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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 land-locked 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 × 29.5 × 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 μg/g body weight, cortisol (Sigma) at 40 μg/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

303 a considerably lower hypo-osmoregulatory ability than the anadromous amago salmon (Fujioka
304 and Fushiki, 1989). We previously reported that Biwa salmon responded to cortisol injection by
305 increasing gill NKA activity but not seawater-type *nka α1b* mRNA levels (Nakajima et al., 2014).
306 In contrast, amago salmon increased both, suggesting that Biwa salmon are less sensitive to
307 cortisol. A comparison of *O. masou* subspecies with different responses to stress may be useful
308 for identifying factors involved in the induction of cortisol or/and the sensitivity to cortisol.
309 Plasma IGFBP-1b was induced by cortisol in masu and Biwa salmon but not in amago
310 salmon. The lack of a cortisol effect was due to the higher levels in the sham group than in the
311 initial group. Although a direct involvement of cortisol in the increased IGFBP-2b levels in the
312 sham group was not demonstrated, cortisol levels in that group might already be high enough to
313 induce IGFBP-1b. Cortisol has been shown to induce IGFBP-1 in mammals and several fish
314 species such as tilapia and channel catfish (Lee et al., 1993; Kajimura et al., 2003; Peterson and
315 Small, 2005). The induction of IGFBP-1 and low-molecular-weight IGFBP (approximately 20–
316 24 kDa) by stress has been reported in many fish species (Kelley et al., 2000, 2001; Shimizu et
317 al., 2011a). However, there are few studies demonstrating the effect of cortisol on IGFBPs, to
318 which the present study adds supportive evidence. In mammals, glucocorticoid action is directed
319 to the glucocorticoid receptor responsive element (GRE) in the proximate promoter region of the
320 *IGFBP-1* gene (Lee et al., 1997). However, the presence of functional GRE in the promoter region
321 of fish *igfbp-1* remains to be identified.
322 The present study suggests that although cortisol induced plasma IGFBP-1b, the effect
323 was reduced by co-injection with GH. Park et al. (2000) examined the effects of GH and prolactins
324 on plasma IGFBPs in hypophysectomized tilapia. In that study, hypophysectomy increased
325 plasma IGFBP (20 kDa) levels, whereas GH injection reduced it, suggesting that GH had a
326 suppressive effect on IGFBP. If tilapia 20-kDa IGFBP is an IGFBP-1, our findings are in
327 agreement with those of Park et al. (2000). The effect of GH appears to be short term, disappearing
328 2 days after injection, in Biwa salmon.
329 Salmonid GH has been shown to reduce *igfbp-1b* mRNA levels and immunoreactive
330 IGFBP-1b released from cultured hepatocytes of coho salmon at a concentration as low as 0.25
331 nM (Pierce et al., 2006). In addition, a direct suppressive effect of GH on IGFBP-1 has been
332 reported in humans (Nørrelund et al., 1999). Our results suggest that porcine GH alone did not
333 reduce IGFBP-1b below the basal levels in contrast with those findings. Shimizu et al. (2007)
334 reported that porcine GH injected into postsmolt coho salmon at a dose of 8 μg/g body weight
335 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 somatotropic 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.

Acknowledgments

We thank Yoshitaka Kataoka, Samegai Trout Farm, Shiga Prefectural Fishery Experiment Station, Japan, for providing Biwa and amago salmon and his help in hormone injection experiment, and Estsuro Yamaha and Eisuke Takahashi, Nanae Freshwater Experimental Station, Field Science Center for Northern Biosphere, Hokkaido University, Japan, for providing masu salmon. We acknowledge Editage (www.editage.com) for English language editing. This work was supported by the grants from the Japan Society for the Promotion of Science (JSPS): the JSPS Bilateral Joint

Research Project (Open Partnership with Norway) Grant Number JPJSBP120189903 and KAKENHI Grant Number 19H0302409.

References

Allard, J.B., Duan, C.M., 2018. IGF-binding proteins: Why do they exist and why are there so many? Front. Endocrinol. 9, 117.

Bach, L.A., 2018. IGF-binding proteins. J. Mol. Endocrinol. 61, T11-T28.

Baxter, R.C., Martin, J.L., 1989. Structure of the Mr 140,000 growth hormone-dependent insulin-like growth factor binding protein complex: determination by reconstitution and affinity-labeling. Proc. Natl. Acad. Sci. USA 86, 6898-6902.

Boeuf, G., Marc, A.M., Prunet, P., Lebail, P.Y., Smal, J., 1994. Stimulation of parr-smolt transformation by hormonal treatment in Atlantic salmon (*Salmo salar* L). Aquaculture 121, 195-208.

Chin, E., Zhou, J., Dai, J., Baxter, R.C., Bondy, C.A., 1994. Cellular localization and regulation of gene expression for components of the insulin-like growth factor ternary binding protein complex. Endocrinology 134, 2498-2504.

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

de la Serrana, D.G., Macqueen, D.J., 2018. Insulin-Like Growth Factor-Binding Proteins of Teleost Fishes. Front. Endocrinol. 9, 80.

Firth, S.M., Baxter, R.C., 2002. Cellular actions of the insulin-like growth factor binding proteins. Endocr. Rev. 23, 824-854.

Fujioka, Y., 1991. Morphological, physiological and ecological studies on biwa salmon. Bull. Shiga Pref. Samegai Trout Farm 3, 1-112. (In Japanese with English abstract)

Fujioka, Y., 2006. Relationships between growth and food intake of juvenile biwa and amago salmon. Bull. Shiga Pref. Fish. Exp. Sta. 51, 43-49. (In Japanese with English abstract)

Fujioka, Y., Fushiki, S., 1989. Seasonal changes in hypoosmoregulatory ability of biwa salmon *Oncorhynchus rhodurus* and amago salmon *O. rhodurus*. Nippon Suisan Gakkaishi 55, 1885-1892.

Fukazawa, Y., Siharath, K., Iguchi, T., Bern, H.A., 1995. In vitro secretion of insulin-like growth factor-binding proteins from liver of striped bass, Morone saxatilis. Gen. Comp. Endocrinol. 99, 239-247.

Gwo, J.-C., Hsu, T.-H., Lin, K.-H., Chou, Y.-C., 2008. Genetic relationship among four subspecies of cherry salmon (*Oncorhynchus masou*) inferred using AFLP. Mol. Phylogenet. Evol. 48, 776-781.

Johnson, J., Silverstein, J., Wolters, W.R., Shimizu, M., Dickhoff, W.W., Shepherd, B.S., 2003. Disparate regulation of insulin-like growth factor-binding proteins in a primitive, ictalurid, teleost (*Ictalurus punctatus*). Gen. Comp. Endocrinol. 134, 122-130.

Kajimura, S., Hirano, T., Visitacion, N., Moriyama, S., Aida, K., Grau, E.G., 2003. Dual mode of cortisol action on GH/IGF-I/IGF binding proteins in the tilapia, *Oreochromis mossambicus*. J. Endocrinol. 178, 91-99.

Kamei, H., Lu, L., Jiao, S., Li, Y., Gyrup, C., Laursen, L.S., Oxvig, C., Zhou, J., Duan, C., 2008. Duplication and diversification of the hypoxia-inducible IGFBP-1 gene in zebrafish. PLoS One 3, e3091.

Kaneko, N., Nilsen, T.O., Tanaka, H., Hara, A., Shimizu, M., 2020. Intact rather than total circulating insulin-like growth factor binding protein-1a is a negative indicator of growth in masu salmon. Am. J. Physiol. Regul. Integ. Comp. Physiol. 318, R329-R337.

Kato, F. 1991. Life history of masu and amago salmon (*Oncorhynchus masou* and *Oncorhynchus rhodurus*). In: Groot, C., Margolis, L. (Eds.), Pacific Salmon Life Histories, University of British Columbia Press, Vancouver, BC, pp. 449-520.

Katz, L.E., Satin-Smith, M.S., Collett-Solberg, P., Baker, L., Stanley, C.A., Cohen, P., 1998. Dual regulation of insulin-like growth factor binding protein-1 levels by insulin and cortisol during fasting. J. Clin. Endocrinol. Metab. 83, 4426-4430.

Kawaguchi, K., Kaneko, N., Fukuda, M., Nakano, Y., Kimura, S., Hara, A., Shimizu, M., 2013. Responses of insulin-like growth factor (IGF)-I and two IGF-binding protein-1 subtypes to fasting and re-feeding, and their relationships with individual growth rates in yearling masu salmon (*Oncorhynchus masou*). Comp. Biochem. Physiol. A 165, 191-198.

Kelley, K.M., Siharath, K., Bern, H.A., 1992. Identification of insulin-like growth factor-binding proteins in the circulation of four teleost fish species. J. Exp. Zool. 263, 220-224.

Kelley, K.M., Desai, P., Roth, J.T., Haigwood, J.T., Arope, S.A., Flores, R.M., Schmidt, K.E., Perez, M., Nicholson, G.S., Song, W.W., 2000. Evolution of endocrine growth regulation: the insulin like growth factors (IGFs), their regulatory binding proteins (IGFBPs), and IGF receptors in fishes and other ectothermic vertebrates. In: Fingerman, M., Nagabhushanam, R. (Eds.), Recent Advances in Marine Biotechnology, Aquaculture, Part B Fishes, 4, Science Publishers, Plymouth, UK, pp. 189-228.

Kelley, K.M., Haigwood, J.T., Perez, M., Galima, M.M., 2001. Serum insulin-like growth factor binding proteins (IGFBPs) as markers for anabolic/catabolic condition in fishes. Comp. Biochem. Physiol. B 129, 229-236.

438 Kubo, T., 1980. Studies on the life history of the “masu” salmon (*Oncorhynchus masou*) in
439 Hokkaido. Sci. Rep. Hokkaido Salmon Hatch. 34, 1–95. (In Japanese with English abstract)
440 Le Roith, D., Bondy, C., Yakar, S., Liu, J.L., Butler, A., 2001. The somatomedin hypothesis: 2001.
441 Endocr. Rev. 22, 53-74.
442 Lee, P.D., Conover, C.A., Powell, D.R., 1993. Regulation and function of insulin-like growth
443 factor-binding protein-1. Proc. Soc. Exp. Biol. Med. 204, 4-29.
444 Lee, P.D., Giudice, L.C., Conover, C.A., Powell, D.R., 1997. Insulin-like growth factor binding
445 protein-1: recent findings and new directions. Proc. Soc. Exp. Biol. Med. 216, 319-357.
446 Mancera, J.M., McCormick, S.D., 2007. Role of prolactin, growth hormone, insulin-like growth
447 factor I and cortisol in teleost osmoregulation. In: Baldisserotto, B., Mancera, J.M.,
448 Kapoor, B.G. (Eds.), Fish Osmoregulation, Science Publishers, Enfield, NH, pp. 497-515.
449 McCormick, S.D., 2001. Endocrine control of osmoregulation in teleost fish. Am. Zool. 41, 781-
450 794.
451 Nakajima, T., Shimura, H., Yamazaki, M., Fujioka, Y., Ura, K., Hara, A., Shimizu, M., 2014. Lack
452 of hormonal stimulation prevents the landlocked Biwa salmon (*Oncorhynchus masou*
453 subspecies) from adapting to seawater. Am. J. Physiol. Regul. Integ. Comp. Physiol. 307,
454 R414-R425.
455 Nørrelund, H., Fisker, S., Vahl, N., Borglum, J., Richelsen, B., Christiansen, J.S., Jorgensen,
456 J.O.L., 1999. Evidence supporting a direct suppressive effect of growth hormone on
457 serum IGFBP-1 levels. Experimental studies in normal, obese and GH-deficient adults.
458 Growth Hormon. IGF Res. 9, 52-60.
459 Ocampo Daza, D., Sundstrom, G., Bergqvist, C.A., Duan, C.M., Larhammar, D., 2011. Evolution
460 of the insulin-like growth factor binding protein (IGFBP) family. Endocrinology 152,
461 2278-2289.
462 Ohlsson, C., Mohan, S., Sjogren, K., Tivesten, A., Isgaard, J., Isaksson, O., Jansson, J.O.,
463 Svensson, J., 2009. The role of liver-derived insulin-like growth factor-I. Endocr. Rev.
464 30, 494-535.
465 Oohara, I., Okazaki, T., 1996. Genetic relationship among three subspecies of Oncorhynchus
466 masou determined by mitochondrial DNA sequence analysis. Zool. Sci. 13, 189-198.
467 Park, R., Shepherd, B.S., Nishioka, R.S., Grau, E.G., Bern, H.A., 2000. Effects of homologous
468 pituitary hormone treatment on serum insulin-like growth-factor-binding proteins
469 (IGFBPs) in hypophysectomized tilapia, *Oreochromis mossambicus*, with special
470 reference to a novel 20-kDa IGFBP. Gen. Comp. Endocrinol. 117, 404-412.

471 Peterson, B.C., Small, B.C., 2005. Effects of exogenous cortisol on the GH/IGF-I/IGFBP network
472 in channel catfish. Domest. Anim. Endocrinol. 28, 391-404.
473 Pierce, A.L., Fukada, H., Dickhoff, W.W., 2005. Metabolic hormones modulate the effect of
474 growth hormone (GH) on insulin-like growth factor-I (IGF-I) mRNA level in primary
475 culture of salmon hepatocytes. J. Endocrinol. 184, 341-349.
476 Pierce, A.L., Shimizu, M., Felli, L., Swanson, P., Dickhoff, W.W., 2006. Metabolic hormones
477 regulate insulin-like growth factor binding protein-1 mRNA levels in primary cultured
478 salmon hepatocytes; lack of inhibition by insulin. J. Endocrinol. 191, 379-386.
479 Rajaram, S., Baylink, D.J., Mohan, S., 1997. Insulin-like growth factor-binding proteins in serum
480 and other biological fluids: regulation and functions. Endocr. Rev. 18, 801-831.
481 Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image
482 analysis. Nat. Methods 9, 671-675.
483 Shimizu, M., Dickhoff, W.W., 2017. Circulating insulin-like growth factor binding proteins in
484 fish: Their identities and physiological regulation. Gen. Comp. Endocrinol. 252, 150-161.
485 Shimizu, M., Swanson, P., Fukada, H., Hara, A., Dickhoff, W.W., 2000. Comparison of extraction
486 methods and assay validation for salmon insulin-like growth factor-I using commercially
487 available components. Gen. Comp. Endocrinol. 119, 26-36.
488 Shimizu, M., Hara, A., Dickhoff, W.W., 2003a. Development of an RIA for salmon 41 kDa IGF-
489 binding protein. J. Endocrinol. 178, 275-283.
490 Shimizu, M., Swanson, P., Hara, A., Dickhoff, W.W., 2003b. Purification of a 41-kDa insulin-like
491 growth factor binding protein from serum of chinook salmon, *Oncorhynchus tshawytscha*.
492 Gen. Comp. Endocrinol. 132, 103-111.
493 Shimizu, M., Dickey, J.T., Fukada, H., Dickhoff, W.W., 2005. Salmon serum 22 kDa insulin-like
494 growth factor-binding protein (IGFBP) is IGFBP-1. J. Endocrinol. 184, 267-276.
495 Shimizu, M., Fukada, H., Hara, A., Dickhoff, W.W., 2007. Response of the salmon somatotropic
496 axis to growth hormone administration under two different salinities. Aquaculture 273,
497 320-328.
498 Shimizu, M., Kishimoto, K., Yamaguchi, T., Nakano, Y., Hara, A., Dickhoff, W.W., 2011a.
499 Circulating salmon 28- and 22-kDa insulin-like growth factor binding proteins (IGFBPs)
500 are co-orthologs of IGFBP-1. Gen. Comp. Endocrinol. 174, 97-106.
501 Shimizu, M., Suzuki, S., Horikoshi, M., Hara, A., Dickhoff, W.W., 2011b. Circulating salmon 41-
502 kDa insulin-like growth factor binding protein (IGFBP) is not IGFBP-3 but an IGFBP-2
503 subtype. Gen. Comp. Endocrinol. 171, 326-331.

Small, B.C., Peterson, B.C., 2005. Establishment of a time-resolved fluoroimmunoassay for measuring plasma insulin-like growth factor I (IGF-I) in fish: effect of fasting on plasma concentrations and tissue mRNA expression of IGF-I and growth hormone (GH) in channel catfish (*Ictalurus punctatus*). Domest. Anim. Endocrinol. 28, 202-215.

Suzuki, S., Takahashi, E., Nilsen, T.O., Kaneko, N., Urabe, H., Ugachi, Y., Yamaha, E., Shimizu, M., 2020. Physiological changes in off-season smolts induced by photoperiod manipulation in masu salmon (*Oncorhynchus masou*). Aquaculture 526, 735353.

Tanaka, H., Oishi, G., Nakano, Y., Mizuta, H., Nagano, Y., Hiramatsu, N., Ando, H., Shimizu, M., 2018. Production of recombinant salmon insulin-like growth factor binding protein-1 subtypes. Gen. Comp. Endocrinol. 257, 184-191.

Unterman, T.G., Oehler, D.T., Murphy, L.J., Lacson, R.G., 1991. Multihormonal regulation of insulin-like growth factor-binding protein-1 in rat H4IIE hepatoma cells: the dominant role of insulin. Endocrinology 128, 2693-2701.

Villafuerte, B.C., Koop, B.L., Pao, C.I., Gu, L., Birdsong, G.G., Phillips, L.S., 1994. Coculture of primary rat hepatocytes and nonparenchymal cells permits expression of insulin-like growth factor binding protein-3 in vitro. Endocrinology 134, 2044-2050.

Wood, A.W., Duan, C., Bern, H.A., 2005. Insulin-like growth factor signaling in fish. Int. Rev. Cytol. 243, 215-285.

Yamamoto, S., Morita, K., Kikko, T., Kawamura, K., Sato, S., Gwo, J.C., 2020. Phylogeography of a salmonid fish, masu salmon *Oncorhynchus masou* subspecies-complex, with disjunct distributions across the temperate northern Pacific. Freshwater Biol. 65, 698-715.

Zapf, J., 1995. Physiological role of the insulin-like growth factor binding proteins. Eur. J. Endocrinol. 132, 645-654.

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.

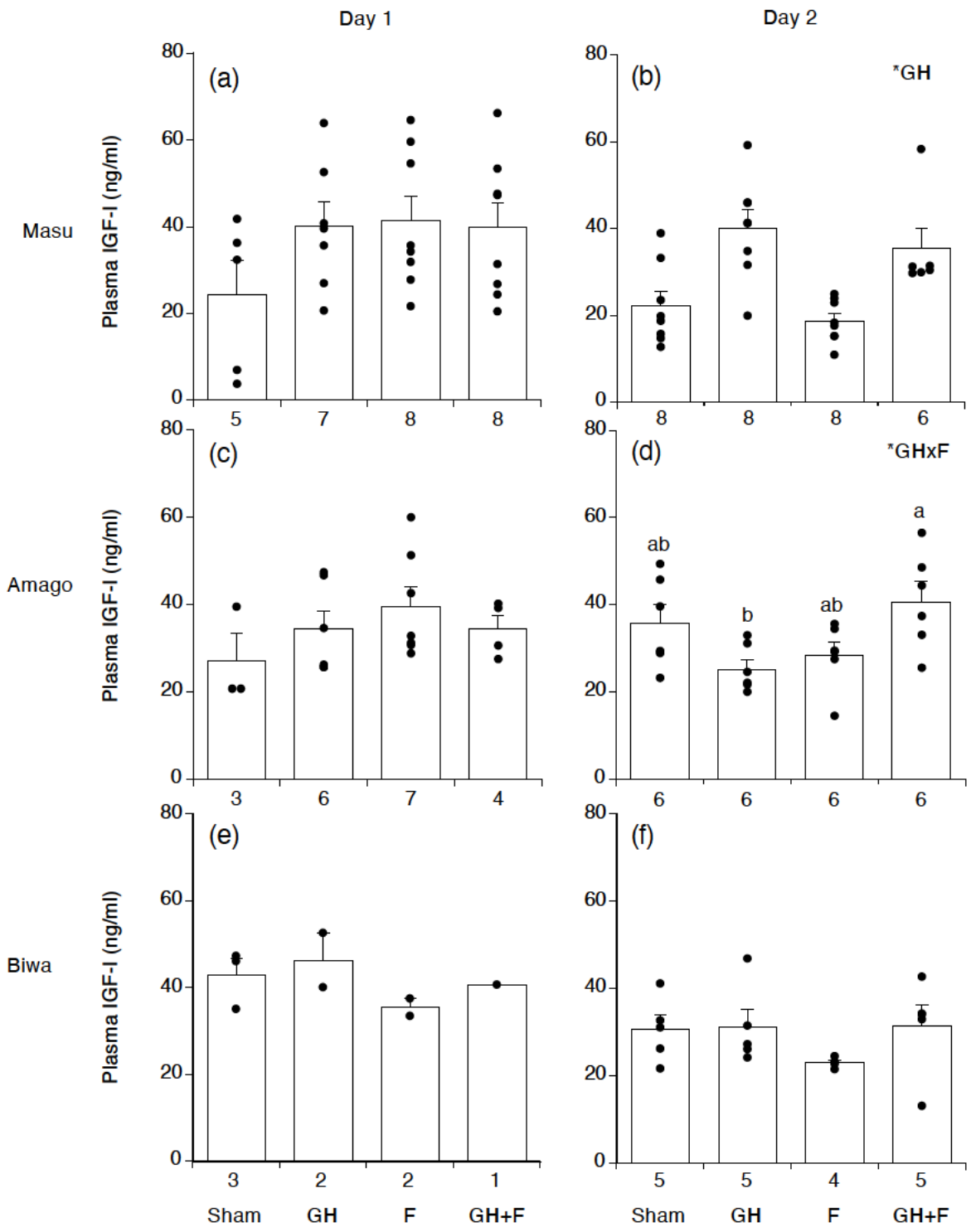
Fig. 3. Effects of GH and cortisol (F) on plasma IGFBP-2b in masu (a,b), amago (c,d) and Biwa salmon (e,f). Underyearlings were injected with porcine GH (8 μg/g body weight) or/and cortisol (40 μg/g body weight) and sampled 1 (a,c,e) and 2 days (b,d,f) after injection. Sham group received vehicle only. 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.

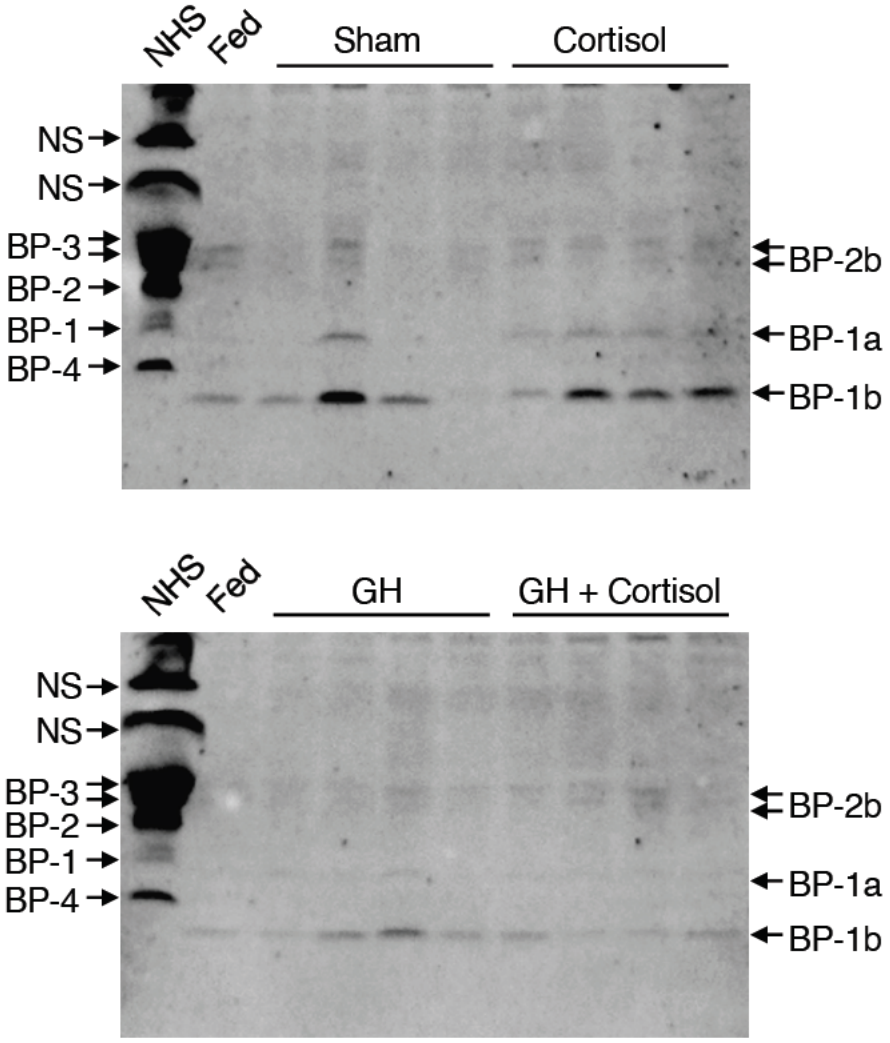
Fig. 4. Effects of GH and cortisol (F) on plasma IGFBP-1a in masu (a,b), amago (c,d) and Biwa salmon (e,f). Underyearlings were injected with porcine GH (8 μg/g body weight) or/and cortisol (40 μg/g body weight) and sampled one (a,c,e) and two days (b,d,f) after injection. Sham group received vehicle 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, $P < 0.05$).

Figure

Yamaguchi et al., Fig. 1




NHS
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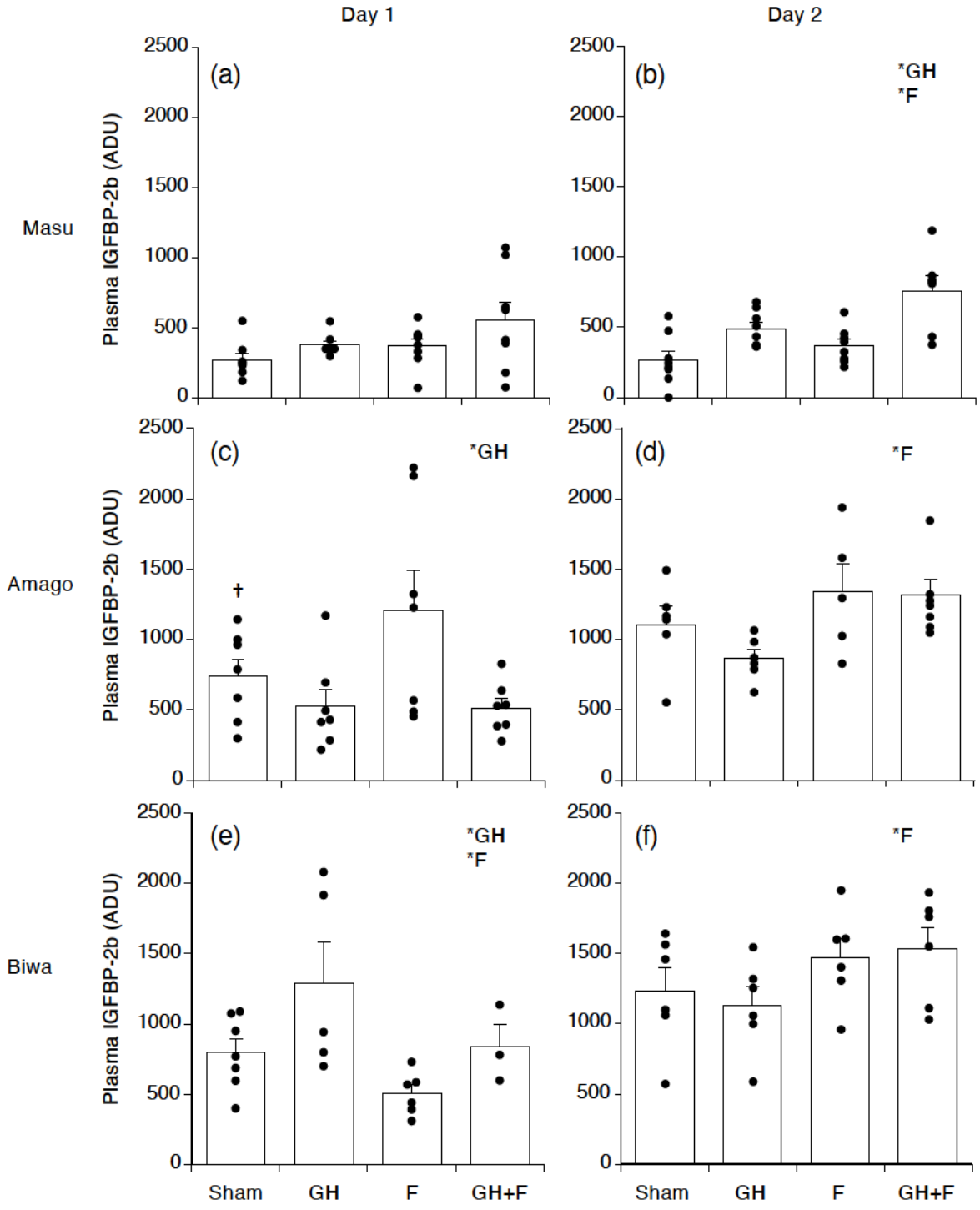
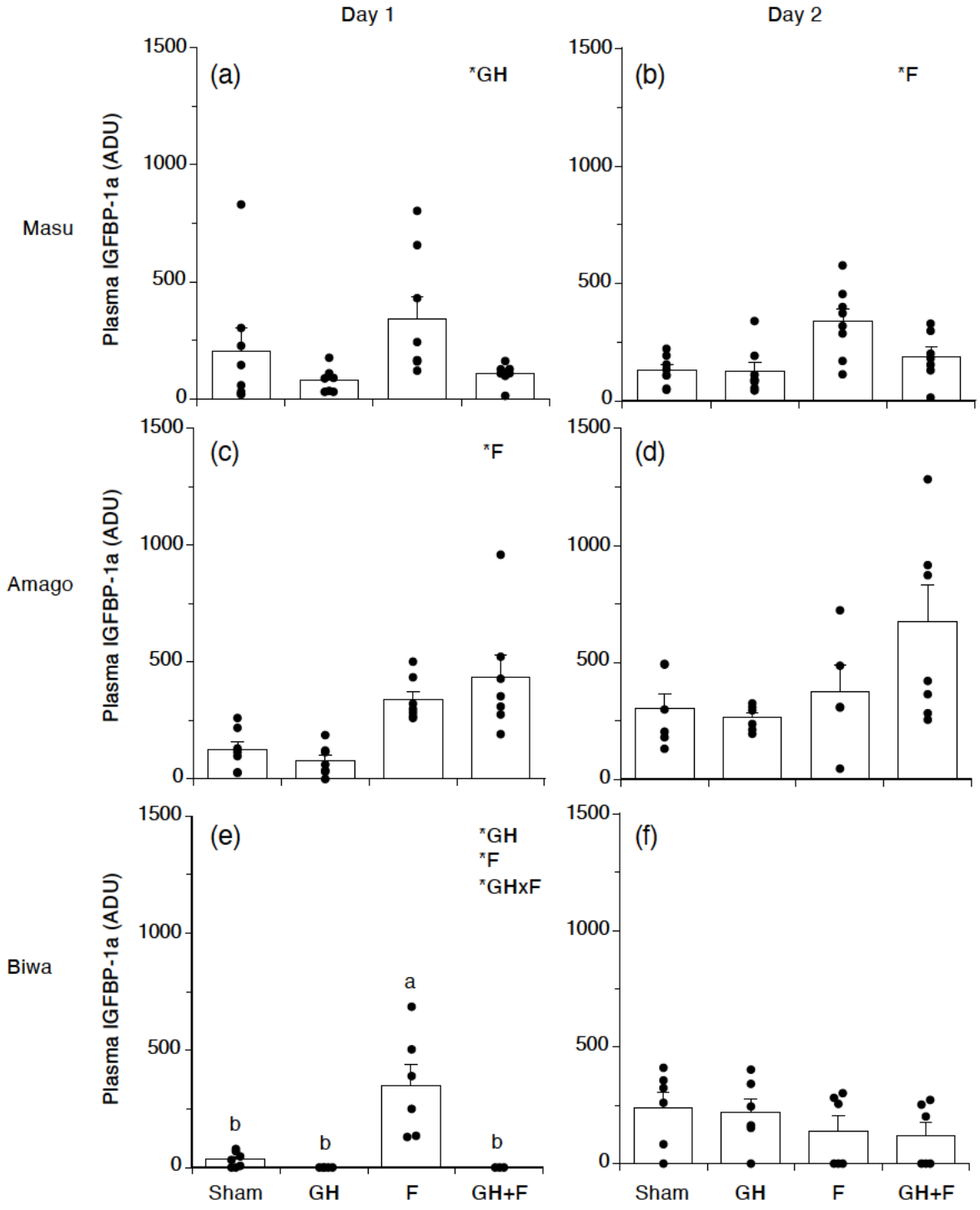

Day 1
Day 2
Masu
Amago
Biwa
Plasma IGFBP-2b (ADU)
(a)
(b)
(c)
(d)
(e)
(f)
*GH
*F
†
0
500
1000
1500
2000
2500
Sham
GH
F
GH+F


Day 1
Day 2
Masu
Amago
Biwa
Plasma IGFBP-1a (ADU)
1500
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(a)
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Sham
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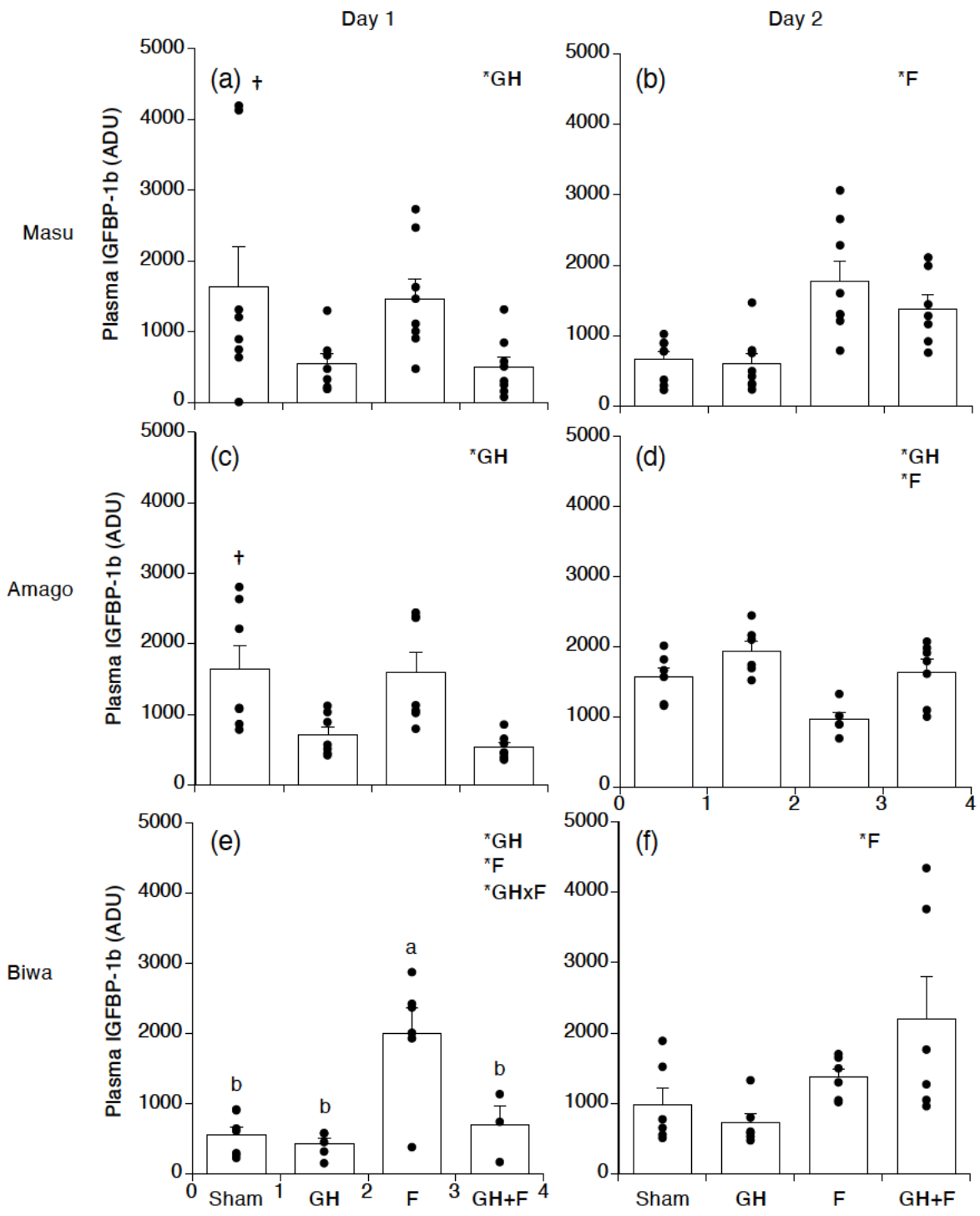
Day 1
Day 2
Masu
Amago
Biwa
Plasma IGFBP-1b (ADU)
(a)
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*GH
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(c)
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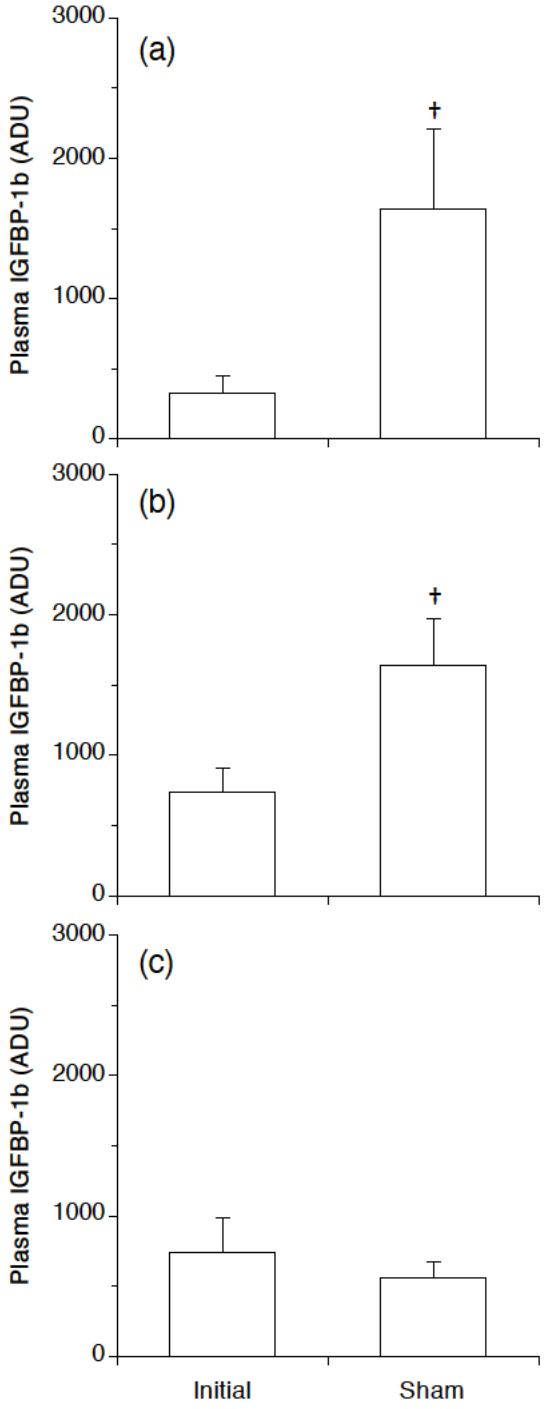

Suppl. Fig. 1. Effects of sham injection on plasma IGFBP-1b in masu (a), amago (b) and Biwa salmon. Underyerlings were untreated (initial) or injected with vehicle (17 mM phosphate, 0.13 M NaCl, pH 7.0 containing 0.08% bovine serum albumin, 15.9% ethanol). Crosses indicate significant differences between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