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Hepatic *insulin-like growth-factor binding protein* (*igfbp*) responses to food restriction in Atlantic salmon smolts

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ABSTRACT

The growth hormone (Gh)/insulin-like growth-factor (Igf) system plays a central role in the regulation of growth in fishes. However, the roles of Igf binding proteins (Igfbps) in coordinating responses to food availability are unresolved, especially in anadromous fishes preparing for seaward migration. We assayed plasma Gh, Igf1, thyroid hormones and cortisol along with *igfbp* mRNA levels in fasted and fed Atlantic salmon (*Salmo salar*). Fish were fasted for 3 or 10 days near the peak of smoltification (late April to early May). Fasting reduced plasma glucose by 3 days and condition factor by 10 days. Plasma Gh, cortisol, and thyroxine (T_4) were not altered in response to fasting, whereas Igf1 and 3,5,3′-triiodo-L-thyronine (T_3) were slightly higher and lower than controls, respectively. Hepatic *igfbp1b1*, *-1b2*, *-2a*, *-2b1* and *-2b2* mRNA levels were not responsive to fasting, but there were marked increases in *igfbp1a1* following 3 and 10 days of fasting. Fasting did not alter hepatic *igf1* or *igf2*; however, muscle *igf1* was diminished by 10 days of fasting. There were no signs that fasting compromised branchial ionoregulatory functions, as indicated by unchanged Na^+/K^+-ATPase activity and ion pump/transporter mRNA levels. We conclude that dynamic hepatic *igfbp1a1* and muscle *igf1* expression participate in the modulation of Gh/Igf signaling in smolts undergoing catabolism.

1. Introduction

Growth performance of teleost fishes, including salmonids, is principally controlled by the actions of the growth hormone (Gh)/insulin-like growth factor (Igf) system (Björnsson, 1997; Duan et al., 2010; Wood et al., 2005). The Gh/Igf system directs the allocation of acquired nutrients toward anabolic processes such as somatic and linear growth. In anadromous salmonids, the Gh/Igf axis also has a role in increasing salinity tolerance that occurs prior to, and during, the downstream migration of smolts (Hoar, 1988; McCormick, 2013). On the other hand, in conditions of prolonged nutrient restriction, the labile nature of Gh/Igf signaling safeguards survival by shifting energy away from anabolic processes to essential physiological processes (Wood et al., 2005). The Gh/Igf system responds to nutrient conditions through a suite of responses that often vary across the organism, permitting adaptive tissue-specific responses to environmental circumstances (Reindl and Sheridan, 2012). By revealing the molecular mechanisms by which Gh/Igf signaling is modulated in both endocrine and paracrine/autocrine fashions, physiologists are positioned to more precisely infer the growth patterns of wild fish populations and optimize rearing strategies for domesticated stocks (Picha et al., 2008; Beckman, 2011).

Gh acts on target cells via transmembrane receptors that initiate JAK/STAT, PI3K and/or MAPK signaling pathways (Reindl and Sheridan, 2012). Endocrine Gh directly stimulates the growth of target tissues by acting as a mitogen (Butler and LeRoith, 2001). Gh indirectly regulates growth through the synthesis and secretion of Igfs (LeRoith et al., 2001). While plasma Igf levels are primarily determined by their rate of secretion from the liver, local production of Igfs may also be important in regulating local and whole animal growth (LeRoith et al., 2001; Wood et al., 2005; Duan et al., 2010). Upon binding receptors, Igfs stimulate growth of tissues such as muscle and bone by controlling cell differentiation, proliferation, migration, and survival (Wood et al., 2005; Castillo et al., 2004; Codina et al., 2008; Capilla et al., 2011). As in mammals, Igf1 has long been considered a somatomedin in teleosts; growing evidence also implicates Igf2 as a key mediator of Gh-regulated growth in fishes (Shamblott et al., 1995; Chen et al.,

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2000; Codina et al., 2008; Pierce et al., 2010, 2011; Azizi et al., in press). Teleosts, like mammals, exhibit a pattern in which plasma Gh levels and hepatic Igf synthesis become uncoupled during times of nutrient restriction, a situation termed "Gh resistance" (Jenkins and Ross, 1996; Björnsson, 1997). This uncoupling seemingly underlies diminished hepatic *igf1* levels when food is limited in the environment (Duan and Plisetskaya, 1993; Chauvigne et al., 2003; Pierce et al., 2005). Comparatively less is known about *igf1* and *igf2* in extrahepatic tissues, and most notably, in muscle, where paracrine/autocrine activities may be modulated by nutritional status (Chauvigne et al., 2003; Bower et al., 2008; Bower and Johnston, 2010).

Insulin-like growth factors interact with an extensive set of binding proteins, termed Igf binding proteins (Igfbps). These proteins affect hormone availability, transport, and receptor binding, and thus modulate the actions of Igfs (Duan et al., 2010). While many of the physiologically relevant actions have been determined for individual Igfbps in mammals (Firth and Baxter, 2002), this is not the case for most Igfbps in fishes. Salmonids express an especially large suite of Igfbps; for instance, Atlantic salmon (*Salmo salar*) express 19 *igfbp* genes (Macqueen et al., 2013). Initial characterizations of these *igfbps* reveal that several isoforms (expressed in muscle) are sensitive to food conditions (Bower et al., 2008; Macqueen et al., 2013), while the regulatory systems controlling the mRNA levels of the full array of *igfbps* across tissues remain unresolved. As metabolic hormones such as Gh, thyroid hormones, and cortisol seemingly modulate *igfbps* in salmonids (Pierce et al., 2006), they represent potential regulators of *igfbps* in Atlantic salmon. An important step toward functionally characterizing the extensive *igfbp* network now identified in Atlantic salmon by Macqueen et al. (2013) is to consider their dynamic responses, along with putative endocrine regulators, to various nutritional conditions.

In addition to mediating growth performance, the Gh/Igf system works in concert with other endocrine systems to the drive parr-smolt transformation, the ontogeny of morphological, physiological, and behavioral phenotypes supporting migration from freshwater to pelagic marine environments (Hoar, 1988; Björnsson, 1997). This is especially true with respect to the acquisition of seawater tolerance via the remodeling of branchial epithelium (Sakamoto et al., 1993). Energized by Na^+/K^+-ATPase, seawater-type ionocytes mediate Na^+ and Cl^- extrusion through the coordinated activities of ion cotransporters and channels, including $Na^+/K^+/2Cl^-$ cotransporter 1 (Nkcc1) and cystic fibrosis transmembrane regulator 1 (Cftr1) (Pelis and McCormick, 2001; Singer et al., 2002). Concurrent with elevated *nkcc1* and *cftr1* levels at the peak of smoltification, Atlantic salmon exhibit a "switch" in the relative levels of two *Na^+/K^+-ATPase α1* (*nka-α1*) subunit-encoding genes and their translated proteins. *nka-α1b* levels are enhanced during smoltification while *nka-α1a* is maintained in freshwater but dramatically decreases after seawater exposure (McCormick et al., 2013). Thus, coordinated increases in *nka-α1b*, *nkcc1*, and *cftr1* underlie the development of seawater tolerance (Tipsmark et al., 2002; Nilsen et al., 2007; McCormick et al., 2013). Since Gh/Igf1 signaling supports seawater tolerance, at least in part, by stimulating Nkcc1/*nkcc1* and *nka-α1b* (Pelis and McCormick, 2001; Tipsmark and Madsen, 2009), nutrition-elicited perturbations of the Gh/Igf system may disrupt the development and/or maintenance of ionoregulatory capacities supporting survival in marine environments.

To understand the physiological ecology of smolts, it is especially important to examine the endocrine responses to fasting for two major reasons. First, it has long been recognized that the parr-smolt transformation is a period when large increases in metabolic rate and lipolysis occur (McCormick and Saunders, 1987), leading to the concept that smolts are "energy deficient" during downstream migration (Stefansson et al., 2003). This is not just a seasonal attribute, as the relative condition (condition factor) of parr increases, and smolts decreases, when both are reared under identical *ad libitum* feeding conditions (McCormick et al., 2007). Second, as smolts migrate downstream and enter estuarine/coastal environments they transition from a largely insect-based diet to marine invertebrates and fish (Andreassen et al., 2001; Renkawitz and Sheehan, 2011). This may result in periods of prolonged food restriction and further energy deficiency as individual smolts learn to prey on new food items (Stefansson et al., 2003).

In the current study, we investigated how the Gh/Igf system, thyroid hormones, and cortisol respond to fasting in Atlantic salmon smolts. Leveraging recent molecular characterizations by Bower et al. (2008) and Macqueen et al. (2013) of the extensive *igfbp* gene family in Atlantic salmon, we paid special attention to the dynamics of *igfbp* transcripts that exhibit robust hepatic expression. In this initial investigation of *igfbp* responses to fasting

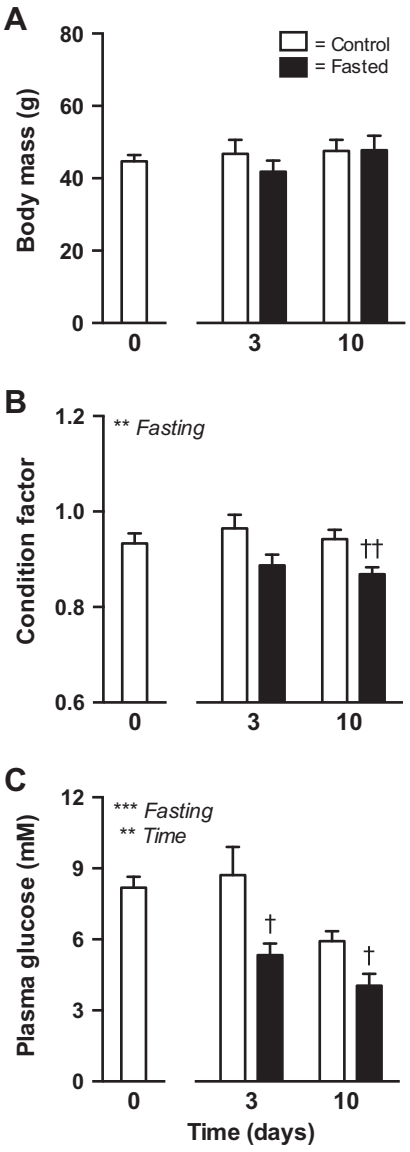

Fig. 1. Effects of fasting on body mass (A), condition factor (B), and plasma glucose (C). Smolts were exposed to continuous feeding (open bars) or fasting (solid bars) and sampled at 3 and 10 days. Significant effects of fasting or time are indicated in respective panels. When there was a significant effect of fasting, post hoc comparisons (Student's t-tests) were made between fed and fasted groups at each time point. $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$. Means ± SEM ($n = 8$).

in smolts, we chose to characterize hepatic responses because the liver is a key player in the Gh/Igf axis, especially with respect to being the primary source of circulating Igfs. We hypothesized that hepatic *igfbps* would also respond in parallel with, and thus potentially mediate, physiological responses to nutrient restriction. Lastly, we assayed branchial ionoregulatory function to evaluate the impacts of nutrient restriction on the development of seawater tolerance.

2. Materials and methods

2.1. Animals

Atlantic salmon (*Salmo salar*) parr were obtained in October of 2014 from the Kensington National Fish Hatchery (Kensington, CT), and held at the Conte Anadromous Fish Research Center (Turners Fall, MA) until undergoing smoltification in the spring of 2014. Prior to the fasting experiment, fish were held in fiberglass tanks receiving flow-through Connecticut River water, maintained under natural photoperiod and ambient river temperatures (2–15 °C). Fish were fed to satiation twice daily with commercial feed (Bio-Oregon, Longview, WA). All experiments were carried out in accordance with US Geological Survey institutional guidelines and an approved IACUC review.

2.2. Experimental design

Atlantic salmon smolts (32–69 g; $n = 40$) of mixed sex were randomly distributed into two 190 L treatment (fed control and fasted groups) tanks maintained at 10 °C (range of daily temperature measurement of 9.4–10.7 °C) with particle and charcoal filtration, continuous aeration, and supplied with dechlorinated tap water at 2 L/h. Fish were acclimated to the experimental tanks for three weeks prior to the beginning of the experiment. Following the acclimation period, four animals were sampled from both tanks as time 0 samples (April 22). Food was then withheld from one tank while animals in the other tank were fed to satiation once daily (10:00 h Eastern Standard Time). At 3 and 10 days, 8 animals

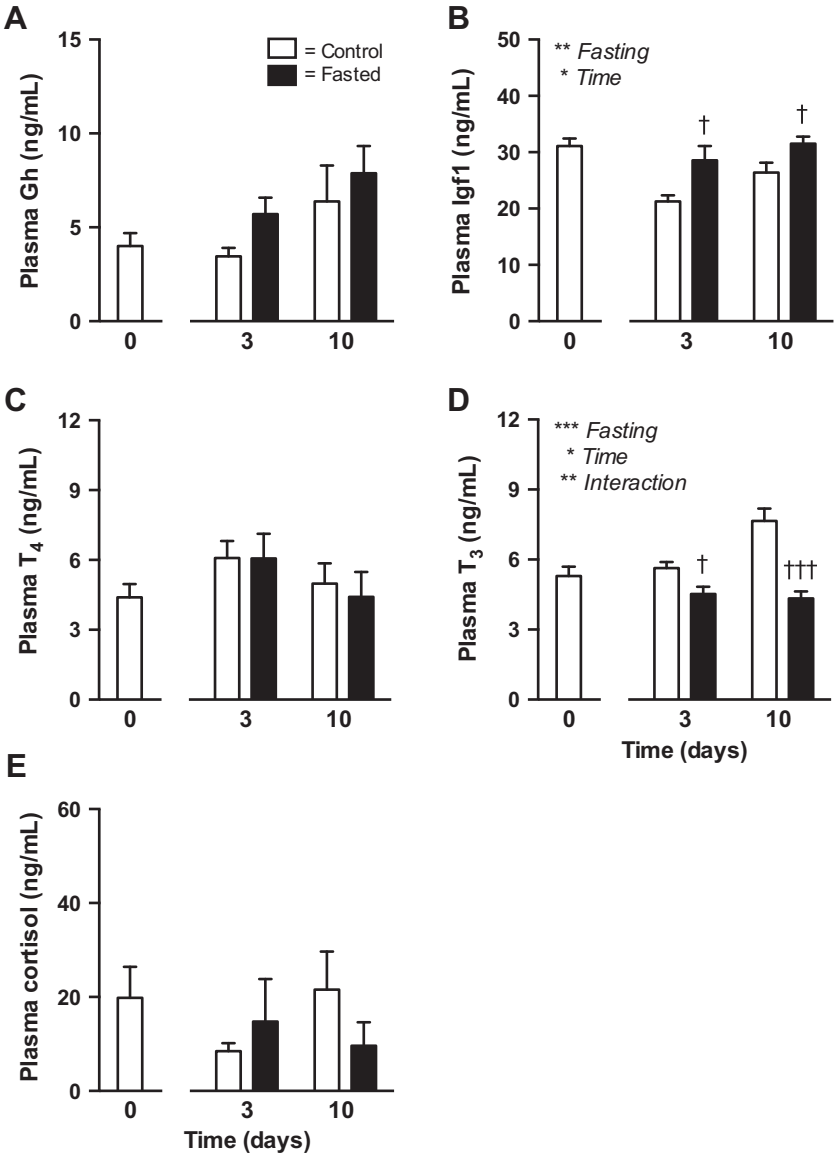

Fig. 2. Effects of fasting on plasma Gh (A), Igf1 (B), T_4 (C), T_3 (D), and cortisol (E). Smolts were exposed to continuous feeding (open bars) or fasting (solid bars) and sampled at 3 and 10 days. Significant effects of fasting, time, or an interaction are indicated in respective panels. When there were significant fasting or interaction effects, post hoc comparisons (Student's t-tests) were made between fed and fasted groups at each time point. $^{\dagger}P < 0.05$ and $^{\dagger\dagger\dagger}P < 0.001$. Means ± SEM ($n = 8$).

were sampled from each treatment tank at 9:00 h. This feeding/sampling schedule was selected to account for well-characterized post-prandial responses by the Gh/Igf system in salmonids (Shimizu et al., 2009; Amaral and Johnston, 2011).

At the time of sampling, fish were netted and anesthetized in buffered MS-222 (100 mg/l; pH 7.0; Sigma, St. Louis, MO). Blood was collected from the caudal vasculature by a needle and syringe treated with ammonium heparin. Blood samples were collected within 5 min of the initial netting. Blood was separated by centrifugation at 4 °C and plasma stored at −80 °C until subsequent analyses. Body mass and standard length were measured for calculation of condition factor: (body mass, g)/(standard length, cm)^3 × 100. Liver, white muscle, and gill tissues were collected and immediately frozen directly on dry ice and stored at −80 °C. Four to six additional gill filaments were placed in ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and stored at −80 °C.

2.3. Plasma and gill analyses

Plasma glucose concentrations were assayed by enzymatic coupling with hexokinase and glucose 6-phosphate dehydrogenase (Glucose Assay Reagent, G3293, Sigma). Plasma Gh levels were measured by a radioimmunoassay (RIA) validated for Atlantic salmon by Björnsson et al. (1994). Plasma Igf1 levels were measured by a RIA validated for salmonids (Moriyama et al., 1994). Thyroxine (T_4) and 3,5,3′-triiodo-L-thyronine (T_3) concentrations were measured by a direct RIA as described by Dickhoff et al. (1978) and modified by McCormick et al. (1995). Plasma cortisol levels were measured by a validated direct competitive enzyme immunoassay as described by Carey and McCormick (1998). Plasma chloride was analyzed by the silver titration method using a Buchler-Cotlove digital chloridometer (Labconco, Kansas City, MO) and external standards. Branchial Na^+/K^+-ATPase activity was determined as described by McCormick (1993). Briefly, ouabain-sensitive Na^+/K^+-ATPase activity was measured by coupling the production of ADP to NADH using lactic dehydrogenase and pyruvate kinase in the presence and absence of 0.5 mmol/l ouabain. Ten microliters of samples were run in duplicate in 96-well microplates at 25 °C and read at a wavelength of 340 nm for 10 min on a BioTek Synergy 2 spectrophotometer (BioTek, Winooski, VT). Protein concentration of the homogenate was determined using a BCA protein assay (Thermo Fisher Scientific, Rockford, IL).

2.4. RNA extraction, cDNA synthesis and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from tissue by the TRI Reagent procedure (MRC, Cincinnati, OH) according to the manufacturer's protocols. RNA concentration and purity were assessed by spectrophotometric absorbance (Nanodrop 1000, Thermo Scientific, Wilmington, DE). First strand cDNA was synthesized with a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). Relative mRNA levels were determined by qRT-PCR using the StepOnePlus real-time PCR system (Life Technologies). We employed previously described primer pairs for *igfbp1a1*, *-1b1*, *-1b2*, *-2a*, *-2b1* and *-2b2* (Macqueen et al., 2013), *igf1*, *igf2*, *rna polymerase 2* (*rnapol2*) and *elongation factor 1α* (*ef1α*) (Bower et al., 2008), *gh receptor* (*ghr*) (Tipsmark and Madsen, 2009), and *nka-α1a*, *nka-α1b*, *nkcc1* and *cftr1* (Nilsen et al., 2007). To date, only single copies of both the *igf1* and *-2* genes have been identified in Atlantic salmon (Macqueen et al., 2013). qRT-PCR reactions were setup in a 15 μl final reaction volume with 400 nM of each primer, 1 μl cDNA and 7.5 μl of 2x SYBR Green PCR Master Mix (Life Technologies). The following cycling parameters were employed: 10 min at 95 °C followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. After verification that levels did not vary across treatments, *rnapol2* (liver) and *ef1α* (muscle and gill) levels were used to normalize target genes (*igfbps* and *igfs*). Reference and target gene levels were calculated by the relative quantification method with PCR efficiency correction (Pfaffl, 2001). Standard curves were prepared from serial dilutions of control liver, muscle, or gill cDNA and included on each plate to calculate the PCR efficiencies for target and normalization genes. Relative mRNA levels are reported as a fold-change from day 0 controls.

2.5. Statistics

All data were analyzed by two-way ANOVA with treatment (fasting) and time as main effects. Significant effects of fasting, time, or an interaction ($P < 0.05$) are indicated in figures: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. When a significant effect of fasting, or an interaction between fasting and time was detected, Student's t-tests were employed at each time point. Significant differences between groups at a given time point are also indicated in figures: $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$. All statistical analyses were performed using GraphPad *Prism 6* (San Diego, CA).

3. Results

3.1. Physical characteristics and plasma glucose

We first described responses to 3 and 10 days of food restriction by assaying morphological and metabolic parameters. There were no significant main effects, or an interaction, on body mass (Fig. 1A) or fork length (data not shown) after 3 and 10 days of fasting. There was, however, a significant main effect of fasting on condition factor (Fig. 1B) and plasma glucose (Fig. 1C). Condition factor

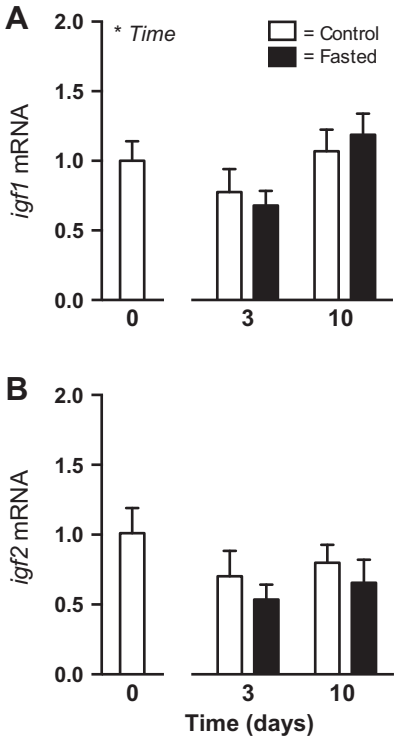

Fig. 3. Effects of fasting on hepatic *igf1* (A) and *igf2* (B) mRNA levels. Smolts were exposed to continuous feeding (open bars) or fasting (solid bars) and sampled at 3 and 10 days. There were no significant main effects or interactions on *igf1* and *igf2* transcripts. Means ± SEM ($n = 8$).

was reduced in fasted animals by 10 days; plasma glucose was reduced from fed controls by 3 days.

3.2. Plasma hormone levels

There were no significant main effects or interactions on plasma Gh, T_4 and cortisol levels (Fig. 2A, C, E). There were significant main effects of fasting and time on plasma Igf1 (Fig. 2B) and T_3 levels (Fig. 2D). At 3 and 10 days of fasting, plasma Igf1 levels were higher than controls while plasma T_3 levels were reduced.

3.3. Hepatic and muscle mRNA levels

There were no clear effects of fasting on hepatic *igf1* (Fig. 3A) and *-2* (Fig. 3B). The mean (±SEM) Ct values for hepatic *igf1* and *-2* were 22.3 ± 0.1 and 21.7 ± 0.1, respectively. Significantly elevated hepatic *igfbp1a1* levels were observed after 3 and 10 days of fasting (Fig. 4A); *igfbp1b1*, *-1b2*, *-2a*, *-2b1*, and *-2b2* levels were not responsive to fasting (Fig. 4B–F). The mean Ct values for hepatic *igfbp1a1*, *-1b1*, *-1b2*, *-2a*, *-2b1* and *-2b2* were 28.0 ± 0.3, 22.5 ± 0.2, 21.5 ± 0.1, 22.7 ± 0.2, 23.4 ± 0.1, and 23.9 ± 0.8, respectively. There was no effect of fasting on hepatic *ghr* levels (data not shown). There was a significant effect of fasting on muscle *igf1* levels; *igf1* was decreased from fed controls after 10 days of fasting (Fig. 5A). There was a significant interaction effect on muscle *igf2* levels; *igf2* levels were modestly elevated in the fasted group at 3 days (Fig. 5B). The mean Ct values for muscle *igf1* and *-2* were 30.9 ± 0.2 and 28.6 ± 0.1, respectively.

3.4. Ionoregulatory parameters

There were no significant fasting or interaction effects on plasma chloride, branchial Na^+/K^+-ATPase activity, or *nka-α1a*, *nka-α1b*, *nkcc1*, and *cftr1* transcript levels (Fig. 6). The mean Ct values for *nka-α1a*, *nka-α1b*, *nkcc1* and *cftr1* were 23.3 ± 0.1, 20.3 ± 0.1, 21.2 ± 0.1, and 23.7 ± 0.1, respectively.

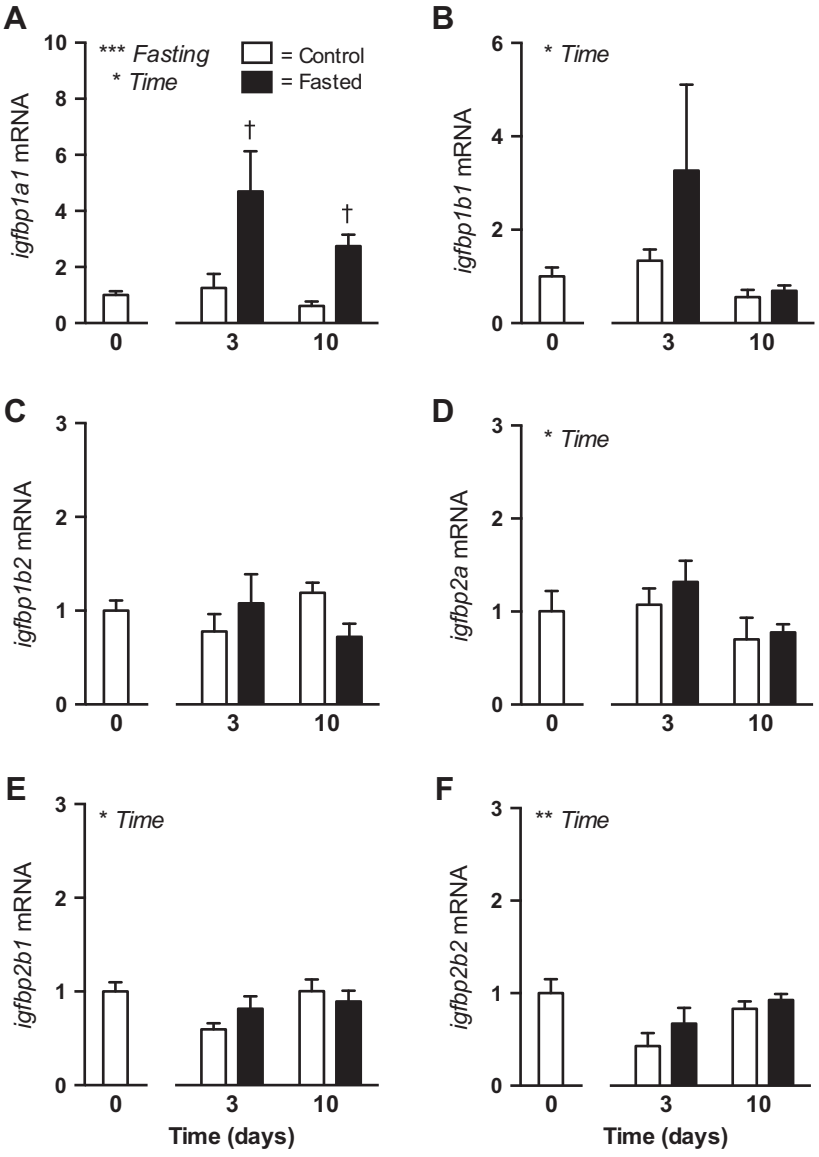

Fig. 4. Effects of fasting on hepatic *igfbp1a1* (A), *-1b1* (B), *-1b2* (C), *-2a* (D), *-2b1* (E), and *-2b2* (F) mRNA levels. Smolts were exposed to continuous feeding (open bars) or fasting (solid bars) and sampled at 3 and 10 days. Significant effects of fasting or time are indicated in respective panels. When there was a significant effect of fasting, post hoc comparisons (Student's t-tests) were made between fed and fasted groups at each time point. $^{\dagger}P < 0.05$. Means ± SEM ($n = 8$).

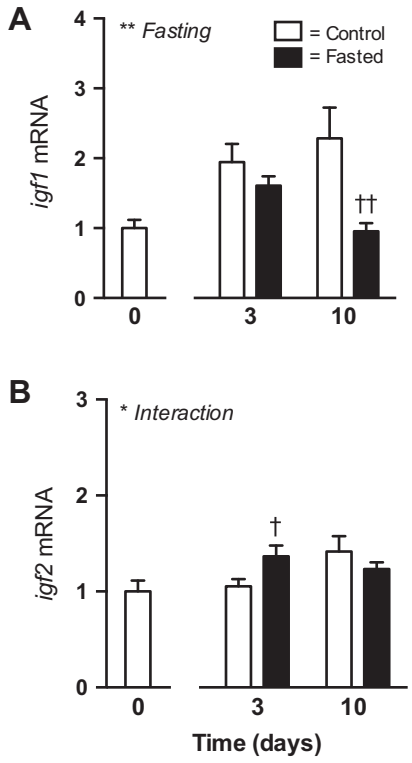

Fig. 5. Effects of fasting on muscle *igf1* (A) and *igf2* (B) mRNA levels. Smolts were exposed to continuous feeding (open bars) or fasting (solid bars) and sampled at 3 and 10 days. Significant effects of fasting or an interaction between time and fasting are indicated in respective panels. When there were significant fasting or interaction effects, post hoc comparisons (Student's t-tests) were made between fed and fasted groups at each time point. $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$. Means ± SEM ($n = 8$).

4. Discussion

Owing to their remarkable life-history strategy, endangered status, and intense aquaculture (Parrish et al., 1998), Atlantic salmon are an important model system from which to resolve how Igfbps regulate growth and development. The smolt stage is of special interest because it entails endogenous increases in Gh and Igf1 that are associated with both increased growth and metabolic rate (McCormick, 2013). Fasting may be a normal occurrence for smolts as they make the transition from freshwater to marine habitats that present external stressors and new food sources (Renkawitz and Sheehan, 2011). Here, we exposed smolts to 10 days of fasting and observed enhanced hepatic *igfbp1a1* in parallel with diminished muscle *igf1* levels. On the other hand, we did not identify any overt effects of nutrient restriction on branchial mediators of ionoregulation.

The catabolic state of the fasted fish in the current study is indicated by decreased plasma glucose levels after 3 days of fasting, followed by a significant decrease in condition factor after 10 days (Fig. 1B, C). In the current study, there was a tendency for increased Gh levels with time in both fed and fasted fish, although somewhat higher Gh levels in the fasted group (Fig. 2A). As the experiment was carried out towards the end of April, this correlates well with the timing of photoperiod-induced increases in plasma Gh levels normally seen during smoltification (Björnsson et al., 1995). It is also well known that Gh levels increase during fasting, but previous studies carried out on salmon smolts report such increases only after ~2 weeks of fasting. For example, in coho (*Oncorhynchus kisutch*) smolts, plasma Gh levels were significantly elevated after 14 days of fasting (Duan and Plisetskaya, 1993), and after 15 days in Atlantic salmon smolts (Wilkinson et al., 2006). Fasting-induced elevation of plasma Gh is thought to be primarily due to an onset of hepatic Gh-resistance caused by down-regulation of the Gh receptor, leading to slower Gh turn-over rate and higher plasma Gh levels. This will lead to decreased Igf1 secretion and plasma Igf1 levels (Gray et al., 1992; Fuentes et al., 2012; Pierce et al., 2005), which, in turn, decreases the negative-feedback inhibition of Igf1 on pituitary Gh secretion, allowing Gh secretion and plasma Gh levels to increase (Beckman, 2011; Fuentes et al., 2012). This Gh/Igf1 response to fasting may be amplified by a concurrently decreased nutrient stimulation of hepatic Igf1 secretion (Duan, 1998).

The slightly higher plasma Igf1 levels during the 10-day fasting period (compared with fed controls) suggest that the fish did not develop Gh-resistance, during which Igf1 levels would otherwise decline. This pattern was similarly observed in Atlantic salmon post-smolts fasted for 14 days at 10 °C (Hevrøy et al., 2011). Wilkinson et al. (2006) observed diminished plasma Igf1 in smolts fasted for 15 days at 16 °C (*igf1* mRNA was not reported). These studies when considered with our current observations suggest that temperature and developmental stage are key determinants of when the onset of "Gh resistance" occurs and that while a fasting paradigm such as ours (10 days) is sufficient to elicit an *igfbp1a1* response, it is not sufficient to elicit a response in *igf1* mRNA expression. A likely explanation for the elevated plasma Igf1 levels may be linked with the elevated hepatic *igfbp1a1* mRNA levels of the fasted fish (Fig. 4A). Provided that *igfbp1a1* mRNA is translated into protein and released into circulation, elevated Igfbp1a1 levels would increase the biological half-life of Igf1 and thus elevate the total plasma Igf1 levels. To our knowledge, whether Igfbps impact the half-life of Igf1 has not been directly tested in fishes. At least in the case of mammalian Igf1, Igfbp1 more than doubled the half-life of circulating Igf1 while simultaneously inhibiting bioactivity *in vivo* (Lewitt et al., 1993).

During the smoltification process, the condition factor of Atlantic salmon decreases markedly, partly due to smoltification-related changes in body shape, but also due to utilization of energy reserves such as lipid stores and liver glycogen (Saunders and Henderson, 1970; Sheridan, 1989). This has led to the concept that smolts are inherently energy deficient (Stefansson et al., 2003) and it could then be hypothesized that further energy withdrawal (such as 3–10 days of fasting) would have limited effect on the Gh/Igf1 system. It will be interesting to learn through future study whether baseline *igfbp1a1* expression is generally higher in smolts compared with other life-history stages. The pluripotency of the Gh/Igf1 system is particularly evident during salmon smoltification, where its important role in maintaining salinity tolerance, critical to survival during seawater entry, has been amply demonstrated (Sakamoto et al., 1993). This may have led to the evolution of regulatory mechanisms that reduce the impact of nutritional status on the Gh/Igf1 system during smolt development.

Atlantic salmon exhibit robust mRNA levels of *igfbp1* and *-2* isoforms in liver (Macqueen et al., 2013), a pattern observed across multiple teleost species (Funkenstein et al., 2002; Kamei et al., 2008; Zhou et al., 2008; Pedroso et al., 2009; Peterson and Waldbieser, 2009; Shimizu et al., 2011a,b; Safian et al., 2012). Of the six hepatic *igfbp1* and *-2* isoforms we assayed, *igfbp1a1* was the sole transcript modulated in response to fasting (Fig. 4). By restricting Igfs from binding cognate receptors, Igfbp1 inhibits somatic growth, development, and glucose metabolism (Lee et al., 1997; Kajimura et al., 2005; Kamei et al., 2008). Thus, the dynamic nature of *igfbp1a1* mRNA levels observed here in Atlantic salmon seems to play a role in associating Igf1/2 activity with adaptive growth patterns during smoltification. Similarly, 22- and 23-kDa Igfbps (putative Igfbp1b paralogs) in chinook (*Oncorhynchus tshawytscha*) and Atlantic salmon were induced by

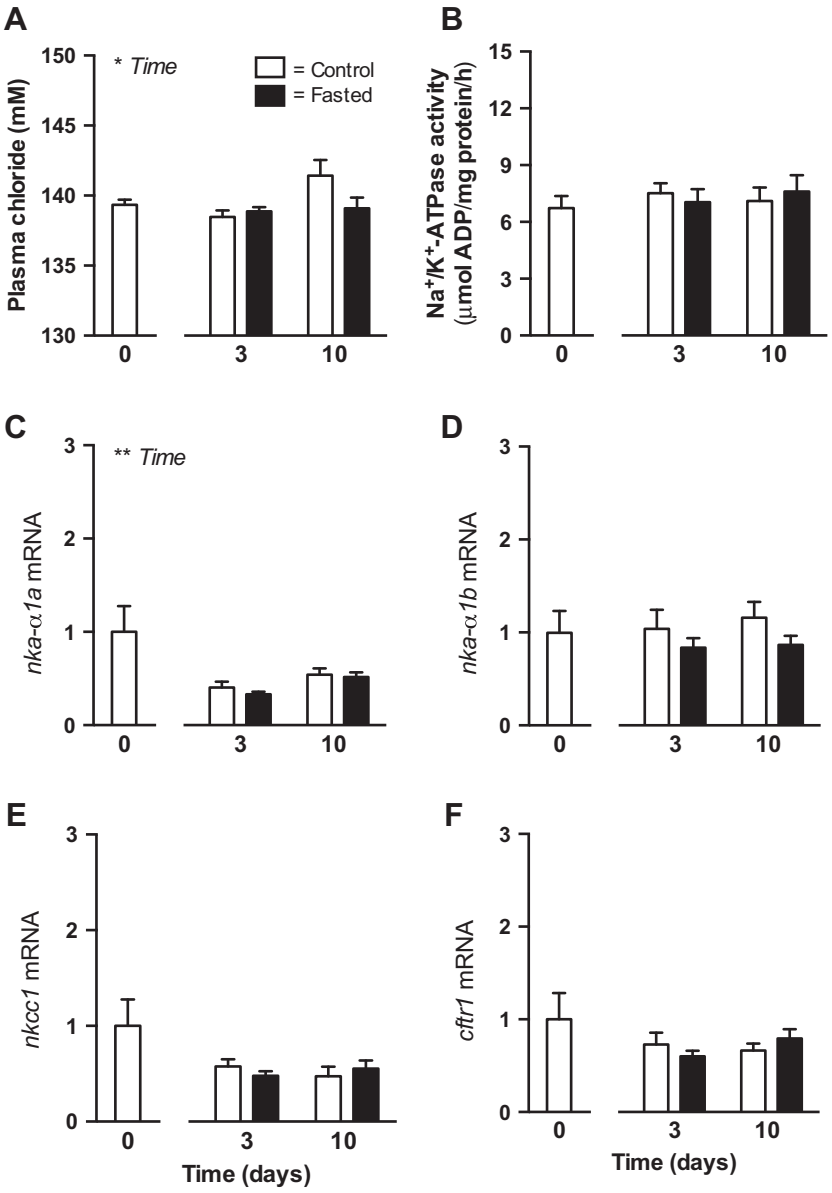

Fig. 6. Effects of fasting on plasma chloride (A), branchial Na^+/K^+-ATPase activity (B) and branchial *nka-α1a* (C), *nka-α1b* (D), *nkcc1* (E), and *cftr1* (F) mRNA levels. Smolts were exposed to continuous feeding (open bars) or fasting (solid bars) and sampled at 3 and 10 days. A significant effect of time is indicated in respective panels. Means ± SEM ($n = 8$).

reduced feeding rations/fasting (Shimizu et al., 2005, 2006, 2009; Hevrøy et al., 2011). Because Igfbp1 restricts Igf1 from binding receptors, we speculate that increased circulating Igf1 levels in fasted animals compared with the controls of this study (Fig. 2B) could be the result of increased hormone half-life through stabilization of Igf1.

While our study suggests that Igfbp1 contributes to the modulation of Igf signaling, the question of how *igfbp* transcription is actually controlled in smolts (and teleosts in general) remains largely unanswered. More specifically, there is a paucity of data regarding how intrinsic signals (hormones) regulate *igfbp* mRNAs (Duan et al., 2010; Reindl and Sheridan, 2012). In mammals, metabolic hormones, including Gh, insulin, glucagon, glucocorticoids, and thyroid hormones regulate Igfbp1 (Lee et al., 1997; Reindl and Sheridan, 2012). In channel catfish (*Ictalurus punctatus*), dietary cortisol induced plasma levels of a ~20-kDa Igfbp (Peterson and Small, 2005). In coho salmon hepatocytes, dexamethasone and glucagon increased, Gh decreased, and T_3 had no effect, on *igfbp1* mRNA levels (Pierce et al., 2006). There is no information to date regarding the effects of these hormones on *igfbp1* mRNAs in Atlantic salmon. In turn, the reductions in plasma T_3 levels with fasting in smolts (Fig. 2D) are noteworthy because thyroid hormones exert permissive actions on growth (Nicoll et al., 1999). T_3 patterns under our experimental conditions were in accord with numerous studies showing depressed plasma thyroid hormone levels in fasted salmonids; these patterns are mediated by alterations in thyroid gland sensitivity to thyroid-stimulating hormone and reduced hepatic deiodinase activity (Eales, 1988; McCormick and Saunders, 1990; Farbridge and Leatherland, 1992). Future *in vivo* and *in vitro* studies are necessary to determine whether T_3 responses to fasting underlie hepatic *igfbp1a1* dynamics in Atlantic salmon.

We did not identify an effect of fasting on hepatic *igf1* or *-2* (Fig. 3); however, we detected a marked reduction in muscle *igf1* following 10 days of fasting (Fig. 5A). While the liver is deemed the principal source of circulating Igfs (Reinecke, 2010; Reindl and Sheridan, 2012), there is robust *in vitro* evidence that Igfs also act in a paracrine/autocrine fashion to stimulate skeletal muscle

growth (Castillo et al., 2004; Codina et al., 2008; Bower and Johnston, 2010; Azizi et al., in press). Accordingly, salmonids exhibit reduced muscle *igf1* and/or *-2* mRNA levels in response to nutrient restriction (Chauvigne et al., 2003; Gabillard et al., 2006; Montserrat et al., 2007; Bower et al., 2008, 2009). Indeed, our current observations are compatible with a role for extrahepatic Igf1 in the attenuation of muscle growth in fasted smolts, a response potentially mediated by the direct sensing of amino acid availability (Bower and Johnston, 2010). Our data also suggest that hepatic and muscle *igf1* expression differs in response to fasting (Figs. 3A and 5A). The mechanisms underlying tissue-specific *igf1* responses to fasting are not fully resolved; however, differential transcriptional responses to Gh, insulin, somatostatin, and other metabolic hormones, seem likely (Reindl and Sheridan, 2012). We observed a modest increase in muscle *igf2* at 3 days (Fig. 5B). Albeit transient, this pattern was unexpected and requires future investigation.

As the Gh/Igf system directs osmoregulatory aspects of parr-smolt transformation (Sakamoto et al., 1993; Björnsson, 1997), we hypothesized that branchial ionoregulatory pathways underlying the development of seawater tolerance would be impacted by fasting induced alterations of the Gh/Igf system. We did not observe any perturbations of gill Na^+/K^+-ATPase activity, or in the levels of mRNAs (*nka-α1b*, *nkcc*, or *cftr1*) that encode components of seawater-type ionocytes (Fig. 6). We selected these targets on the basis that their activities/mRNA levels during the peak of smoltification predict osmoregulatory performance upon seawater exposure (Nilsen et al., 2007; McCormick et al., 2013). To date, there has been limited study of the impact of reduced ration on osmoregulation in smolting salmonids. Imsland et al. (2011) found only moderate (15%) decreases in gill Na^+/K^+-ATPase activity in Atlantic salmon smolts after 12 days of food withdrawal, but much larger decreases (75%) after an additional 14 days. Such moderate decreases in gill Na^+/K^+-ATPase activity after 12 days are consistent with the absence of changes in osmoregulatory parameters observed in the present study after 10 days of fasting.

In summary, the responses we observed in Atlantic salmon smolts provide further support that hepatic *igfbp1* isoforms are enhanced under catabolic conditions in representatives of various teleost groups, including Siluriformes, Cyprinidae, and Cichlidae (Kamei et al., 2008; Peterson and Waldbieser, 2009; Breves et al., 2014), as well as Salmonidae (Shimizu et al., 2006). We also documented a marked attenuation of muscle *igf1* with fasting, an autocrine/paracrine response similarly conserved across teleosts. Because Atlantic salmon express an expansive suite of *igfbp* genes across tissues, the physiological phenomenon of parr-smolt transformation is well suited to further examine how *igfbp* transcriptional patterns mediate an array of Gh/Igf-mediated processes.

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