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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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Abstract

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

Keywords

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

1. Introduction

Rainbow trout (*Oncorhynchus mykiss*) is an important target species for aquaculture; approximately 848,000 tons of rainbow trout was produced worldwide in 2018 (FAO, 2020). In Norway, it accounts for about 6% of aquaculture production. Although this species has traditionally been cultured in freshwater, there is an increasing interest in culturing them in seawater or brackish water to occupy the unused area available from Atlantic salmon (*Salmo salar*) aquaculture. However, notable mortalities and retarded growth have occasionally been observed in practice, probably due to their premature transfer to seawater (Morro et al., 2019, 2020, 2021). Such phenomenon is called "stunting" reported in Atlantic salmon and coho salmon (*O. kisutch*) (Björnsson et al., 1988; Young et al., 1989). Although seawater tolerance/adaptability 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 the knowledge gap about the physiological and endocrine changes that take place during the smoltification process in rainbow trout.

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

During smoltification, salmonids acquire another characteristic, which is an enhanced growth capacity before and after entering seawater (Dickhoff et al., 1997). The GH-IGF-1 system is responsible for regulating the growth of vertebrates, including fish (Wood et al., 2005; Reinecke, 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 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).

In vertebrates, most IGF-1 molecules in circulation are bound to one of the six IGF-binding proteins (IGFBPs) (Rajaram et al., 1997; Bach, 2018). IGFBPs prolong the half-life of circulating IGF-1 and regulate the availability of IGF-1 to its receptor. In salmonids, three to four IGFBPs have been detected in the circulatory system (Shimizu and Dickhoff, 2017). Among these, IGFBP-2b is the major carrier of circulating IGF-1 in salmonids, including rainbow trout (Shimizu et al., 2011; Cleveland et al., 2018; 2020). IGFBP-2b is presumed to deliver IGF-1 to target tissues while protecting it from degradation and glomerular filtration in the kidney (Shimizu and Dickhoff, 2017). Moreover, circulating IGFBP-2b levels are also correlated with growth, to the same extent as IGF-1 (Beckman et al., 2004a,b). Since circulating IGF-1 during smoltification is presumably involved in both stimulating hypo-osmoregulatory ability and promoting growth, 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 salmon (Shimizu et al., 2003). This study has reported that circulating IGFBP-2b showed a peak in March, coinciding with the first peak of circulating IGF-1, and remained constant thereafter, while IGF-1 showed a second peak (Shimizu et al., 2003). Such different expression profiles warrant simultaneous monitoring of IGFBP-2b along with IGF-1 to assess their roles during smoltification.

Smoltification is a season-dependent developmental event influenced by environmental factors such as photoperiod and water temperature (Wedemeyer et al., 1980; Björnsson et al., 2011). Photoperiod is a “zeitgeber” of smoltification, whereas water temperature affects the rate and degree of smoltification (McCormick et al., 1995, 2000, 2002). In natural environments, smoltification generally occurs during spring in response to the expansion of the photoperiod. A prolonged photoperiod increases the secretion of GH from the pituitary gland and stimulates the gills to activate NKA directly or indirectly through IGF-1 (Björnsson et al., 1989; McCormick et al., 1995). There is an interaction between the developmental stage and physiological status of juvenile salmon and the environment. Handeland et al. (2013) suggested that juvenile Atlantic salmon exhibits an increased gill NKA activity when a certain size is exceeded. Based on these findings, the timing and degree of smoltification are environmentally controlled to produce “off-season” smolts for sea cage aquaculture of Atlantic salmon (Thrush et al., 1994; Duston and 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 manipulation is central and has been successfully applied for Atlantic salmon. However, photoperiod manipulation may not be operative for culturing rainbow trout in seawater. This uncertainty arises from the fact that there are two life-history types in this species: anadromous steelhead and non-anadromous rainbow trout (Kendall et al., 2015). Although the two forms may emerge through phenotypic plasticity, the life-history patterns of this species are predominantly determined by genetics. Johnsson et al. (1994) compared seasonal variation in seawater adaptability among steelhead, rainbow trout and their hybrid and found that the hybrid reduced the seasonality. Yada et al. (2014) also showed that steelhead exhibited an increase in gill NKA activity during spring, while no such change was observed in rainbow trout. Thus, it is important to know what extent rainbow trout used for sea cage aquaculture exhibit changes associated with smoltification in response to environmental cues.

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

2. Materials and Methods

2.1. Rearing experiment 1: Effects of photoperiod

The plasma samples were obtained from Morro et al. (2019). The procedure of the rearing experiment has been described in Morro et al. (2019) in detail. Briefly, yearling rainbow trout with an initial weight of 78 ± 16.7 g were acclimated in 2×2 m rearing tanks (2500 L) under ambient water temperature and constant light (LL photoperiod) for two weeks in a trout facility of Lerøy Vest AS (Bjørsvik, Hordaland, Norway). Fish were slightly overfed using a commercial dry diet. Thereafter, from February to July, 720 untagged fish were subjected to one of the following four photoperiod regimes under ambient water temperature and well-fed conditions (Fig. 1): constant light, LL (18 weeks); advanced phase photoperiod, APP (6 weeks of light 12 h: dark 12 h (LD12:12) followed by 12 weeks of LD24:0); delayed phase photoperiod, DPP (four weeks of LD24:0 followed by six weeks at LD12:12 and eight weeks of LD24:0); and simulated natural photoperiod, SNP (initial photoperiod was LD12:12 and light period was increased by 45 min every week until LD24:0 was attained). Two tanks were used for each treatment and eight 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 treatment
159 on March 3, March 31, April 13, May 11, June 6, and July 5 were used for analysis.
160 For sampling, fish were quickly dip-netted out of the tanks and euthanized by a lethal
161 overdose of isoeugenol (AQUI-S®, AquaTactics Fish Health, WA, USA). The weight and length
162 were recorded for each fish. Blood samples were extracted using a heparinized syringe and
163 centrifuged at 3000 ×*g* for 5 min to obtain plasma, which was frozen at −80 °C. The data on gill
164 NKA activity from Morro et al. (2019) was used for reproducing a graph and correlation analysis
165 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;
170 Kameda-gun, Hokkaido, Japan). They were fed a commercial diet (Marubeni Nisshin Feed Co.
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) × 100/FL $(cm)^3$. The specific growth rate (SGR) was calculated in
184 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
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 ×*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*

In the first experiment, the data on gill NKA activity were from Morro et al. (2019), which measured according to the procedure provided by McCormick (1993). In the second experiment, gill NKA activity was measured according to a previous study (Quabius et al., 1997) with a minor 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 the ouabain-sensitive fraction of ATP hydrolysis, expressed as Pi (μmol) per mg protein per hour.

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).

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.

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

2.6. Statistical analysis

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

3. Results

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).

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

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 ($P < 0.0001$).

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

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

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

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

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

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

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

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

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

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

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

partitioning IGF-1 between the gills and somatic tissues, and IGFBP-2b is thus a good parameter to monitor the degree of putative smoltification in rainbow trout.

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607

608 **Figure legends**

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

612

613 Fig. 2. Effects of photoperiods on gill Na^+,K^+-ATPase (NKA) activity (a), plasma IGF-1 levels
614 (b) and plasma IGFBP-2b band intensity (c) in yearling rainbow trout. Fish were reared under
615 advanced phase photoperiod (APP), simulated natural photoperiod (SNP), continuous light (LL)
616 or delayed phase photoperiod (DPP) in freshwater. Values are expressed as means ± SE (n =
617 8/treatment/time point). At a given time point, groups without letter or sharing the same letters
618 are not significantly different from each other (Tukey's HSD, $P < 0.05$). The graph of gill NKA
619 is reproduced from Morro et al. (2019) with permission.

620

621 Fig. 3 Correlations of gill Na^+,K^+-ATPase (NKA) activity with body weight (BW; a), plasma IGF-
622 1 levels (b) and plasma IGFBP-2b intensity (c) in yearling rainbow trout under simulated natural
623 photoperiod during March-May.

624

625 Fig. 4. Profiles of fork length (a), body weight (b) and condition factor (c) in yearling rainbow
626 trout with or without food restriction. Values are expressed as means ± SE (n =
627 16/treatment/time point, except n = 10–12 in July). Asterisks indicate significant difference
628 between two feeding treatments at a given time point (t-test, $P < 0.05$).

629

630 Fig. 5. Profiles of gill Na^+,K^+-ATPase (NKA) activity (a) and serum IGF-1 levels (b) in yearling
631 rainbow trout with or without food restriction. Values are expressed as means ± SE (n =
632 16/treatment/time point, except n = 10–12 in July). Asterisks indicate significant difference
633 between two feeding treatments at a given time point (Wilcoxon test, $P < 0.05$).

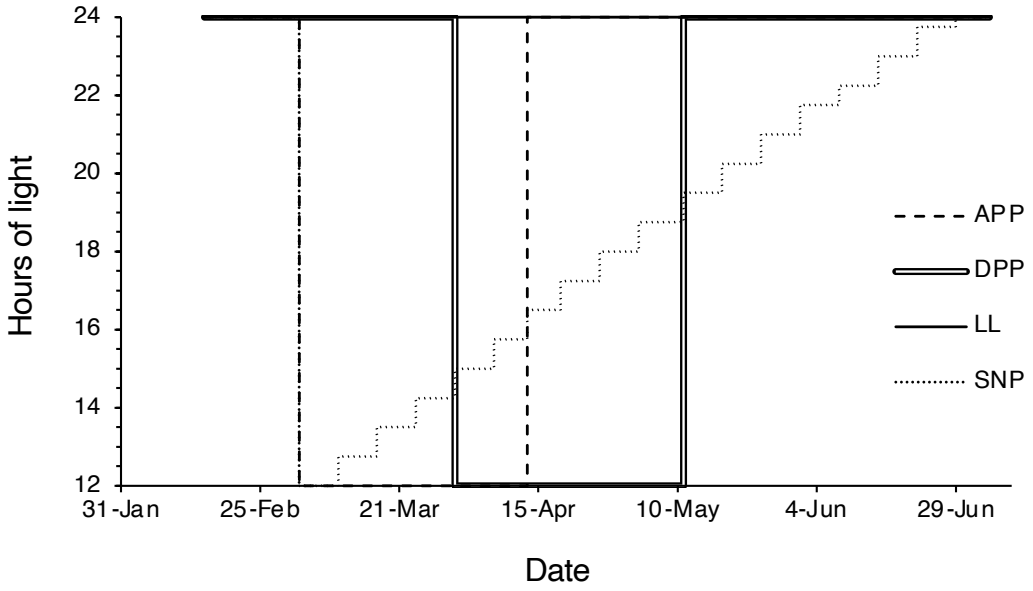
634

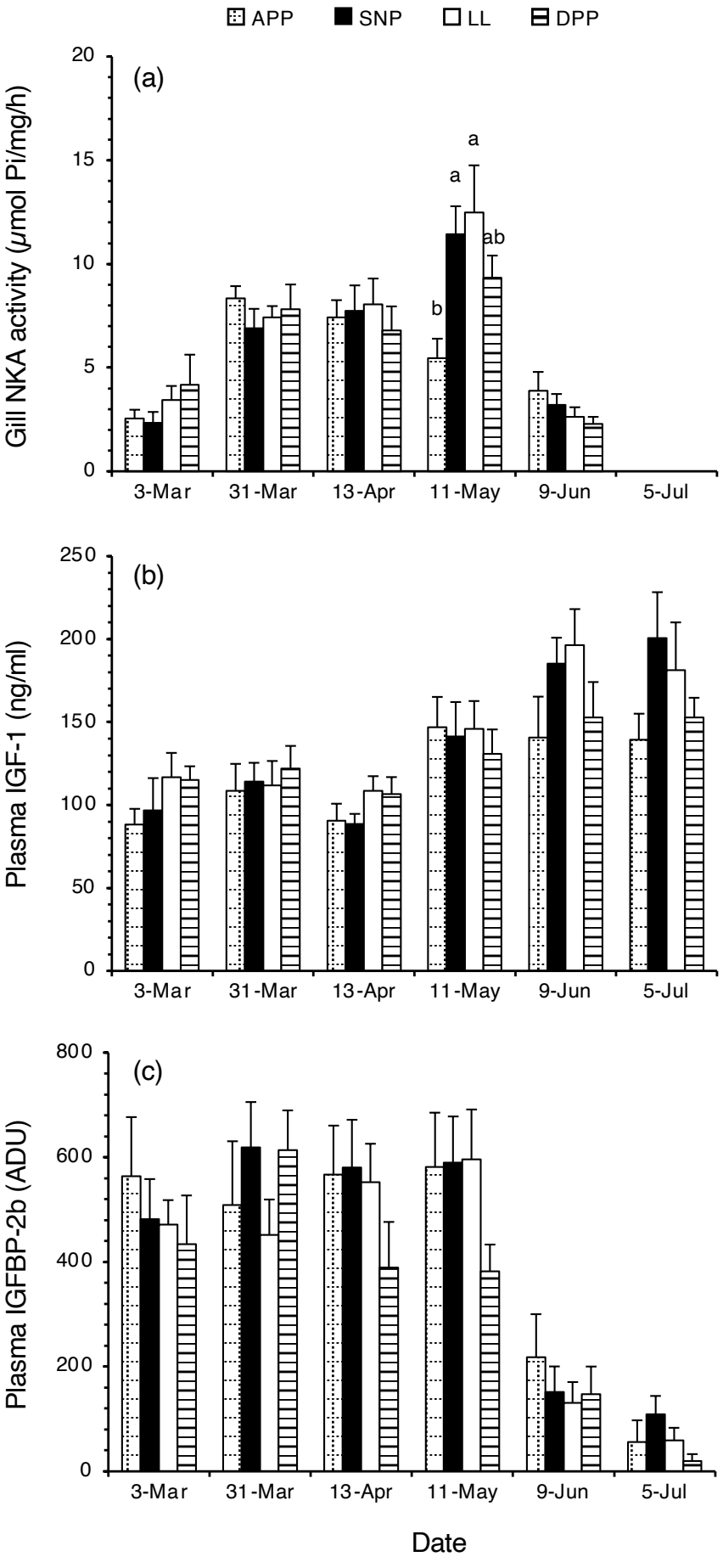
635 Fig. 6. IGFBP patterns in serum of rainbow trout fed to satiation (Satiated) and at restricted ration
636 (Restricted) in April. Two microliters of serum was separated by 12.5% SDS-PAGE under non-
637 reducing conditions, electroblotted onto a nitrocellulose membrane and subjected with ligand
638 blotting using digoxigenin-labeled human IGF-I (50 ng) and antiserum against digoxigenin
639 (1:20,000). Arrows indicate migration positions of human (left) NHS and trout (right) IGFBP
640 bands. NHS: normal human serum; NS: non-specific

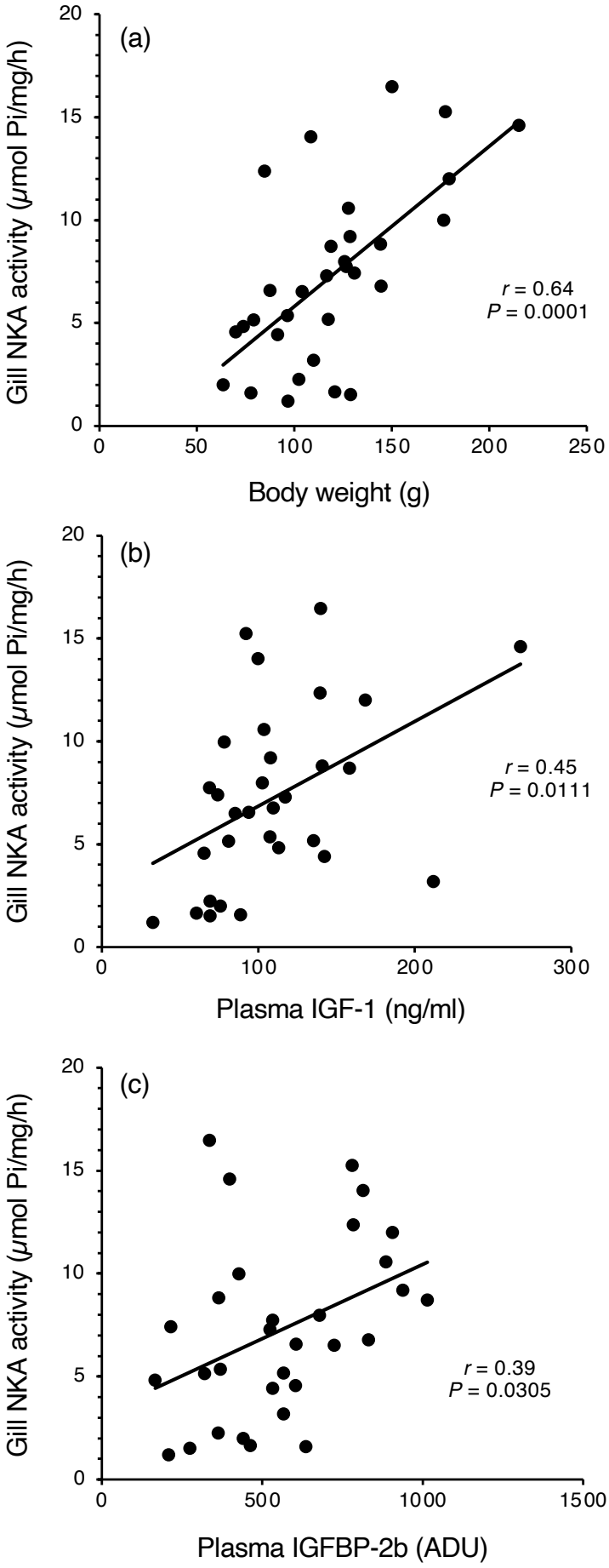
641

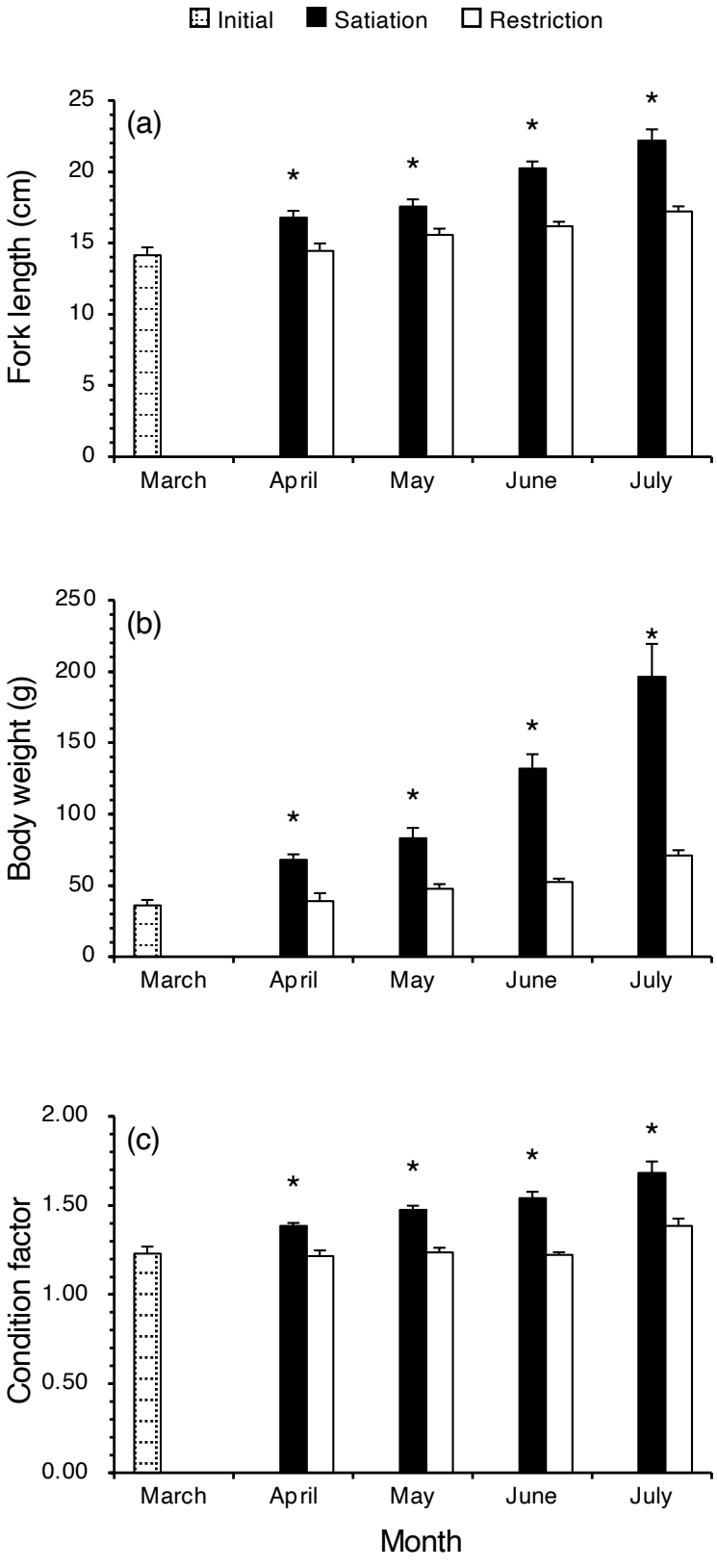
642 Fig. 7. Profiles of the band intensities of serum IGFBP-2b (a), 32-kDa IGFBP and IGFBP-1b (c)
643 in yearling rainbow trout with or without food restriction. Values are expressed as means ± SE

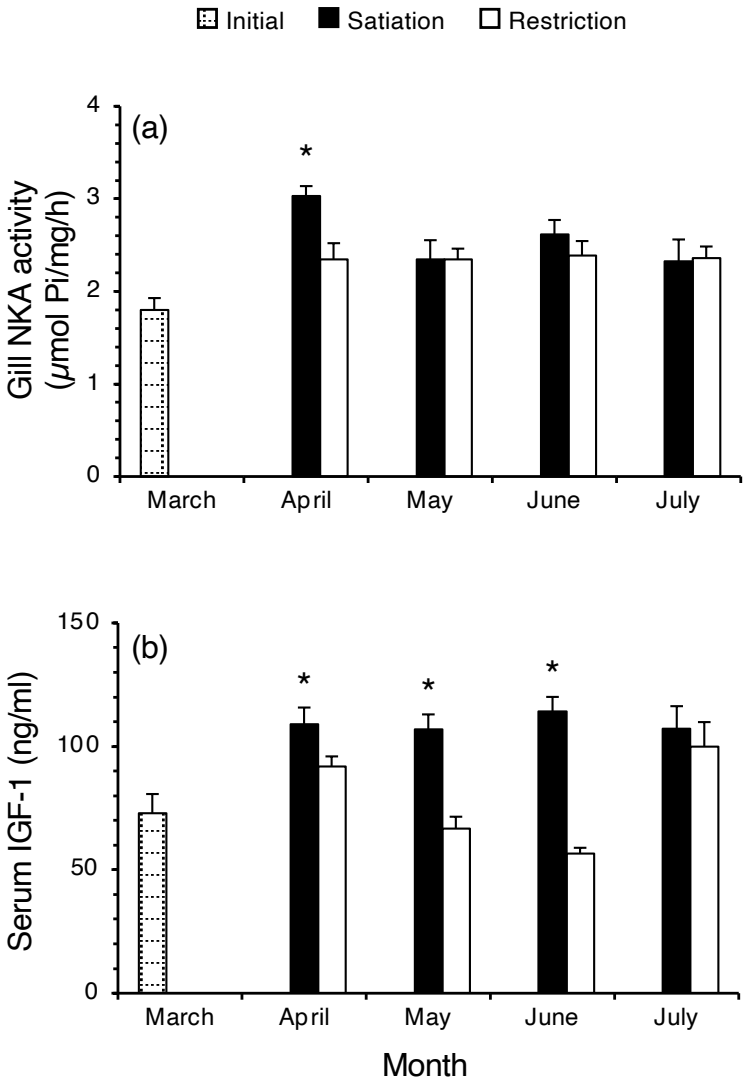
644 (n = 16/treatment/time point, except n = 10–12 in July). Asterisks indicate significant difference
645 between two feeding treatments at a given time point (Wilcoxon test, $P < 0.05$).

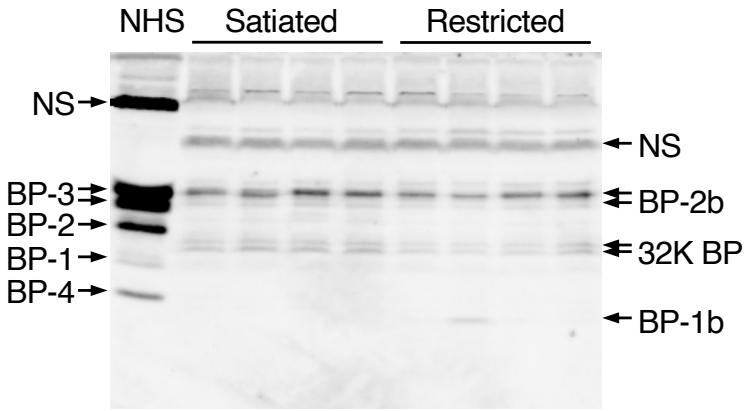

24
22
20
18
16
14
12
Hours of light
31-Jan
25-Feb
21-Mar
15-Apr
10-May
4-Jun
29-Jun
Date
APP
DPP
LL
SNP

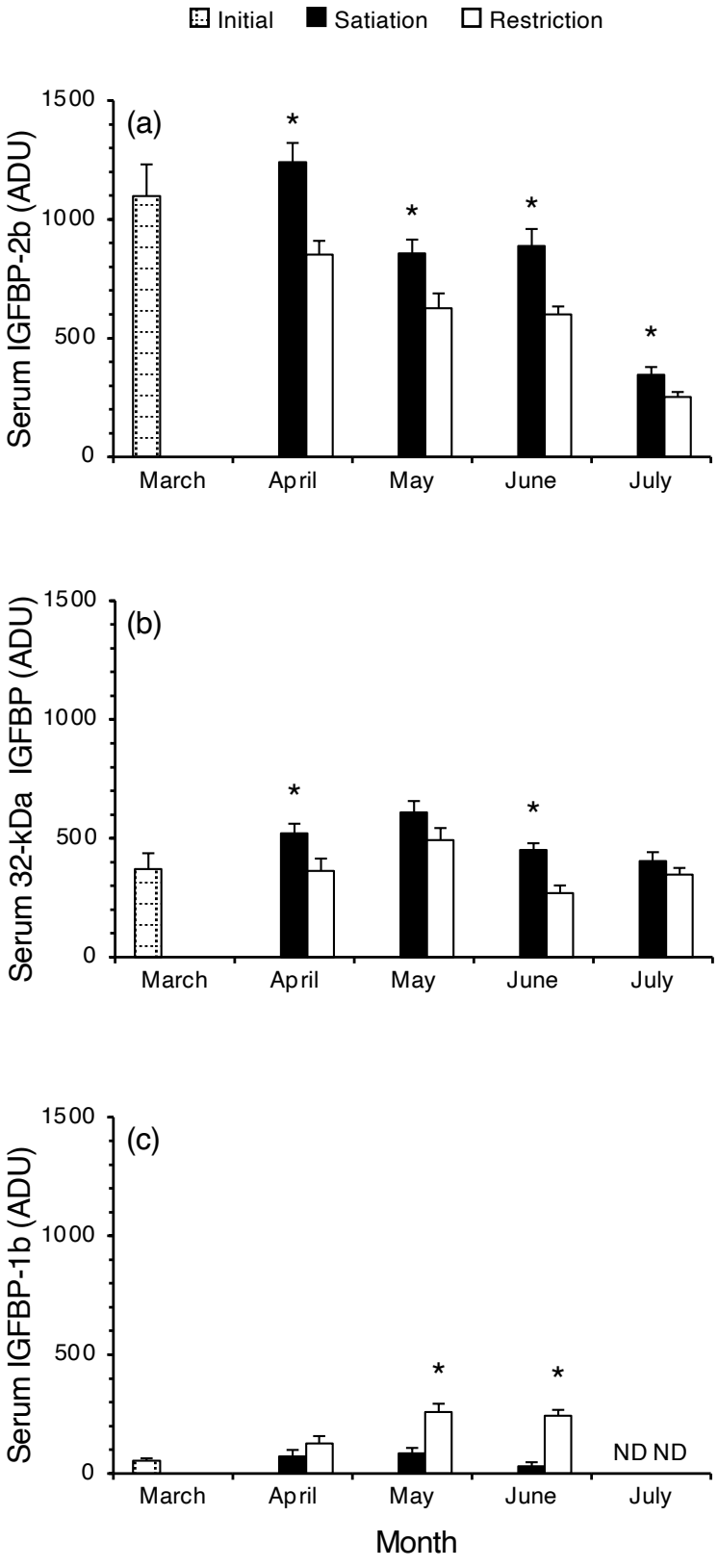

APP
SNP
LL
DPP
(a)
Gill NKA activity (μmol Pi/mg/h)
20
15
10
5
0
a
a
ab
b
3-Mar
31-Mar
13-Apr
11-May
9-Jun
5-Jul
(b)
Plasma IGF-1 (ng/ml)
250
200
150
100
50
0
3-Mar
31-Mar
13-Apr
11-May
9-Jun
5-Jul
(c)
Plasma IGFBP-2b (ADU)
800
600
400
200
0
3-Mar
31-Mar
13-Apr
11-May
9-Jun
5-Jul
Date


(a)
Gill NKA activity (μmol Pi/mg/h)
Body weight (g)
r = 0.64
P = 0.0001
(b)
Gill NKA activity (μmol Pi/mg/h)
Plasma IGF-1 (ng/ml)
r = 0.45
P = 0.0111
(c)
Gill NKA activity (μmol Pi/mg/h)
Plasma IGFBP-2b (ADU)
r = 0.39
P = 0.0305


Initial
Satiation
Restriction
(a)
Fork length (cm)
25
20
15
10
5
0
March
April
May
June
July
(b)
Body weight (g)
250
200
150
100
50
0
(c)
Condition factor
2.00
1.50
1.00
0.50
0.00
Month


Initial
Satiation
Restriction
(a)
Gill NKA activity
(μmol Pi/mg/h)
0
1
2
3
4
*
March
April
May
June
July
(b)
Serum IGF-1 (ng/ml)
0
50
100
150
*
*
*
March
April
May
June
July
Month


NHS
Satiated
Restricted
NS
BP-3
BP-2
BP-1
BP-4
NS
BP-2b
32K BP
BP-1b


Initial
Satiation
Restriction
(a)
Serum IGFBP-2b (ADU)
1500
1000
500
0
March
April
May
June
July
(b)
Serum 32-kDa IGFBP (ADU)
(c)
Serum IGFBP-1b (ADU)
ND ND
Month

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.