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Title:

Effects of growth hormone and cortisol administration on plasma insulin-like growth factor binding proteins in juveniles of three subspecies of masu salmon (*Oncorhynchus masou*)

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Abstract

In this study, we examined the effects of porcine growth hormone (GH) and cortisol on plasma insulin-like growth factor binding proteins (IGFBPs) in juveniles of three subspecies of *Oncorhynchus masou* (masu, amago, and Biwa salmon). Ligand blotting using digoxigenin-labeled human IGF-I was used to detect and semi-quantify three major circulating IGFBP bands at 41, 28, and 22 kDa, corresponding to IGFBP-2b, -1a, and -1b, respectively. GH increased plasma IGFBP-2b concentration in masu and Biwa salmon but suppressed it in amago salmon. Plasma IGFBP-2b levels were increased by cortisol in the three subspecies. Cortisol induced plasma IGFBP-1a in the three subspecies, whereas GH had a suppressive effect in masu and Biwa salmon. Sham and cortisol injections increased plasma IGFBP-1b levels after 1 day in masu and amago salmon, suggesting that IGFBP-1b is induced following exposure to stressors via cortisol. Increased IGFBP-1b levels were restored to basal levels when co-injected with GH in Biwa salmon, and the same trend was seen in masu and amago salmon. However, the suppressive effect of GH disappeared 2 days after injection in the three subspecies. Despite some differences among subspecies, the findings suggest that cortisol is a primary inducer of plasma IGFBP-1b; however, GH counteracts it in the short term. Therefore, GH has the potential to modulate the degree of increase in circulating IGFBP-1b levels during acute stress.

Keywords

Insulin-like growth factor binding protein subtypes; Hormonal control; Growth hormone action; Ligand blotting; Acute stress; *Oncorhynchus masou* subspecies-complex

1. Introduction

The growth hormone (GH)/insulin-like growth factor (IGF)-I system is central to growth regulation in vertebrates. GH stimulates the hepatic production of IGF-I, which mediates GH actions (Daughaday and Rotwein, 1989). In mammals, IGF-I synthesis also occurs in peripheral tissues, where it acts as a local growth factor essential for normal postnatal growth (Le Roith et al., 2001; Ohlsson et al., 2009). However, IGF-I activity is regulated by multiple IGF-binding proteins (IGFBPs). These proteins either inhibit or potentiate the action of IGF-I, depending on their type, post-translational modification, and cellular environment (Firth and Baxter, 2002; Bach, 2018). This additional layer of regulation of IGF-I by IGFBPs is important for fine-tuning growth speed and energy partitioning under conditions of malnutrition and stress (Allard and Duan, 2018).

In mammals, six members of the IGFBP family are secreted into the bloodstream (Rajaram et al., 1997; Bach, 2018): IGFBP-3 and IGFBP-1 are the two main, best-characterized forms. The most abundant IGFBP in the mammalian circulation is IGFBP-3, which transports 75–80% of IGF-I by forming a ternary complex with the ligand and an acid-labile subunit (ALS; Baxter and Martin, 1989; Zapf, 1995; Rajaram et al., 1997). The concentration of IGFBP-1 in the circulation is much lower than that of IGFBP-3; however, it shows large fluctuations in response to meals and metabolic states and may play a role in regulating free IGF-I (Lee et al., 1993, 1997).

Teleosts have extra copies of the six IGFBP genes due to an additional round of whole genome duplication specific to this lineage. For instance, sticklebacks (*Gasterosteus aculeatus*) have two co-orthologs of each member of the IGFBP family, except for IGFBP-4, resulting in 11 IGFBPs (Ocampo Daza et al., 2011). Salmonids possess up to 22 genes for IGFBPs because this group experienced an autotetraploidization event (de la Serrana and Macqueen, 2018). The retention of most duplicated copies in such cases suggests that IGFBPs play an important role in growth via regulating IGF-I activity or/and exhibiting IGF-independent effects.

Two to three IGFBPs are typically detected in the plasma/serum of teleosts; however, their identities have been the subject of debate for approximately two decades (Kelley et al., 2000; Wood et al., 2005; Shimizu and Dickhoff, 2017). In Chinook salmon (*Oncorhynchus tshawytscha*), three major plasma/serum IGFBP bands at 41, 28, and 22 kDa have been identified as IGFBP-2b, -1a, and -1b, respectively (Shimizu et al., 2005, 2011a,b). In salmon, there is no evidence of the presence of IGFBP-3 in the circulation; however, very low levels of *igfbp-3* mRNA have been detected in several tissues (Shimizu et al., 2011b). Although salmonids are the only group in which circulating IGFBPs have been specifically identified, such proteins will likely be identified in other teleosts.

Because vertebrate growth is achieved by integrating internal conditions and external stimuli via endocrine systems, IGFBPs respond to many hormones and adjust the availability of IGF-I to target tissues. Insulin, GH, cortisol, and sex steroids have been shown to regulate certain IGFBP types (Rajaram et al., 1997). In mammals, the major inducer of IGFBP-3, IGF-I, and ALS expression is GH. After GH stimulation, Kupffer cells are responsible for synthesizing IGFBP-3, whereas hepatocytes are the production site for IGF-I and ALS (Daughaday and Rotwein, 1989; Chin et al., 1994; Villafuerte et al., 1994). Insulin and cortisol are the two major hormones that regulate IGFBP-1 in mammals. Insulin suppresses the hepatic production of IGFBP-1, whereas cortisol induces it, although cortisol action is secondary to that of insulin (Unterman et al., 1991; Katz et al., 1998).

Fish IGFBPs are also regulated by hormones. Fukazawa et al. (1995) examined the effects of several hormones on the secretion of IGFBPs from the liver pieces of striped bass (*Morone saxatilis*). Kelley et al. (1992, 2001) reported that a synthetic glucocorticoid, dexamethasone (Dex), induced two low-molecular-weight IGFBPs (25–30 kDa and 20–25 kDa) into the circulation. Salmon IGFBP-2b, which corresponds to the 41-kDa form in the circulation, is physiologically equivalent to mammalian IGFBP-3. It is regulated by GH and acts as a major carrier of circulating IGF-I (Shimizu et al., 2003a,b, 2007, 2011b). In tilapia (*Oreochromis mossambicus*), cortisol suppresses plasma IGFBP (40 kDa) and IGF-I (Kajimura et al., 2003). Salmonid IGFBP-1a and -1b and low-molecular-weight IGFBPs in the plasma/serum of other fishes are induced by stress from events such as handling, confinement, and salinity changes, most likely via cortisol (Kelley et al., 2001; Johnson et al., 2003; Peterson and Small, 2005; Shimizu et al., 2005, 2011a). Cortisol and GH cooperate to enhance the hypo-osmoregulatory ability in salmon and other euryhaline fishes (McCormick, 2001; Mancera and McCormick, 2007); therefore, determining whether they have a combined effect on growth, particularly on circulating IGFBPs, is an important question that needs to be addressed.

Oncorhynchus masou is one of eight Pacific salmon species distributed only on the Asian side of the Pacific Ocean. This species forms a subspecies complex comprising four subspecies: masu salmon (*O. masou masou*), amago salmon (*O. masou ishikawae*), Biwa salmon (*O. masou* subsp.), and Formosa salmon (*O. masou formosanus*), which differ in distribution and life history patterns (Oohara and Okazaki, 1996; Yamamoto et al., 2020). The majority of masu and amago salmon populations are anadromous, migrating downstream to the ocean in the second spring and the first fall of their lives, respectively. The Biwa salmon subspecies has been landlocked in the Lake Biwa watershed for approximately 500,000 years and thus has lost its ability

to adapt to seawater (Fujioka and Fushiki, 1989). The Formosa salmon subspecies is an endangered land-locked subspecies found only in the headwaters of the Tachia River on Taiwan Island (Gwo et al., 2010). We have focused on masu salmon as a model to unravel the mechanisms of endocrine control of growth, development of hypo-osmoregulatory ability, and their interaction (Kawaguchi et al., 2013; Tanaka et al., 2018; Kaneko et al., 2020; Suzuki et al., 2020) because it is a new target species for sea cage aquaculture along the Japanese coasts. The availability of two subspecies (amago and Biwa salmon) that differ in their life-history patterns provides a unique opportunity for a comparative study. Previously, we compared the responses of gill Na⁺,K⁺-ATPase (NKA) and mRNAs of *nka* α -subunit isoforms to GH and cortisol and reported differences between subspecies (Nakajima et al., 2014). In the present study, we examined the combined effect of GH and cortisol on plasma IGFBPs and IGF-I in juvenile masu salmon by injecting these hormones in a manner similar to that previously used for juvenile amago and Biwa salmon (Nakajima et al., 2014). We then compared the responses of plasma IGFBPs in masu salmon with those in the other two subspecies.

2. Materials and Methods

2.1. Fish

Underyearling masu salmon used in the present study were a captive broodstock maintained at Nanae Fresh-Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University (Kameda-gun, Hokkaido, Japan). Fish were reared at 10°C under a natural photoperiod and fed a commercial diet ad libitum once per day (Marubeni Nisshin Feed Co. Ltd., Tokyo, Japan).

Underyearling amago and Biwa salmon were reared in freshwater at 10.5°C in tanks under a natural photoperiod at Samegai Trout Farm, Shiga Prefecture Fishery Experiment Station (Maibara, Shiga, Japan). Amago salmon were from a captive broodstock originating from an anadromous strain in Gifu, whereas Biwa salmon were the offspring of adults returning from Lake Biwa to a river. Fish were fed commercial diets (Nihon Nosan Inc., Kanagawa, Japan) at 2% of their body weight three to four times per day.

2.2. Hormone injections

The following experiments and samplings were carried out in accordance with the guidelines of the Animal Care and Use Committees of Shiga Prefecture Fishery Experiment Station and Hokkaido University.

Underyearling masu salmon (9.0 ± 0.2 cm, 7.6 ± 0.8 g) were transferred to the indoor rearing facility at the Faculty of Fisheries Sciences, Hokkaido University (Hakodate, Hokkaido, Japan) in May 2015. Fish were acclimated in glass tanks ($60 \times 29.5 \times 36$ cm) filled with 60 L freshwater in a temperature-controlled room (10°C). Each tank had a closed circulation system with a filtration unit in the upper half (GEX, Osaka, Japan). Until the start of the experiment, the fish were fed a commercial diet, as previously described. After fasting overnight, fish were lightly anesthetized and injected intraperitoneally once with either porcine GH (Sigma, St. Louis, MO, USA) at $8 \mu\text{g/g}$ body weight, cortisol (Sigma) at $40 \mu\text{g/g}$ body weight, or both (GH + cortisol). As a control, some injected fish received vehicle (17 mM phosphate, 0.13 M NaCl, pH7.0 containing 0.08% bovine serum albumin, 15.9% ethanol) only (sham-injected group). On both one and two days after injection, eight fish per treatment were anesthetized by 3.3% 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan) and measured for fork length and body weight. Gill arches were excised, and a block of gill filaments was immediately frozen on dry ice and stored at -80°C until NKA activity analysis. Blood was obtained by severing the tail and collecting blood into a heparinized glass tube (Hirschmann Laborgeräte, Eberstadt, Germany). Blood was then centrifuged at $8,050 g$ at 4°C for 10 min; plasma was then stored at -80°C until use.

Underyearling amago salmon (6.2 ± 0.1 cm, 2.4 ± 0.2 g) and Biwa salmon (6.2 ± 0.2 cm, 2.2 ± 0.2 g) were injected with GH and cortisol in mid-May 2010. The injection and sampling procedures were the same as described above and detailed in Nakajima et al. (2014).

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

Prior to the assay for IGF-I, plasma was extracted with an acid-ethanol as described in Shimizu et al. (2000). Then, IGF-I was quantified by TR-FIA based on the method described in Small and Peterson (2005) using recombinant salmon/trout IGF-I (GroPep Bioreagents Pty Ltd., Adelaide, SA, Australia) as a standard.

2.4. Electrophoresis and western blotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a 3% stacking gel and 12.5% separating gel. Plasma samples were treated with an equal volume of sample and buffer containing 2% SDS and 10% glycerol at 85°C for 5 min. Gels were run in a solution of 50 mM Tris, 400 mM glycine, and 0.1% SDS at 50 V in the stacking gel and at 100 V in the separating gel until the bromophenol blue dye front reached the bottom of the gel. Gels were stained with

0.1% Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA, USA). The molecular mass was estimated using Precision Marker (Bio-Rad).

Western ligand blotting with digoxigenin-labeled human IGF-I (DIG-hIGF-I) was carried out as described by Shimizu et al. (2000). After electroblotting, the nitrocellulose membrane was incubated with 10–50 ng/mL DIG-hIGF-I for 2 h at room temperature. It was then incubated with antibodies against DIG conjugated with horseradish peroxidase (Roche, Indianapolis, IN, USA) at a dilution of 1:1500–2500 for 1 h at room temperature. Visualization of IGFBP was achieved by use of enhanced chemiluminescent Western blotting reagents (Amersham Life Science, Arlington Heights, IL, USA) and a luminescent image analyzer (LAS-1000 mini; Fuji Film, Tokyo, Japan). Plasma IGFBP levels were semi-quantified using Image-J version 1.440 (Schneider et al., 2012), normalized to the human IGFBP-4 band intensity, and expressed as an arbitrary density unit (ADU).

2.5. Statistical analysis

On each day, the effects of GH and cortisol on plasma IGFBPs were analyzed by two-way ANOVA using the JMP program (SAS Institute Inc., Cary, NC, USA). When significant interactions were found, differences between groups were further qualified by the Tukey-Kramer test. Levels of plasma IGFBPs in the initial group on day zero were compared with those on day one by the *t*-test. Differences between groups were considered significant at $P < 0.05$.

3. Results

3.1. Response of plasma IGF-I to hormone injections

In masu salmon, there were no effects of GH and cortisol 1 day after injection, but GH had a main effect on maintaining plasma IGF-I levels 2 days after injection (Fig. 1a,b). In amago and Biwa salmon, there were no effects of GH and cortisol, except for an interactive effect ($P = 0.0049$) on day 2 in amago salmon (Fig. 1c–f).

3.2. Detection of three plasma IGFBPs by ligand blotting

Ligand blotting using labeled hIGF-I from masu salmon plasma detected IGFBP-2b, -1a, and -1b bands at 41, 28, and 22 kDa, respectively (Fig. 2). IGFBP-2b appeared as doublet bands that were semi-quantified together.

3.3. Response of plasma IGFBP-2b to hormone injections

The hormone injections did not affect plasma IGFBP-2b levels in masu salmon after 1 day (Fig. 3a). However, both hormones increased plasma IGFBP-2b levels 2 days after injection (GH: $P < 0.0001$; cortisol: $P = 0.0091$; Fig. 3b). GH decreased and increased plasma IGFBP-2b levels in amago and Biwa salmon, respectively (amago: $P = 0.0137$; Biwa: $P = 0.0281$; Fig. 3c,e). In amago salmon, cortisol increased plasma IGFBP-2b levels 2 days after injection ($P = 0.0155$, Fig. 3d). In Biwa salmon, cortisol had a main inhibitory effect on IGFBP-2b levels 1 day after injection ($P = 0.0463$, Fig. 3e), whereas its effect was stimulatory 2 days after injection ($P = 0.0422$, Fig. 3f).

3.4. Response of plasma IGFBP-1a to hormone injections

In masu salmon, plasma IGFBP-1a levels were reduced 1 day after GH injection ($P = 0.0162$), whereas a stimulatory effect of cortisol was observed 2 days after injection ($P = 0.0018$; Fig. 4a,b). Cortisol also increased plasma IGFBP-1a levels in amago and Biwa salmon 1 day after injection (amago: $P < 0.0001$; Biwa: $P = 0.0113$; Fig. 4c,e). In Biwa salmon, the stimulatory effect of cortisol was neutralized by co-injection with GH, which also had a main inhibitory effect ($P = 0.0031$; Fig. 4e). On day 2, no effect of GH or cortisol was seen on plasma IGFBP-1a levels in amago and Biwa salmon (Fig. 4d,f).

3.5. Response of plasma IGFBP-1b to hormone injections

In masu and amago salmon, plasma IGFBP-1b levels increased 1 day after sham injection with vehicle (Suppl. Fig. 1), and plasma IGFBP-1b levels in the cortisol group were similar to those in the sham group (Fig. 5a,c). GH had a main suppressive effect on plasma IGFBP-1b levels 1 day after injection in both masu and amago salmon (masu: $P = 0.0057$; amago: $P = 0.0002$). On day 2, cortisol maintained high plasma IGFBP-1b levels in masu salmon, whereas it had a main suppressive effect in amago salmon ($P = 0.0055$; Fig. 5b,d). In Biwa salmon, sham injection had no effect on plasma IGFBP-1b levels (Suppl. Fig. 1), whereas cortisol increased it ($P = 0.0028$; Fig. 5e). Treatment with GH had a main inhibitory effect ($P = 0.0086$) and restored IGFBP-1b levels to similar levels to those in the sham group (Fig. 5e); however, this effect disappeared 2 days after injection (Fig. 5f).

4. Discussion

The *O. masou* subspecies-complex exhibits large variations in life-history patterns, especially the timing and degree of the development of hypo-osmoregulatory ability (Kubo, 1980; Kato, 1991; Fujioka, 1991). There has been increasing interest in aquaculture of the three subspecies (i.e.,

masu, amago, and Biwa salmon) in seawater and freshwater in regions of their distribution in Japan. However, little is known about the differences in their growth characteristics owing to their allopatric distribution and the presence of anadromous and non-anadromous strains. Fujioka (2006) compared growth patterns between amago and Biwa salmon in a common garden setting and showed that growth in underyearling Biwa salmon was delayed because of a reduction in appetite from July, which corresponded to the timing of their downstream migration to Lake Biwa. This growth retardation may be adaptive to the scarcity of their prey item, the amphipod *Jesogammarus annandalei*, in the lake during that period (Fujioka, 2006). We have been focusing on anadromous strains of masu salmon to investigate the growth regulation by IGF-I and IGFBPs and their involvement in the development of hypo-osmoregulatory ability (i.e., smoltification) (Kawaguchi et al., 2013; Tanaka et al., 2018; Kaneko et al., 2020; Suzuki et al., 2020). We previously compared the hypo-osmoregulatory ability of juvenile amago and Biwa salmon by injecting them with GH and cortisol (Nakajima et al., 2014). In the present study, we compared the responses of circulating IGFBPs in the three subspecies of *O. masou* by injecting juvenile masu salmon with GH and cortisol.

A single injection of porcine GH increased plasma IGF-I levels in the masu salmon. The dose of porcine GH (8 µg/g body weight) was chosen based on the results from postsmolt coho salmon (*O. kisutch*; Shimizu et al., 2007). However, there was no clear induction of plasma IGF-I by GH in the amago and Biwa salmon. Several samples from the amago and Biwa salmon were not measured for IGF-I because the volume of plasma was limited, and analysis of IGFBPs was prioritized. Therefore, it is not known whether IGF-I in amago and Biwa salmon was less sensitive to GH or the number of plasma samples analyzed for IGF-I was too small to detect the effect. Therefore, our discussion on IGF-I responses is limited to masu salmon. In the present study, there was no effect of cortisol on serum IGF-I levels in masu salmon. This result is in accordance with the finding by Pierce et al. (2005) that Dex had no direct effect on decreasing *igf-1* mRNA levels in cultured salmon hepatocytes. In tilapia, a single cortisol injection decreased plasma IGF-I levels and *igf-1* mRNA in the liver in 2 days (Kajimura et al., 2003). Thus, the IGF-I response to cortisol differs between species.

The effect of GH on plasma IGFBP-2b levels differed among the three subspecies; GH stimulated plasma IGFBP-2b levels in masu and Biwa salmon but not in amago salmon. The induction by GH of plasma IGFBP-2b has previously been reported in coho salmon (*O. kisutch*; Shimizu et al., 2003a, 2007). In tilapia, hypophysectomy reduced plasma 40-kDa IGFBP levels, and injection with a homologous GH increased them (Park et al., 2000). In contrast, in channel

catfish (*Ictalurus punctatus*), injection of bovine GH reduced plasma 44- and 47-kDa IGFBPs (Johnson et al., 2003). These results suggest that the effect of GH on plasma levels of IGFBP-2b and 40–47-kDa IGFBPs is species specific. Cortisol unexpectedly increased plasma IGFBP-2b levels 2 days after injection in the three subspecies; however, a suppressive effect of cortisol was seen in Biwa salmon on day 1. In tilapia, cortisol has a suppressive effect on plasma doublet IGFBPs (40–42 kDa) within 24 h (Kajimura et al., 2003). The inconsistencies in the response of salmon IGFBP-2b and fish 40–47 kDa IGFBPs to GH and cortisol within and between species should be investigated in future studies.

The intensity of the plasma IGFBP-1a band (28 kDa) was increased by cortisol in the three subspecies, which is in accordance with the finding in tilapia that a 30-kDa IGFBP was induced by cortisol injection (Kajimura et al., 2003). Circulating IGFBP-1a in salmon is often undetectable or faint under normal conditions; however, its levels are increased under severe stress (Shimizu et al., 2011a). Our results suggest that the stress-related increase in IGFBP-1a was caused by cortisol, although we did not measure plasma cortisol levels. In Biwa salmon, co-injection with GH diminished the increased levels of IGFBP-1a, suggesting a counteractive effect of GH on cortisol. The same trend was seen in masu salmon but not in amago salmon; however, the reason for this finding is unknown.

A transient increase in serum IGFBP-1b but not IGFBP-1a in masu and amago salmon subjected to sham injection suggests that IGFBP-1b is more sensitive to stress than IGFBP-1a. We previously reported a difference in the sensitivity between IGFBP-1a and -1b to osmotic stress in Chinook salmon parr transferred prematurely to seawater: plasma IGFBP-1b was induced faster than IGFBP-1a (Shimizu et al., 2011a). Moreover, Kawaguchi et al. (2013) reported that although both serum IGFBP-1a and -1b were induced by fasting, IGFBP-1b responded earlier than IGFBP-1a. In zebrafish (*Danio rerio*) embryos, both IGFBP-1a and -1b were inhibitory to IGF-I action, but the degree of inhibition by IGFBP-1a was stronger (Kamei et al., 2008). We previously produced recombinant proteins for masu salmon IGFBP-1a and -1b and showed that IGFBP-1a was effective in inhibiting IGF-I action in a primary pituitary culture system (Tanaka et al., 2018). These findings suggest that IGFBP-1b is more sensitive to stress, whereas IGFBP-1a is more potent in inhibiting IGF-I action under severe catabolic conditions. Such subfunction partitioning may allow fish to fine-tune their growth speed and energy allocation under changing environments, as discussed by Allard and Duan (2018).

The lack of response of Biwa salmon plasma IGFBP-1b to sham injections suggests a subspecies difference in sensitivity to acute stress. Biwa salmon is a land-locked subspecies with

a considerably lower hypo-osmoregulatory ability than the anadromous amago salmon (Fujioka and Fushiki, 1989). We previously reported that Biwa salmon responded to cortisol injection by increasing gill NKA activity but not seawater-type *nka α1b* mRNA levels (Nakajima et al., 2014). In contrast, amago salmon increased both, suggesting that Biwa salmon are less sensitive to cortisol. A comparison of *O. masou* subspecies with different responses to stress may be useful for identifying factors involved in the induction of cortisol or/and the sensitivity to cortisol.

Plasma IGFBP-1b was induced by cortisol in masu and Biwa salmon but not in amago salmon. The lack of a cortisol effect was due to the higher levels in the sham group than in the initial group. Although a direct involvement of cortisol in the increased IGFBP-2b levels in the sham group was not demonstrated, cortisol levels in that group might already be high enough to induce IGFBP-1b. Cortisol has been shown to induce IGFBP-1 in mammals and several fish species such as tilapia and channel catfish (Lee et al., 1993; Kajimura et al., 2003; Peterson and Small, 2005). The induction of IGFBP-1 and low-molecular-weight IGFBP (approximately 20–24 kDa) by stress has been reported in many fish species (Kelley et al., 2000, 2001; Shimizu et al., 2011a). However, there are few studies demonstrating the effect of cortisol on IGFBPs, to which the present study adds supportive evidence. In mammals, glucocorticoid action is directed to the glucocorticoid receptor responsive element (GRE) in the proximate promoter region of the *IGFBP-1* gene (Lee et al., 1997). However, the presence of functional GRE in the promoter region of fish *igfbp-1* remains to be identified.

The present study suggests that although cortisol induced plasma IGFBP-1b, the effect was reduced by co-injection with GH. Park et al. (2000) examined the effects of GH and prolactins on plasma IGFBPs in hypophysectomized tilapia. In that study, hypophysectomy increased plasma IGFBP (20 kDa) levels, whereas GH injection reduced it, suggesting that GH had a suppressive effect on IGFBP. If tilapia 20-kDa IGFBP is an IGFBP-1, our findings are in agreement with those of Park et al. (2000). The effect of GH appears to be short term, disappearing 2 days after injection, in Biwa salmon.

Salmonid GH has been shown to reduce *igfbp-1b* mRNA levels and immunoreactive IGFBP-1b released from cultured hepatocytes of coho salmon at a concentration as low as 0.25 nM (Pierce et al., 2006). In addition, a direct suppressive effect of GH on IGFBP-1 has been reported in humans (Nørrelund et al., 1999). Our results suggest that porcine GH alone did not reduce IGFBP-1b below the basal levels in contrast with those findings. Shimizu et al. (2007) reported that porcine GH injected into postsmolt coho salmon at a dose of 8 µg/g body weight was retained in the circulation at approximately 380 ng/mL 1 day after injection. Assuming that

the effectiveness of mammalian GH is one-tenth that of homologous GH, as reported in Atlantic salmon (*Salmo salar*; Boeuf et al., 1994), circulating porcine GH would have been approximately 1.7 nM 1 day after injection, which is higher than the effective dose required to reduce hepatic *igfbp-1b* (Pierce et al., 2006). It is still unknown whether this contrast is attributed to species or stage differences, or in vitro versus in vivo experiments, or a combination of these. The same authors also examined the combined effects of salmon GH and Dex on *igfbp-1b* mRNA in primary cultured coho salmon hepatocytes. In that study, Dex strongly increased *igfbp-1b* levels by approximately eight-fold. Co-treatment with GH reduced Dex-stimulated *igfbp-1b* levels, although they remained higher than the control levels (Pierce et al., 2006). The findings of the present study provide further evidence of the counteractive effect of GH on IGFBP-1b in vivo.

The significance of the counteractive effects of GH and cortisol on IGFBP-1b and -1a needs to be addressed in future studies. However, the results of the present study suggest that GH plays an important role in integrating the somatotrophic and stress axes in salmonids by regulating IGFBP-1b and -1a, which are likely inhibitory to IGF-I action. Insulin, the primary suppressor of IGFBP-1 synthesis and secretion in mammals, is not effective to reduce *igfbp-1b* in salmon hepatocytes (Pierce et al., 2006). Thus, GH may be a major suppressor of IGFBP-1b in salmon rather than insulin. Such regulation may be important in regulating the availability of IGF-I under stressful conditions.

Despite some differences among the three subspecies of *O. masou*, our results indicate that cortisol is a primary inducer of IGFBP-1b and -1a, whereas GH counteracts the cortisol action in the short term. Our findings suggest that GH plays a role in integrating growth and stress response by suppressing the cortisol-stimulated increase in IGFBP-1b and -1a. In addition, the *O. masou* subspecies-complex offers a unique opportunity to investigate the endocrine control of growth in relation to other life-history traits such as stress response and hypo-osmoregulatory ability.

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Figure legends

Fig. 1. Effects of GH and cortisol (F) on plasma IGF-I in masu (a,b), amago (c,d) and Biwa salmon (e,f). Underyearlings were injected with porcine GH (8 µg/g body weight) or/and cortisol (40 µg/g body weight) and sampled 1 (a,c,e) and 2 days (b,d,f) after injection. Sham group received vehicle only. Values are expressed as means ± SE (*n* for each treatment is shown under each bar). Individual data are also shown as dots. Symbols sharing the same letters are not significantly different from each other (Tukey-Kramer test, *P* < 0.05).

Fig. 2. Representative ligand blots for IGFbps in plasma of masu salmon treated with GH or/and cortisol for one day. Two microliters of plasma were separated by 12.5% SDS-PAGE under non-reducing conditions, electroblotted onto nitrocellulose membranes and subjected with ligand blotting using digoxigenin-labeled human IGF-I (50 ng) and antiserum against digoxigenin (1:20,000). Arrowheads indicate migration positions of human (left) and salmon (right) IGFBP bands. NS: non-specific; NHS: normal human serum; Fed: serum from fed masu salmon as a reference.

Fig. 3. Effects of GH and cortisol (F) on plasma IGFBP-2b in masu (a,b), amago (c,d) and Biwa salmon (e,f). Underyearlings were injected with porcine GH (8 µg/g body weight) or/and cortisol (40 µg/g body weight) and sampled 1 (a,c,e) and 2 days (b,d,f) after injection. Sham group received vehicle only. Band intensities of IGFBP-2b on ligand blotting were semi-quantified as arbitrary density unit (ADU). Values are expressed as means ± SE (*n* = 5-8, except *n* = 3 in the GH+F group in Biwa salmon on day 1). Individual data are also shown as dots. A cross indicates a significantly lower levels in the sham group compared to the initial (no injection) group. Asterisks indicate main effects.

Fig. 4. Effects of GH and cortisol (F) on plasma IGFBP-1a in masu (a,b), amago (c,d) and Biwa salmon (e,f). Underyearlings were injected with porcine GH (8 µg/g body weight) or/and cortisol (40 µg/g body weight) and sampled one (a,c,e) and two days (b,d,f) after injection. Sham group received vehicle only. Band intensities of IGFBP-1a on ligand blotting were semi-quantified as arbitrary density unit (ADU). Values are expressed as means ± SE (*n* = 5-8, except *n* = 3 in the GH+F group in Biwa salmon on day 1). Individual data are also shown as dots. Asterisks indicate main effects or an interaction.

Fig. 5. Effects of GH and cortisol (F) on plasma IGFBP-1b in masu (a,b), amago (c,d) and Biwa salmon (e,f). Underyearlings were injected with porcine GH (8 µg/g body weight) or/and cortisol (40 µg/g body weight) and sampled one (a,c,e) and two days (b,d,f) after injection. Sham group received vehicle only. Band intensities of IGFBP-1b on ligand blotting were semi-quantified as arbitrary density unit (ADU). Values are expressed as means ± SE ($n = 5-8$, except $n = 3$ in the GH+F group in Biwa salmon on day 1). Individual data are also shown as dots. Crosses indicate significantly higher levels in the sham group compared to the initial (no injection) group. Asterisks indicate main effects or an interaction. Symbols sharing the same letters are not significantly different from each other (Tukey-Kramer test, $P < 0.05$).

Suppl. Fig. 1. Effects of sham injection on plasma IGFBP-1b in masu (a), amago (b) and Biwa salmon. Underyearlings were untreated (initial) or injected with vehicle (17 mM phosphate, 0.13 M NaCl, pH 7.0 containing 0.08% bovine serum albumin, 15.9% ethanol). Crosses indicate significant differences between groups (t -test, $P < 0.05$).