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

4

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17

18 **Abstract**

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

35

36 **Keywords**

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

39 **1. Introduction**

40 The growth hormone (GH)/insulin-like growth factor (IGF)-I system is central to growth
41 regulation in vertebrates. GH stimulates the hepatic production of IGF-I, which mediates GH
42 actions (Daughaday and Rotwein, 1989). In mammals, IGF-I synthesis also occurs in peripheral
43 tissues, where it acts as a local growth factor essential for normal postnatal growth (Le Roith et
44 al., 2001; Ohlsson et al., 2009). However, IGF-I activity is regulated by multiple IGF-binding
45 proteins (IGFBPs). These proteins either inhibit or potentiate the action of IGF-I, depending on
46 their type, post-translational modification, and cellular environment (Firth and Baxter, 2002; Bach,
47 2018). This additional layer of regulation of IGF-I by IGFBPs is important for fine-tuning growth
48 speed and energy partitioning under conditions of malnutrition and stress (Allard and Duan, 2018).

49 In mammals, six members of the IGFBP family are secreted into the bloodstream
50 (Rajaram et al., 1997; Bach, 2018): IGFBP-3 and IGFBP-1 are the two main, best-characterized
51 forms. The most abundant IGFBP in the mammalian circulation is IGFBP-3, which transports
52 75–80% of IGF-I by forming a ternary complex with the ligand and an acid-labile subunit (ALS;
53 Baxter and Martin, 1989; Zapf, 1995; Rajaram et al., 1997). The concentration of IGFBP-1 in the
54 circulation is much lower than that of IGFBP-3; however, it shows large fluctuations in response
55 to meals and metabolic states and may play a role in regulating free IGF-I (Lee et al., 1993, 1997).

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

63 Two to three IGFBPs are typically detected in the plasma/serum of teleosts; however,
64 their identities have been the subject of debate for approximately two decades (Kelley et al., 2000;
65 Wood et al., 2005; Shimizu and Dickhoff, 2017). In Chinook salmon (*Oncorhynchus tshawytscha*),
66 three major plasma/serum IGFBP bands at 41, 28, and 22 kDa have been identified as IGFBP-2b,
67 -1a, and -1b, respectively (Shimizu et al., 2005, 2011a,b). In salmon, there is no evidence of the
68 presence of IGFBP-3 in the circulation; however, very low levels of *igfbp-3* mRNA have been
69 detected in several tissues (Shimizu et al., 2011b). Although salmonids are the only group in
70 which circulating IGFBPs have been specifically identified, such proteins will likely be identified
71 in other teleosts.

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

82 Fish IGFbps are also regulated by hormones. Fukazawa et al. (1995) examined the
83 effects of several hormones on the secretion of IGFbps from the liver pieces of striped bass
84 (*Morone saxatilis*). Kelley et al. (1992, 2001) reported that a synthetic glucocorticoid,
85 dexamethasone (Dex), induced two low-molecular-weight IGFbps (25–30 kDa and 20–25 kDa)
86 into the circulation. Salmon IGFbp-2b, which corresponds to the 41-kDa form in the circulation,
87 is physiologically equivalent to mammalian IGFbp-3. It is regulated by GH and acts as a major
88 carrier of circulating IGF-I (Shimizu et al., 2003a,b, 2007, 2011b). In tilapia (*Oreochromis*
89 *mossambicus*), cortisol suppresses plasma IGFbp (40 kDa) and IGF-I (Kajimura et al., 2003).
90 Salmonid IGFbp-1a and -1b and low-molecular-weight IGFbps in the plasma/serum of other
91 fishes are induced by stress from events such as handling, confinement, and salinity changes,
92 most likely via cortisol (Kelley et al., 2001; Johnson et al., 2003; Peterson and Small, 2005;
93 Shimizu et al., 2005, 2011a). Cortisol and GH cooperate to enhance the hypo-osmoregulatory
94 ability in salmon and other euryhaline fishes (McCormick, 2001; Mancera and McCormick,
95 2007); therefore, determining whether they have a combined effect on growth, particularly on
96 circulating IGFbps, is an important question that needs to be addressed.

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

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

119

120 **2. Materials and Methods**

121 *2.1. Fish*

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

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

133

134 *2.2. Hormone injections*

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

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

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

158

159 2.3. Time-resolved fluoroimmunoassay (TR-FIA) for IGF-I

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

164

165 2.4. Electrophoresis and western blotting

166 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a 3% stacking gel and
167 12.5% separating gel. Plasma samples were treated with an equal volume of sample and buffer
168 containing 2% SDS and 10% glycerol at 85°C for 5 min. Gels were run in a solution of 50 mM
169 Tris, 400 mM glycine, and 0.1% SDS at 50 V in the stacking gel and at 100 V in the separating
170 gel until the bromophenol blue dye front reached the bottom of the gel. Gels were stained with

171 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA, USA). The molecular mass was
172 estimated using Precision Marker (Bio-Rad).

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

183

184 2.5. Statistical analysis

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

190

191 3. Results

192 3.1. Response of plasma IGF-I to hormone injections

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

197

198 3.2. Detection of three plasma IGFbps by ligand blotting

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

202

203 3.3. Response of plasma IGFBP-2b to hormone injections

204 The hormone injections did not affect plasma IGFBP-2b levels in masu salmon after 1 day (Fig.
205 3a). However, both hormones increased plasma IGFBP-2b levels 2 days after injection (GH: $P <$
206 0.0001 ; cortisol: $P = 0.0091$; Fig. 3b). GH decreased and increased plasma IGFBP-2b levels in
207 amago and Biwa salmon, respectively (amago: $P = 0.0137$; Biwa: $P = 0.0281$; Fig. 3c,e). In amago
208 salmon, cortisol increased plasma IGFBP-2b levels 2 days after injection ($P = 0.0155$, Fig. 3d).
209 In Biwa salmon, cortisol had a main inhibitory effect on IGFBP-2b levels 1 day after injection (P
210 $= 0.0463$, Fig. 3e), whereas its effect was stimulatory 2 days after injection ($P = 0.0422$, Fig. 3f).

211

212 *3.4. Response of plasma IGFBP-1a to hormone injections*

213 In masu salmon, plasma IGFBP-1a levels were reduced 1 day after GH injection ($P = 0.0162$),
214 whereas a stimulatory effect of cortisol was observed 2 days after injection ($P = 0.0018$; Fig. 4a,b).
215 Cortisol also increased plasma IGFBP-1a levels in amago and Biwa salmon 1 day after injection
216 (amago: $P < 0.0001$; Biwa: $P = 0.0113$; Fig. 4c,e). In Biwa salmon, the stimulatory effect of
217 cortisol was neutralized by co-injection with GH, which also had a main inhibitory effect ($P =$
218 0.0031 ; Fig. 4e). On day 2, no effect of GH or cortisol was seen on plasma IGFBP-1a levels in
219 amago and Biwa salmon (Fig. 4d,f).

220

221 *3.5. Response of plasma IGFBP-1b to hormone injections*

222 In masu and amago salmon, plasma IGFBP-1b levels increased 1 day after sham injection with
223 vehicle (Suppl. Fig. 1), and plasma IGFBP-1b levels in the cortisol group were similar to those in
224 the sham group (Fig. 5a,c). GH had a main suppressive effect on plasma IGFBP-1b levels 1 day
225 after injection in both masu and amago salmon (masu: $P = 0.0057$; amago: $P = 0.0002$). On day
226 2, cortisol maintained high plasma IGFBP-1b levels in masu salmon, whereas it had a main
227 suppressive effect in amago salmon ($P = 0.0055$; Fig. 5b,d). In Biwa salmon, sham injection had
228 no effect on plasma IGFBP-1b levels (Suppl. Fig. 1), whereas cortisol increased it ($P = 0.0028$;
229 Fig. 5e). Treatment with GH had a main inhibitory effect ($P = 0.0086$) and restored IGFBP-1b
230 levels to similar levels to those in the sham group (Fig. 5e); however, this effect disappeared 2
231 days after injection (Fig. 5f).

232

233 **4. Discussion**

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

237 masu, amago, and Biwa salmon) in seawater and freshwater in regions of their distribution in
238 Japan. However, little is known about the differences in their growth characteristics owing to their
239 allopatric distribution and the presence of anadromous and non-anadromous strains. Fujioka
240 (2006) compared growth patterns between amago and Biwa salmon in a common garden setting
241 and showed that growth in underyearling Biwa salmon was delayed because of a reduction in
242 appetite from July, which corresponded to the timing of their downstream migration to Lake Biwa.
243 This growth retardation may be adaptive to the scarcity of their prey item, the amphipod
244 *Jesogammarus annandalei*, in the lake during that period (Fujioka, 2006). We have been focusing
245 on anadromous strains of masu salmon to investigate the growth regulation by IGF-I and IGFBPs
246 and their involvement in the development of hypo-osmoregulatory ability (i.e., smoltification)
247 (Kawaguchi et al., 2013; Tanaka et al., 2018; Kaneko et al., 2020; Suzuki et al., 2020). We
248 previously compared the hypo-osmoregulatory ability of juvenile amago and Biwa salmon by
249 injecting them with GH and cortisol (Nakajima et al., 2014). In the present study, we compared
250 the responses of circulating IGFBPs in the three subspecies of *O. masou* by injecting juvenile
251 masu salmon with GH and cortisol.

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

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

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

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

287 A transient increase in serum IGFBP-1b but not IGFBP-1a in masu and amago salmon
288 subjected to sham injection suggests that IGFBP-1b is more sensitive to stress than IGFBP-1a.
289 We previously reported a difference in the sensitivity between IGFBP-1a and -1b to osmotic stress
290 in Chinook salmon parr transferred prematurely to seawater: plasma IGFBP-1b was induced
291 faster than IGFBP-1a (Shimizu et al., 2011a). Moreover, Kawaguchi et al. (2013) reported that
292 although both serum IGFBP-1a and -1b were induced by fasting, IGFBP-1b responded earlier
293 than IGFBP-1a. In zebrafish (*Danio rerio*) embryos, both IGFBP-1a and -1b were inhibitory to
294 IGF-I action, but the degree of inhibition by IGFBP-1a was stronger (Kamei et al., 2008). We
295 previously produced recombinant proteins for masu salmon IGFBP-1a and -1b and showed that
296 IGFBP-1a was effective in inhibiting IGF-I action in a primary pituitary culture system (Tanaka
297 et al., 2018). These findings suggest that IGFBP-1b is more sensitive to stress, whereas IGFBP-
298 1a is more potent in inhibiting IGF-I action under severe catabolic conditions. Such subfunction
299 partitioning may allow fish to fine-tune their growth speed and energy allocation under changing
300 environments, as discussed by Allard and Duan (2018).

301 The lack of response of Biwa salmon plasma IGFBP-1b to sham injections suggests a
302 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 α 1b* 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 μ g/g body weight
335 was retained in the circulation at approximately 380 ng/mL 1 day after injection. Assuming that

336 the effectiveness of mammalian GH is one-tenth that of homologous GH, as reported in Atlantic
337 salmon (*Salmo salar*; Boeuf et al., 1994), circulating porcine GH would have been approximately
338 1.7 nM 1 day after injection, which is higher than the effective dose required to reduce hepatic
339 *igfbp-1b* (Pierce et al., 2006). It is still unknown whether this contrast is attributed to species or
340 stage differences, or in vitro versus in vivo experiments, or a combination of these. The same
341 authors also examined the combined effects of salmon GH and Dex on *igfbp-1b* mRNA in primary
342 cultured coho salmon hepatocytes. In that study, Dex strongly increased *igfbp-1b* levels by
343 approximately eight-fold. Co-treatment with GH reduced Dex-stimulated *igfbp-1b* levels,
344 although they remained higher than the control levels (Pierce et al., 2006). The findings of the
345 present study provide further evidence of the counteractive effect of GH on IGFBP-1b in vivo.

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

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

361

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371

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527

528 **Figure legends**

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

535

536 Fig. 2. Representative ligand blots for IGFbps in plasma of masu salmon treated with GH or/and
537 cortisol for one day. Two microliters of plasma were separated by 12.5% SDS-PAGE under non-
538 reducing conditions, electroblotted onto nitrocellulose membranes and subjected with ligand
539 blotting using digoxigenin-labeled human IGF-I (50 ng) and antiserum against digoxigenin
540 (1:20,000). Arrowheads indicate migration positions of human (left) and salmon (right) IGFBP
541 bands. NS: non-specific; NHS: normal human serum; Fed: serum from fed masu salmon as a
542 reference.

543

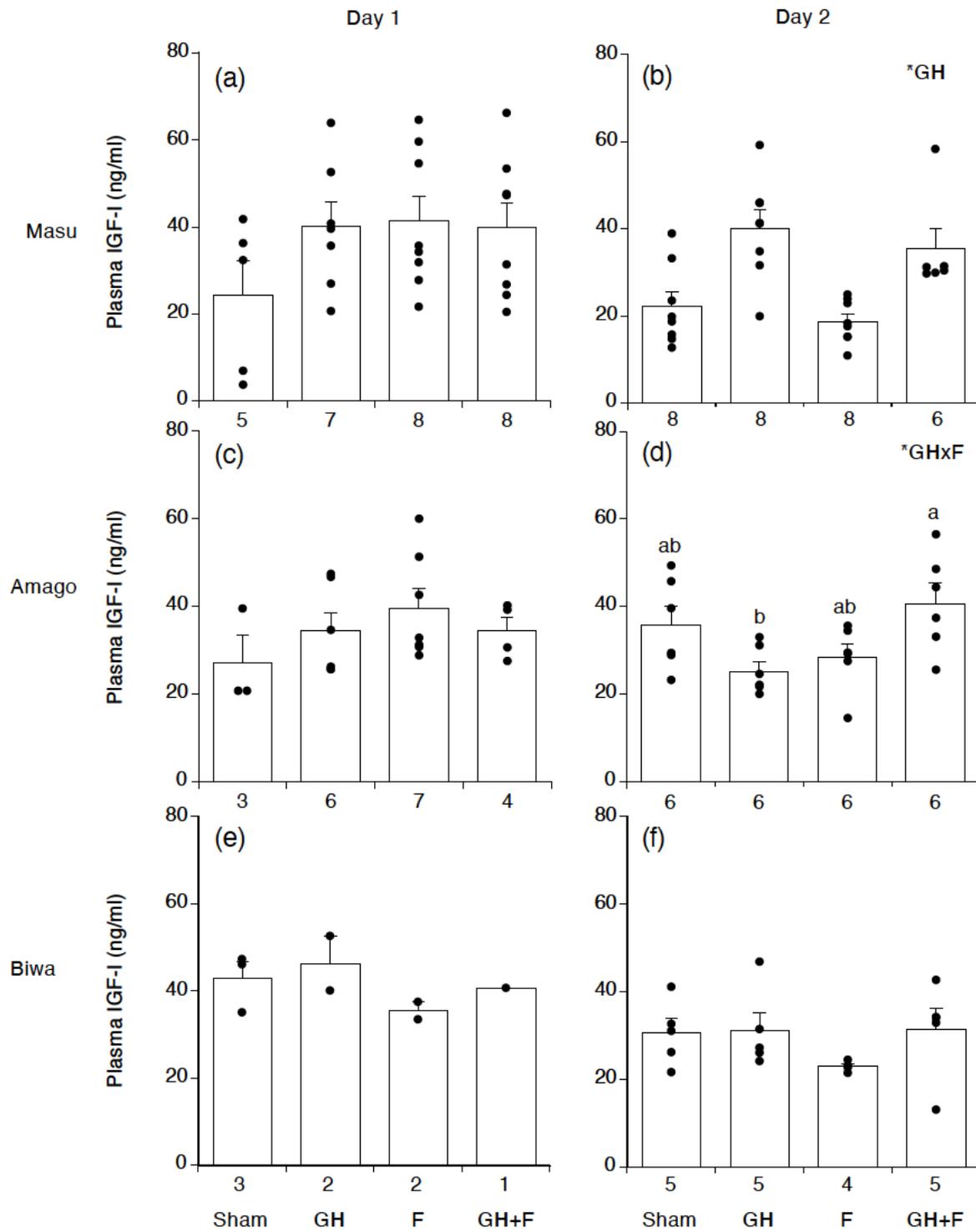
544 Fig. 3. Effects of GH and cortisol (F) on plasma IGFBP-2b in masu (a,b), amago (c,d) and Biwa
545 salmon (e,f). Underyearlings were injected with porcine GH (8 µg/g body weight) or/and cortisol
546 (40 µg/g body weight) and sampled 1 (a,c,e) and 2 days (b,d,f) after injection. Sham group
547 received vehicle only. Band intensities of IGFBP-2b on ligand blotting were semi-quantified as
548 arbitrary density unit (ADU). Values are expressed as means ± SE (*n* = 5-8, except *n* = 3 in the
549 GH+F group in Biwa salmon on day 1). Individual data are also shown as dots. A cross indicates
550 a significantly lower levels in the sham group compared to the initial (no injection) group.
551 Asterisks indicate main effects.

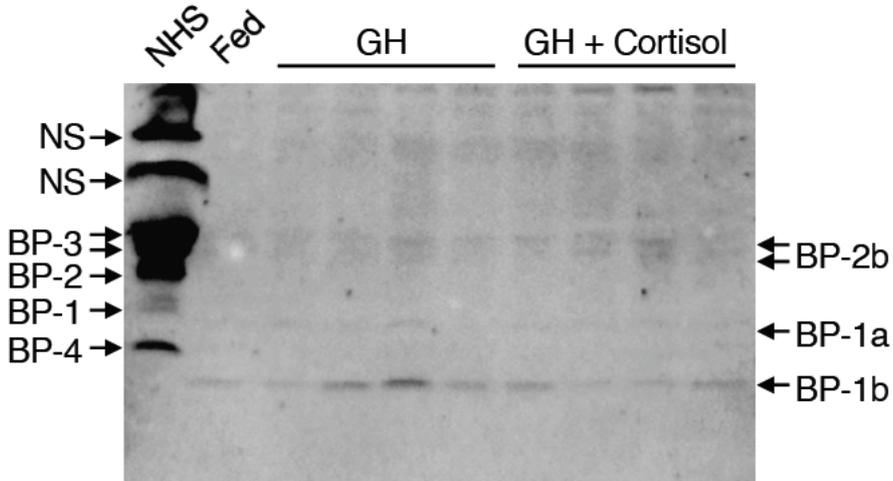
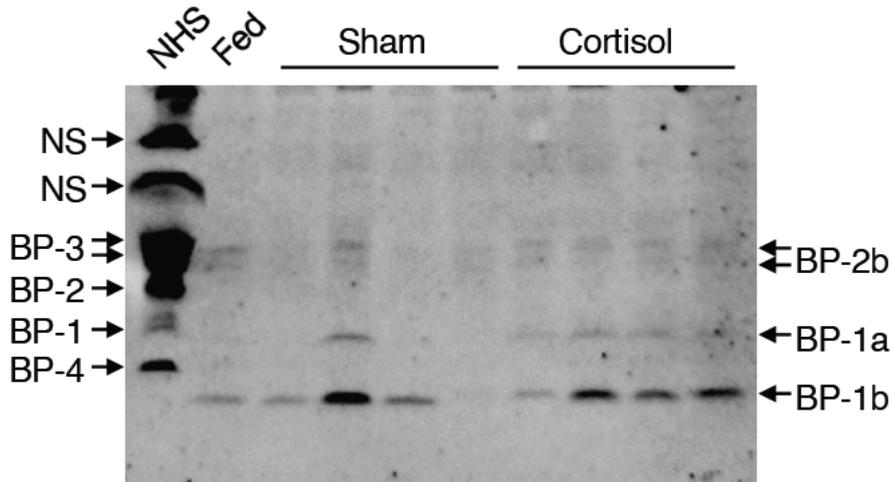
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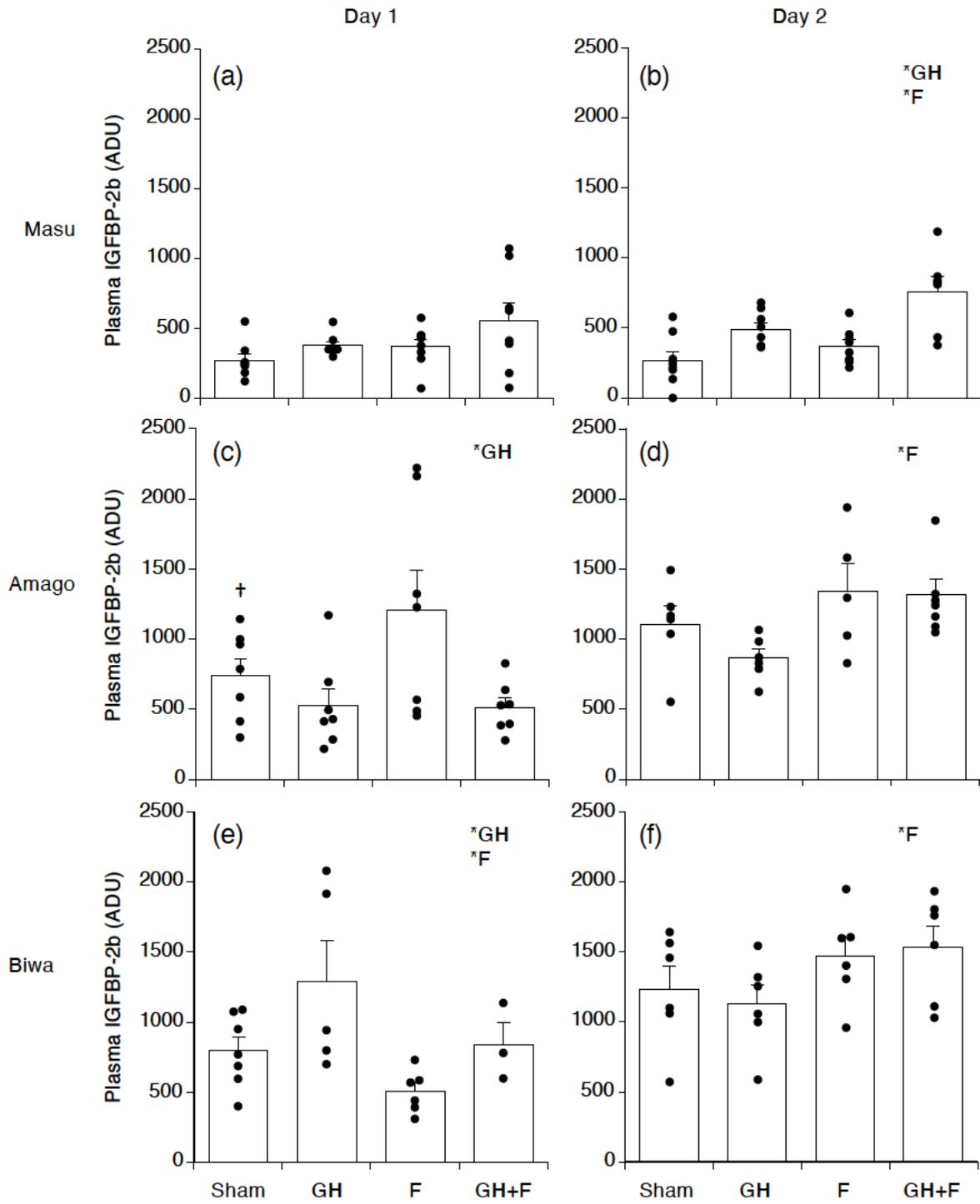
553 Fig. 4. Effects of GH and cortisol (F) on plasma IGFBP-1a in masu (a,b), amago (c,d) and Biwa
554 salmon (e,f). Underyearlings were injected with porcine GH (8 µg/g body weight) or/and cortisol
555 (40 µg/g body weight) and sampled one (a,c,e) and two days (b,d,f) after injection. Sham group
556 received vehicle only. Band intensities of IGFBP-1a on ligand blotting were semi-quantified as
557 arbitrary density unit (ADU). Values are expressed as means ± SE (*n* = 5-8, except *n* = 3 in the
558 GH+F group in Biwa salmon on day 1). Individual data are also shown as dots. Asterisks indicate
559 main effects or an interaction.

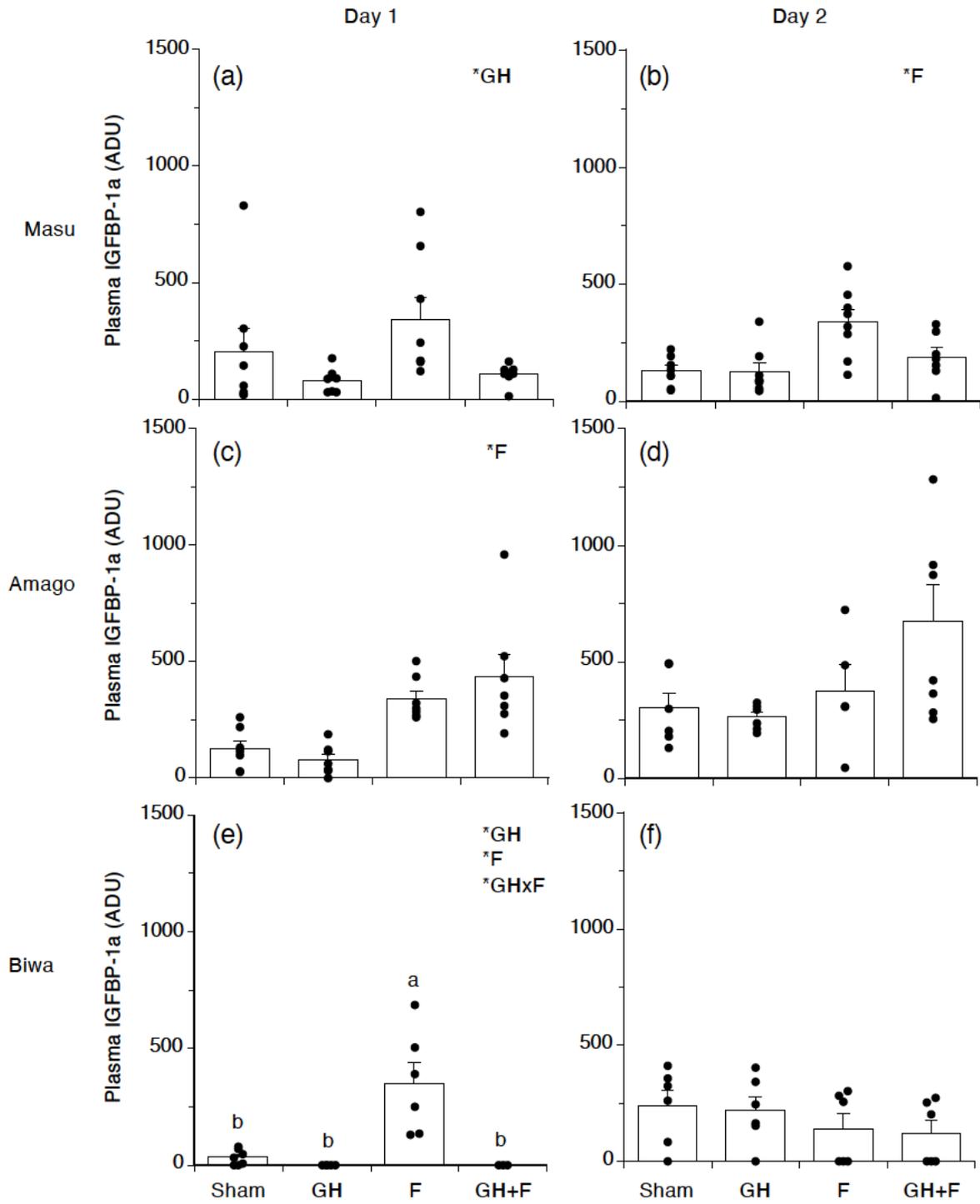
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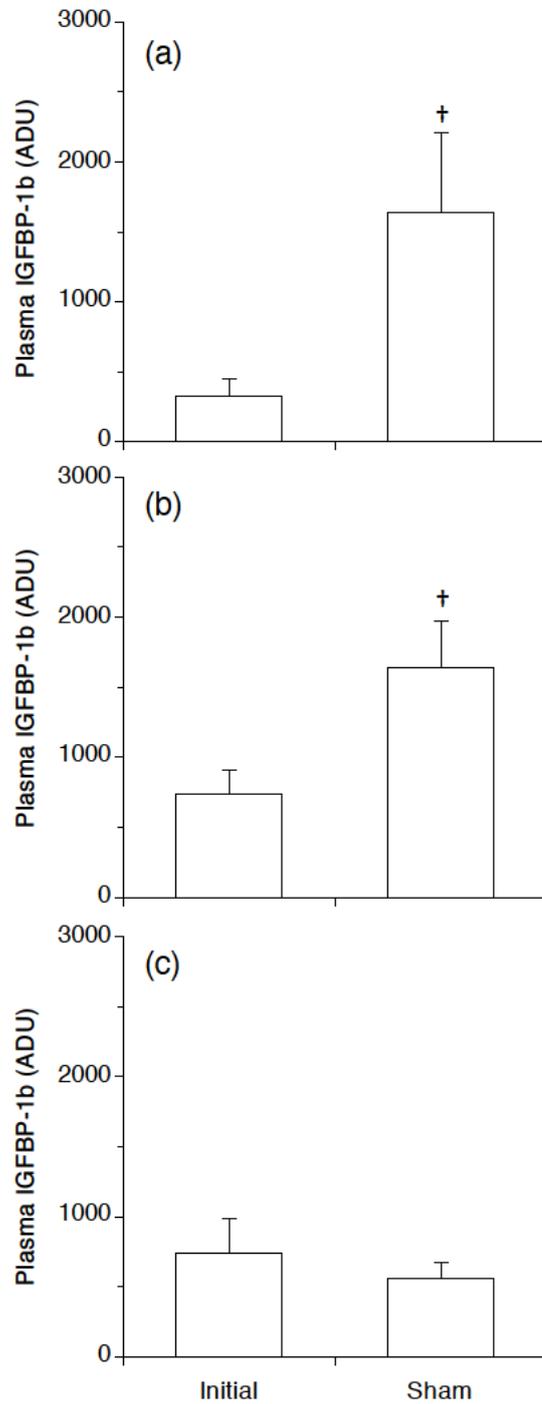
561 Fig. 5. Effects of GH and cortisol (F) on plasma IGFBP-1b in masu (a,b), amago (c,d) and Biwa
562 salmon (e,f). Underyearlings were injected with porcine GH (8 µg/g body weight) or/and cortisol
563 (40 µg/g body weight) and sampled one (a,c,e) and two days (b,d,f) after injection. Sham group
564 received vehicle only. Band intensities of IGFBP-1b on ligand blotting were semi-quantified as
565 arbitrary density unit (ADU). Values are expressed as means ± SE ($n = 5-8$, except $n = 3$ in the
566 GH+F group in Biwa salmon on day 1). Individual data are also shown as dots. Crosses indicate
567 significantly higher levels in the sham group compared to the initial (no injection) group.
568 Asterisks indicate main effects or an interaction. Symbols sharing the same letters are not
569 significantly different from each other (Tukey-Kramer test, $P < 0.05$).
570











Suppl. Fig. 1. Effects of sham injection on plasma IGFBP-1b in masu (a), amago (b) and Biwa salmon. Undererlings 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, $P < 0.05$).