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Effects of different photoperiod regimes on the smoltification and seawater adaptation of seawater-farmed rainbow trout (*Oncorhynchus mykiss*): Insights from Na^+, K^+–ATPase activity and transcription of osmoregulation and growth regulation genes

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ABSTRACT

Photoperiod is thought to be the main *zeitgeber* that induces smoltification in salmonids. However, its effects on the smoltification of rainbow trout (*Oncorhynchus mykiss*) are not fully understood and no published data documents the effects of the photoperiod regime currently used commercially, continuous light (LL). The present study compared the effect of four different photoperiod regimes (i.e. advanced phase photoperiod (APP), delayed phase photoperiod (DPP), LL and simulated natural photoperiod (SNP)) on the smoltification and growth of juvenile rainbow trout during their freshwater phase of winter-spring and the following summer post smolt phase. Smoltification was evaluated by monitoring gill Na^+,K^+–ATPase (NKA) activity and transcription of NKA α-subunit isoforms *1a* and *1b*, and *Na^+,K^+,2Cl^- cotransporter 1a*. Growth was measured as specific growth rate of both length and weight, and through molecular growth proxies such as the levels of circulating insulin-like growth factor 1 (IGF-I) in plasma and transcription of *igf-I*, *igf binding protein 1b* (*igfbp1b*), *growth hormone receptor 1* (*ghr1*) and *cathepsin L* (*ctsl*) in the liver. Results indicate that APP induces a longer smolt window and higher levels of plasma IGF-I in both freshwater and seawater (two months post transfer), while DPP led to a shorter smolt window, lower plasma IGF-I levels in freshwater and seawater, an earlier decrease in liver *igf-I* and *ctsl* transcription in freshwater (as seen by modelling over time) and lower specific growth rate in freshwater. The transcription analysis of osmoregulatory genes complemented NKA activity and allowed for the detection of a transient response to light and of differences between the osmoregulatory capacity of parr and desmolted fish. Furthermore, an upregulation of the liver transcription of *igf-I*, *ghr1* and *ctsl* was found in all treatments during the smolt window, which corresponded to the periods with highest growth. Finally, both plasma IGF-I and liver *igf-I* in seawater were found to be significantly correlated to fish growth in seawater. However, our data did not show that plasma IGF-I prior to seawater transfer could be used as a reliable predictor of growth in seawater. Overall, and especially when compared with other salmonid species, photoperiod seems to be a weaker inducer of smoltification in rainbow trout, according to the parameters that were tested, suggesting that other environmental cues might be more important drivers of this process.

1. Introduction

Anadromous salmonids migrate to seawater in order to meet their energy demands for maintenance, growth and reproduction. However, juveniles do not have the biological traits needed for life in seawater and therefore, require to go through a series of simultaneous, yet often independent, changes that prepare salmonids to life in seawater, collectively known as smoltification (Björnsson et al., 2011; Hoar, 1988).

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These changes are biochemical in nature, such as the alteration of the haemoglobin isoforms, which increases oxygen carrying-capacity of blood (Fyhn et al., 1991); physiological, such as the increase of gill Na^+,K^+–ATPase (NKA) activity, which is the main enzyme involved in ion absorption and secretion (Mancera and McCormick, 2007; McCormick, 2001); morphological, such as the transition from dark, rounded parr to silvery, streamlined smolts; and behavioural, including the shift from bottom-dwelling, aggressive and territorial parr to pelagic, schooling and downstream migrating smolts (Riley et al., 2014). Once anadromous fish reach a threshold size (Kendall et al., 2015), smoltification is triggered by environmental cues, such as changes in photoperiod, water temperature and salinity, which in turn alter the pituitary, thyroid and inter-renal tissues (Prunet et al., 1989). These tissues are key orchestrators of the seawater-adaptation changes. Seawater-adaptation changes are reversible and last during a short period of time (smolt window). If fish do not reach seawater within the smolt window changes are lost (desmoltification) (Stefansson et al., 1998).

Over the last three decades there has been an increase in the aquaculture production of the sea-run phenotype of rainbow trout (*Oncorhynchus mykiss*), which reached over 87,000 t in Norway alone in 2016 (Food and Agriculture Organization of the United Nations, 2016). Industrial interest of this phenotype is based on its resistance to infectious pancreatic necrosis (Okamoto et al., 1993; Ozaki et al., 2001) and its preference for brackish water compared to Atlantic salmon (*Salmo salar*) (Altinok and Grizzle, 2001). This preference for brackish seawater gives the possibility to grow rainbow trout in locations that are not as suitable for Atlantic salmon production and that are less prone to sea lice (*Lepeophtheirus salmonis*) infestations. However, there is limited knowledge on the environmental control of rainbow trout smoltification and there is a need for appropriate tools to assess rainbow trout smolt status. In fact, the aquaculture industry has reported that rearing protocols developed for the all-season production of Atlantic salmon might not be suitable for other salmonids. Reported issues include high mortality and fish that experience sub-optimal growth (growth-stunted phenotype) after seawater transfer, especially in summer post smolts, similarly to growth-stunted Atlantic salmon (Stephen and Ribble, 1995; Stien et al., 2013; Vindas et al., 2016). These problems are likely related to a current lack of understanding of how vital intrinsic (e.g. critical size, genetically determined phenotypic plasticity) and extrinsic (e.g. salinity, temperature, light) factors impact smoltification in rainbow trout, leading to suboptimal rearing conditions and/or mismatched timing of seawater transfer, as reported for other salmonids (Folmar et al., 1982). Currently, for rainbow trout, seawater tolerance is regarded only as size dependent and no studies have analysed if phenotype plasticity is linked to particular genotypes. Regarding extrinsic factors, smoltification is currently induced for all year production by rearing the rainbow trout under continuous light (LL) photoperiod without much supporting evidence for such practice while the impact of other potentially related factors such as temperature and salinity are unknown.

Photoperiod is known to play a major role in the smoltification of anadromous salmonids, with short days (winter signal) followed by increasingly longer days (spring signal) acting as a *zeitgeber* that indicates the proximity to the summer season and the necessity to migrate, thus triggering smoltification (Brauer, 1982; Saunders et al., 1985; Zaugg and Wagner, 1973). The manipulation of this environmental factor is the most common tool for the all year production of anadromous salmonid aquaculture (Handeland and Stefansson, 2001). Extensive literature on the manipulation of photoperiod to induce smoltification is available for Atlantic salmon (Handeland et al., 2013; McCormick and Moriyama, 2000; Stefansson et al., 2007) and this knowledge is applied commercially by using dynamic photoperiod regimes optimised for each of the four harvest times in a year (Good et al., 2016). On the other hand, for rainbow trout it is unclear whether the photoperiod that is currently being used in commercial farms, LL, is the most appropriate to produce smolts, although there is evidence that long day photoperiods (light/dark (LD) 18:6) stimulate growth in freshwater rainbow trout through the action of insulin-like growth factor 1 (IGF-I) (Taylor et al., 2005).

Optimizing rearing conditions for rainbow trout, as well as identifying and implementing novel markers for the evaluation of the smolt status and the early detection of fish that will grow sub-optimally when transferred to seawater is crucial towards the improvement of both fish welfare and production. In this sense, a significant correlation between (IGF-I) abundance in fish plasma and growth has been previously reported for several fish species (Beckman et al., 2001). However, the regulation of plasma IGF-I through the transcription of *igf-I*, which is highest in liver, has been studied mainly in relation to fish growth and information on the effects of photoperiod on its regulation is incomplete. Similarly, the transcription of other key growth-regulating genes in liver, such as *insulin-like growth factor binding protein 1b* (*igfbp1b*), that results in a protein that likely inhibits IGF-I from interacting with its receptor, and *growth hormone receptor 1* (*ghr1*), which translates into the transmembrane receptor that activates the pathway that results in IGF-I production by the liver (Reindl and Sheridan, 2012) have not been studied in rainbow trout. Moreover, in a smoltification context, *cathepsin L* (*ctsl*) might prove an interesting marker, since it is a lysosomal endopeptidase involved in the turnover of cells and tissues, which is critical during the smoltification process (Björnsson et al., 2012). The analysis of these growth-related factors is interesting not only from a mechanistic perspective but also within the context of this research, as they could be good candidates as growth-predictor markers.

From a smoltification perspective, *Na^+,K^+–ATPase α-subunit isoform 1a* (*nkaα1a*), expressed in lamellar chloride cells in the gills, and *Na^+,K^+–ATPase α-subunit isoform 1b* (*nkaα1b*) and *Na^+,K^+, $2Cl^-$ co-transporter 1a* (*nkcc1a*), found primarily in filamental chloride cells in the gills, have been reported in rainbow trout (Katoh et al., 2008; McCormick et al., 2009; Richards et al., 2003). Studies in Atlantic salmon and rainbow trout showed that their transcription complements NKA activity, with *nkaα1b* and *nkcc1a* increasing in response to a seawater challenge while *nkaα1a* decreases, suggesting that the first two play a role in seawater tolerance while the third one is needed in freshwater (Flores and Shrimpton, 2012; McCormick et al., 2013; Nilsen et al., 2007). This is currently being exploited by the Atlantic salmon industry, as the transcription of these genes is increasingly replacing the analysis of NKA activity as quick smoltification markers (Nilsen et al., 2007). However, their suitability as smoltification markers for rainbow trout is currently unknown.

Therefore, the objectives of this study were (1) to test the effect of different photoperiod regimes on the smoltification of rainbow trout through the measurement of the NKA activity over a five month period in freshwater, (2) to evaluate the suitability of gill *nkaα1a*, *nkaα1b*, *nkcc1a* transcription during this period as smoltification markers complementing NKA activity, (3) to analyse IGF-I abundance in plasma as a growth predictor in fish undergoing different photoperiod regimes and (4) to measure the transcription of key genes from the somatotropic axis in liver, *igf-I*, *igfbp1b*, *ghr1* and *ctsl*, to understand their role on growth regulation, the effect that different photoperiods have on them and evaluate their suitability together with plasma IGF-I as growth proxies.

2. Materials and methods

2.1. Fish and rearing conditions

Juvenile rainbow trout (*Oncorhynchus mykiss*) with an initial weight of 78 ± 16.7 g were used in this experiment. Fish were slightly overfed using a standard commercial dry diet (Skretting AS) from automatic feeders according to temperature and fish size. Fish were kept indoors in tanks equipped with timer-controlled LED lights in a trout facility from Lerøy Vest AS (Bjørsvik, Hordaland, Norway). The fish were kept at ambient temperature, water flow at 0.4 L/kg/min and O_2 was above

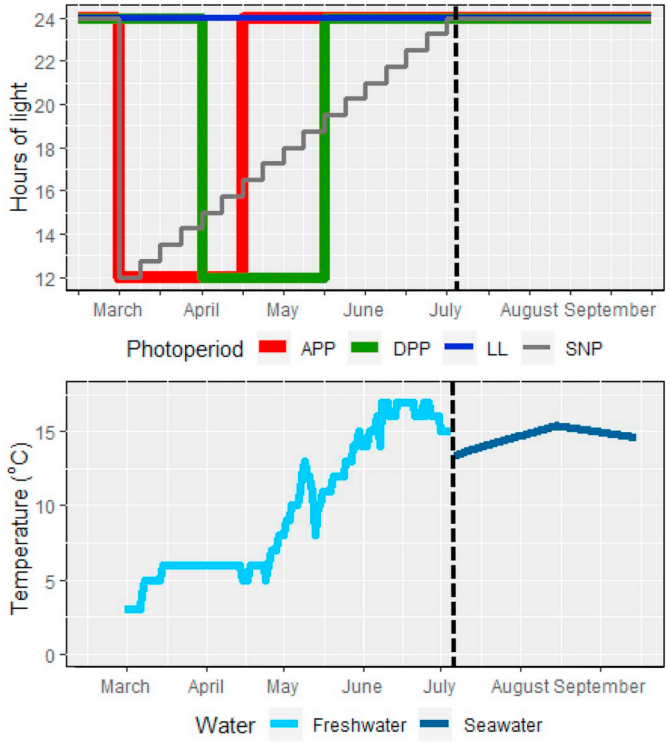

Fig. 1. Photoperiod treatment and temperature during the experiment. Schematic representation of the number of hours of light for each of the four different photoperiod treatments (a) and water temperature during the experimentation period. Dashed lines indicate seawater transfer. APP = advanced phase photoperiod, DPP = delayed phase photoperiod, LL = continuous light, SNP = simulated natural photoperiod.

80% in the outlet.

2.2. Experimental design

Prior to the freshwater experimentation phase, fish were kept in 2 × 2 m rearing tanks (2500 l) under natural temperature and LL photoperiod for 2 weeks. On 18th February 2016 (mid-February), 160 fish were individually Carlin tagged for recording individual growth rates during both the freshwater and seawater phase of the experiment. Fish were randomly distributed into eight tanks, resulting in 20 tagged and 90 untagged fish per tank. After two weeks of acclimation the photoperiod regimes were initiated. The experimental design included four different photoperiod treatments from mid-February until mid-July, as shown in Fig. 1a: Constant Light, LL (18 weeks), Advanced Phase Photoperiod (APP; 6 weeks of LD12:12 (Light 12 h:Dark 12 h) followed by 12 weeks of LD24:0), Delayed Phase Photoperiod (DPP; 4 weeks of LD24:0 followed by 6 weeks at LD12:12 and 8 weeks at LD24:0) and Simulated Natural Photoperiod (SNP; starting at LD12:12 and increasing light time by 45 min every week until reaching LD24:0). Weight and length were recorded in tagged fish once per month during the freshwater phase (February to July). On the 5th of July 2016, the remaining non-tagged fish from all experimental groups were individually tagged and length and weight recorded before being randomly distributed into four replicate tanks supplied with seawater and kept at LL in a common garden experiment to strengthen growth studies. Weight and length were recorded once more at the end-point sampling on the 14th of September. Water temperature was recorded once per day. During the freshwater phase it was measured on-site. For the seawater phase, it was retrieved from the records of a Marine Harvest facility (Stord, Hordaland, Norway) (Fig. 1b).

Experimental work was ethically reviewed, approved and registered by the Norwegian Animal Research Authority (NARA) and by the Animal Welfare and Ethical Review Body (AWERB 088), University of Stirling, UK.

2.3. Sampling

On the 18th of February 2016, ten fish per tank were sampled to secure a common biological starting point prior to experimental photoperiod treatments being initiated. Lethal samplings of six fish per tank (12 per group) were conducted every two weeks during the freshwater phase. Samplings took place on 3rd of March, 17th of March, 31st of March, 13th of April, 27th of April, 11th of May, 25th of May, 9th of June, 22nd of June and 5th of July. A final lethal sampling was done 9 weeks after seawater transfer (14th of September).

Fish were quickly dip-netted out of the tanks and euthanized by a lethal overdose of isoeugenol (AQUI-S). For each fish, weight and length were recorded. Blood was extracted using heparinised syringes and centrifuged at 3000 × *g* for 5 min to obtain plasma, which was frozen at −80 °C. The first gill arch from each side of the fish were dissected out and preserved at −80 °C; one in SEI buffer (Sucrose 250 mM, Na_2EDTA 10 mM, Imidazole 50 mM (all Sigma-Aldrich)) and the other one in RNAlater (ThermoFisher Scientific). Liver samples were also preserved in RNAlater according to manufacturer's guidelines (overnight at 4 °C and frozen at -80 °C).

2.4. Gill NKA activity

Between March and July (freshwater phase) gill NKA activity of all fish sampled (12 per group) were analysed. For the seawater phase (final sampling), only the 50 fish above the third quartile in length (31.7 cm) with the highest condition factor and the 50 fish below the first quartile in length (29.0 cm) with the lowest condition factor were analysed.

NKA activity was measured according to McCormick's methodology, which couples the hydrolysis of ATP to the enzymatic production of NAD^+ through the involvement of the enzymes pyruvate kinase and lactate dehydrogenase, and uses the NKA inhibitor ouabain to trace the baseline (McCormick, 1993). Kinetic assay readings were carried out at 340 nm for 10 min (60 cycles) at 25 °C in a Sunrise-basic (Tecan) spectrophotometer. Total amount of protein in the homogenate was analysed using a bicinchoninic acid (BCA) assay run in triplicate. NKA values were determined as the ouabain sensitive fraction of the ATP hydrolysis, expressed as μmol ADP mg $protein^{-1}$ h^{-1}.

2.5. Real-time polymerase chain reaction (RT-PCR)

All freshwater samples were analysed for gill *nkaα1a*, *nkaα1b* and *nkcc1a* and, with the exception of the samples from the first sampling in February, for liver *igf-I*, *igfbp1b*, *ghr1* and *ctsl* mRNA abundance. For the seawater phase only the 50 fish above the third quartile (31.7 cm) in length with the highest condition factor and the 50 fish below the first quartile in length (29.0 cm) with the lowest condition factor were analysed for liver *igf-I*, *igfbp1b*, *ghr1* and *ctsl* mRNA abundance.

Before total RNA isolation of samples, 20–25 mg of tissue was homogenized in RLT buffer (Qiagen) with zirconium oxide beads (1.4 μm) using a homogenizer (5000 rpm, 15 min) (Precellys 24, Bertin Technologies). Subsequent total RNA isolation was carried out using the Qiasymphony RNA kit in the QIAsymphony SP automatic system following manufacturer instructions (Qiagen).

Total RNA concentration and purity was measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Purity was confirmed with 260/280 and 260/230 ratios above 1.8. A selected number of samples were assessed for RNA integrity on RNA 6000 Nano LabChip® kit using the Agilent 2100 Bioanalyzer (Agilent Technologies). Integrity was confirmed with RIN values higher than 8.

Complementary DNA was reversely transcribed using 1.5 μg (gill) or 1.4 μg (liver) of total RNA using oligo(dT_{20}) primer and the Superscript

Table 1
Primers used for RT-PCR analysis and accession numbers of the gene sequences (GenBank).

Gene name	Forward primer (5′ > 3′)	Reverse primer (5′ > 3′)	Accession number
nkaa1a	CCAGGATCACTCAATGTCACTCT	CAAAGGCAAATGGGTTTAATATCAT	CK878443
nkaa1b	GCTACATCTCAACCAACAACATTACAC	TGCAGCTGAGTGCACCAT	CK879688
nkcc1a	GATGATCTGCGGCCATGTTC	CTGGTCATTGGACAGTTCTTTG	AJ417890
igf-I	TGCGGAGAGAGAGGCTTTTA	AGCACTCGTCCACAATACCA	M81904
igfbp1b	AGTTCACCAACTTCTACCTACC	GACGACTCACACTGCTTGGC	AF403539
ghr1	CGTCCTCATCCTTCCAGTTTTA	GTTCTGTGAGGTTCTGGAAAAC	AF403539
ctsl	CAACTACCTGCAGGCACCTA	ACATGATCCCTGGTCCTTGAC	AF358668
efa1	CCCCTCCAGGATGTCTACAAA	CACACGGCCCACGGGTACT	AF498320

III kit (Thermo Fisher Scientific) using a MicrolabSTARlet Liquid Handling Workstation (Hamilton Robotics).

RT-PCR was carried out in a CFX-96 Real-Time PCR detection system platform (Bio-Rad) using the following PCR conditions: 3 min at 95 °C, 34 cycles of 15 s at 95 °C and 1 min at 60 °C and a melting curve step at the end (10 s at 95 °C, 5 s at 65–95 °C with increments of 0.5 °C and 5 s at 95 °C). For each assay, triplicate two-fold cDNA dilution series from pooled samples (1:5–1:160) were used to determine amplification efficiencies. Samples were run in 25 µl duplicates using iTaq universal SYBR green supermix (Bio-Rad), 0.20 µM of each primer and 5 µl of diluted cDNA (dilution 1:50 for gill and 1:30 for liver). Each plate included a negative control as well as a common pooled sample used for the intercalibration of assays among plates. The relative transcription levels of the genes were normalized following the efficiency corrected method (Pfaffl, 2001) using *ef1α* as an endogenous reference gene (Olsvik et al., 2005). Primers used in this study are summarized in Table 1.

2.6. Time-resolved fluoroimmunoassay for plasma IGF-I

Circulating IGF-I levels were measured in plasma collected from 58 randomly selected tagged fish (n = 16 SNP; n = 16 APP, n = 17 LL and n = 9 DPP) at the beginning (July) and at the end (September) of the seawater period.

Time-resolved competitive fluoro-immunoassay (TR-FIA) protocol was used to measure plasma IGF-I concentration (Small and Peterson, 2005). Prior to the assay, plasma IGF-I was dissociated from the binding protein with acid-ethanol (Shimizu et al., 2000). Briefly, 96-well DELFIA pre-coated goat anti-rabbit IgG Microtitration plates (Perkin Elmer) were washed with 200 µl DELFIA wash buffer before each well received 20 µl anti-barramundi IGF-I rabbit antiserum (GroPep; diluted 1:8000) and 100 µl of standard-recombinant salmon IGF-I (GroPep) or 20 µl extracted sample (Cleveland et al., 2018). Standards and samples were diluted in Assay Buffer (Perkin Elmer). Plates were incubated overnight with shaking (600 rpm at 4 °C). Europium labeled (0.05 ng μl^{-1}) IGF-I was added to each well and the plate incubated overnight under agitation (600 rpm at 4 °C). The plate was washed six times with 200 µl Washing Buffer (Perkin Elmer) before adding 200 µl DELFIA enhancement solution (PerkinElmer) to each well. After shaking at 600 rpm for 10 min at room temperature, time-resolved fluorescence was measured by a fluorometer (ARVO X4; PerkinElmer) with emission and read wavelengths at 340 and 615 nm, respectively. Parallel displacement of dilutions of extracted plasma from rainbow trout with the standard was confirmed (Supp. Fig. 1). The half-maximal displacement (ED_{50}) occurred at 0.77 ± 0.02 ng/ml (mean ± SEM, n = 4). The ED_{80} and ED_{20} were 2.08 ± 0.05 ng/ml (n = 4) and 0.29 ± 0.01 ng/ml (n = 4), respectively. The minimum detection limit of the assay, defined as the mean count of the zero standard minus two standard deviations, was 0.06 ± 0.03 ng/ml (n = 4). The intra- and inter-assay coefficients of variation were 5.4 ± 0.2% (n = 4) and 9.8 ± 0.9% (n = 4), respectively.

2.7. Growth calculations

Specific growth rate in length (SGRL) was calculated using the formula:

$$100 \times \frac{Ln\ (Length_{Final}) - Ln\ (Length_{Initial})}{Number\ of\ days}$$

Similarly for specific growth rate in weight (SGRW):

$$100 \times \frac{Ln\ (Weight_{Final}) - Ln\ (Weight_{Initial})}{Number\ of\ days}$$

The condition factor was calculated with Fulton's formula:

$$100 \times \frac{Weight}{Length^3}$$

2.8. Data analysis and representation

Statistical tests were performed using R statistical software. Data representation was carried out using R package ggplot2 (Wickham, 2009).

One-way ANOVA was performed at each time point to find differences among treatments (effect of photoperiod) and also performed on the whole time-series for each treatment (effect of time). Data was transformed by either natural logarithm or square root to satisfy the normal distribution and homogeneity of variance assumptions, tested with the Shapiro and Bartlett tests, respectively. Significant comparisons ($p < .05$) were followed by Tukey's posthoc test to identify different treatments.

Linear relationship among variables was determined by linear regression using the QR method. Significance values ($p < .05$) were obtained by testing the null hypothesis: the slope of the least squares linear fit to the data is equal to 0.

2.8.1. Quadratic model fit

Measurements for any particular photoperiod treatment that followed a clear parabolic trend were fitted to a quadratic model. Estimated parameters were: initial value, *init*, maximum value, *Ymax*, and time when *Ymax* occurred, *Tmax*. These were estimated using the formula:

$$a \times Time^2 + b \times Time + c,$$

where:

$$a = (c - Ymax)/(Tmax^2),$$

$$b = -2 \times a \times Tmax,$$

$$c = init$$

Calculated estimates of a particular measurement were considered different between treatments (photoperiod effect) if the estimates did not overlap (value ± S.E.) between two treatments.

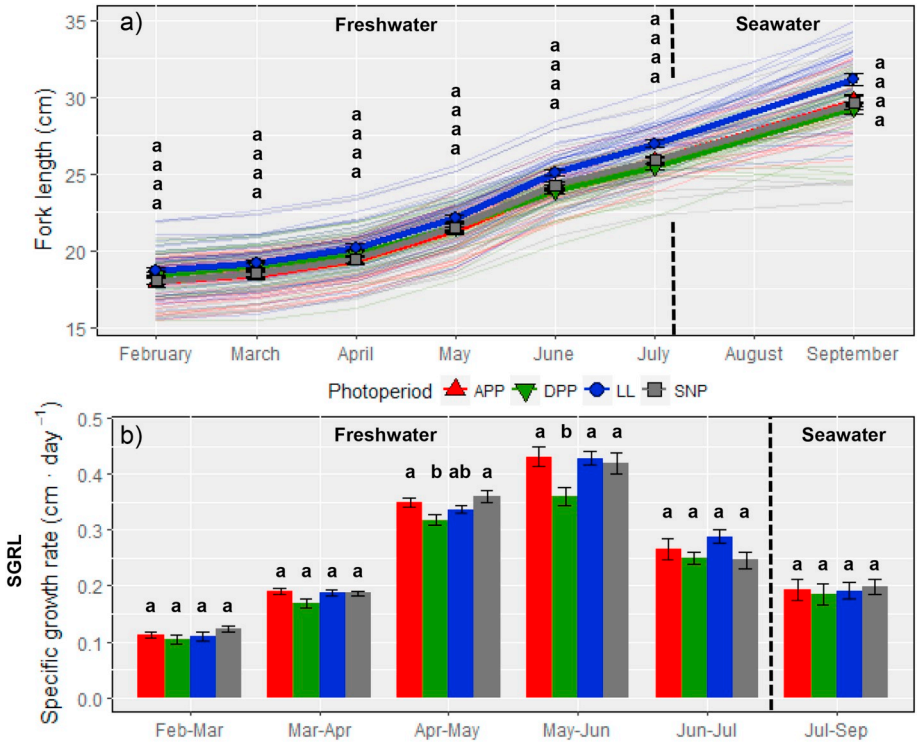

Fig. 2. Fork length (cm, ± 1 s.e.) of juvenile rainbow trout reared in freshwater under four different photoperiod treatments and transferred to seawater on the 5th of July. Mean fork length (points and thick lines) and individual growth trajectory (thin lines) of each tagged fish, measured once per month (a) and mean specific growth rate in length between samplings (b). Samplings took place on 24th of February, 18th of March, 14th of April, 12th of May, 10th of June, 5th of July and 14th of September. Error bars indicate standard error. The dashed line indicates seawater transfer. Different letters indicate statistical differences within a time point ($p < .05$). APP = advanced phase photoperiod, DPP = delayed phase photoperiod, LL = continuous light, SNP = simulated natural photoperiod.

3. Results

3.1. Fish growth

3.1.1. Freshwater

When analysing tagged fish, it is possible to visualize the growth trajectory of each fish (Fig. 2a) and to calculate specific growth rate (i.e. SGRL and SGRW). SGRL was low during the first three months, after which it increased steadily until June, before decreasing again until the end of the experiment (Fig. 2b). Though no significant differences were found for either length or weight (Fig. 2a, Supp. Fig. 2a) differences were present in both SGRL and SGRW. The overall SGRL during the freshwater phase (February to July) was significantly lower ($p < .01$, df: 3, F: 4.53) in DPP compared to the other three treatments (data not shown). Month per month, the SGRL of fish kept at DPP was significantly lower than for fish kept at APP in April–May and May–June, for fish kept at SNP in April–May and May–June, and finally compared to fish kept at LL but only in May–June (Fig. 2b). A very similar result was found for SGRW (Supp. Fig. 2b). Results related to length were given priority over those related to weight for simplicity in further analysis but since the two were so similar (i.e. highly correlated, data not shown, $p < .001$, slope = 23.86), the results for one can be extrapolated to the other.

3.1.2. Seawater

After nine weeks in seawater at LL, no differences in length (Fig. 2a), SGRL (Fig. 2b), weight (Supp. Fig. 2a) or SGRW (Supp. Fig. 2b) were found among fish that had been reared in different photoperiod treatments during their freshwater phase.

There was a significant correlation between the overall freshwater SGRL (February to July) and seawater SGRL (data not shown, $p < .01$, slope = 0.07). However, the worst performing fish in seawater (SGRL below 0.10 cm * day^{-1}) were not consistently the fish with the worst freshwater SGRL (0.25 ± 0.010 cm * day^{-1}) in the total population (0.27 ± 0.004 cm * day^{-1}).

3.2. The effect of different photoperiod regimes on NKA activity

3.2.1. Freshwater

Fish from all photoperiod treatments experienced a significant increase in gill NKA activity from mid-March to April, followed by a plateau from April until mid-May/June which was followed by a sharp decrease (Fig. 3a). However, while NKA activity in fish kept at LL, SNP and DPP peaked in mid-May and started to decrease in June, NKA activity in fish kept at APP peaked two weeks later (June) and decreased also later in mid-June. At the final sampling point in July, NKA activity in fish from all treatments reached comparable low values, similar to those recorded in March (full statistical analysis available in Supp. Table 1). Significant differences among treatments were only found in early June when NKA activity in fish kept at APP was significantly higher than in fish kept at DPP (Tukey test, $p < .001$).

3.2.2. Seawater

The NKA activity in seawater (September) showed no differences among fish reared in any of the freshwater photoperiod treatments (Data not shown; APP: 2.7 ± 0.29, DPP: 3.2 ± 0.53, LL: 2.8 ± 0.30, SNP: 2.5 ± 0.31 µmol ADP mg protein^{-1} h^{-1}).

3.3. Transcription of *nkaα1a, nkaα1b and nkcc1a* complement the NKA results

Freshwater transcription of *nkaα1b* and *nkcc1a*, changed in a similar fashion to NKA activity while the transcription of *nkaα1a* changed in an inversely manner with highest values recorded from March to April instead of May to June (Fig. 3b-d). Taking into consideration the whole freshwater period, all three genes, *nkaα1a*, *nkaα1b* and *nkcc1a*, correlated significantly with NKA activity (data not shown, $p < .001$, slope = −0.15; $p < .01$, slope = 0.13; $p < .001$, slope = 0.002; respectively).

The relative gill *nkaα1a* mRNA abundance was highest at the start of the trial, decreasing after mid-April and reaching minimum values in mid-May and June (Fig. 3b). In March, the transcription of this gene in fish kept at LD24:0 (LL, DPP) was significantly higher than in those kept

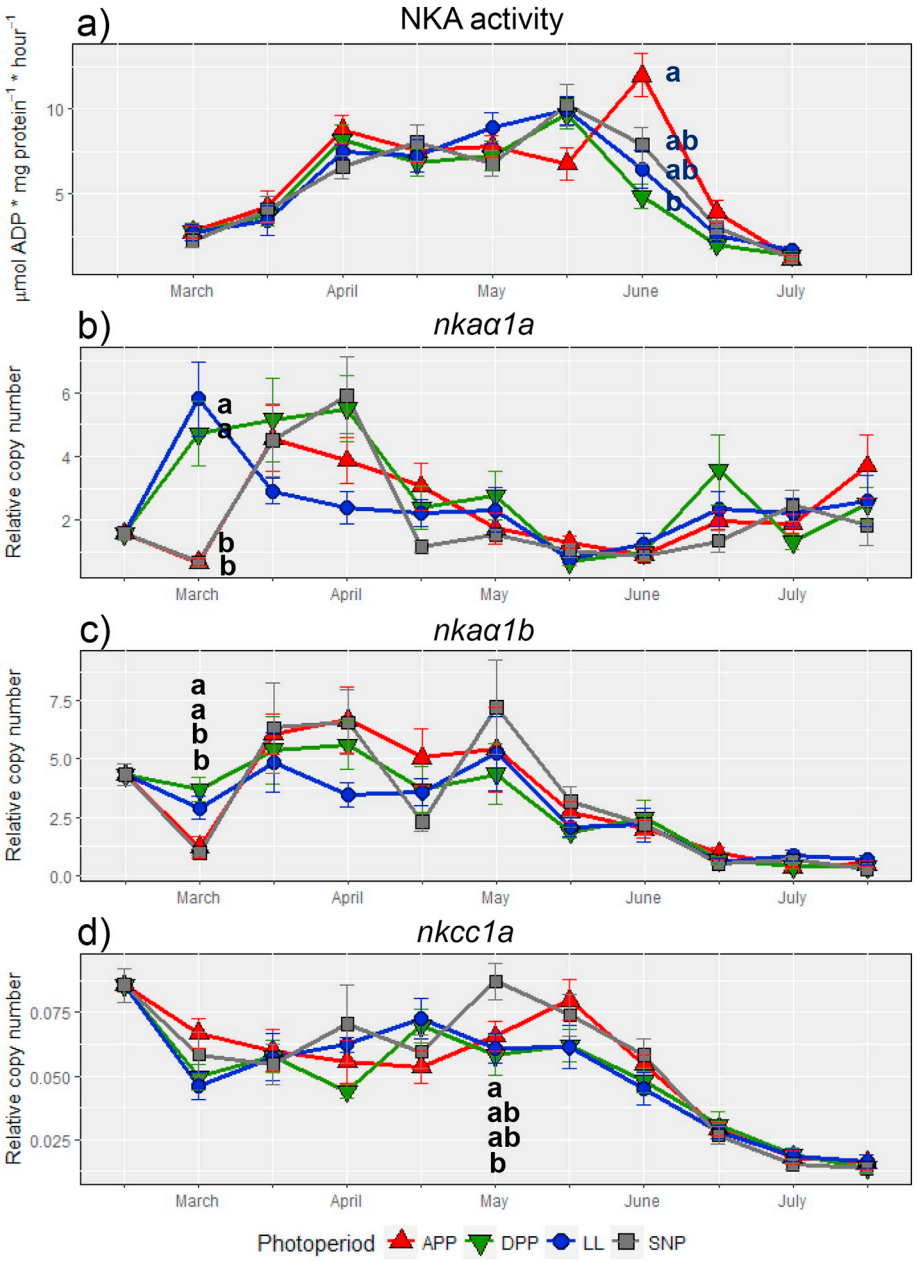

Fig. 3. NKA activity (a) and relative gene transcription of related genes, *nkaa1a* (b), *nkaa1b* (c) and *nkcc1a* (d) of juvenile rainbow trout in freshwater under four photoperiod treatments. Error bars indicate standard error. Different letters indicate statistical differences with a time point ($p < .05$). Samplings took place on 3rd of March, 17th of March, 31st of March, 13th of April, 27th of April, 11th of May, 25th of May, 9th of June, 22nd of June and 5th of July. The dashed line indicates seawater transfer. Different letters indicate statistical differences ($p < .05$). APP = advanced phase photoperiod, DPP = delayed phase photoperiod, LL = continuous light, SNP = simulated natural photoperiod. NKA = Na^+,K^+–ATPase, *nkaα1a = nka α subunit 1a*, *nkaα1b = nka α subunit 1b*, *nkcc1a = nk 2 cl^- cotransporter.*

at a photoperiod that changed to LD12:12 (SNP, APP) ($p < .001$, df: 3, F: 18.72).

For all the different treatments, *nkaα1b* increased in mid-March and decreased in mid-May, earlier than NKA activity in both cases (Fig. 3c). Similarly to *nkaα1a*, fish kept at LL (LL, DPP) was higher than those at LD12:12 (SNP, APP) in March ($p < .001$, df: 3, F: 11.56).

The transcription of *nkcc1a* was stable (no statistical differences on time) until it decreased at the same time as the NKA activity, in mid-June (Fig. 3d). After decreasing, its values were significantly lower than in February (Fig. 3d; Supp. Table 1). Transcription of *nkcc1a* in fish kept at SNP was significantly higher than in fish kept at DPP in May (Tukey test, $p < .05$).

3.4. *igf-I, igfbp1b, ghr1 and ctsl transcription and plasma IGF-I abundance in response to different photoperiod regimes*

3.4.1. *Freshwater*

In freshwater, the transcription of *igf-I*, *ghr1* and *ctsl* followed a similar trend as gill NKA enzyme activity while *igfbp1b* showed less variation over time (Fig. 4). Independently of photoperiod treatment, *igf-I* increased between March and April and then decreased in mid-June. This trend was also observed in *ghr1*, which increased sharply in mid-May before decreasing again in mid-June and in *ctsl*, which slowly increased until mid-May and decreased in June.

Statistical differences among groups were found mostly at the beginning of the experiment, with *igf-I* in March being higher in fish kept at DPP compared to APP, LL and SNP. Similarly *ghr1* in March was higher in fish kept at DPP compared to those at SNP. During the two following months *igfbp1b* was higher in fish kept at DPP compared to those kept at SNP. Finally, *ctsl* was higher in fish kept at DPP and LL compared to those kept at APP in mid-March and in mid-June *ctsl* was higher in fish kept APP compared to those at LL.

Plasma IGF-I levels in July, were significantly higher in APP compared to the other treatments ($p < .001$, df: 3, F: 6.68) (Supp. Fig. 3a).

3.4.2. *Seawater*

In the seawater phase, no significant differences among fish reared in any of the freshwater photoperiod treatments were found for any of

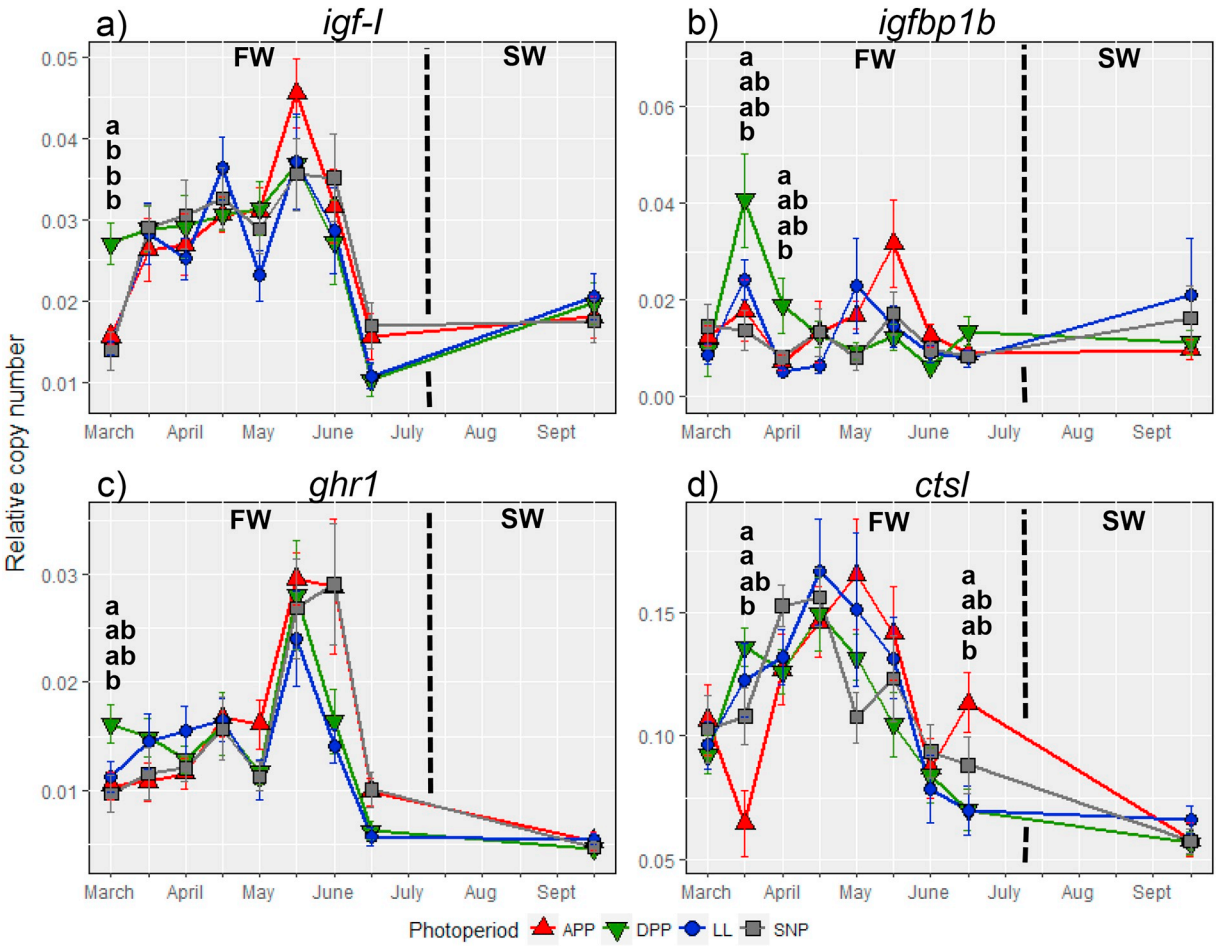

Fig. 4. Transcription of genes of the somatotropic axis, *igf-I* (a), *igfbp1b* (b), *ghr1* (c), and *ctsl* (d), of juvenile rainbow trout reared in freshwater under four different photoperiod treatments and transferred to seawater on the 5th of July. Error bars indicate standard error. Samplings took place on 3rd of March, 17th of March, 31st of March, 13th of April, 27th of April, 11th of May, 25th of May, 9th of June, 22nd of June, 5th of July and 14th of September. Different letters indicate statistical differences within a time point ($p < .05$). The dashed line indicates seawater transfer. No letters indicate lack of significant differences. APP = advanced phase photoperiod, DPP = delayed phase photoperiod, LL = continuous light, SNP = simulated natural photoperiod, *ctsl* = *cathepsin* L, *igf-I* = *insulin-like growth factor 1*, *igfbp1b* = *igf binding protein 1b*, *ghr1* = *growth hormone receptor 1*, FW = freshwater, SW = seawater.

the genes analysed (Fig. 4).

In September, plasma IGF-I levels were significantly higher in fish that had been kept at APP during freshwater phase compared to fish that had been kept at DPP and LL (Supp. Fig. 3b) ($p < .001$, df: 3, F: 6.98).

3.5. Quadratic model fit

Freshwater SGRL over time for each photoperiod treatment was fitted to a quadratic model (Fig. 6a, Supp. Table 2). According to the estimated model there were no differences among treatments for the initial values (*init*). However, the maximum value (*Ymax)*, was lower in DPP ($0.33 \pm 0.008\,cm * day^{-1}$) compared to the other treatments (ranging from 0.356 to $0.379\,cm * day^{-1}$). Moreover, the day when the maximum value occurred (*Tmax*) was later for LL (at day 155 ± 2.5) than for SNP (at day 147 ± 2.1).

Similarly, freshwater NKA activity over time for each photoperiod treatment was also fitted to a quadratic model (Fig. 6b, Supp. Table 2). According to the estimated model there were no differences among treatments for the initial values (*init)* and the day when the maximum value occurred (*Tmax*). However, the maximum value (*Ymax)*, was higher in APP ($9.0 \pm 0.48\,\mu mol\ ADP\ mg\ protein^{-1}\ h^{-1}$) compared to DPP ($7.9 \pm 0.39\,\mu mol\ ADP\ mg\ protein^{-1}\ h^{-1}$).

The transcription of *igf-I* and *ctsl* during the freshwater period followed a parabolic trend similar to NKA activity data, thus each treatment was fitted to a quadratic model. Estimates for *igf-I* indicate that *Tmax* occurs earlier for DPP (at day 102.7 ± 3.99) than for any of the other treatments (ranging from 108.1 to 117.79 days) (Fig. 6c, Supp. Table 2). Similarly, for *ctsl* transcription *Tmax* occurs earlier in DPP (at day 101.4 ± 3.23) than in APP (at day 114.2 ± 4.87) (Supp. Table 2).

3.6. Relationship between plasma IGF-I, liver igf-I, igfbp1b, ghr, ctsl and growth in seawater

Tagged fish were used to study the relationship between growth in seawater (assessed through the SGRL between the time of transfer in July and the end-point sampling after two months in September) plasma IGF-I (July and September) and liver gene transcription (September).

The relationship of SGRL in September with the plasma IGF-I in July was not significant (Fig. 5a, $p < .05$, slope = 96.67). However, SGRL in September was significantly correlated with plasma IGF-I in September (Fig. 5b, $p < .01$, slope = 431.21). Moreover, a significant correlation between the liver *igf-I* transcription in September and the SGRL in seawater was found (Fig. 5c, $p < .001$, slope = 0.08). Correlations of SGRL with *igfbp1b*, *ghr1* and *ctsl* were not significant.

A significant correlation was also found for the plasma IGF-I in July

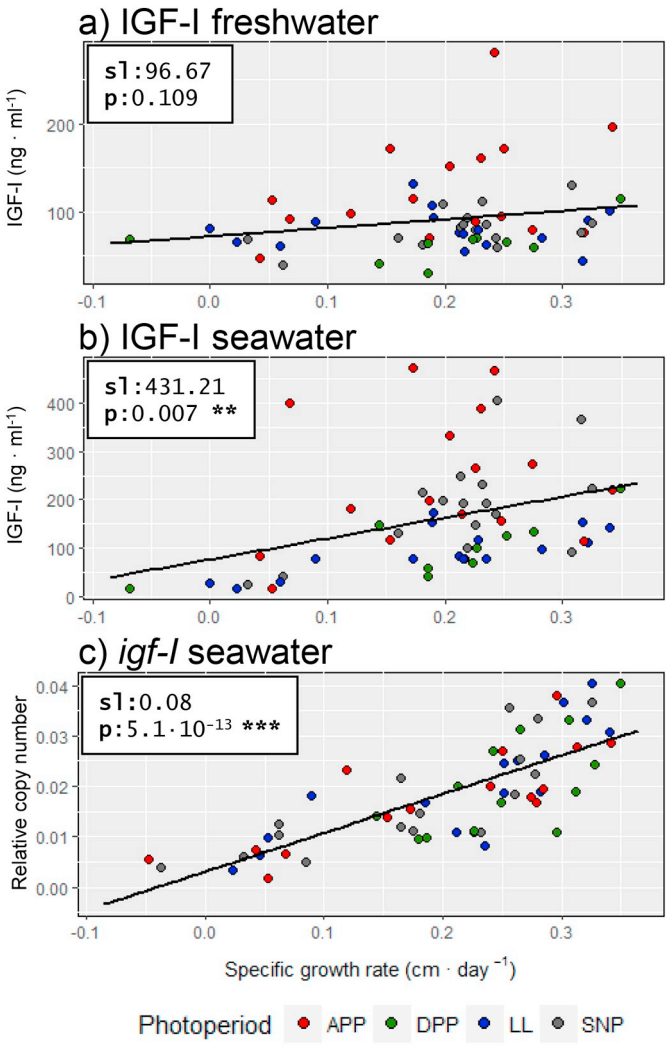

Fig. 5. Relationship between specific growth rate (length) and plasma IGF-I or liver *igf-I* transcription of rainbow trout sampled in seawater on the 14th of September. Relationship between the specific growth rate ($cm * day^{-1}$) between July and September with plasma IGF-I prior seawater transfer (July) (a), plasma IGF-I in seawater (September) (b) and liver *igf-I* transcription in seawater (September) (c). APP = advanced phase photoperiod, DPP = delayed phase photoperiod, LL = continuous light, SNP = simulated natural photoperiod, IGF-I = insulin-like growth hormone 1, p = *p*-value, sl = slope.

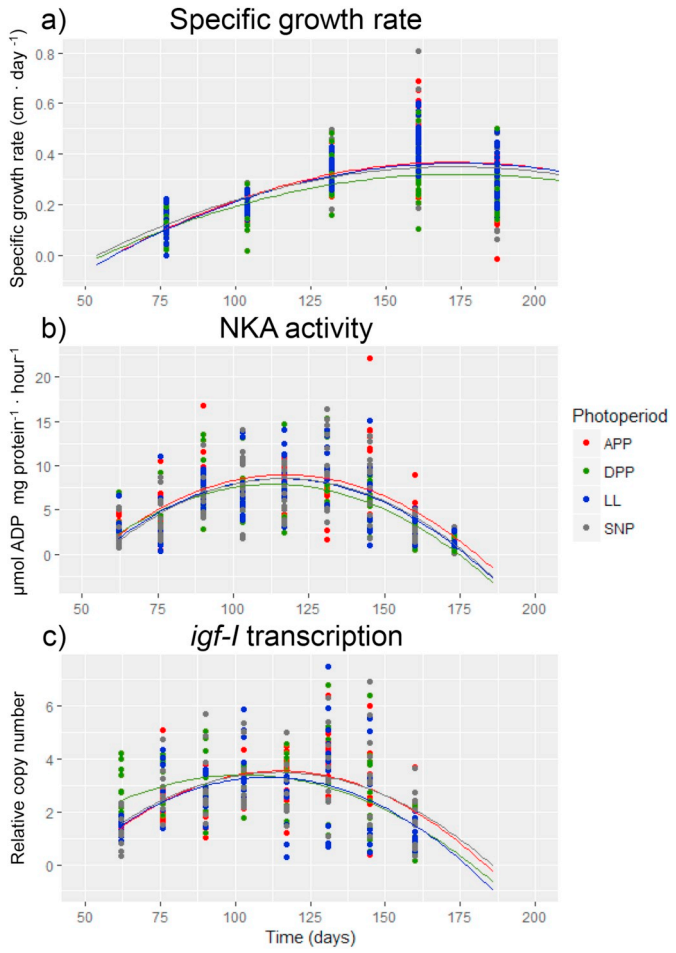

Fig. 6. Quadratic model fit to specific growth rate in length (SGRL) (a), Na^+,K^+–ATPase (NKA) activity (b) and *insulin-like growth factor 1* (*igf-I*) gene transcription (c) during the freshwater phase of juvenile rainbow trout under four different photoperiod treatments. Samplings for SGRL took place on 24th of February (day 55), 18th of March (day 78), 14th of April (day 105), 12th of May (day 133), 10th of June (day 162), 5th of July (day 188). Samplings for NKA activity and *igf-I* transcription took place on 3rd of March (day 63), 17th of March (day 77), 31st of March (day 91), 13th of April (day 104), 27th of April (day 118), 11th of May (day 132), 25th of May (day 146), 9th of June (day 161), 22nd of June (day 174).

and the plasma IGF-I in September ($p < .001$, slope = 0.17). However, the correlation between plasma IGF-I and liver *igf-I* in September was not significant ($p = .16$, slope = $2.7 * 10^{-5}$).

4. Discussion

In salmon aquaculture it is common to use different photoperiod regimes to produce robust smolts throughout the year while ensuring optimal growth and welfare of fish (Handeland and Stefansson, 2001). In contrast, the preferred photoperiod protocol for production of rainbow trout smolts is LL, despite little evidence to support that LL is indeed best suited photoperiod to induce smoltification related traits in this species. This study aimed at evaluating the effect of different photoperiods on smoltification through the use of both traditional and well established smolt assessment tools, such as NKA activity measurement, and less established molecular tools, like the transcription of osmoregulatory genes through RT-PCR. According to results from this study, there is no reason to disregard LL as a suitable photoperiod regime for rainbow trout smoltification, although APP might be more beneficial. Moreover, since a growth-stunted phenotype of fish has been reported in rainbow trout once transferred to seawater, liver transcription of *igf-I*, *igfbp1b*, *ghr1* and *ctsl*, and the abundance of circulating IGF-I in plasma were measured and evaluated in relation to both photoperiod treatment received in freshwater and somatic growth during the seawater phase. This experiment was performed on the winter to summer period, since a majority of the problems encountered by the industry occur in summer post smolts. In this study, a strong correlation between growth in seawater and IGF-I (both circulating in plasma and in liver transcription) was found, highlighting that low levels of this hormone (among others that were not tested; a hormonal dysregulation) is one of the factors involved in the development of the growth-stunted phenotype.

Growth was lower in fish kept at DPP compared to fish kept at the other photoperiod treatments during spring in freshwater and after 9 weeks in seawater. However, in contrast to the results obtained in similar studies with Atlantic salmon (Handeland and Stefansson, 2001), differences in the smoltification process in response to the different photoperiod treatments were generally mild, with few significant differences in NKA activity. In the present study, different photoperiods only caused a minor desynchronization of the biological clock of

rainbow trout in spring. In fact, rainbow trout can smolt in total darkness (Wagner, 1974a), hinting that light regime is not their main *zeitgeber* for smoltification. Although all four treatments generated a smolt window in April, its duration was slightly longer for APP and shorter for DPP than in the rest of the treatments, particularly in June. In this context, a longer smolt window could have an impact to fish farmers, allowing them to be less constrained by time in something as crucial as the seawater transfer of the fish. The difference between these two treatments was further shown by the NKA quadratic model, which showed that the overall activity of NKA was higher in APP than in DPP, suggesting that fish at APP become more seawater-ready. Nonetheless, the differences in the duration of the smolt window in the different treatments was small and, in mid-June, all treatments converged at low NKA activities, suggesting that this drop in hypoosmoregulatory capacity (desmoltification) is induced by other factors that were not considered in this study, such as water temperature or simply a biological clock that is poorly affected by exogenous stimuli.

Although it was tightly related to the NKA activity during the whole freshwater period, the transcription of *nkaα1a*, *nkaα1b* and *nkcc1a* offered further detail into the smoltification process. In this context, the transcription of both *nkaα1a* and *nkaα1b* in March allowed for the detection of a transient transcriptional response of SNP and APP to the switch from LD12:12 to LL, which the NKA activity did not show. This response may decrease the osmoregulation capacity of the fish, which are likely the effects of an transient response to photoperiod, as it has been previously observed on the immune response (Leonardi and Klempau, 2003; Valenzuela et al., 2008). Nonetheless, this effect was only transitory and transcription recovered in the two week period between samplings. During the smolt window, even though NKA activity stayed relatively stable at high values, there were changes that affected the NKA pumps at the gene expression level. The transcription of *nkaα1a* suggests that fish would have experienced their highest freshwater preference up to April, after which it decreased, reaching a minimum in mid-May, towards the end of the smolt window. On the other hand, *nkaα1b* and *nkcc1a* stayed relatively stable at high values until there was a significant decrease in mid-June for both, suggesting that the fish were able to respond optimally to a seawater challenge until mid-June. During the desmoltification phase, the transcription of *nkaα1b* and *nkcc1a* decreased accordingly to the NKA activity, while *nkaα1a* rose back, showing that fish were abandoning their migratory instinct and readapting to a life in freshwater. At this point, the transcription of the two seawater genes was significantly lower than at the start of the experiment in February, while *nkaα1a* transcription was at similar levels. This could not be appreciated from the NKA activity, and it suggests that the osmotic stress of a sudden seawater challenge would be stronger for desmolted than for parr rainbow trout. Indeed, this would explain the mechanism behind the results of Wagner (1974b), who showed that parr rainbow trout in December can have a higher survival rate to a direct seawater transfer than desmolts in June, and it provides further evidence that the desmoltification process is not simply the reversal to the parr state.

In mid-June rainbow trout from all treatments had desmolted, as their NKA activity and transcription of both *nkaα1b* and *nkcc1a* decreased to low levels while the transcription of *nkaα1a* increased. Since fish lose the characteristics of a smolt, it has been suggested that transferring Atlantic salmon to seawater at this point would be suboptimal (Arnesen et al., 2003). Regardless, it was decided to proceed with the seawater allocation in order to understand the effects of a suboptimal seawater transfer on rainbow trout. This approach offers the possibility to research the mechanism of the growth-stunted fish development. In fact, although mortality upon seawater transfer was negligible, approx. 14% of fish experienced very little to no growth (SGRL below 0.10 $cm * day^{-1}$) during the summer months in seawater (July to September). These results are therefore in line with previous similar studies (Wagner, 1974b) and with the frequencies reported by Norwegian fish farmers. These two phenotypes might be linked to the two classes that are found in all migration studies in rainbow trout: sea-run and freshwater-resident phenotypes are always present, even in the same cohort (Christie et al., 2011; Kendall et al., 2015). Although the proportion of each phenotype is influenced by genotype (Hecht et al., 2013; Nichols et al., 2008), individual condition (size, growth rate, energy storage) (McMillan et al., 2012) and environmental factors (Sloat et al., 2014), the current knowledge is not enough to completely explain their smoltification patterns (Kendall et al., 2015). Efforts to produce a strain with a single phenotype, either selecting the sea-run (Christie et al., 2011; Sharpe et al., 2007; Sloat and Reeves, 2014) or the freshwater-resident (Hayes et al., 2012; Thrower and Joyce, 2005), have proved unsuccessful. Therefore, it is likely that the fish that performed poorly in seawater would have been natural freshwater-residents, while the others would have been the sea-run phenotype. Another explanation could be related to fish personality, with several studies not related to seawater transfer showing that rainbow trout exhibit dominant and subordinate behaviour (DiBattista et al., 2006; Kostyniuk et al., 2018). The latter experience stress and reduced growth, although this has not been shown in aquaculture production setups, with bigger tanks and bigger numbers of fish per tank (which might difficult the establishment of dominance) and longer experiment durations. However, in the present study, no behavioural tests were performed on the fish.

The GH-IGF1-axis is involved in the control of both growth and smoltification (Mancera and McCormick, 2007; Shimomura et al., 2012; Shrimpton et al., 2000). The mechanism of the GH-IGF-I axis on growth, through the activation of GHR by GH and the subsequent production of hepatic IGF-I, the activity of which is regulated by IGFbp1b (Shimizu et al., 2011), among others, has been studied in multiple fish groups (Beckman, 2011). On the other hand, in relation to smoltification, it has been proposed that GH, through GHR and IGF-I stimulation, is related to an increase in gill salt secretion capacity through the increment of the number of seawater chloride cells (Mancera and McCormick, 2007; Reinecke, 2010). In these cells, the isoforms NKAα1b and NKCC1a are involved in osmoregulation, increasing the NKA activity and seawater tolerance (McCormick, 2001; Poppinga et al., 2007). On the other hand, *ctsl* is lysosomal endopeptidase which is involved in the initiation of protein degradation (Joseph et al., 1988; Lysenko et al., 2017), relevant during turnover of cells and tissues during smoltification. The present study showed that the photoperiod treatment has relatively little effect on the regulation of the transcription of *igf-I*, *igfbp1bI, ghr*, and *ctsl* in liver. In March, due to reasons that cannot be traced back but are likely not related to the photoperiod treatment, DPP showed an increased transcription of *igf-I* and *ghr1*. Interestingly, this growth-enhancing condition might be quickly stopped by an increase of the transcript of *igfbp1b* in mid-March and April, which would decrease the effect of IGF-I, therefore the growth of DPP fish was not higher than in the other treatments and the transcription of the measured genes was soon returned to normal levels. Regarding *ctsl*, it transiently decreased in mid-March for the two treatments that changed to LD12:12, SNP and APP, the latter one being more affected. This is likely related to the transient stress response to photoperiod that was also seen for *nkaα1a* and *nkaα1b* and, in the same way, the transcription recovered in the two week period until the next sampling. Months after, in mid-June, *ctsl* transcription of APP was higher than that of LL. However, the SGRL was not bigger in APP than in LL during the June–July or July–September periods, which puts into question the validity of the transcription of this gene as a growth proxy. When modelled, only DPP treatment showed differences on the transcription of *igf-I* and *ctsl*, as seen through the model fit, since it started decreasing earlier than other treatments, which is consistent with the lower SGRL recorded in this group. In any case, regardless of the photoperiod, the relationship between *igf-I*, *ghr1* and *ctsl* and growth seemed clear: their trend is consistent with the SGRL in tagged fish, with high gene transcriptions corresponding to periods of high SGRL, which is especially clear in May and June, although with a phase delay.

When compared to *igf-I* transcription, SGRL has a phase delay of about 40 days, as would be expected when comparing a quick process (gene transcription) with a slow one (growth). It is likely that the liver transcription of *igf-I*, *ghr1* and *ctsl*, among other genes, are responsible for promoting the growth of rainbow trout in the medium to long term (Beckman et al., 2004; Beckman, 2011; Picha et al., 2008). This suggests that the smoltification process anticipates a period of high growth during the post smolt phase, triggering the earlier transcription of growth-promoting genes. During desmoltification, the transcription of these genes is down-regulated as the fish would not become post smolts and this period of intense growth would no longer occur. Finally, although they both *igf-I* transcription and NKA activity increased in mid-March, *igf-I* transcription reached the high plateau of the smolt window sooner than *nkaα1b* or NKA activity, suggesting that indeed IGF-I drives the increase in NKA activity (McCormick, 2001; Poppinga et al., 2007). Moreover, NKA activity and *igf-I* transcription seemed to follow a very similar trend (*Tmax* for NKA activity: 111.72 to 119.12, *Tmax* for *igf-1* transcription: 98.73 to 117.79 days) (Sakamoto et al., 1995; Shimomura et al., 2012).

Unlike the case of the gene transcription of *igf-I*, *ghr*, *ctsl* and *igfbp1b*, the photoperiod treatment did affect the circulating IGF-I in plasma at the end of the freshwater phase (it was not measured prior to this point), when APP had significantly higher abundances than the other treatments, and in seawater in September, when it was higher in APP compared to DPP and LL. Similarly, Taylor et al. (2005) reported higher growth and plasma IGF-I levels in the LD18:6 treatment. It is possible that the rhythmicity of the APP provides the adequate *zeitgeber* for rainbow trout development, while DPP does the opposite. However, this differences due to the photoperiod were not found in the liver *igf-I* transcription. Assessing the relationship between plasma IGF-I and SGRL in seawater (July–September), the data do not allow to accept plasma IGF-I in July as a reliable predictor of the growth to come during the next two months in seawater. However, overall SGRL in freshwater was a predictor of SGRL in seawater, as previously observed (Johnsson et al., 1997).Moreover, both the plasma IGF-I and the liver *igf-I* transcription had significant correlations with SGRL in tagged fish showing that they are involved in the regulation of growth in seawater for rainbow trout.

In conclusion, the effects of photoperiod on rainbow trout in winter-spring are relatively small when compared with the effects on other salmonids, as seen from the minor differences in NKA activity, osmoregulatory genes and the studied transcription of growth-related genes among treatments. To exemplify this, after 16 weeks of photoperiod treatment, Atlantic salmon kept at SNP showed an NKA activity of around 10 μmol ADP mg protein^{-1} h^{-1} while those fish kept at LL presented values of around 4 (Handeland and Stefansson, 2001). While given the general lack of differences between APP, LL and SNP it is difficult to pinpoint the most appropriate photoperiod regime for rainbow trout, APP induced a longer smolt window and fish showed higher values of plasma IGF-I. On the other hand, DPP was consistently the worst treatment, as shown by the shorter length of the smolt window, reduced NKA activity, earlier decrease in the transcription of *igf-I* and *ctsl*, lower abundance of plasma IGF-I and lower SGRL and SGRW. From a growth perspective, although *igf-I*, *ghr1* and *ctsl* transcription were, mostly, not significantly affected by the light regime, they showed a clear increase during the smolt window, suggesting that during this phase the endocrine system of anadromous salmonids is preparing for the imminent period of high growth in seawater. Finally, while plasma IGF-I and liver *igf-I* transcription in seawater were clearly related to the regulation of growth in seawater, IGF-I prior seawater transfer was not a reliable predictor of growth. Since the present work seems to indicate that the photoperiod is not a strong inducer of smoltification for the species, future work should investigate the effect of other environmental cues, like temperature and salinity as smoltification inducers in rainbow trout.

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