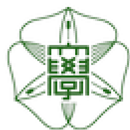
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1 **Title:**

2 Evaluation of circulating insulin-like growth factor (IGF)-I and IGF-binding proteins as growth
3 indices in rainbow trout (*Oncorhynchus mykiss*)

4

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19

20 **Abstract**
21 Circulating insulin-like growth factor (IGF)-I has been proposed as a growth index in several
22 teleosts, including salmonids, and its level in circulation is stabilized by multiple IGF-binding
23 proteins (IGFBPs). Three IGFBPs, IGFBP-2b, -1a, and -1b, are consistently detected in salmonid
24 blood and are suggested to be indices of positive or negative growth, although their applicability
25 to rainbow trout (*Oncorhynchus mykiss*) is unclear. The present study examined the usefulness of
26 IGFBPs along with IGF-I as a physiological indicator of growth rate in rainbow trout through a
27 rearing experiment. Two groups of underyearling rainbow trout were pit-tagged and either fed or
28 fasted for 33 days. A third group was fasted for 22 days, followed by refeeding for 11 days. Serum
29 IGF-I levels were reduced after fasting for 22 days, but refeeding did not retore its levels to those
30 of the fed control. Nevertheless, there was a positive relationship between serum IGF-I levels and
31 individual growth rates over 33 days of experimentation, confirming its validity as a growth index.
32 Ligand blotting using labeled human IGF-I revealed two IGFBP bands at 43 and 32 kDa, which
33 corresponded to IGFBP-2b and an unidentified form, respectively. In contrast, bands
34 corresponding to IGFBP-1a and -1b, which usually increase after fasting, were hardly detected,
35 even in the fasted fish. The responses of circulating IGFBP-2b to fasting and refeeding were
36 similar to those of circulating IGF-I and positively correlated with growth rate and IGF-I levels.
37 The intensity of the serum 32-kDa IGFBP band was higher in constantly fed fish than in the fasted
38 fish; however, its correlation with growth rate was weaker than those of IGF-I and IGFBP-2b.
39 The present study shows that IGF-I and IGFBP-2b can be used as growth indices for rainbow
40 trout. In contrast, circulating IGFBP-1a and -1b may not serve as negative growth indices in
41 rainbow trout under regular aquaculture conditions because they are rarely detected by ligand
42 blotting or respond to fasting/refeeding.
43

44 **Keywords**
45 aquaculture, compensatory growth, somatotropic axis, immunoassay, individual growth

1. Introduction

Insulin-like growth factor (IGF)-I is a 7.5 kDa polypeptide, which is structurally similar to proinsulin and plays an important role in cell proliferation, differentiation, and survival. IGF-I is produced in virtually all tissues and exerts its actions through endocrine, paracrine, and autocrine signaling in vertebrates (Daughaday and Rotwein, 1989; LeRoith et al., 2001; Wood et al., 2005; Ohlsson, 2009). Endocrine IGF-I is produced mainly by the liver upon growth hormone (GH) stimulus and mediates GH actions in various tissues (Daughaday and Rotwein, 1989; Ohlsson, 2009). Unlike insulin, circulating IGF-I levels are stabilized by the presence of IGF-binding proteins (IGFBPs), which prolong the half-life of IGF-I by protecting against glomerular filtration in the kidney and enzymatic degradation (Rajaram et al., 1997). They also regulate the availability of IGF-I to target tissues by either inhibiting or promoting the binding to the receptor (Firth and Baxter, 2002), making them important growth regulators in vertebrates.

Six IGFBPs have been identified and characterized in mammals (Shimasaki and Lin, 1991; Bach, 2018). Teleosts generally possess two paralogs of each member of the IGFBPs, except IGFBP-4 due to a third round of whole-genome duplication (WGD) that occurred after their divergence from other vertebrates (Ocampo Daza et al. 2011). Salmonids experienced an additional round of WGD and have up to four copies of each IGFBP, which resulted in the presence of 22 paralogous genes (Macqueen et al. 2013; de la Serrana and Macqueen, 2018). The retention of the most duplicated IGFBP paralogs suggests that the fine-tuning of IGF-I activity by IGFBPs had an adaptive value in salmonids and teleosts (Allard and Duan, 2018; de la Serrana and Macqueen, 2018).

In addition to understanding the roles and functions of IGF-I and IGFBPs in teleosts, there is an increasing interest in utilizing them as growth indices (Picha et al., 2008; Beckman, 2011). This is based on the findings that circulating IGF-I levels are generally positively correlated with individual growth rates in postsmolt coho salmon (*Oncorhynchus kisutch*; Beckman et al., 2004a,b,c). This relationship has also been observed in other salmonids and fish species, including masu salmon (*O. masou*: Kaneko et al., 2020), chum salmon (*O. keta*: Kaneko et al., 2015), Atlantic salmon (*Salmo salar*: Breves et al., 2020), olive rockfish (*Sebastes serranoides*: Hack et al., 2018), copper rockfish (*S. caurinus*: Hack et al., 2019), and cabezon (*Scorpaenichthys marmoratus*: Strobel et al., 2020). This characteristic makes circulating IGF-I a useful index for assessing and evaluating the growth status of fish under captivity and in the field.

Some IGFBPs are also good markers of anabolic and catabolic conditions in fish and could be used as quantitative growth indices (Kelley et al., 2001; Kaneko et al., 2020). Three major IGFBP bands at 40–50, 25–30 and 20–25 kDa were consistently detected in the plasma/serum of several fish species by ligand blotting using labeled IGF-I (Kelley et al., 2001;

82 Shimizu and Dickhoff, 2017); these three IGFBPs have been identified as IGFBP-2b, -1a, and -
83 1b, respectively (Shimizu et al., 2011a,b). Salmon IGFBP-2b is believed to be the main carrier of
84 circulating IGF-I, and its level was positively correlated with individual growth rate as well as
85 IGF-I levels (Shimizu et al., 2003; Beckman et al., 2004a). In contrast, fish IGFBP-1s have been
86 shown to inhibit IGF-I actions in zebrafish (*Danio rerio*) and salmonids (Maures et al. 2001;
87 Bauchat et al., 2001; Kamei et al., 2008; Tanaka et al., 2018; Hasegawa et al., 2020). In salmonids,
88 IGFBP-1a and -1b levels increased by fasting and stress and negatively correlated with individual
89 growth rates (Kaneko et al., 2020). These findings support the use of circulating IGFBPs in fish
90 as positive or inverse growth indices.
91 Rainbow trout (*O. mykiss*) is an important species for aquaculture and is the second most
92 aquacultured salmonid after Atlantic salmon (FAO, 2020). It is reared in a wide variety of salinity,
93 temperature, photoperiod, rearing density, and water quality using open, semi-closed, or closed
94 systems. Selective breeding has greatly improved the growth performance of rainbow trout under
95 captivity (Leeds et al., 2016) and alternative feeds have been developed for sustainable
96 aquaculture of this species and other fishes (Jalili et al., 2013; Hua et al., 2019). Therefore,
97 evaluating the growth performance of rainbow trout under different environmental and feeding
98 conditions is critical.
99 Despite its importance in aquaculture and fish physiology, the validation of circulating
100 IGF-I and IGFBPs as growth indices in rainbow trout is incomplete. Taylor et al. (2005, 2008)
101 first reported the relationship between plasma IGF-I and growth rate in rainbow trout. Rainbow
102 trout is one of the first species to be reported for the presence of IGFBPs in fish (Niu et al., 1993).
103 Multiple IGFBP bands were detected at 50, 42, 32, and 21 kDa by ligand blotting, and some of
104 them responded to salinity change or handling stress (Shepherd et al., 2005, 2011). We have
105 recently reported a positive correlation between serum IGF-I levels and serum IGFBP-2b band
106 intensity in rainbow trout, suggesting that IGFBP-2b in trout is also a major carrier of circulating
107 IGF-I (Cleveland et al., 2018, 2020). However, our initial screening of serum IGFBP-1a and -1b
108 suggested that they might not be major circulating forms (Cleveland et al., 2018; 2020). Despite
109 the information available for the regulation of circulating IGF-I and IGFBPs in rainbow trout, no
110 study has comprehensively examined their relationships with feeding status and individual growth
111 rates in this species. The objectives of the present study were to confirm the validity of serum
112 IGF-I as a positive growth index in rainbow trout and to assess the use of circulating IGFBPs as
113 growth indices through a fasting/refeeding experiment of individually tagged fish.
114
115 **2. Materials and Methods**
116 *2.1. Fish and experimental design*
117 Fertilized eggs of rainbow trout were obtained from Troutlodge (Bonny Lake, WA) and

118 transferred to an indoor rearing facility in FRD Japan (Saitama, Japan). Juvenile trout were reared
119 in 1 and 3-ton tanks connected to a recirculating aquaculture system (RAS) with nitrification and
120 denitrification systems at 15 °C in 0.5 psu under a photoperiod regime of LD 24:0. Fish were fed
121 a commercial diet containing 42% crude protein and 19% crude lipid (Nosan Corporation,
122 Kanagawa, Japan) ad libitum twice daily. When fish exceeded 200 g, they were transferred to
123 another rearing facility in FRD Japan (Chiba, Japan) and reared in a 7-ton indoor-pond installed
124 with a RAS. Water temperature, photoperiod, and feeding conditions were the same as described
125 above, with a salinity of 4 psu. On November 4, 2020, 48 eight-month-old rainbow trout with an
126 average body length (BL), the length from the tip of the snout to the posterior end of the body
127 covered with scales, of 24.1 ± 0.2 cm and body weight (BW) of 265.4 ± 7.8 g were anesthetized
128 in 0.02% Eugenol (FA100, DS Pharma Animal Health Co., Ltd, Osaka, Japan) and individually
129 marked with passive integrated transponder (PIT) tag (12.5 × 2.1 mm, Biomark, Boise, ID, USA).
130 Sixteen fish were randomly placed into one of the three 200 L tanks (DAILITE, Tokyo, Japan)
131 and assigned to the fed, fasted, or refed groups. The fed group was given a commercial diet daily
132 to satiety, whereas the fasted group was fasted throughout the experimental period for 33 days.
133 The refed group was fasted for first 22 days and then fed to satiety for the following 11 days.
134 During the experimental period, four fish died and six fish lost their tag ID. Rearing and handling
135 fish were carried out in accordance with the guidelines of the Hokkaido University Animal Care
136 and Use Committee (#30-3).
137
138 *2.2. Sampling procedure*
139 At 0, 22, and 33 days after the beginning of the experiment, all individuals were anesthetized,
140 read for PIT-tag, and measured for BL and BW. The condition factor (K) was calculated as
141 follows: (BW (g)) × 100 / (BL (cm))^3. The specific growth rate (SGR) was calculated as follows:
142 SGR (%/day) = ln ($s_2 - s_1$) × ($d_2 - d_1$)$^{-1}$ × 100, where s_2 is length or weight on day 2, s_1 is length
143 or weight on day 1, and $d_2 - d_1$ is the number of days between measurements. Sixteen fish from
144 each treatment group were divided into two categories: serial or single blood collection. Serial
145 blood sampling was conducted for eight fish from each treatment on days 0, 22, and 33, whereas
146 a single blood collection was performed for the other eight fish on day 33. Blood was withdrawn
147 using a syringe from the caudal veins, allowed to clot overnight at 4 °C, and then centrifuged at
148 10,000 × *g* for 10 min. Serum was stored at -30 °C until use.
149
150 *2.3. Time-resolved fluoroimmunoassay (TR-FIA) for IGF-I*
151 To measure IGF-I, serum was extracted with acid–ethanol as described by Shimizu et al. (2000).
152 IGF-I was quantified by TR-FIA based on the method described by Small and Peterson (2005),
153 using recombinant salmon/trout IGF-I (GroPep, Adelaide, SA, Australia) as the standard. Time-

154 resolved fluorescence was measured using a Wallac ARVO X4 (PerkinElmer, Waltham, MA,
155 USA).
156
157 *2.4. Ligand blotting*
158 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a
159 3% stacking gel and 12.5% separating gel. Serum samples were treated with an equal volume of
160 sample and buffer containing 2% SDS and 10% glycerol at 85 °C for 5 min. Gels were placed in
161 a solution of 50 mM Tris, 400 mM glycine, and 0.1% SDS at 8 mA for the stacking gel and 12
162 mA for the separating gel until the bromophenol blue dye front reached the bottom of the gel.
163 Ligand blotting with digoxigenin-labeled human IGF-I (DIG-hIGF-I) was performed
164 according to a previously described protocol (Shimizu et al. 2000). The nitrocellulose membranes
165 were incubated overnight with DIG-hIGF-I and then incubated with antibodies against DIG
166 conjugated horseradish peroxidase (Roche, Indianapolis, IN, USA) at a dilution of 1:1500–2500
167 for 1.5 h at room temperature (20–25 °C). IGFBP was visualized using enhanced
168 chemiluminescence (ECL) western blotting reagents (Amersham Life Science, Arlington Heights,
169 IL, USA). The intensities of serum IGFBP bands were semi-quantified using ImageJ version
170 1.440 (Schneider et al., 2012), normalized to the human IGFBP-4 band intensity, and expressed
171 as an arbitrary density unit (ADU).
172
173 *2.5. Statistical analysis*
174 The effects of tank/feeding treatment and time were analyzed by two-way analysis of variance
175 (ANOVA) using the JMP program (SAS Institute Inc., Cary, NC, USA). When significant effects
176 were found, differences were further identified by one-way ANOVA followed by Tukey's
177 honestly significant difference (HSD) test, with differences considered significant at $P < 0.05$.
178 Simple regression analysis was also conducted using JMP software, and the relationships were
179 considered significant at $P < 0.05$.
180
181 **3. Results**
182 *3.1. Body size*
183 There were main effects of tank/feeding treatment and time and their interaction on BL, BW and
184 K (Fig. 1). Control-fed fish grew for 33 days of the experimental period (Fig. 1). Fasting for 22
185 days suppressed BW and K but not BL. Refeeding for 11 days had positive effects on BW
186 compared to those of time-matched fasted group, whereas no difference was seen in BL and K.
187 However, refeeding did not restore BW to values similar to those of fed fish. There were no effects
188 of serial sampling on these parameters.
189

190 *3.2. SGR*

191 SGRs in length and body weight (SGRL and SGRW) of the fasted group were lower than those
192 of the fed group after 22 days of fasting (Table 1). Serial blood sampling negatively impacted
193 SGRL in the fasted group ($P < 0.05$) which in turn resulted in a relatively low SGRL compared
194 to the refed group despite the same fasting treatment during days 0-22. Refeeding for 11 days
195 after 22 days of fasting increased the SGRW but not the SGRL. SGRs during 33 days in the refed
196 group were higher than those in the fasted group but lower than those in the fed group.
197

198 *3.3. Serum IGF-I*

199 There were main effects of tank/feeding treatment and time and their interaction on serum IGF-I
200 (Fig. 2). Serum IGF-I levels were reduced after 22 days of fasting. Refeeding for 11 days had no
201 effect on serum IGF-I levels. There was no effect of serial sampling on serum IGF-I (data not
202 shown).
203

204 *3.4. Serum IGFBPs*

205 Multiple IGFBP bands were visualized in the serum of rainbow trout by ligand blotting using
206 DIG-labeled hIGF-I (Fig. 3). The IGFBP-2b bands at 41-45 kDa were consistently detected in all
207 groups, whereas the IGFBP-1a and -1b bands at 28 and 22 kDa, respectively, were rarely detected.
208 An unidentified IGFBP was detected at a molecular weight of 32 kDa. The 32-kDa IGFBP band
209 was not detected in five out of 18 samples on day 22 and seven out of 38 samples on day 33 (data
210 not shown).

211 The intensities of the bands of IGFBP-2b and 32 kDa IGFBP were semi-quantified and
212 compared among the treatments (Fig. 4). There were main effects of tank/feeding treatment and
213 time and their interaction on serum IGFBP-2b (Fig. 4a). The intensity of the IGFBP-2b band in
214 fed fish increased over the experimental period of 33 days. Fish fasted for 33 days had a lower
215 band intensity than that of fed fish. Refeeding for 11 days had no effect on the serum IGFBP-2b
216 band intensity. There was a main effect of time on serum 32-kDa IGFBP where the intensity of
217 the 32-kDa IGFBP band was reduced over the experimental period (Fig. 4b). Fasting nor
218 refeeding had no significant effects on the serum 32-kDa IGFBP band intensity. There were no
219 effects of serial sampling on the band intensity of IGFBP-2b and 32-kDa IGFBP (data not shown).
220

221 *3.5. Relationship with SGRs*

222 There were positive correlations between serum IGF-I levels at day 33 and SGRs over 33 days
223 (Fig. 5a,b). The serum IGFBP-2b and 32-kDa IGFBP band intensities also positively correlated
224 with both SGRL and SGRW, whereas the coefficient of correlation appeared to be higher with
225 IGFBP-2b than with 32-kDa IGFBP (Fig.5c–f). When the correlations with SGRs during days

226 22–33 were compared among treatments, serum IGF-I, IGFBP-2b, and 32-kDa IGFBP showed
227 positive correlations, except for the 32-kDa IGFBP with SGRL (Table 2). In the serially collected
228 blood from fed fish, serum IGF-I levels on day 22 positively correlated with future SGRs during
229 22–33 days (SGRL: $r^2 = 0.49$, $P = 0.0117$; SGRW: $r^2 = 0.83$, $P < 0.0001$), which were comparable
230 to those between serum IGF-I levels on day 33 and past SGRs during 22–33 days (SGRL: $r^2 =$
231 0.65, $P = 0.0016$; SGRW: $r^2 = 0.80$, $P < 0.0001$).
232

233 **4. Discussion**

234 In the present study, rainbow trout was subjected to fasting followed by refeeding to investigate
235 responses of body conditions and the circulating IGF-I/IGFBP. After 22 days of fasting, BW, BL,
236 and K in the fasted group were lower than those in the fed group, consistent with previous results
237 (Gabillard et al., 2006; Medeirous et al., 2020). Refeeding for 11 days had positive effects on
238 restoring BW and K but not BL when compared to the fasted group; however, none of the
239 parameters matched those of the fed group, showing that 11 days of refeeding was insufficient to
240 restore body size and conditions. Such slow recovery of growth from fasting has been previously
241 reported in rainbow trout (Gabillard et al., 2006). Surprisingly, the SGRW of the refed group in
242 the present study was not higher than that of the fed group as compensatory growth was often
243 observed in fish after food restriction/deprivation followed by increasing the feeding ration (Ali
244 et al., 2003). Despite the lack of compensatory growth in refed fish, the treatments in the present
245 study (fed, fasted, and refed) resulted in different levels of SGR over 33 days.

246 Circulating IGF-I showed a response similar to that of BL, whose decreased levels after
247 the 22 day fasting period did not increase even after 11 days of refeeding. The reduction in serum
248 IGF-I levels after a few weeks of fasting is compatible with other studies on salmonids, including
249 rainbow trout (Medeiros et al., 2020; Gabillard et al, 2006; Caldarone et al. 2016; Kaneko et al.
250 2019). However, the degree of recovery of circulating IGF-I after refeeding may depend on the
251 strain/species, developmental stage and/or experimental setting. Gabillard et al. (2006) reported
252 that plasma IGF-I levels in rainbow trout fasted for one month started increasing four days after
253 refeeding and reached similar levels as the initial group after 14 days. In contrast, when rainbow
254 trout were fasted for three weeks and refed for one week, there was no increase in serum IGF-I
255 levels (Cleveland et al., 2020). In a study using yearling masu salmon that fasted for one month,
256 two weeks of refeeding increased serum IGF-I levels, although the levels were still lower than
257 those of fed controls (Kaneko et al., 2020). We previously reported that in coho salmon,
258 continuously fed fish exhibited increased plasma IGF-I levels after a meal in 24 h, whereas fish
259 previously fasted for three weeks did not (Shimizu et al., 2009). Thus, in addition to species
260 differences, the fasting period also affects the response of circulating IGF-I to food intake. Further
261 research to fully elucidate the underlying mechanism of growth after food deprivation in rainbow

262 trout needs analyses of the GH and IGF-I receptor abundance and signaling pathways.
263 Despite the relatively low recovery of average serum IGF-I levels after refeeding, there
264 were positive relationships between serum IGF-I and SGRs in body length/weight during 22–33
265 days as well as during 0–33 days. Taylor et al. (2005, 2008) found a positive relationship between
266 average plasma IGF-I levels and average SGR in adult rainbow trout. In contrast, Morro et al.
267 (2019) reported that plasma IGF-I positively correlated with individual SGR of rainbow trout in
268 seawater but not in freshwater, although the trout were exposed to different photoperiod regimes
269 in freshwater. The results of the present study are similar to those of the study by Taylor et al.
270 (2005, 2008) and confirm the positive relationship of individual fish under varying feeding
271 conditions. Moreover, in the present study, half of the experimental fish were sequentially
272 sampled for blood at days 0, 22, and 33. When fed and fasted groups were combined, serum IGF-
273 I levels at day 22 and day 33 correlated with SGRL during days 22–33, showing that serum IGF-
274 I levels projected future growth rate as long as feeding and other conditions were unaltered. Such
275 links with both past and future growth rates have been suggested in coho salmon (Pierce et al.,
276 2001) and is consistent with the growth-promoting action of IGF-I in salmon (McCormick et al.,
277 1992). The results of the present study further strengthen the validity of circulating IGF-I as a
278 "current" growth index in fish.
279 The response of serum IGFBP-2b was similar to that of serum IGF-I, and the intensity
280 of IGFBP-2b band was positively correlated with serum IGF-I levels, supporting the notion that
281 IGFBP-2b is a major carrier of circulating IGF-I (Shimizu et al., 2003; Shimizu and Dickhoff,
282 2017). However, IGFBP-2b not responding to refeeding for 11 days in rainbow trout was atypical.
283 We previously showed that IGFBP-2b retained the ability to increase in response to a single meal
284 after three weeks of fasting, whereas IGF-I did not in coho salmon (Shimizu et al., 2009). Low
285 sensitivities of IGF-I and IGFBP-2b to refeeding may be a characteristic of rainbow trout. Thus,
286 slow recovery of the endocrine parameters after fasting demands further studies on the mechanism
287 of compensatory growth using rainbow trout.
288 Despite the low sensitivity of serum IGFBP-2b to refeeding, it was positively correlated
289 with SGR, similar to serum IGF-I. Our results are consistent with previous findings in coho
290 salmon that plasma IGFBP-2b was also useful as a growth index (Beckman et al., 2004a,b,c). The
291 present study also semi-quantified the intensity of IGFBP-2b band, which possesses IGF-binding
292 ability (i.e. "intact" form), by ligand blotting, and previous studies quantified immunoreactive
293 IGFBP-2b, which contained intact and fragmentated forms, by radioimmunoassay (RIA;
294 Beckman et al., 2004b,c). Despite the difference in the detection principle, the two methods
295 revealed similar relationships between IGFBP-2b and SGR, suggesting that most IGFBP-2b
296 circulates in an intact form in immature rainbow trout. However, the degree of IGFBP-2b
297 fragmentation in different stages, status, and species is important because the release of IGF-I

298 from IGFBP by enzymatic degradation is an important mechanism for delivering IGF-I to its
299 receptor (Firth and Baxter, 2002). Although quantitative immunoassays such as RIA or TR-FIA
300 are desirable for IGFBP-2b, the present study showed ligand blotting as a promising assay to
301 show the robust relationship with SGRs under different feeding regimes and its utility as a growth
302 index in rainbow trout.

303 The response of the 32-kDa IGFBP appeared to be more sensitive to feeding changes
304 because it was reduced after fasting and restored by refeeding. The 32-kDa IGFBP is a fourth
305 circulating form, along with IGFBP-1a, -1b and -1b, detected in the circulation of coho salmon,
306 masu salmon, and rainbow trout (Shimizu and Dickhoff, 2017; Cleveland et al., 2018, 2020;
307 Hayashi et al., in press). Our result that the 32-kDa IGFBP was positively influenced by feeding
308 is consistent with that of Cleveland et al. (2020). However, although serum 32-kDa IGFBP
309 responded to refeeding better than serum IGFBP-2b, its correlation with SGR was lower than that
310 of IGFBP-2b. Based on these results, we hypothesize that the 32-kDa IGFBP has a higher
311 sensitivity to feeding ration and plays a role in complementing with IGFBP-2b to protect IGF-I
312 in the short-term, which will be addressed in a future study.

313 One of the significant findings from the present study is that IGFBP-1a and -1b were
314 rarely detected in the serum of rainbow trout in all feeding treatments for up to one month. Several
315 studies on salmon have shown that IGFBP-1a and/or -1b were useful as inverse growth indices in
316 salmon (Shimizu et al., 2006; Kawaguchi et al. 2013; Kaneko et al. 2019; Kaneko et al. 2020),
317 although this has not been demonstrated in rainbow trout. In the plasma/serum of rainbow trout,
318 up to five IGFBP bands at 50, 42, 32, 30, and 21 kDa have been detected by ligand blotting (Niu
319 and Le Bail, 1993; Bauchat et al., 2001; Shephered et al., 2005). The IGFBP bands at 30 and 21
320 kDa appeared to correspond to IGFBP-1a and -1b, respectively (Shimizu et al., 2011). Moreover,
321 these IGFBPs are induced in the blood by cortisol (Shimizu et al., 2011a). Thus, two IGFBP-1s
322 are present in the circulation of rainbow trout. Indeed, plasma IGFBP-1b levels in rainbow trout
323 were measurable by TR-FIA and responded to changes in water temperature and seawater transfer
324 (Hevrøy et al., 2015; Morro et al., 2020). However, in the present study, ligand blotting detected
325 IGFBP-1a and -1b very rarely and weakly, which did not appear to respond to feeding status. This
326 result contrasts with those of previous studies using masu salmon (Kawaguchi et al., 2013;
327 Kaneko et al., 2020) but agrees with our previous study using rainbow trout, although its
328 experimental design was incomplete (Cleveland et al., 2020). The poor reactivity of IGFBP-1s to
329 nutritional change was also reported at the mRNA level. In an experiment using rainbow trout,
330 *igfbp-1* mRNA was detected in both liver and muscle but did not respond to fasting for 30 days
331 (Gabillard et al., 2006). The apparent lack of the IGFBP-1 response to fasting in rainbow trout
332 might be due to genetic alterations after selective breeding for high growth in fish farms (Tymchuk
333 and Devlin, 2005) where fish are fed to satiation and rarely experience long-term fasting.

Although the cause and significance are subjects of future study, IGFBP-1s play little role in regulating growth in domesticated rainbow trout under normal aquaculture conditions and are not useful as inverse growth indices unless fish are severely stressed.

In summary, the present study confirmed the validity of circulating IGF-I and IGFBP-2b as positive growth indices for rainbow trout. In contrast, IGFBP-1a and -1b are not reliable inverse growth indices under aquaculture conditions because of their low levels and low sensitivity to feeding status. The slow recovery of growth and IGF-I/IGFBP-2b from fasting and the apparent lack of growth regulation by IGFBP-1s make rainbow trout a unique comparative model to investigate the mechanism of growth regulation in fish.

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529

530 **Figure legends**

531 Fig. 1 . Effects of fasting and refeeding on body length (BL; a), body weight (BW; b) and
532 condition factor (K; c). Values are expressed as means ± SE (day 0: n = 48. day 22: n = 40 and
533 day 33: n = 38). Groups sharing the same letters are not significantly different from each other
534 (Tukey's HSD, $P < 0.05$).

535

536 Fig. 2. Effects of fasting and refeeding on serum IGF-I levels. Values are expressed as means ±
537 SE (day 0: n = 22. day 22: n = 18 and day 33: n = 38). Groups sharing the same letters are not
538 significantly different from each other (Tukey's HSD, $P < 0.05$).

539

540 Fig. 3. IGFBP patterns in serum of fed and fasted rainbow trout. Rainbow trout were fed or fasted
541 for 33 days or were refed for 11 days after 22 days of fasting. Two microliters of serum was
542 separated by 12.5% SDS-PAGE under non-reducing conditions, electroblotted onto a
543 nitrocellulose membrane and subjected with ligand blotting using digoxigenin-labeled human
544 IGF-I (50 ng) and antiserum against digoxigenin (1:20,000). Arrows indicate migration positions
545 of human (left) NHS and trout (right) IGFBP bands. NHS: normal human serum.

546

547 Fig. 4. Effects of fasting and refeeding on band intensities of serum IGFBP-2b (a) and 32-kDa
548 IGFBP (b). The intensities of the IGFBP bands on ligand blotting were semi-quantified and
549 expressed as arbitrary density unit (ADU). Values are expressed as means ± SE (day 0: n = 22.
550 day 22: n = 18 and day 33: n = 38). Groups sharing the same letters are not significantly different
551 from each other (Tukey's HSD, $P < 0.05$).

552

553 Fig. 5. Correlations of serum IGF-I (a.b), IGFBP-2b (c,d) and 32-kDa IGFBP (e,f) with SGRs in
554 length (a,c,e) and weight (b, d,f) during 33 days of the experimental period. Data are from fed,
555 fasted and refed fish (n = 38).

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Izutsu et al., Fig. 1

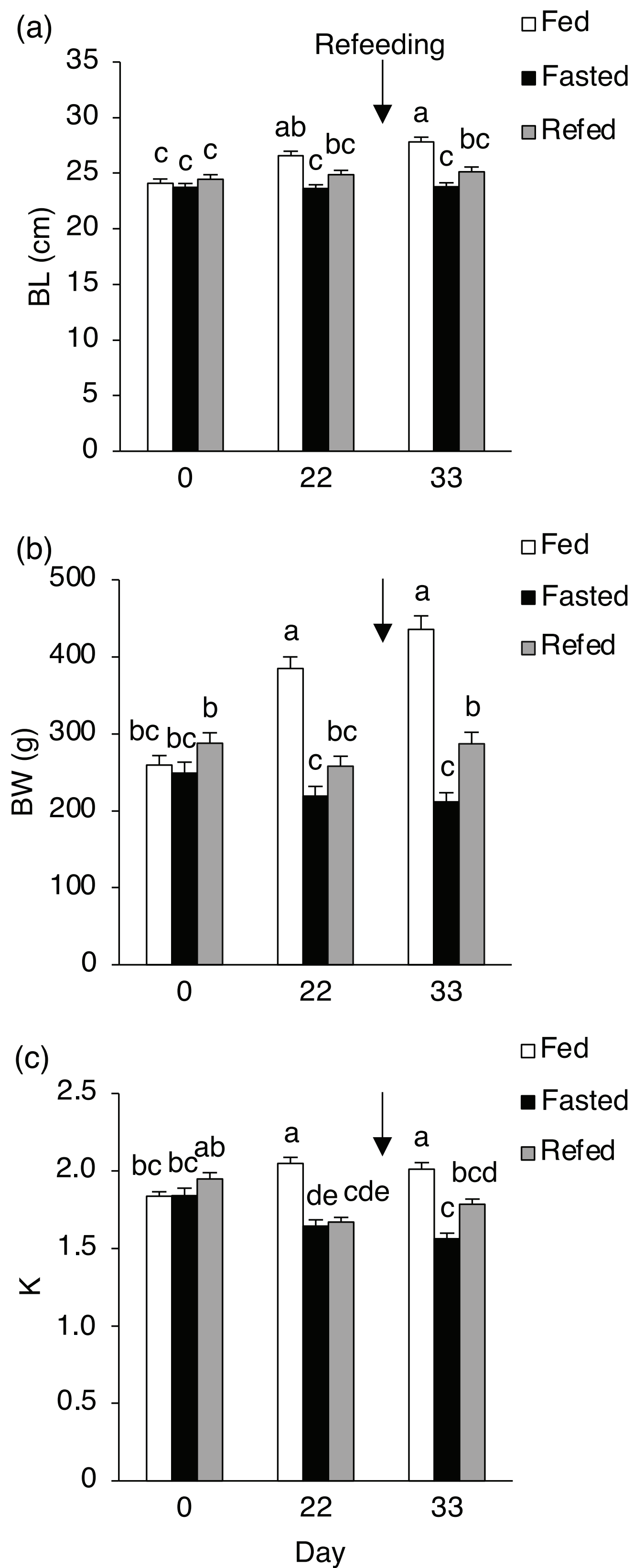

Figure 2

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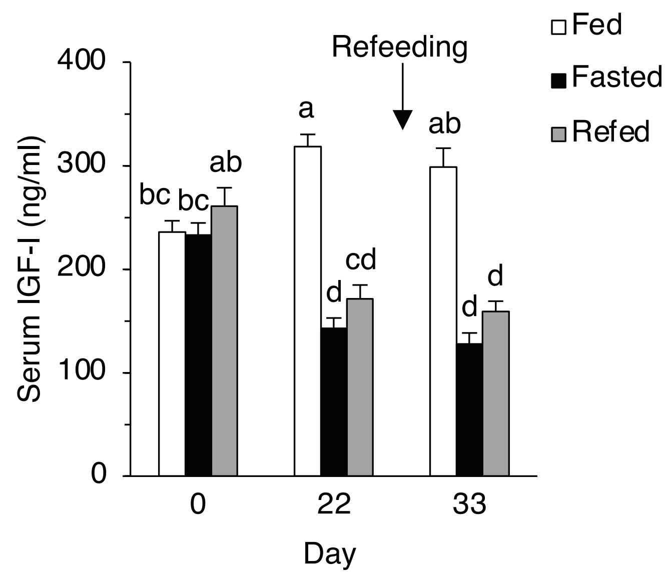

Figure 3

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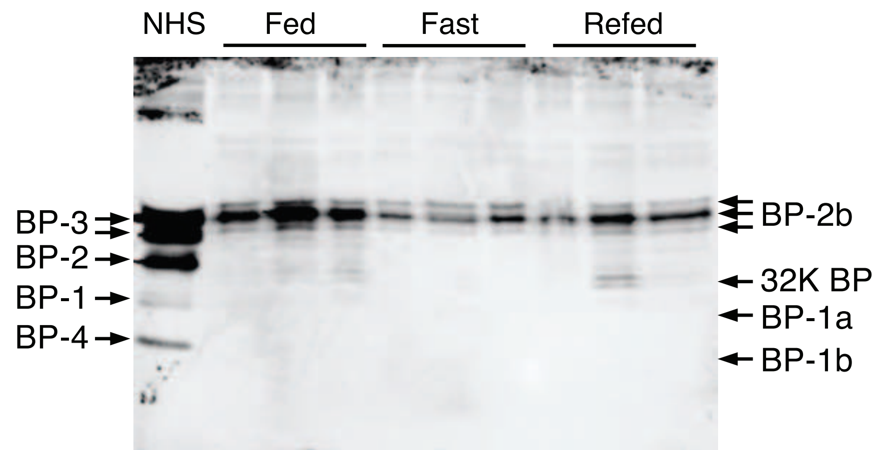

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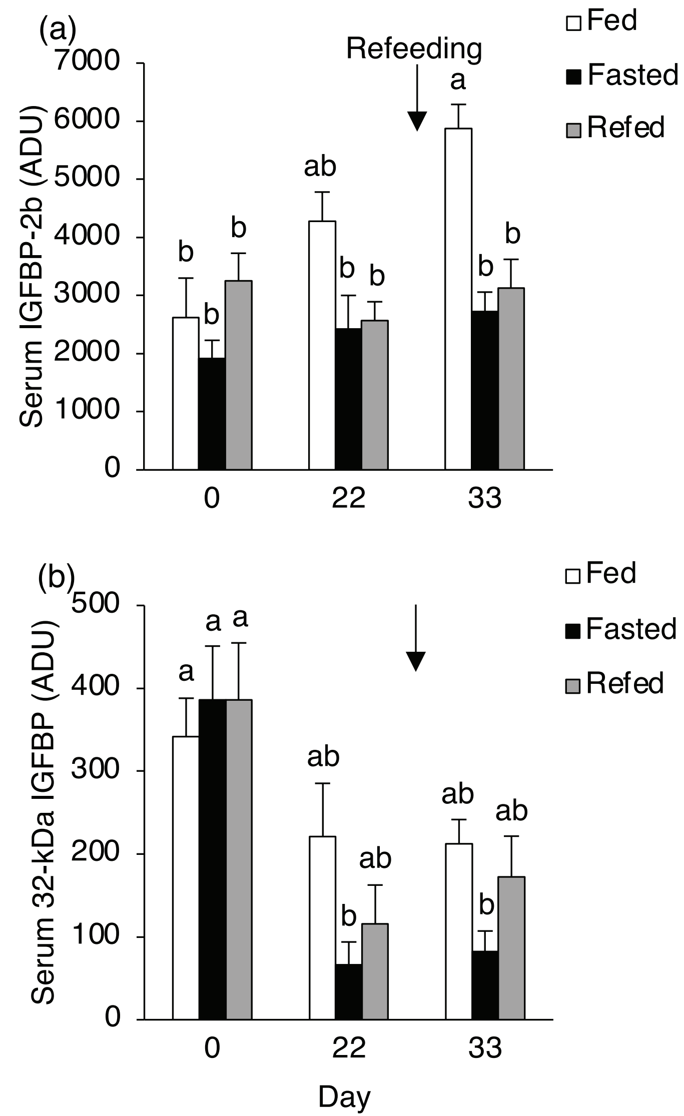

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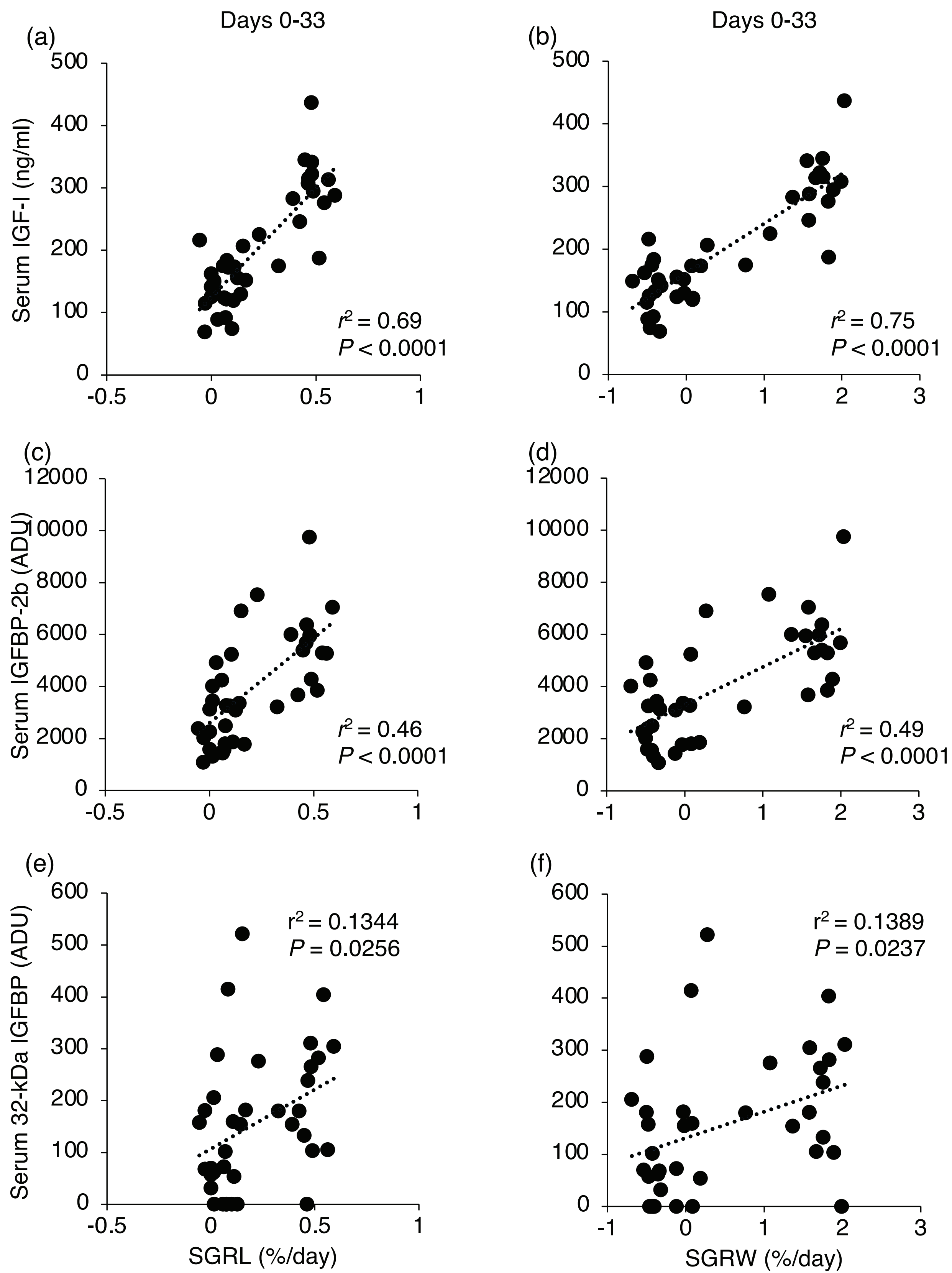

Table 1. Specific growth rates (SGR) in length (L) and body weight (W).

		Days 0-22	Days 22–33	Days 0-33
SGRL (%/day)	Fed	0.46 ± 0.03[a]	0.47 ± 0.05[a]	0.47 ± 0.02[A]
	Fasted	0.01 ± 0.01[b]	0.05 ± 0.03[b]	0.02 ± 0.01[C]
	Refed	0.10 ± 0.02[b]	0.15 ± 0.09[b]	0.12 ± 0.03[B]
SGRW (%/day)	Fed	1.93 ± 0.09[a]	1.26 ± 0.07[b]	1.69 ± 0.07[A]
	Fasted	-0.50 ± 0.05[c]	-0.34 ± 0.06[c]	-0.45 ± 0.03[C]
	Refed	-0.51 ± 0.04[c]	1.17 ± 0.27[b]	0.06 ± 0.09[B]

At a given time interval, groups without a letter or sharing the same letters are not significantly different from each other (Tukey's HSD, $P < 0.05$).

Table 2 Click here to access/download;Table;Table 2.docx

Table 2. Correlations between endocrine parameters and specific growth rates (SGR) in length (L) and weight (W) during days 22-33.

	SGRL (22-33 days)	SGRW (22-33 days)
IGF-I	$r^2 = 0.39$	$r^2 = 0.29$
	$P < 0.0001$	$P = 0.0006$
IGFBP-2b	$r^2 = 0.34$	$r^2 = 0.24$
	$P < 0.0001$	$P = 0.0021$
32-kDa IGFBP	–	$r^2 = 0.15$
	ns	$P = 0.0184$

ns: not significant.