropped in June (Fig. 5c). Gill *igf1rb* was low in March but increased in April, maintained high levels in May and decreased in June (Fig. 5d). Muscle igf1ra and igf1rb mRNA decreased from May to June and April to June, respectively (Fig. 5e,f).

## 4. Discussion

Yearling masu salmon used in the present study were reared in freshwater under ambient water temperature and photoperiod. Based on the maximum activation gill NKA activity, the peak of smoltification was considered in May. Indeed, masu salmon in Hokkaido typically migrate to the ocean in May and local hatcheries release smolt during this period. In June, gill NKA activity dramatically dropped, which is a sign of desmoltification (smolt-parr reversion) [19,55]. Thus the sampling period spanned smoltification and desmoltification of this species.

Profiles of circulating IGF-I during smoltification have been reported in several salmonids. A general trend is that plasma IGF-I shows higher levels during March-May and a drop in following months [1,2,20,21,30,49]. However, there are exceptions that IGF-I levels were unchanged or even decreased during smoltification [28,33]. Obviously, species/strain difference combined with various rearing conditions affects the profiles. In the present study, serum IGF-I levels continued to increase during and after smoltification. There was a strong positive relationship between serum IGF-I and fish size, suggesting that endocrine IGF-I is important for promoting growth during smoltification as seen in post-smolt period [6]. However, it is difficult to know how much of circulating IGF-I was used for growth and seawater adaptation, respectively, since smoltification concerts there two biological processes. In addition, IGF-I may enhance seawater adaptability indirectly though enlarging body size which is a factor affecting seawater tolerance [18]. In order to better analyze the relationship between endocrine IGF-I and gill NKA activity in the course of smoltification (March to May), size effect was eliminated by standardizing the both parameters to body length. This standardization method was applied to analyze the diurnal variation of circulating IGF-I in post-smolt coho salmon otherwise size variation could mask significant IGF-I variation [50]. In the present study, an attempt was to exclude IGF-I fraction that was related to size. However, it should be noted that standardizing with fish size does not fully eliminate IGF-I fraction for recent growth (i.e. growth rate). As a result, there was still a positive relationship between IGF-I and gill NKA activity after standardization to body length. This suggests that endocrine IGF-I acts, independent of absolute size, on the gill to activate NKA in masu salmon. Positive effects of systemically administrated IGF-I on gill NKA as well as whole-body seawater adaptability have been reported in trouts [29,47], which supports our notion that endocrine IGF-I is important in the development of seawater adaptability. On the other hand, important role of local (gill) IGF-I has been also reported [42,44]. We propose that both endocrine and local IGF-I regulate NKA activity in masu

salmon.

Liver is the major site of IGF-I production [10,22,34]. Indeed, the levels of *igf1* mRNA in the liver was the highest among the tissues examined. Surprisingly, there is only a few studies measuring liver *igf1* mRNA during smotificiation [13,45]. A general trend of these studies is that liver *igf1* mRNA showed a peak during April to May. The increase may be a response to an elevated plasma GH level, agreeing the general belief that GH is the major regulator of *igf1* expression in the liver. In the present study, liver *igf1* mRNA was higher in March than in April and May. Although we did not measure circulating GH levels, Mizuno et al. [31] reported a GH peak in March in masu salmon from the same river system. Thus the relatively high levels of liver *igf1* mRNA seen in March was likely due to a simulation by GH. A positive relationship between mRNA and circulating levels has been reported in Chinook salmon (*O. tshawytscha*) [36]. In contrast, circulating IGF-I showed no correlation with liver *igf1* mRNA. A change in the stability of *igf1* mRNA or/and rate of translation might account for it. Such discrepancy between liver *igf1* and circulating IGF-I have been reported in fasted hybrid striped bass (*Morone chrysops* x *Morone saxatilis*) [35]. Other possibilities may be that increase in IGF-I secretion into bloodstream was masked by active uptake by the receptor in target tissues as seen for GH [39] or changes in circulating IGF-binding proteins might alter the half-life of IGF-I [60]. However, these are just speculations and we have no empirical explanation for this discrepancy at present.

In the present study, gill *igf1* mRNA levels increased in March and maintained high levels during April and May when gill NKA activity peaked. The increase in gill *igf1* mRNA during smoltification has been observed in coho salmon (*O. kisutch*) [13,45] and Atlantic salmon (*Salmo salar*) [33]. Gill *igf1* mRNA levels sharply decreased to the basal levels in June in the present study, which is coincident with a drop in NKA activity. In addition, there was a positive correlation between gill *igf1* mRNA and gill NKA activity. All of the data indicate a role of local (gill) IGF-I in regulating NKA activity. This is in good agreement with the results from seawater transfer experiments [38,57]. In addition, Nilssen et al. [33] found that a landlocked Atlantic salmon having depressed development of seawater adaptability showed no increase in gill *igf1* mRNA during smoltification, which further supports the importance of local IGF-I.

There was no relation between muscle *igf1* mRNA and gill NKA activity. Moreover, muscle *igf1* mRNA did not correlate with fish size neigther. Instead, its change appeared to be inversely related with water temperature. Water temperature is a factor influencing the GH-IGF-I system [15]. Gabillard et al. [14] examined effects of water temperature on the GH-IGF-I system in rainbow trout and found that muscle *igf1* mRNA was highest in fish in the lowest water

temperature (8ºC) while the reverse was the case for liver *igf1* mRNA. Given that circulating IGF-I shows a good correlation with body size, endocrine IGF-I rather than local IGF-I may be responsible for promoting muscle growth during smoltification of masu salmon.

Desmoltification is an alternative strategy for smolts that have been prevented from going to the ocean to re-adapt to freshwater life [19,55]. Desmoltification abandons many, but not all, changes acquired during smoltification including increased NKA activity in the gills. Endocrine systems involved in smoltification are also inactivated during this period. An environmental cue for desmoltification is increasing water temperature [19,54,55,59]. However, little is known about the consequence or the mechanism of the inactivation. In the present study, a balance between serum IGF-I levels and gill NKA activity in June samples was quite different from that of other months: high IGF-I levels despite of low gill NKA activity in June. We hypothesized that the activity of the IGF-I signal pathway, especially IGF-I receptor, changed during this period and caused the "imbalance". In order to test this hypothesis, we analyzed transcript levels of IGF-I receptor (IGF-IR) by qPCR. There are two subtypes of IGF-IR reported in salmonids [16] and we analyzed both subtypes. As a result, we found that both *igf1ra* and *igf1rb* mRNA in the gill significantly decreased from May to June. Moreover, their mRNA levels in the liver and muscle were also relatively low in fish in June. Assuming that *igf1r* transcript levels are related to protein levels, the down-regulation of the both *igf1r* subtypes should lead to a decrease in the IGF-binding capacity in the gills. A decline of the IGF-I binding would then result in the retention of IGF-I in the circulation. Although what factors regulate *igf1r* mRNA is not known at present, somatostatin or/and endocrine IGF-I may be involved in the receptor down-regulation. Somatostatins (SS) are short polypeptide hormones modulating the GH and IGF-I actions in extrapituitary tissues as well as GH release from the pituitary gland [48]. A series of studies mainly using rainbow trout suggest that SS can regulate sensitivity of target tissues to GH and IGF-I [48]. Very and Sheridan [58] demonstrated that *in vivo* implantation of rainbow trout (*O. mykiss*) with SS-14 reduced *igf1r* mRNA as well as IGF-binding capacity in the gills. High levels of IGF-I are also capable of down-regulating IGF-binding capacity in isolated trout cardiomyocytes [32]. More work needs to be done to further unravel the mechanism of down-regulation of gill IGF-IR and NKA activity during desmoltification.

In conclusion, the present study established profiles of circulating IGF-I and *igf* mRNA levels in the liver, gill and white muscle during smoltification in masu salmon. Correlation analyses suggest the increase in the gill NKA activity in the course of smoltification of hatchery-reared masu salmon was supported by both endocrine and local IGF-I. It is also

suggested that the decrease in the gill NKA activity during desmoltification in freshwater was due at least in part to the down-regulation of IGF-IR.

**Acknowledgments**

We thank Brian R. Beckman and Larissa Felli, Northwest Fisheries Science Center, NOAA Fisheries, Seattle, WA, for their help in measuring serum IGF-I. We also thank Etsuro Yamaha and Shizuo Kimura, Nanae Freshwater Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, for providing masu salmon used for cDNA cloning. This work was supported by Grant-in-Aids for Scientific Research from Japan Society for the Promotion of Science (#21580214), and grants from the Akiyama Life Science Foundation and Northern Advancement Center for Science & Technology (Noastec).

**References**

[1] Ø. Aas-Hansen, H.K. Johnsen, M.M. Vijayan, E.H. Jørgensen, Development of seawater tolerance and concurrent hormonal changes in fed and fasted Arctic charr at two temperature regimes, Aquaculture 222 (2003) 135-148.

[2] T. Ágústsson, K. Sundell, T. Sakamoto, V. Johansson, M. Ando, B.Th. Björnsson, Growth hormone endocrinology of Atlantic salmon (*Salmo salar*): pituitary gene expression, hormone storage, secretion and plasma levels during parr-smolt transformation, J. Endocrinol. 170 (2001) 227-234.

[3] B.R. Beckman, W.W. Dickhoff, W.S. Zaugg, C. Sharpe, S. Hirtzel, R. Schrock, D.A. Larsen, R.D. Ewing, A. Palmisano, C.B. Schreck, C.V.W. Mahnken, Growth, smoltification, and smolt-to-adult return of spring chinook salmon from hatcheries on the Deschutes River, Oregon, Trans. Am. Fish. Soc. 128 (1999) 1125-1150.

[4] B.R. Beckman, D.A. Larsen, S. Moriyama, B. Lee-Pawlak, W.W. Dickhoff, Insulin-like growth factor-I and environmental modulation of growth during smoltification of spring chinook salmon (*Oncorhynchus tshawystscha*), Gen. Comp. Endocrinol. 109 (1998) 325-335.

[5] B.R. Beckman, K.D. Shearer, K.A. Cooper, W.W. Dickhoff, Relationship of insulin-like growth factor-I and insulin to size and adiposity of under-yearling chinook salmon, Comp. Biochem. Physiol. 129A (2001) 585-593.

[6] B.R. Beckman, M. Shimizu, B. Gadberry, K. Cooper, Response of the somatotropic axis of juvenile coho salmon to alterations in plane of nutrition with an analysis of the relationships

among growth rate and circulating IGF-I and 41 kDa IGFBP, Gen. Comp. Endocrinol. 135 (2004) 334-344.

[7] B.T. Björnsson, T. Ogasawara, T. Hirano, J.P. Bolton, H.A. Bern, Elevated growth hormone levels in stunted Atlantic salmon, *Salmo salar*. Aquaculture 73 (1988) 275-281.

[8] B.T. Björnsson, S.O. Stefansson, S.D. McCormick, Environmental endocrinology of salmon smoltification. Gen. Comp. Endocrinol. 170 (2011) 290-298.

[9] N.I. Bower, X. Li, R. Taylor, I.A. Johnston, Switching to fast growth: the insulin-like growth factor (IGF) system in skeletal muscle of Atlantic salmon, J. Exp. Biol. 211 (2008) 3859-3870.

[10] W.H. Daughaday, R. Rotwein, Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. Endocr. Rev. 10 (1989) 68-91.

[11] W.W. Dickhoff, B.R. Beckman, D.A. Larsen, C. Duan, S. Moriyama, The role of growth in endocrine regulation of salmon smoltification, Fish Physiol. Biochem. 17 (1997) 231-236.

[12] W.W. Dickhoff, B.R. Beckman, D.A. Larsen, C.V.W. Mahnken, Quality assessment of hatchery-reared spring chinook salmon smolts in the Columbia River basin, Am. Fish. Soc. Sympo. 15 (1995) 292-302.

[13] S.J. Duguay, P. Swanson, W.W. Dickhoff, Differential expression and hormonal regulation of alternatively spliced IGF-I mRNA transcripts in salmon, J. Mol. Endocrinol. 12 (1994) 25-37.

[14] J.C. Gabillard, C. Weil, P.Y. Rescan, I. Navarro, J. Gutiérrez, P.Y. Le Bail, Effects of environmental temperature on IGF1, IGF2, and IGF type I receptor expression in rainbow trout (*Oncorhynchus mykiss*), Gen. Comp. Endocrinol. 133 (2003) 233-242.

[15] J.C. Gabillard, C. Weil, P.Y. Rescan, I. Navarro, J. Gutiérrez, P.Y. Le Bail, Does the GH/IGF system mediate the effect of water temperature on fish growth? A review, Cybium 29 (2005) 107-117.

[16] M.W. Greene, T.T. Chen, Characterization of teleost insulin receptor family members. II. Developmental expression of insulin-like growth factor type I receptor messenger RNAs in rainbow trout, Gen. Comp. Endocrinol. 115 (1999) 270-281.

[17] S. Hirose, T. Kaneko, N. Naito, Y. Takei, Molecular biology of major components of chloride cells, Comp. Biochem. Physiol. 136B (2003) 593-620.

[18] W.S. Hoar, Smolt transformation: evolution, behavior, and physiology. J. Fish. Res. Board Can. 33 (1976) 1234-1252.

[19] W.S. Hoar, The physiology of smolting salmonids, in:W.S. Hoar, D. Randall (Eds.), Fish Physiology, vol 11B, Academic Press, Orland, FL, 1988, pp. 275-343.

[20] E.H. Jørgensen, Ø. Aas-Hansen, S. Moriyama, M. Iwata, J.E.T. Strand, The parr-smolt transformation of Arctic charr is comparable to that of Altantic salmon, Aquaculture 273 (2007) 227-234.

[21] D.A. Larsen, B.R. Beckman, W.W. Dickhoff, The effect of low temperature and fasting during the winter on metabolic stores and endocrine physiology (insulin, insulin-like growth factor-I, and thyroxine) of coho salmon, *Oncorhynchus kisutch*, Gen. Comp. Endocrinol. 123 (2001) 308-323.

[22] D. Le Roith, C. Bondy, S. Yakar, J.-L. Liu, A. Butler, The somatomedin hypothesis: 2001. Endocr. Rev. 22 (2001) 53-74.

[23] S.S. Madsen, The role of cortisol and growth hormone in seawater adaptation and development of hypoosmoregulatory mechanisms in sea trout parr (*Salmo trutta trutta*), Gen Comp Endcrinol 79 (1990) 1-11.

[24] S.S. Madsen, H.A. Bern, In vitro effects of insulin-like growth factor-I on gill Na+, K+-ATPase in coho salmon, *Oncorhynchus kisutch*, J. Endocrinol. 138 (1993) 23-30.

[25] S.S. Madsen, M.K. Jensen, J. Nøhr, K. Kristiansen, Expression of Na+-K+-ATPase in the brown trout, *Salmo trutta*: in vivo modulation by hormones and seawater. A. J. Physiol. Regul. Integr. Comp. Physiol. 269 (1995) R1339-1345.

[26] J.M. Mancera, S.D. McCormick, Role of prolactin, growth hormone, insulin-like growth factor I and cortisol in teleost osmoregulation, in: Baldisserotto, B., Mancera, J.M., Kapoor, B.G. (Eds.), Fish Osmoregulation. Science Publishers, Enfield, NH, 2007, pp. 497-515.

[27] S.D. McCormick, Endocrine control of osmoregulation in teleost fish, Amer. Zool. 41 (2001) 781-794.

[28] S.D. McCormick, M.F. O'Dea, A.M. Moeckel, B.T. Björnsson, Endocrine and physiological changes in Atlantic salmon smolts following hatchery release, Aquaculture 222 (2003) 45-57.

[29] S.D. McCormick, T. Sakamoto, S. Hasegawa, T. Hirano, Osmoregulatory actions of insulin-like growth factor-I in rainbow trout (*Oncorhynchus mykiss*), J. Endocrinol. 130 (1991) 87-92.

[30] S.D. McCormick, J.M. Shrimpton, S. Moriyama, B.T. Björnsson, Effects of an advanced temperature cycle on smolt development and endocrinology indicate that temperature is not a zeitgeber for smolting in Atlantic salmon, J. Exp. Biol. 205 (2002) 3553-3560.

[31] S. Mizuno, K. Ura, Y. Onodera, H. Fukada, N. Misaka, A. Hara, S. Adachi, K. Yamauchi, Changes in transcript levels of gill cortisol receptor during smoltification in wild masu salmon, *Oncorhynchus masou*, Zool. Sci. 18 (2001) 853-860.

[32] T.W. Moon, C. Castejon, N. Banos, M.A. Maestro, E.M. Plisetskaya, J. Gutiérrez, I. Navarro, Insulin and IGF-I binding in isolated trout cardiomyocytes, Gen. Comp. Endocrinol. 103 (1996) 264-272.

[33] T.O. Nilsen, L.O. Ebbesson, P. Kiilerich, B.T. Björnsson, S.S. Madsen, S.D. McCormick, S.O. Stefansson, Endocrine systems in juvenile anadromous and landlocked Atlantic salmon (*Salmo salar*): seasonal development and seawater acclimation, Gen. Comp. Endocrinol. 155 (2008) 762-772.

[34] C. Ohlsson, S. Mohan, K. Sjögren, Å. Tivesten, J. Isgaard, O. Isaksson, J.-O. Jansson, J. Svensson, The role of liver-derived insulin-like growth factor-I. Endocr. Rev. 30 (2009) 494-535.

[35] M.E. Picha, J.T. Silverstein, R.J. Borski, Discordant regulation of hepatic IGF-I mRNA and circulating IGF-I during compensatory growth in a teleost, the hybrid striped bass (*Morone chrysops* x *Morone saxatilis*), Gen Comp Endocrinol 147 (2006) 196-205.

[36] A.L. Pierce, M. Shimizu, B.R. Beckman, D.M. Baker, W.W. Dickhoff, Time course of the GH/IGF axis response to fasting and increased ration in chinook salmon (*Oncorhynchus tshawytscha*), Gen. Comp. Endocrinol. 140 (2005) 192-202.

[37] M. Pisam, A. Rambourg, Mitochondria-rich cells in the gill epithelium of teleost fishes: an ultrastructural approach. Int. Rev. Cytol. 130 (1991) 191-232.

[38] J. Poppinga, J. Kittilson, S.D. McCormick, M.A. Sheridan, Effects of somatostatin on the growth hormone-insulin-like growth factor axis and seawater adaptation of rainbow trout (*Oncorhynchus mykiss*), Aquaculture 273 (2007) 312-319.

[39] J. Pérez-Sánchez, H. Martí-Palanca, S.J. Kaushik, Ration size and protein intake affect circulating growth hormone concentration, hepatic growth hormone binding and plasma insulin-like growth factor-I immunoreactivity in a marine teleost, the gilthead sea bream (Sparus aurata). J. Nutr. 125 (1995) 546-552.

[40] P. Prunet, M. Pisam, J.P. Claireaux, G. Boeuf, A. Rambourg, Effects of growth hormone on gill chloride cells in juvenile Atlantic salmon (*Salmo salar*), Am. J. Physiol. Regul. Integr. Comp. Physiol. 266 (1994) R850-R857.

[41] E.S. Quabius, P.H.M. Balm, S.E.W. Bonga, Interrenal stress responsiveness of tilapia (*Oreochromis mossambicus*) is impaired by dietary exposure to PCB 126, Gen. Comp.

435 Endocrinol. 108 (1997) 472-482.
436 [42] M. Reinecke, Influences of the environment on the endocrine and paracrine fish growth
437 hormone-insulin-like growth factor-I system, J Fish Biol 76 (2010) 1233-1254.
438 [43] N.H. Richman, W.S. Zaugg, Effects of cortisol and growth hormone on osmoregulation in
439 pre- and desmoltified coho salmon (*Oncorhynchus kisutch*), Gen. Comp. Endocrinol. 65
440 (1987) 189-198.
441 [44] T. Sakamoto, T. Hirano, Expression of insulin-like growth factor I gene in osmoregulatory
442 organs during seawater adaptation of the salmonid fish: possible mode of osmoregulatory
443 action of growth hormone, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 1912-1916.
444 [45] T. Sakamoto, T. Hirano, S.S. Madsen, R.S. Nishioka, H.A. Bern, Insulin-like growth factor I
445 gene expression during parr-smolt transformation of coho salmon, Zool. Sci. 12 (1995)
446 249-252.
447 [46] T. Sakamoto, S.D. McCormick, Prolactin and growth hormone in fish osmoregulation, Gen.
448 Comp. Endocrinol. 147 (2006) 24-30.
449 [47] M. Seidelin, S.S. Madsen, A. Byrialsen, K. Kristiansen, Effects of insulin-like growth
450 factor-I and cortisol on Na+,K+-ATPase expression in osmoregulatory tissues of brown trout
451 (*Salmo trutta*), Gen. Comp. Endocrinol. 113 (1999) 331-342.
452 [48] M.A. Sheridan, A.L. Hagemeister, Somatostatin and somatostatin receptors in fish growth,
453 Gen. Comp. Endocrinol. 167 (2010) 360-365.
454 [49] M. Shimizu, B.R. Beckman, A. Hara, W.W. Dickhoff, Measurement of circulating salmon
455 IGF binding protein-1: assay development, response to feeding ration and temperature, and
456 relation to growth parameters, J. Endocrinol. 188 (2006) 101-110.
457 [50] M. Shimizu, K.A. Cooper, W.W. Dickhoff, B.R. Beckman, Postprandial changes in plasma
458 growth hormone, insulin, insulin-like growth factor (IGF)-I, and IGF-binding proteins in
459 coho salmon fasted for varying periods, Am. J. Physiol. Regul. Integr. Comp. Physiol. 297
460 (2009) R352-361.
461 [51] M. Shimizu, K. Kishimoto, T. Yamaguchi, Y. Nakano, A. Hara, W.W. Dickhoff, Circulating
462 salmon 28- and 22-kDa insulin-like growth factor binding proteins (IGFBPs) are
463 co-orthologs of IGFBP-1, Gen. Comp. Endocrinol. 174 (2011) 97-106.
464 [52] M. Shimizu, P. Swanson, H. Fukada, A. Hara, W.W. Dickhoff, Comparison of extraction
465 methods and assay validation for salmon insulin-like growth factor-I using commercially
466 available components, Gen. Comp. Endocrinol. 119 (2000) 26-36.
467 [53] B.C. Small, B.C. Peterson, Establishment of a time-resolved fluoroimmunoassay for

measuring plasma insulin-like growth factor I (IGF-I) in fish: effect of fasting on plasma concentrations and tissue mRNA expression of IGF-I and growth hormone (GH) in channel catfish (*Ictalurus punctatus*), Domest. Anim. Endocrinol. 28 (2005) 202-215.

[54] S.O. Stefansson, Å.I. Berge, G.S. Gunnarsson, Changes in seawater tolerance and gill Na+,K+-ATPase activity during desmoltification in Atlantic salmon kept in freshwater at different temperatures, Aquaculture 168 (1998) 271-277.

[55] S.O. Stefansson, B.T. Björnsson, L.O.E. Ebbesson, S.D. McCormick, Smoltification, in: Finn, R.N., Kapoor, B.G. (Eds.), Fish Larval Physiology. Science Publishers, Enfield, NH, 2008, pp. 639-681.

[56] S.O. Stefansson, T.O. Nilsen, L.O.E. Ebbesson, A. Wargelius, S.S. Madsen, B.Th. Bjornsson, S.D. McCormick, Molecular mechanisms of continuous light inhibition of Atlantic salmon parr-smolt transformation, Aquaculture 273 (2007) 235-245.

[57] C.K. Tipsmark, J.A. Luckenbach, S.S. Madsen, R.J. Borski, IGF-I and branchial IGF receptor expression and localization during salinity acclimation in striped bass, Am J Physiol Regul Integr Comp Physiol 292 (2007) R535-543.

[58] N.M. Very, M.A. Sheridan, Somatostatin inhibits insulin-like growth factor-I receptor expression in the gill of a teleost fish (*Oncorhynchus mykiss*), FEBS Lett. 581 (2007) 4773-4777.

[59] G.A. Wedemeyer, R.L. Saunders, W.C. Clarke, Environmental factors affecting smoltification and early marine survival of anadormous salmonids, Mar. Fish. Rev. 42 (1980) 1-14.

[60] J. Zapf, C. Hauri, M. Waldvogel, E.R. Froesch, Acute metabolic effects and half-lives of intravenously administrated insulinlike growth factors I and II in normal and hypophysectomized rats, J. Clin. Invest. 77 (1986) 1768-1775.

493 **Figure legends**

494 Fig. 1 Changes in water temperature (a), fork length (b), body weight (c) and condition factor (d)
495 during smoltification of masu salmon. Values are expressed as means ± SE (*n* = 6-7) except water
496 temperature. Symbols sharing the same letters are not significantly different from each other.
497
498 Fig. 2 Changes in gill $Na^+$,$K^+$-ATPase activity during smolfication of masu salmon. Values are
499 expressed as means ± SE (*n* = 6-7). Symbols sharing the same letters are not significantly
500 different from each other.
501
502 Fig. 3 Changes in *igf1* mRNA levels in liver (a), gill (b) and white muscle (c), and serum IGF-I
503 levels (d) during smoltification of masu salmon. Values are expressed as means ± SE (*n* = 6-7).
504 Symbols sharing the same letters are not significantly different from each other.
505
506 Fi.g 4 Relationships between serum IGF-I and $Na^+$,$K^+$-ATPase (a), and gill *igf1* and
507 $Na^+$,$K^+$-ATPase activity (b). As serum IGF-I and gill $Na^+$,$K^+$-ATPase activity were correlated
508 with fork length, both parameters were standardized (std) to body length to eliminate size
509 influence.
510
511 Fig. 5 Changes in *igf1ra* (a,c,e) and *igf1rb* (b,c,f) mRNA levels in the liver (a,b), gill (c,d) and
512 white muscle (e,f) during smoltification of masu salmon. Values are expressed as means ± SE (*n* =
513 6-7). Symbols sharing the same letters are not significantly different from each other.
514

## Tables

Table 1 Primer sequences used for real-time PCR (qPCR) analysis

| Gene | Forward primer 5'-3' | Reverse primer 5'-3' | Product size (bp) |
|---|---|---|---|
| IGF-I | TCTCCAAAACGAGCCTGCG | CACAGCACATCGCACTCTTGA | 207 bp |
| EF-1α | GAATCGGCCATGCCCGGTGAC | GGATGATGACCTGAGCGGTG | 142 bp |
| IGF-IRa | AAGAGAACACATCCAGCCAGGT | TTGTTGGCGTTGAGGTATGC | 97 bp |
| IGF-IRb | CTGCCAGCATGAGAGAGAGAATA | TAGGACTGGGACGGATCTTTAG | 196 bp |

## Tables

Table 2 Correlation coefficient ($r^2$) among fish size, IGF-I, NKA and IGF-IR during March and May before standardization with size

| | FL | BW | K | Liver *igf1* | Gill *igf1* | Muscle *igf1* | Serum IGF-I | NKA | Liver *igflra* | Liver *igflrb* | Gill *igf1ra* | Gill *igf1rb* | Muscle *igflra* | Muscle *igflrb* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FL | — | 0.95 | *0.47* | ns | ns | *0.27* | 0.58 | 0.51 | *0.23* | *0.27* | ns | ns | ns | ns |
| BW | 0.95 | — | *0.27* | ns | ns | *0.31* | 0.51 | 0.48 | *0.25* | *0.34* | ns | ns | ns | ns |
| K | *0.47* | *0.27* | — | ns | ns | ns | *0.31* | *0.24* | ns | ns | ns | ns | ns | ns |
| Liver *igf1* | ns | ns | ns | — | ns | ns | ns | *0.27* | 0.28 | ns | ns | *0.23* | ns | ns |
| Gill *igf1* | ns | ns | ns | ns | — | ns | ns | ns | ns | ns | 0.48 | ns | ns | ns |
| Muscle *igf1* | *0.27* | *0.31* | ns | ns | ns | — | ns | ns | ns | ns | ns | ns | ns | ns |
| Serum IGF-I | 0.58 | 0.51 | *0.31* | ns | ns | ns | — | 0.74 | *0.26* | ns | ns | ns | ns | *0.25* |
| NKA | 0.51 | 0.48 | *0.24* | *0.27* | ns | ns | 0.74 | — | *0.30* | ns | 0.31 | ns | ns | *0.24* |
| Liver *igflra* | *0.23* | *0.25* | ns | 0.28 | ns | ns | *0.26* | *0.30* | — | 0.56 | ns | ns | ns | ns |
| Liverr *igflrb* | *0.27* | *0.34* | ns | ns | ns | ns | ns | ns | 0.56 | — | ns | ns | ns | ns |
| Gill *igf1ra* | ns | ns | ns | ns | 0.48 | ns | ns | 0.31 | ns | ns | — | ns | ns | ns |
| Gill *igf1rb* | ns | ns | ns | *0.23* | ns | ns | ns | ns | ns | ns | ns | — | ns | ns |
| Muscle *igf1ra* | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | — | ns |
| Muscle *igf1rb* | ns | ns | ns | ns | ns | ns | *0.25* | *0.24* | ns | ns | ns | ns | ns | — |

Numbers in italic are negative correlations. ns: not significant.

## Tables

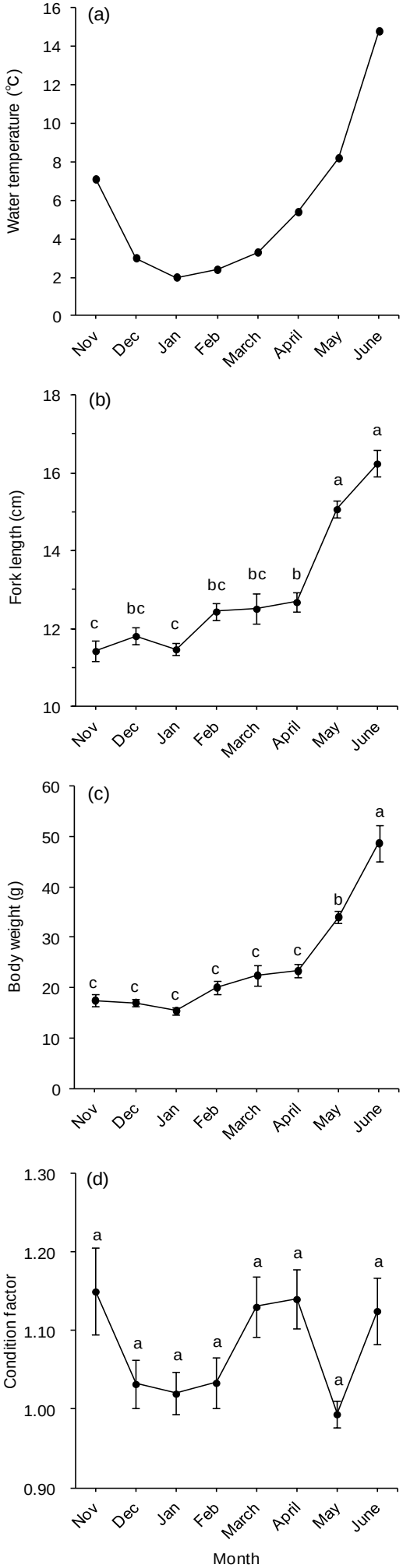
Table 3 Correlation coefficient ($r^2$) among fish size, IGF-I, NKA and IGF-IR during March and May after standardization with size

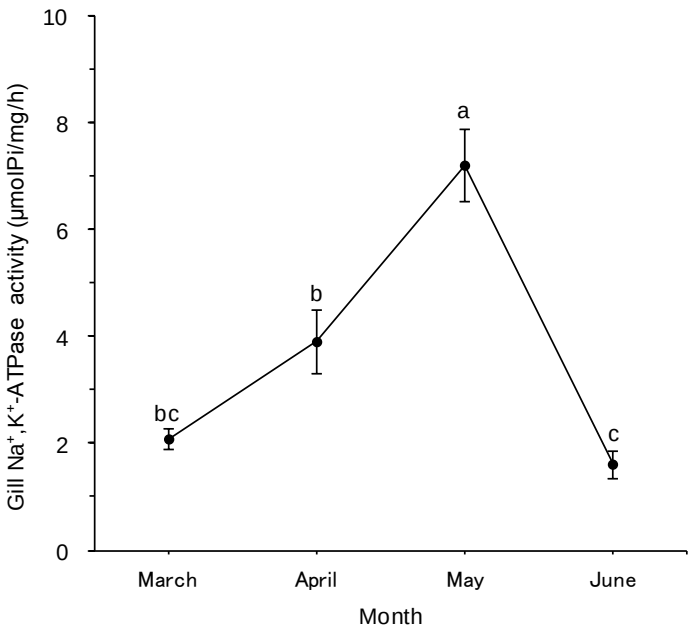
| | FL | **BW** | **K** | Liver *igf1* | Gill *igf1* | **Muscle *igf1*** | **Serum IGF-I** | **NKA** | **Liver *igflra*** | **Liver *igflrb*** | Gill *igf1ra* | Gill *igf1rb* | Muscle *igflra* | Muscle *igflrb* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FL | — | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| **BW** | ns | — | 0.93 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| **K** | ns | 0.93 | — | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Liver *igf1* | ns | ns | ns | — | ns | ns | ns | ns | ns | ns | ns | 0.23 | ns | ns |
| Gill *igf1* | ns | ns | ns | ns | — | ns | ns | 0.25 | ns | ns | 0.48 | ns | ns | ns |
| **Muscle *igf1*** | ns | ns | ns | ns | ns | — | ns | ns | ns | ns | ns | ns | ns | ns |
| **Serum IGF-I** | ns | ns | ns | ns | ns | ns | — | 0.49 | ns | ns | 0.24 | ns | ns | ns |
| **NKA** | ns | ns | ns | ns | 0.25 | ns | 0.49 | — | ns | ns | 0.44 | ns | ns | ns |
| **Liver *igflra*** | ns | ns | ns | ns | ns | ns | ns | ns | — | 0.45 | ns | ns | ns | ns |
| **Liverr *igflrb*** | ns | ns | ns | ns | ns | ns | ns | ns | 0.45 | — | ns | ns | ns | ns |
| Gill *igf1ra* | ns | ns | ns | ns | 0.48 | ns | 0.24 | 0.44 | ns | ns | — | ns | ns | ns |
| Gill *igf1rb* | ns | ns | ns | 0.23 | ns | ns | ns | ns | ns | ns | ns | — | ns | ns |
| Muscle *igf1ra* | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | — | ns |
| Muscle *igf1rb* | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | — |

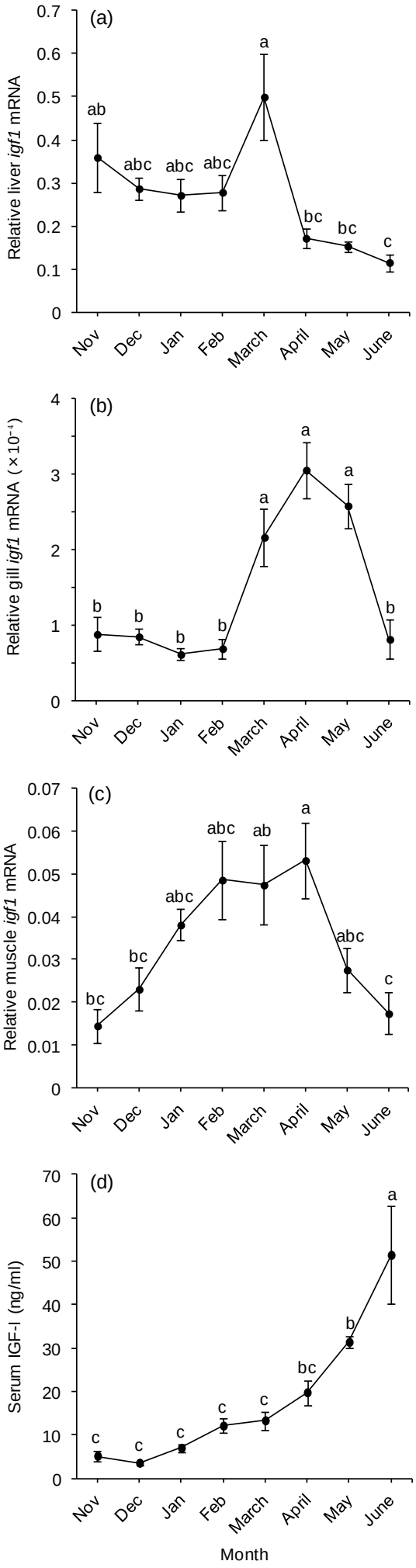
Parameters in bold have been standardized by fork length. ns: not significant.

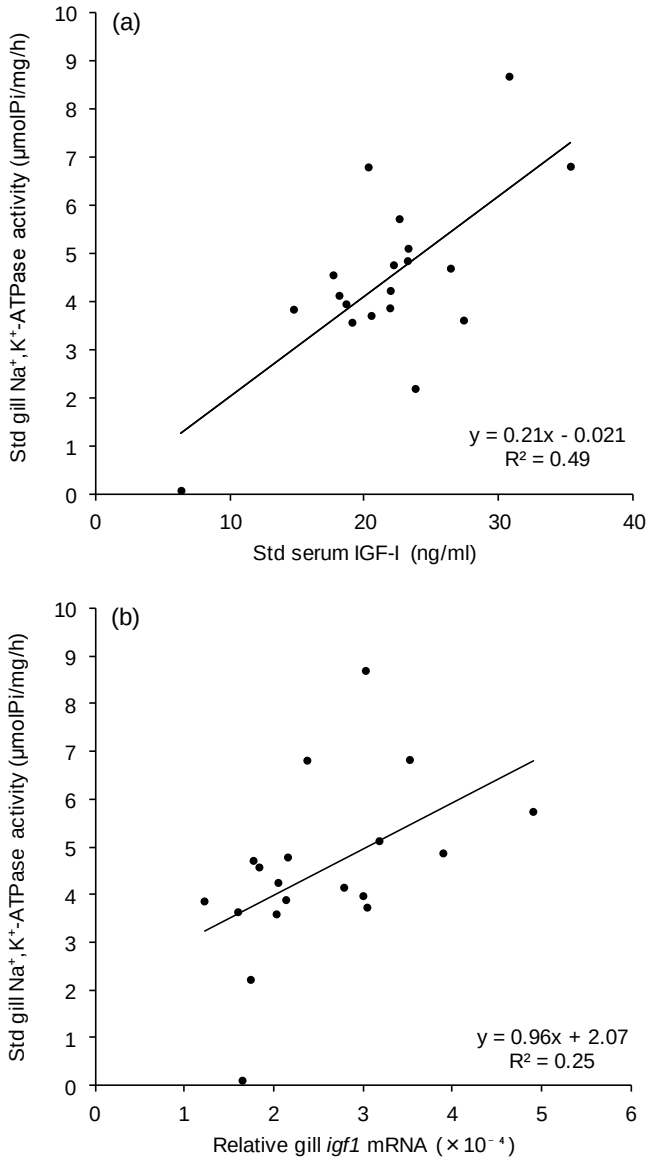
**Figures**

Shimomura et al. Fig. 1



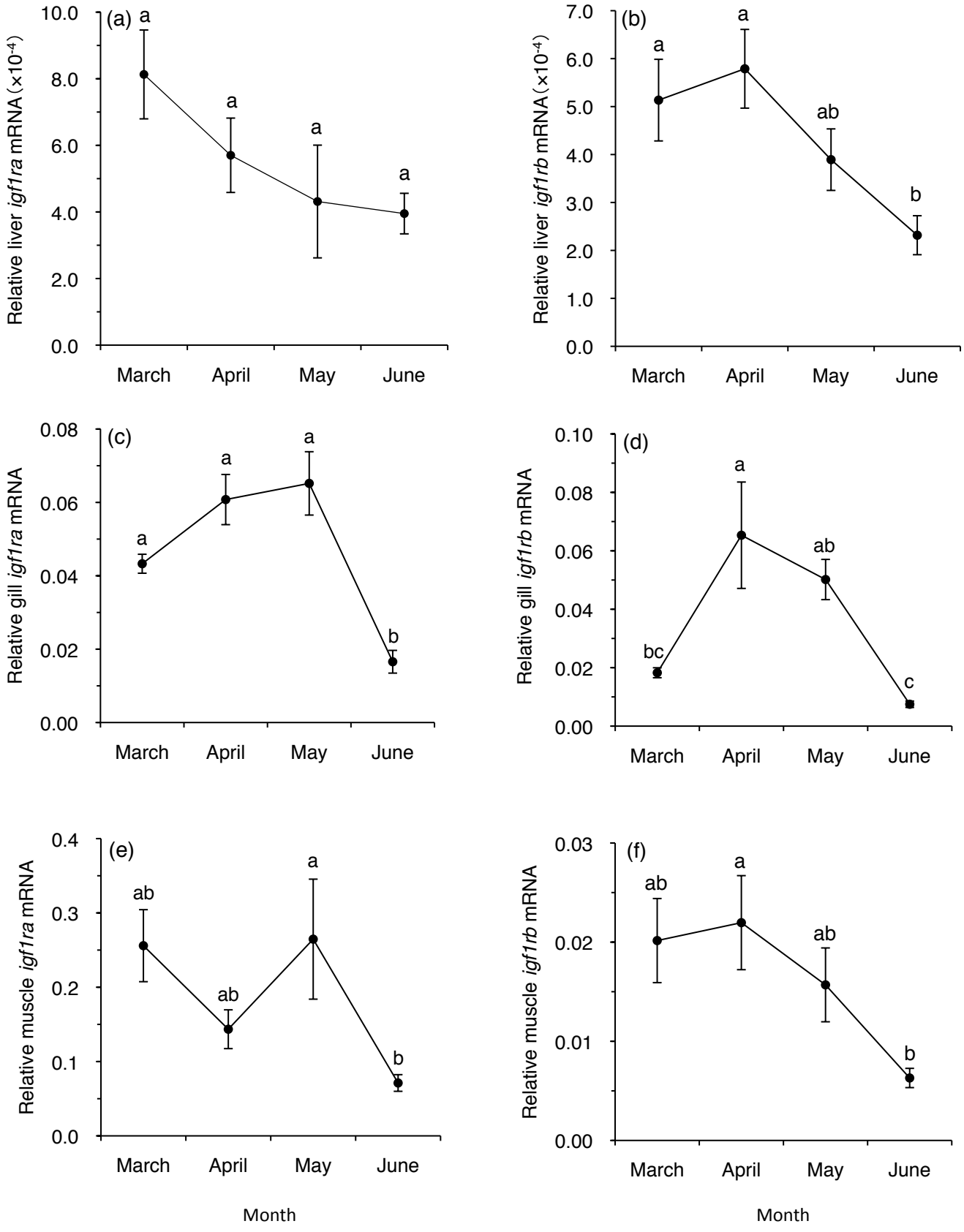

10
8
6
4
2
0
Gill Na+,K+-ATPase activity (μmolPi/mg/h)
a
b
bc
c
March
April
May
June
Month

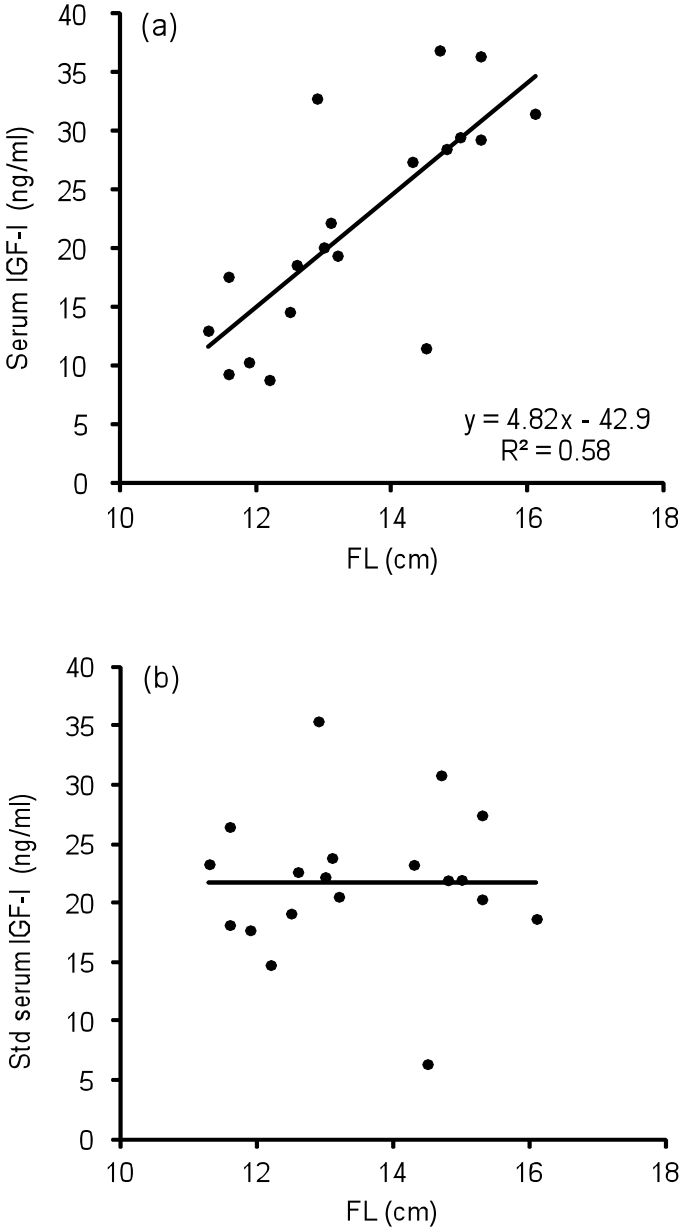

(a)
Relative liver igf1 mRNA
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ab
abc
abc
abc
a
bc
bc
c
Nov
Dec
Jan
Feb
March
April
May
June
(b)
Relative gill igf1 mRNA (×10⁻⁴)
4
3
2
1
0
b
b
b
b
a
a
a
b
(c)
Relative muscle igf1 mRNA
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
bc
bc
abc
abc
ab
a
abc
c
(d)
Serum IGF-I (ng/ml)
70
60
50
40
30
20
10
0
c
c
c
c
c
bc
b
a
Month


(a)
Std gill Na+,K+-ATPase activity (μmolPi/mg/h)
Std serum IGF-I (ng/ml)
y = 0.21x - 0.021
R² = 0.49
(b)
Std gill Na+,K+-ATPase activity (μmolPi/mg/h)
Relative gill igf1 mRNA (×10⁻⁴)
y = 0.96x + 2.07
R² = 0.25

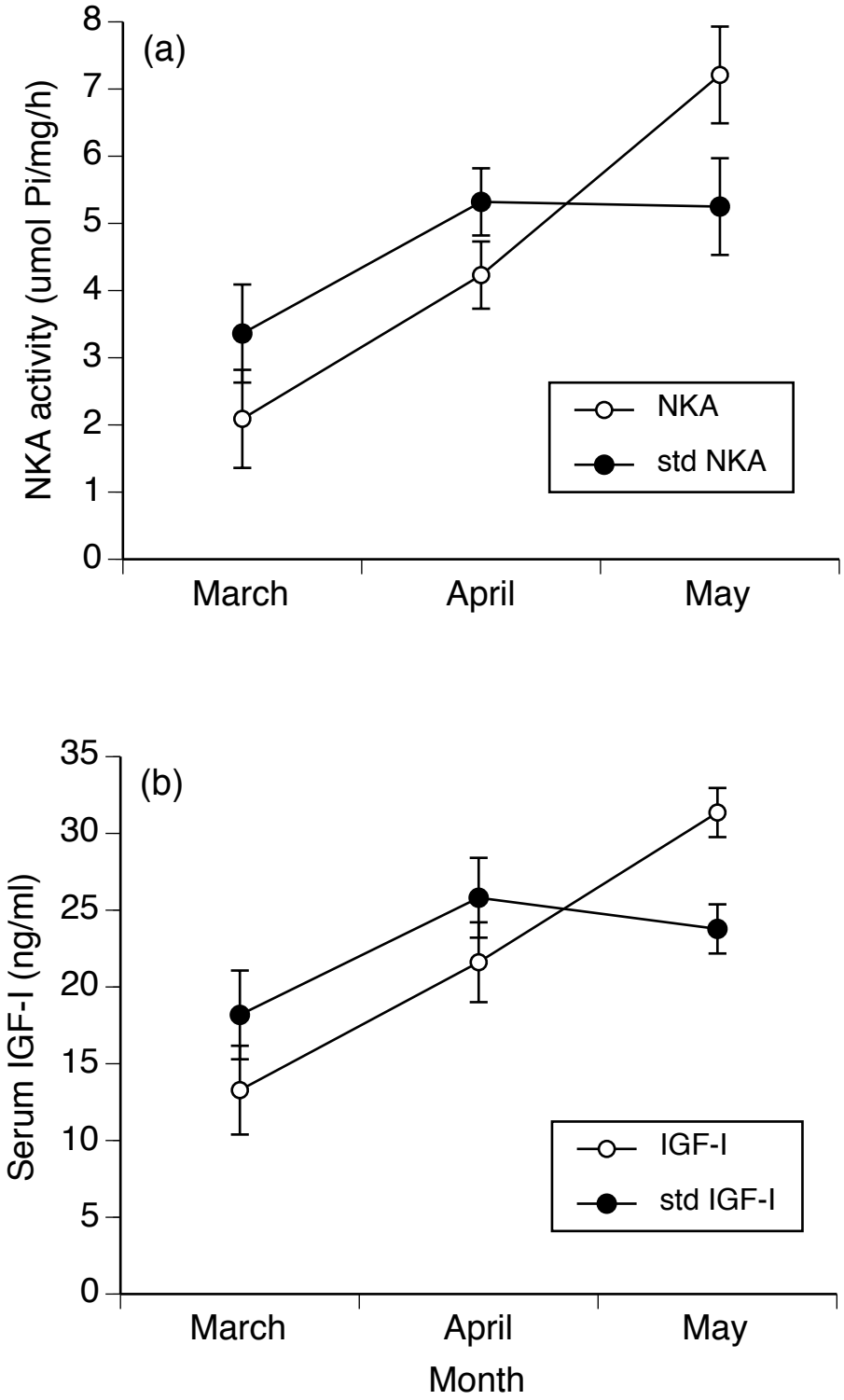
**Figures**

Shimomura et al. Fig. 5






Supplemental Fig. S1 Relationships between fork length and serum IGF-I before (a) and after (b) standardization by fork length. As serum IGF-I was correlated with fork length, serum IGF-I values were standardized (std) to fork length to eliminate size influence.




Supplemental Fig. S2 Profiles of gill Na+,K+-ATPase activity (a) and serum IGF-I (b) before (white circle) and after (black circle) standardization by fork length.