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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*)

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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: $(\text{body weight}) \times 1000 / (\text{fork length})^3$. Blood was withdrawn by a syringe from the caudal vein, allowed to clot overnight at 4°C and centrifuged at 8,050g 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-1 α (EF-1 α)

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].

Primer sets designed for Atlantic salmon (*Salmon salar*) IGF-I and EF-1 α (Genbank ID: EF432852 and BG933853 [9]) (Table 1) were applied to masu salmon. Reverse transcriptase (RT)-PCR was performed with a Veriti Thermal Cycler (Applied Biosystems, Carlsbad, CA) and AmpliTaq Gold® 360 Mater Mix (Applied Biosystems, Foster City, CA). PCR cycles consisted 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 sec; 1 cycle of 72°C for 7 min. PCR products were cloned into the pGEM-T Easy Vector Systems (Promega, Madison, WI) and positive clones were sequenced as described in Shimizu et al. [51].

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from the tissues as described in Shimizu et al. [51]. One and half μ g RNA was reverse-transcribed using SuperScript VILO cDNA Synthesis kit (Invitrogen, Carlsbad, CA) in a 10 μ l reaction according to the manufacturer's instruction. cDNA was stored at -30°C until use.

2.4. Real-time quantitative PCR (qPCR)

Primer sets for qPCR of IGF-I and EF-1 α were designed based on the cloned masu salmon cDNA sequences using MacVector Ver 9 (MacVector Inc., Cary, NC). One primer in each assay was placed across an exon/exon boundary predicted from the gene structure of zebrafish from Ensembl data base (<http://asia.ensembl.org/index.html>). The primers for IGF-I target the signal peptide region.

RT-PCRs using these primers were performed to prepare assay standards. PCR products run on 1.5% agarose gel were excised and purified using QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA). Copy numbers of the purified amplicon were calculated from the molecular weight of the amplicon and concentration. The standard cDNA were serially diluted from 1×10^7 to 3×10^2 copies.

qPCR was set up using Power SYBR Green PCR Master Mix (Applied Biosystems) in a reaction volume of 20 μ l with primer concentration of 100 nM. The reaction mixture contained 0.1-2 μ l cDNA template. qPCR was run on a 7300 Sequence Detector (Applied Biosystems) using the manufacturer's recommended cycling conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles at 95°C for 15 se and 60°C for 1 min. Measured values were normalized by those of *ef1a* and expressed as relative values.

Primers specific to type I IGF receptor subtypes (IGF-IRa and IGF-IRb) were 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 *ef1a*.

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

Prior to the assay, serum IGF-I was extracted with an acid-ethanol as described in Shimizu et al. [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 modifications. 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 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 = $4.82 \times \text{fork length} - 42.9$, $r^2 = 0.58$) 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: $\text{standardized hormone value}_i = \text{hormone value}_i - [(\text{length}_i - \text{length mean}) \times \text{slope}]$, where hormone value_i is the individual hormone level of a given fish, length_i 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 ($\text{NKA} = 1.25 \times \text{fork length} - 12.3$, $r^2 = 0.51$) 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 concert these two biological processes. In addition, IGF-I may enhance seawater adaptability indirectly through 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 administered 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 smoltification [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 stimulation 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 neither. 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 (8°C) 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.

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Figure legends

Fig. 1 Changes in water temperature (a), fork length (b), body weight (c) and condition factor (d) during smoltification of masu salmon. Values are expressed as means $\pm$ SE ($n = 6-7$) except water temperature. Symbols sharing the same letters are not significantly different from each other.

Fig. 2 Changes in gill Na^+, K^+ -ATPase activity during smoltification of masu salmon. Values are expressed as means $\pm$ SE ($n = 6-7$). Symbols sharing the same letters are not significantly different from each other.

Fig. 3 Changes in *igf1* mRNA levels in liver (a), gill (b) and white muscle (c), and serum IGF-I levels (d) during smoltification of masu salmon. Values are expressed as means $\pm$ SE ($n = 6-7$). Symbols sharing the same letters are not significantly different from each other.

Fig. 4 Relationships between serum IGF-I and Na^+, K^+ -ATPase (a), and gill *igf1* and Na^+, K^+ -ATPase activity (b). As serum IGF-I and gill Na^+, K^+ -ATPase activity were correlated with fork length, both parameters were standardized (std) to body length to eliminate size influence.

Fig. 5 Changes in *igf1ra* (a,c,e) and *igf1rb* (b,c,f) mRNA levels in the liver (a,b), gill (c,d) and white muscle (e,f) during smoltification of masu salmon. Values are expressed as means $\pm$ SE ($n = 6-7$). Symbols sharing the same letters are not significantly different from each other.

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-1 α	GAATCGGCCATGCCCCGGTGAC	GGATGATGACCTGAGCGGTG	142 bp
IGF-IRa	AAGAGAACACATCCAGCCAGGT	TTGTTGGCGTTGAGGTATGC	97 bp
IGF-IRb	CTGCCAGCATGAGAGAGAGAATA	TAGGACTGGGACGGATCTTTAG	196 bp

Table 2 Correlation coefficient (r²) among fish size, IGF-I, NKA and IGF-IR during March and May before standardization with size

	FL	BW	K	Liver <i>igfI</i>	Gill <i>igfI</i>	Muscle <i>igfI</i>	Serum IGF-I	NKA	Liver <i>igflra</i>	Liver <i>igflrb</i>	Gill <i>igflra</i>	Gill <i>igflrb</i>	Muscle <i>igflra</i>	Muscle <i>igflrb</i>
FL	—	0.95	<i>0.47</i>	ns	ns	<i>0.27</i>	0.58	0.51	<i>0.23</i>	<i>0.27</i>	ns	ns	ns	ns
BW	0.95	—	<i>0.27</i>	ns	ns	<i>0.31</i>	0.51	0.48	<i>0.25</i>	<i>0.34</i>	ns	ns	ns	ns
K	<i>0.47</i>	<i>0.27</i>	—	ns	ns	ns	<i>0.31</i>	<i>0.24</i>	ns	ns	ns	ns	ns	ns
Liver <i>igfI</i>	ns	ns	ns	—	ns	ns	ns	<i>0.27</i>	0.28	ns	ns	<i>0.23</i>	ns	ns
Gill <i>igfI</i>	ns	ns	ns	ns	—	ns	ns	ns	ns	ns	0.48	ns	ns	ns
Muscle <i>igfI</i>	<i>0.27</i>	<i>0.31</i>	ns	ns	ns	—	ns	ns	ns	ns	ns	ns	ns	ns
Serum IGF-I	0.58	0.51	<i>0.31</i>	ns	ns	ns	—	0.74	<i>0.26</i>	ns	ns	ns	ns	<i>0.25</i>
NKA	0.51	0.48	<i>0.24</i>	<i>0.27</i>	ns	ns	0.74	—	<i>0.30</i>	ns	0.31	ns	ns	<i>0.24</i>
Liver <i>igflra</i>	<i>0.23</i>	<i>0.25</i>	ns	0.28	ns	ns	<i>0.26</i>	<i>0.30</i>	—	0.56	ns	ns	ns	ns
Liver <i>igflrb</i>	<i>0.27</i>	<i>0.34</i>	ns	ns	ns	ns	ns	ns	0.56	—	ns	ns	ns	ns
Gill <i>igflra</i>	ns	ns	ns	ns	0.48	ns	ns	0.31	ns	ns	—	ns	ns	ns
Gill <i>igflrb</i>	ns	ns	ns	<i>0.23</i>	ns	ns	ns	ns	ns	ns	ns	—	ns	ns
Muscle <i>igflra</i>	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	—	ns
Muscle <i>igflrb</i>	ns	ns	ns	ns	ns	ns	<i>0.25</i>	<i>0.24</i>	ns	ns	ns	ns	ns	—

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

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 <i>igf1</i>	Gill <i>igf1</i>	Muscle <i>igf1</i>	Serum IGF-I	NKA	Liver <i>igflra</i>	Liver <i>igflrb</i>	Gill <i>igflra</i>	Gill <i>igflrb</i>	Muscle <i>igflra</i>	Muscle <i>igflrb</i>
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 <i>igf1</i>	ns	ns	ns	—	ns	ns	ns	ns	ns	ns	ns	0.23	ns	ns
Gill <i>igf1</i>	ns	ns	ns	ns	—	ns	ns	0.25	ns	ns	0.48	ns	ns	ns
Muscle <i>igf1</i>	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 <i>igflra</i>	ns	ns	ns	ns	ns	ns	ns	ns	—	0.45	ns	ns	ns	ns
Liver <i>igflrb</i>	ns	ns	ns	ns	ns	ns	ns	ns	0.45	—	ns	ns	ns	ns
Gill <i>igflra</i>	ns	ns	ns	ns	0.48	ns	0.24	0.44	ns	ns	—	ns	ns	ns
Gill <i>igflrb</i>	ns	ns	ns	0.23	ns	ns	ns	ns	ns	ns	ns	—	ns	ns
Muscle <i>igflra</i>	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	—	ns
Muscle <i>igflrb</i>	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.

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.