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Title	Changes in circulating insulin-like growth factor-1 and its binding proteins in yearling rainbow trout during spring under natural and manipulated photoperiods and their relationships with gill Na+, K+-ATPase and body size
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26 Abstract

27 Smoltification in salmonids occurs during spring in response to increasing photoperiod to prepare 28 for marine life. Smoltification is associated with increased hypo-osmoregulatory ability and 29 enhanced growth potential, mediated by growth hormone and insulin-like growth factor (IGF)-1. 30 Rainbow trout is uniquely insensitive to the induction of smoltification-associated changes by 31 photoperiod, such as the activation of gill Na⁺,K⁺-ATPase (NKA). We measured the circulating 32 IGF-1 and IGF-binding protein (IGFBP)-2b levels in yearling rainbow trout exposed to natural 33 and manipulated photoperiods during spring and correlated these with gill NKA activity and body 34 size. Although the effect of photoperiod manipulation on body size and circulating IGF-1 and 35 IGFBP-2b was negligible, they were positively correlated with gill NKA activity in fish under 36 simulated natural photoperiod. We next pit-tagged yearling rainbow trout and fed them a restricted 37 ration or to satiation under a natural photoperiod. In April, gill NKA activity was higher in the 38 satiation group than in the restricted group and positively correlated with body size and growth 39 rate. In addition, circulating IGFBP-2b was positively correlated with gill NKA, size and growth, 40 whereas circulating IGF-1 was correlated only with size and growth. The relationship between 41 circulating IGF-1 and growth intensified from May to June, suggesting that the IGF-1-growth 42 relationship was disrupted in April when gill NKA was activated. Two additional IGFBPs were 43 related to growth parameters but not to gill NKA activity. The present study suggests that 44 circulating IGFBP-2b and IGF-1 mediate the size-dependent activation of gill NKA in yearling 45 rainbow trout during spring.

46

47 Keywords

48 Body size; Feeding restriction; Hypo-osmoregulatory ability; IGFBP-2b; Smoltification

49

50 **1. Introduction**

51 Rainbow trout (Oncorhynchus mykiss) is an important target species for aquaculture; 52 approximately 848,000 tons of rainbow trout was produced worldwide in 2018 (FAO, 2020). In 53 Norway, it accounts for about 6% of aquaculture production. Although this species has 54 traditionally been cultured in freshwater, there is an increasing interest in culturing them in 55 seawater or brackish water to occupy the unused area available from Atlantic salmon (Salmo 56 salar) aquaculture. However, notable mortalities and retarded growth have occasionally been 57 observed in practice, probably due to their premature transfer to seawater (Morro et al., 2019, 58 2020, 2021). Such phenomenon is called "stunting" reported in Atlantic salmon and coho salmon 59 (O. kisutch) (Björnsson et al., 1988; Young et al., 1989). Although seawater tolerance/adaptability 60 and growth after transfer to seawater in rainbow trout are generally higher in larger fish (Landness, 1976; Jackson, 1981; Johnsson and Clarke, 1988; Kaneko et al., 2019), there is a need for filling 61 62 the knowledge gap about the physiological and endocrine changes that take place during the 63 smoltification process in rainbow trout.

64 Juveniles of anadromous salmonids undergo parr-smolt transformation (smoltification) 65 in freshwater to be able to sustain marine life. Smoltification involves a series of morphological, 66 physiological, and biochemical changes (Wedemeyer et al., 1980; Hoar, 1988; Stefansson et al., 2008; Björnsson et al., 2011; McCormick, 2013). Acquisition of hypo-osmoregulatory ability is 67 68 one of the most significant physiological changes associated with smoltification. Growth 69 hormone (GH) and cortisol are two major hormones that play important roles in this process, by 70 stimulating the gills to transform the ionocytes from freshwater-type to seawater-type. The 71 seawater-type ionocytes have a well-developed tubular system and abundant Na⁺,K⁺-ATPase 72 (NKA) to extrude monovalent ions such as sodium and chloride (Mancera and McCormick, 2007; 73 Hiroi and McCormick, 2012). The action of GH is partially mediated by insulin-like growth factor 74 (IGF)-1 (Daughaday and Rotwein, 1989). IGF-1 is predominantly produced by the liver after 75 stimulation by GH released from the pituitary gland. In addition, peripheral tissues including the 76 gills also produce IGF-1 (Wood et al., 2005; Reinecke, 2010). IGF-1 can stimulate gill NKA 77 activity and enhance the hypo-osmoregulatory ability of the entire organism, as shown by in vitro 78 and in vivo studies involving salmonids, respectively (McCormick et al., 1991; Madsen and Bern, 79 1993).

80 During smoltification, salmonids acquire another characteristic, which is an enhanced 81 growth capacity before and after entering seawater (Dickhoff et al., 1997). The GH-IGF-1 system 82 is responsible for regulating the growth of vertebrates, including fish (Wood et al., 2005; Reinecke, 83 2010). The importance of local IGF-1 in growth regulation has been emphasized in mammals (LeRoith et al., 2001; Ohlsson et al., 2009). However, circulating IGF-1 in salmonids and other 84 85

fish is also reported to be positively correlated with individual growth rate in general, thus making

- a useful index of growth (Beckman et al., 2004a,b; Picha et al., 2008; Beckman, 2011; Hack et
 al., 2018). In addition, circulating IGF-1 levels increase or maximize during smoltification
 (Beckman et al., 1998; Larsen, 2001; Shimomura et al., 2012). The possible involvement of
 circulating IGF-1 in both enhancing hypo-osmoregulatory ability and growth makes it a good
 endocrine parameter for monitoring the status of smoltification (Dickhoff et al., 1997; Beckman
 et al., 1998; Kaneko et al., 2015; Suzuki et al., 2020).
- 92 In vertebrates, most IGF-1 molecules in circulation are bound to one of the six IGF-93 binding proteins (IGFBPs) (Rajaram et al., 1997; Bach, 2018). IGFBPs prolong the half-life of 94 circulating IGF-1 and regulate the availability of IGF-1 to its receptor. In salmonids, three to four 95 IGFBPs have been detected in the circulatory system (Shimizu and Dickhoff, 2017). Among these, 96 IGFBP-2b is the major carrier of circulating IGF-1 in salmonids, including rainbow trout 97 (Shimizu et al., 2011; Cleveland et al., 2018; 2020). IGFBP-2b is presumed to deliver IGF-1 to 98 target tissues while protecting it from degradation and glomerular filtration in the kidney (Shimizu 99 and Dickhoff, 2017). Moreover, circulating IGFBP-2b levels are also correlated with growth, to 100 the same extent as IGF-1 (Beckman et al., 2004a,b). Since circulating IGF-1 during smoltification 101 is presumably involved in both stimulating hypo-osmoregulatory ability and promoting growth, 102 it is possible that IGFBP-2b partitions circulating IGF-1 between gills and muscle/bone. However, only a single study has measured circulating IGFBP-2b levels during smoltification in coho 103 104 salmon (Shimizu et al., 2003). This study has reported that circulating IGFBP-2b showed a peak 105 in March, coinciding with the first peak of circulating IGF-1, and remained constant thereafter, 106 while IGF-1 showed a second peak (Shimizu et al., 2003). Such different expression profiles 107 warrant simultaneous monitoring of IGFBP-2b along with IGF-1 to assess their roles during 108 smoltification.
- 109 Smoltification is a season-dependent developmental event influenced by environmental 110 factors such as photoperiod and water temperature (Wedemeyer et al., 1980; Björnsson et al., 111 2011). Photoperiod is a "zeitgeber" of smoltification, whereas water temperature affects the rate 112 and degree of smoltification (McCormick et al., 1995, 2000, 2002). In natural environments, 113 smoltification generally occurs during spring in response to the expansion of the photoperiod. A 114 prolonged photoperiod increases the secretion of GH from the pituitary gland and stimulates the 115 gills to activate NKA directly or indirectly through IGF-1 (Björnsson et al., 1989; McCormick et 116 al., 1995). There is an interaction between the developmental stage and physiological status of 117 juvenile salmon and the environment. Handeland et al. (2013) suggested that juvenile Atlantic 118 salmon exhibits an increased gill NKA activity when a certain size is exceeded. Based on these 119 findings, the timing and degree of smoltification are environmentally controlled to produce "off-120 season" smolts for sea cage aquaculture of Atlantic salmon (Thrush et al., 1994; Duston and 121 Saunders, 1995; Handeland and Stefansson, 2002; Berrill et al., 2006; Striberny et al., 2021).

Among the many strategies for producing high-quality off-season smolts, photoperiod 122 manipulation is central and has been successfully applied for Atlantic salmon. However, 123 124 photoperiod manipulation may not be operative for culturing rainbow trout in seawater. This 125 uncertainty arises from the fact that there are two life-history types in this species: anadromous 126 steelhead and non-anadromous rainbow trout (Kendall et al., 2015). Although the two forms may 127 emerge through phenotypic plasticity, the life-history patterns of this species are predominantly 128 determined by genetics. Johnsson et al. (1994) compared seasonal variation in seawater 129 adaptability among steelhead, rainbow trout and their hybrid and found that the hybrid reduced 130 the seasonality. Yada et al. (2014) also showed that steelhead exhibited an increase in gill NKA 131 activity during spring, while no such change was observed in rainbow trout. Thus, it is important 132 to know what extent rainbow trout used for sea cage aquaculture exhibit changes associated with 133 smoltification in response to environmental cues.

134 We previously revealed that the effects of photoperiod manipulation on the parameters 135 related to smoltification, especially hypo-osmoregulatory ability and growth in seawater, were 136 relatively weak in rainbow trout (Morro et al., 2019). However, the circulating IGF-1 and IGFBP-137 2b levels during the freshwater phase have not been analyzed. Thus, the present study is aimed to 138 further advance the previous study (Morro et al. 2019) by assessing circulating IGF-1 and IGFBP-139 2b in rainbow trout during the predicted smoltification period in that study. Furthermore, we 140 attempt to activate gill NKA activity through manipulating body size by feeding and relate it with 141 circulating IGF-1 and IGFBP-2b.

142

143 **2. Materials and Methods**

144 2.1. Rearing experiment 1: Effects of photoperiod

145 The plasma samples were obtained from Morro et al. (2019). The procedure of the rearing 146 experiment has been described in Morro et al. (2019) in detail. Briefly, yearling rainbow trout 147 with an initial weight of 78 \pm 16.7 g were acclimated in 2×2 m rearing tanks (2500 L) under 148 ambient water temperature and constant light (LL photoperiod) for two weeks in a trout facility 149 of Lerøy Vest AS (Bjørsvik, Hordaland, Norway). Fish were slightly overfed using a commercial 150 dry diet. Thereafter, from February to July, 720 untagged fish were subjected to one of the 151 following four photoperiod regimes under ambient water temperature and well-fed conditions 152 (Fig. 1): constant light, LL (18 weeks); advanced phase photoperiod, APP (6 weeks of light 12 h: 153 dark 12 h (LD12:12) followed by 12 weeks of LD24:0); delayed phase photoperiod, DPP (four 154 weeks of LD24:0 followed by six weeks at LD12:12 and eight weeks of LD24:0); and simulated 155 natural photoperiod, SNP (initial photoperiod was LD12:12 and light period was increased by 45 156 min every week until LD24:0 was attained). Two tanks were used for each treatment and eight 157 fish from each tank (16 fish/treatment) were sampled at 10 occasions during March 3 and July 5, 158 2016. In the present study, the samples and data of the first eight fish out of 16 fish per treatment159 on March 3, March 31, April 13, May 11, June 6, and July 5 were used for analysis.

For sampling, fish were quickly dip-netted out of the tanks and euthanized by a lethal overdose of isoeugenol (AQUI-S[®], AquaTactics Fish Health, WA, USA). The weight and length were recorded for each fish. Blood samples were extracted using a heparinized syringe and centrifuged at 3000 ×g for 5 min to obtain plasma, which was frozen at -80 °C. The data on gill NKA activity from Morro et al. (2019) was used for reproducing a graph and correlation analysis with permission.

166

167 2.2. Rearing experiment 2: Effects of feeding restriction

168 A captive brood stock of yearling rainbow trout (30 g) was reared at Nanae Fresh-Water 169 Laboratory, Field Science Center for Northern Biosphere, Hokkaido University (41°90'N; Kameda-gun, Hokkaido, Japan). They were fed a commercial diet (Marubeni Nisshin Feed Co. 170 171 Ltd., Tokyo, Japan) until satiation in February 2021. In mid-March, eight fish were sampled as 172 the initial group, as described below. One-hundred and eight fish were lightly anesthetized in 173 water containing 2-phenoxyethanol (Kanto Chemical Co., Inc., Tokyo, Japan), individually 174 marked with passive integrated transponder tags (Biomark, Inc. Boise, ID, USA), and evaluated 175 for the initial fork length (FL) and body weight (BW). The fish were randomly placed into one of 176 two tanks/groups, wherein one group was fed to satiation every day and the other group received 177 a restricted feeding ration (25% body weight/day during March, decreased to 12.5% body 178 weight/day that continued until July). Fish were reared under a natural photoperiod in 500 L 179 circular tanks supplied with flow-through well water (10 °C) until mid-July. The experiment was 180 carried out in accordance with the guidelines of the Hokkaido University Field Science Center 181 Animal Care and Use Committee (Approval No. 30-3).

182 The FL and BW of all the fish were measured every month. The condition factor (K) was 183 calculated as: BW (g) \times 100/FL (cm)³. The specific growth rate (SGR) was calculated in length/weight (SGRL/SGRW) as: SGR (% day) = $\ln (s_2 - s_1) \times \ln (d_2 - d_1)^{-1} \times 100$, where s_2 is the 184 185 length or weight on day 2, s_1 is the length or weight on day 1, and $d_2 - d_1$ is the number of days 186 between measurements. Sixteen fish per treatment tank (10–12 fish in July) were sampled every 187 month for gills and blood. Fish were anesthetized in water containing 2-phenoxyethanol, and 188 blood was withdrawn from the caudal vein using a syringe and allowed to clot at 4 °C overnight. 189 Serum was collected after centrifugation at 9,730 \times g for 10 min and stored at -80 °C until further 190 use. The gill filaments from the first arch were collected, frozen immediately on dry ice, and 191 stored at -80 °C until use.

192

193 2.3. Measurement of gill NKA activity

- 194 In the first experiment, the data on gill NKA activity were from Morro et al. (2019), which
- 195 measured according to the procedure provided by McCormick (1993). In the second experiment,
- 196 gill NKA activity was measured according to a previous study (Quabius et al., 1997) with a minor
- 197 modification (i.e., an incorrectly reported concentration (0.66 mM) of sulfuric acid was rectified
- to 0.66 M). The total amount of protein in the homogenate was analyzed using a bicinchoninic
- acid (BCA) protein assay kit (Thermo Scientific, IL, USA). The NKA values were determined as
- $200 \qquad \text{the ouabain-sensitive fraction of ATP hydrolysis, expressed as Pi~(\mu mol) per mg protein per hour.}$
- 201

202 2.4. Time-resolved fluoroimmunoassay (TR-FIA) for IGF-1

To measure IGF-1, serum was first extracted using acid–ethanol, as described by Shimizu et al.
(2000). Thereafter, IGF-1 was quantified using a time-resolved fluoroimmunoassay (TR-FIA),
based on the method described by Small and Peterson (2005). Recombinant salmon/trout IGF-1
(GroPep Bioreagents Pty Ltd, Adelaide, Australia) was used as standard. Time-resolved
fluorescence was measured using a Wallac ARVO X4 Multilabel Counter (PerkinElmer, Inc.,
Waltham, MA, USA).

209

210 2.5. Ligand blotting for IGFBPs

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 3% stacking gel and 12.5% separating gel. Plasma or serum samples (2 µl plasma/serum diluted with 8 µl phosphate-buffered saline) were treated with an equal volume of buffer containing 2% SDS and 10% glycerol at 85 °C for 5 min. Then, the gels were immersed in a solution of 50 mM Tris, 400 mM glycine, and 0.1% SDS, and electrophoresis was carried out at 8 mA for the stacking gel and 12 mA for the separating gel until the bromophenol blue dye-front reached the bottom of the gel.

218 Ligand blotting with digoxigenin-labeled human IGF-1 (DIG-hIGF-1) was performed 219 according to a previously described protocol (Shimizu et al., 2000). The nitrocellulose membranes 220 were incubated overnight with DIG-hIGF-1 and then incubated with antibodies against DIG-221 conjugated horseradish peroxidase (Roche, Indianapolis, IN, USA) at a dilution of 1:1500-2500 222 for 1.5 h at room temperature (20-25 °C). IGFBP was visualized using enhanced 223 chemiluminescence (ECL) western blotting reagents (Amersham Life Science, Arlington Heights, 224 IL, USA). The intensities of serum IGFBP bands were semi-quantified using ImageJ version 225 1.440 (Schneider et al., 2012) and expressed as an arbitrary density unit (ADU). Each blot run 226 normal human serum and the human IGFBP-4 band was used as an inter-assay control to 227 normalize ADU values of trout IGFBPs.

228

229 2.6. Statistical analysis

For the rearing experiment 1, the effects of photoperiod manipulation were analyzed by 230 231 repeated measures analysis of variation (mixed model) by designating replicated tanks as 232 experimental units using the JMP software (SAS Institute Inc., Cary, NC, USA). When significant 233 effects or interactions were found, differences among groups or time points were further identified 234 by one-way analysis of variation (ANOVA) followed by Tukey's honestly significant difference 235 (HSD) test, with differences considered significant at P < 0.05. The data on K and serum IGFBP-236 2 were analyzed by Shapiro-Wilk test because not all data had normal distribution. For the rearing 237 experiment 2, the effects of feeding restriction at each time point were analyzed by Student's t-238 test, with differences considered significant at P < 0.05. When normal distribution or 239 homoscedasticity of the data was violated, Wilcoxon test were performed. Changes over time 240 were assessed by one-way ANOVA or Shapiro-Wilk test depending on the distribution and 241 variation. Simple linear fitting was also conducted using JMP software, and the relationships were 242 considered significant at P < 0.05. Differences in correlation coefficients (r) were tested by 243 comparing the distributions of the data after Fisher's z-transformation.

244

3. Results

246 3.1. Effects of photoperiod manipulation

FL and BW increased over time (P < 0.0001), whereas K remained constant. There were no statistical differences among the four photoperiod treatments in FL, BW, and K in each month except the variation in BW in May (P = 0.0356; Table 1).

250 There was a seasonal fluctuation in gill NKA (P < 0.0001; Fig. 2a). Gill NKA activity 251 was relatively low from March to April and no variation was noted among photoperiod treatments. 252 However, the activity increased in May ($P \le 0.0001$). The gill NKA activity was similar among 253 groups and became low in June (P < 0.0001). Circulating IGF-1 levels were similar among groups 254 and increased from April to July (P < 0.0001; Fig. 2b). Ligand blotting using DIG-hIGF-1 255 revealed the IGFBP-2b band at 43-45 kDa whereas other IGFBP bands were faint (Suppl. Fig. 1). 256 Thus, only IGFBP-2b was semi-quantified. The band intensities of plasma IGFBP-2b were also 257 similar among treatment groups and decreased from May to June (P < 0.0001; Fig. 2c).

Linear fitting was used to examine the correlations of gill NKA and morphological and endocrine parameters for each treatment during March–May. Gill NKA activity was positively correlated with BW in fish subjected to SNP treatment (Fig. 3a). DPP treatment also showed a positive correlation (r = 0.44, P = 0.0160) between gill NKA activity and BW while APP or LL did not (Data not shown). In the SNP-treated fish, gill NKA was also correlated with the plasma IGF-1 levels and the band intensity of plasma IGFBP-2b (Fig. 3b,c).

264

265 *3.2. Effects of variation in feeding ration*

Food restriction resulted in lower FL, BW, and K values compared to satiation throughout the experimental period (P < 0.001; Fig. 4). SGRL and SGRW of the restricted group were also lower than those in the satiation group (P < 0.001; Table 2). Due to further reduction in feeding ration in April, SGRs were significantly decreased in the restricted group (P < 0.0001). From May to July, SGRs in the satiation group decreased (P < 0.0001).

271 Feeding to satiation had a positive effect on gill NKA activity in April (P = 0.0010; Fig. 272 5a). During May–July, gill NKA activities in both groups were similar. Serum IGF-1 levels were 273 higher in the satiation group in comparison with the restriction group (P < 0.001 - 0.0332) except 274 in July and were constant between April and July (Fig. 5b). In contrast, the serum IGF-1 levels in 275 the restricted group increased from June to July (P = 0.0001), regardless of a constant feeding 276 ration. Ligand blotting detected the unidentified 32-kDa IGFBP and IGFBP-1b bands along with 277 the IGFBP-2b band (Fig. 6) and they were semi-quantified. The band intensity of serum IGFBP-278 2b was higher in the satiation group throughout the experimental period (P = 0.0014 - 0.0443; Fig. 279 6a), compared to the restriction group. Both groups showed seasonal changes, being high in April 280 and decreasing thereafter (P < 0.0001 - 0.0002). The band intensity of 32-kDa IGFBP was higher 281 in the satiated group in April (P = 0.0204) and June (P = 0.0021) whereas that of IGFBP-1b was 282 high in the restricted group in May (P = 0.0003) and June (P < 0.0001; Fig. 6b,c).

283 The gill NKA activity in April was positively correlated with the morphological 284 parameters and the band intensity of serum IGFBP-2b, but not with the serum IGF-1 levels (Table 285 3). Such correlations disappeared during May–July, except that between the gill NKA activity and 286 IGFBP-2b in June. Positive correlations were observed between the serum IGF-1 levels and SGRs 287 during April-June, while the highest correlation coefficients were recorded between the serum 288 IGF-1 levels, and SGRs and morphological parameters in June (Table 3). The band intensity of 289 serum IGFBP-2b showed the highest correlation coefficient with gill NKA in April, among other 290 morphological parameters (Table 3). In June, the band intensity of serum IGFBP-2b was 291 correlated with all parameters, while the relationship between body size and gill NKA was not 292 observed in May and July. The band intensity of serum 32-kDa IGFBP was positively correlated 293 with that of serum IGFBP-2b during April–June and growth parameters in June (Table 3). Serum 294 IGFBP-1b showed negative correlations to growth rates and serum IGF-1 during May-June 295 (Table 3).

296

4. Discussion

In the present study, we first established the profiles of circulating IGF-1 in freshwater rainbow trout under different photoperiod regimes using plasma samples obtained from the study by Morro et al. (2019). Taylor et al. (2005) were the first to examine the effect of photoperiod on circulating

301 IGF-1 in rainbow trout and reported that the constant long-days (LD 18:6) from June increased

302 the plasma IGF-1 levels and growth in September. In the present study, surprisingly, photoperiod 303 manipulation, including LL 24:0, did not affect the circulating IGF-1 levels during March–July. 304 This apparent conflict may be attributed to seasonal differences (summer-winter vs. spring-305 summer) and/or the day length of the long-day photoperiod (i.e., LD 18:6 vs. LL 24:0). Even 306 though no significant effect of photoperiod was noted in the present study, circulating IGF-1 levels 307 were found to increase over time. A similar increase in the circulating IGF-1 levels from summer 308 to autumn has been previously observed in coho salmon and rainbow trout; however, it was not 309 accompanied by an increased growth rate (Beckman et al., 2004a; Taylor et al., 2005). The 310 significance of the increased IGF-1 levels from summer to autumn is currently unknown.

311 We further semi-quantified the band intensity of plasma IGFBP-2b using ligand blotting. 312 Although a radioimmunoassay (RIA) was previously established for salmon IGFBP-2b (Shimizu 313 et al., 2003), it is currently not functional because of the limited availability of a radiolabeled 314 tracer and a restriction on the use of radioisotopes (Shimizu, personal communication). To 315 circumvent these limitations, ligand blotting using DIG-labeled hIGF-1 was used to detect 316 IGFBP-2b, which exhibits IGF-binding ability after SDS treatment. The band intensity of IGFBP-317 2b was not altered by photoperiod manipulation, but showed a decrease from May to June, 318 accompanied by a decrease in gill NKA activity. In contrast to our results, an increase in 319 circulating IGFBP-2b in September has been reported in postsmolt coho salmon (Beckman et al., 320 2004), which may be due to species-based differences. Considering the role of IGFBP-2b as the 321 main carrier of IGF-1 (Shimizu and Dickhoff, 2017), the increase in circulating IGF-1 and 322 decrease in IGFBP-2b implies an increase in the availability of IGF-1 to target tissue. However, 323 the growth parameters of these fish did not increase during June-July (Morro et al., 2019). The 324 cause and significance of the decrease in IGFBP-2b band intensity are not known at present and 325 need to be investigated in future studies.

326 Morro et al. (2019) reported that the effect of photoperiod on the activation of gill NKA 327 in rainbow trout was relatively weak. We examined whether body size/condition, IGF-1 or/and 328 IGFBP-2b are involved in the activation of gill NKA under different photoperiod regimes. For 329 this purpose, the data obtained between March and May were pooled, encompassing the period 330 of gill NKA activation for each photoperiod treatment, while the data obtained in June were 331 excluded to avoid a possible physiological shift during desmoltification (reversion to parr; Hoar, 332 1989). There were positive correlations between gill NKA activity and body size in fish under 333 SNP, LL, and DPP, but not under APP conditions. In addition, the plasma IGF-1 levels and 334 IGFBP-2b band intensity were positively correlated with gill NKA in the SNP-treated fish. These 335 results suggest that the size-dependent increase in gill NKA activity under SNP is mediated via IGF-1 and IGFBP-2b. 336

337

To assess the relationships of circulating IGF-1 and IGFBP-2b to the development of

338 gill NKA activity and body size and growth rate, individually pit-tagged rainbow trout were reared 339 under two feeding regimes: fed to satiation and at restricted ration. Feeding manipulation 340 separated the two groups based on FL, BW, K, and SGRs since April. From March to April, gill 341 NKA activity increased, and the values were higher in fish fed to satiation than in fish fed with 342 restricted ration. These results confirm that body size and/or growth affect gill NKA activity in 343 yearling rainbow trout in spring. Our finding is in good agreement with previous studies showing 344 that seawater tolerance/adaptability of rainbow trout is size-dependent (Landness, 1976; Jackson, 345 1981; Johnsson and Clarke, 1988; Kaneko et al., 2019). Such size-dependent increase in gill NKA 346 activity has also been reported in coho salmon and Atlantic salmon (Shrimpton, 1996; Handeland 347 et al., 2013). Overall, the findings of the present study further highlight the contribution of size 348 in activating gill NKA. However, the peak values of gill NKA activity observed in the present 349 study are remarkably lower as compared to those obtained from the previous experiment, 350 presumably due to the differences in the methods employed (i.e. Quabius et al., 1997 and 351 McCormick et al., 1993). The method provided by Quabius et al. (1997) revealed an increase in 352 gill NKA activity from 0.5 µmol Pi/mg/h to 5.0 µmol Pi/mg/h during smoltification of yearling 353 masu salmon (Suzuki et al., 2020). Thus, in this respect, gill NKA activity observed in the satiated 354 group in April (3.0 µmol Pi/mg/h) was relatively high and significant. Notably, the increase in gill 355 NKA activity in the satiated group was transient and observed only in April. Later, the activity 356 became similar to that in restricted fish, which suggests that the independent effect of feeding 357 ration was moderate on this size range of fish.

358 Although we confirmed that body size during spring is a factor affecting gill NKA in 359 yearling rainbow trout, whether body size or growth rate has a more determinative effect is yet to 360 be elaborated. In addition, our results also suggest that the size-dependent increase in gill NKA 361 depends on the season and developmental stage, because the size of the fish with a restricted diet 362 in July was similar to that of the satiated fish in April, without any increase gill NKA activity. 363 Zydlewski et al. (2014) reported that advancing photoperiod and fish size were significant factors 364 explaining the variation of gill NKA activity in coastal cutthroat trout (O. clarkii), a species 365 closely related to rainbow trout. Our results are in line with their finding, and it is possible that 366 feeding manipulation at an earlier stage would have a stronger effect on the enhancement of gill 367 NKA activity in rainbow trout.

Serum IGF-1 levels and the IGFBP-2b band intensity were higher in the satiation group than in the restricted group from April to June, as expected from previous reports on postsmolt coho salmon (Beckman et al., 2004a,b). However, the effects of food restriction disappeared in July due to an increase in IGF-1 in the restricted group and a decrease in IGFBP-2b in both groups. These changes are similar to those observed in the photoperiod manipulation experiment, suggesting it is a common seasonal response in this species. Moreover, results of the correlation analysis revealed that relationships between morphological and endocrine parameters in July were
 very different from those during April–June. Such physiological shift during June–July might
 reflect the desmoltification process.

377 Unexpectedly, gill NKA activity was correlated with the IGFBP-2b levels, but not with 378 circulating IGF-1 levels. The present study is the first to report a positive correlation between gill 379 NKA activity and circulating IGFBP-2b levels. This suggests that IGFBP-2b is important for the 380 partitioning of IGF-1 to stimulate the gills in addition to directing it to muscles and bones to 381 promote growth during smoltification. There was a variance in the relationship between 382 circulating IGF-1 and growth, which was weak (r = 0.37) in April when gill NKA was high and 383 strong (P < 0.001) in June (r = 0.87) when gill NKA was stable. Similarly, in smolting masu 384 salmon, the IGF-receptor 1a and 1b mRNA levels were relatively high in May and June 385 (Shimomura et al., 2012). The disruption of the IGF-1–growth relationship suggests that IGF-1 is 386 partitioned between the activation of gill NKA and promotion of growth during this period. 387 However, the roles of local IGF-1 and IGFBPs should also be considered because their mRNA 388 levels in the gills are also altered during smoltification (Shimomura et al., 2012; Breves et al., 389 2017).

390 Two additional serum IGFBP showed responses to food restriction but had no relation 391 to gill NKA activity. The 32-kDa IGFBP is a fourth IGFBP detected in some salmonids including 392 rainbow trout (Cleveland et al., 2020). The band intensity of the 32-kDa IGFBP was generally 393 high in the satiated group and positively correlated with growth parameters in June. In addition, 394 there was a consistent positive relationship between serum 32-kDa IGFBP and IGFBP-2b during 395 April-June, which is in line with the previous finding in Cleveland et al. (2020) and suggests that 396 it has a role similar or cooperative to IGFBP-2b. However, it showed no relationship with gill 397 NKA activity. Serum IGFBP-1b is believed to be inhibitory to IGF-1 action in fish and induced 398 under catabolic conditions such as fasting and stress (Kelley et al., 2001; Shimizu and Dickfhoff, 399 2017; Hasegawa et al., 2020). In rainbow trout, IGFBP-1b is generally hardly detected (Cleveland 400 et al., 2018; 2020). In the feeding manipulation experiment, the IGFBP-1b band was weak but 401 detectable, which was stronger in the restricted group than in the satiated group and showed 402 negative relationships with growth parameters in May and June but not with gill NKA activity. In 403 masu salmon (O. masou), circulating IGFBP-1b level was positively correlated with gill NKA 404 during smotlfitication (Fukuda et al., 2015), suggesting that there is a species difference in the 405 role of IGFBP-1b during this period. Overall, the lack of relationship of serum 32-kDa IGFBP 406 and IGFBP-1b with gill NKA activity further highlights the possible involvement of IGFBP-2b 407 in the development of gill NKA in rainbow trout.

In conclusion, our results suggest the size-dependent increase in gill NKA in yearling
 rainbow trout during spring is mediated in part by the action of IGFBP-2b most likely through

410 partitioning IGF-1 between the gills and somatic tissues, and IGFBP-2b is thus a good parameter

- 411 to monitor the degree of putative smoltification in rainbow trout.
- 412

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425 **6. References**

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607

608 Figure legends

Fig. 1. Schematic representation of the number of hours of light for each of the four different
treatments. APP: advanced phase photoperiod, DPP: delayed phase photoperiod, LL: continuous
light, SNP: simulated natural photoperiod.

612

Fig. 2. Effects of photoperiods on gill Na⁺,K⁺-ATPase (NKA) activity (a), plasma IGF-1 levels (b) and plasma IGFBP-2b band intensity (c) in yearling rainbow trout. Fish were reared under advanced phase photoperiod (APP), simulated natural photoperiod (SNP), continuous light (LL) or delayed phase photoperiod (DPP) in freshwater. Values are expressed as means \pm SE (n =8/treatment/time point). At a given time point, groups without letter or sharing the same letters are not significantly different from each other (Tukey's HSD, P < 0.05). The graph of gill NKA is reproduced from Morro et al. (2019) with permission.

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Fig. 3 Correlations of gill Na⁺,K⁺-ATPase (NKA) activity with body weight (BW; a), plasma IGF1 levels (b) and plasma IGFBP-2b intensity (c) in yearling rainbow trout under simulated natural
photoperiod during March-May.

624

Fig. 4. Profiles of fork length (a), body weight (b) and condition factor (c) in yearling rainbow trout with or without food restriction. Values are expressed as means \pm SE (n =16/treatment/time point, except n = 10-12 in July). Asterisks indicate significant difference between two feeding treatments at a given time point (*t*-test, P < 0.05).

629

Fig. 5. Profiles of gill Na⁺,K⁺-ATPase (NKA) activity (a) and serum IGF-1 levels (b) in yearling rainbow trout with or without food restriction. Values are expressed as means \pm SE (n =16/treatment/time point, except n = 10–12 in July). Asterisks indicate significant difference between two feeding treatments at a given time point (Wilcoxon test, P < 0.05).

634

Fig. 6. IGFBP patterns in serum of rainbow trout fed to satiation (Satiated) and at restricted ration
(Restricted) in April. Two microliters of serum was separated by 12.5% SDS-PAGE under nonreducing conditions, electroblotted onto a nitrocellulose membrane and subjected with ligand
blotting using digoxigenin-labeled human IGF-I (50 ng) and antiserum against digoxigenin
(1:20,000). Arrows indicate migration positions of human (left) NHS and trout (right) IGFBP
bands. NHS: normal human serum; NS: non-specific

641

642 Fig. 7. Profiles of the band intensities of serum IGFBP-2b (a), 32-kDa IGFBP and IGFBP-1b (c)

643 in yearling rainbow trout with or without food restriction. Values are expressed as means \pm SE

- (n = 16/treatment/time point, except n = 10-12 in July). Asterisks indicate significant difference
- 645 between two feeding treatments at a given time point (Wilcoxon test, P < 0.05).







Date





□ Initial ■ Satiation □ Restriction



□ Initial ■ Satiation □ Restriction

Cui et al., Fig. 6







Suppl. Fig. 1. IGFBP patterns in plasma of rainbow trout reared under different photoperiod regimes in May. Two microliters of plasma was separated by 12.5% SDS-PAGE under non-reducing conditions, electroblotted onto a nitrocellulose membrane and subjected with ligand blotting using digoxigenin-labeled human IGF-I (50 ng) and antiserum against digoxigenin (1:20,000). Arrows indicate migration positions of human (left) NHS and trout (right) IGFBP bands. NHS: normal human serum; APP: advanced phase photoperiod; SNP: simulated natural photoperiod; LL: continuous light; DPP: delayed phase photoperiod; NS: non-specific.