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Effects of temperature and photoperiod on rainbow trout (*Oncorhynchus mykiss*) smoltification and haematopoiesis

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1. Introduction

Most freshwater fish would not be able to cope with the osmotic stress intrinsic to seawater, quickly dehydrating and dying, and so are bound to freshwater ecosystems (Quinn et al., 2016). However, anadromous salmonids have developed a strategy to minimize osmotic counter effects, thus allowing them to live in both freshwater, ideal for safe spawning, hatching and early growth stages, and seawater environments, rich in energy resources for fast growth (Hendry et al., 2004; Lima and Dill, 1990).

Anadromous salmonids develop the attributes necessary for life in seawater through a process known as smoltification. Once anadromous salmonids 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 many biochemical (e.g. haemoglobin, Fyhn et al., 1991), physiological (e.g. increased metabolism, Björnsson et al., 2011), morphological (e.g. dark and rounded to silvery and streamlined, Winans and Nishioka, 1987) and behavioural changes (e.g. bottom-dwelling, aggressive and territorial to pelagic, schooling and downstream migrating, McCormick et al., 1998; Riley et al., 2014), all designed to improve seawater performance and survival. Among these changes is the development of hypo-osmoregulatory capacity. This is possible because salmonids have a repertoire of Na^+, K^+–ATPase (NKA) pumps. Each of these pumps have differences in function, peak in expression at different times and are predominantly found in one of the gill cell types (Flores and Shrimpton, 2012; Nilsen et al., 2007). Thus, NKA α-subunit isoform 1a (NKAα1a) is a freshwater pump present in lamellar chloride cells, the function of which is to generate a hyper-osmotic gradient that results in ion uptake, and is highest during the parr (juvenile) stage of the fish. On the other hand, the seawater pumps NKA α-subunit isoform 1b (NKAα1b) and Na^+, K^+, $2Cl^-$ co-transporter 1a (NKCC1a) are found primarily in filamental chloride cells, their function is to generate a hypo-osmotic inner environment that results in ion secretion, and peak during the smolt (migrating) stage and in seawater (McCormick et al., 2013). All smoltification changes are reversible and last during what is known as the smolt window (time of maximal seawater-readiness). If the fish do not reach seawater during the smolt window, these changes are lost in the process known as desmoltification (Stefansson et al., 1998).

Both photoperiod and temperature have been investigated in aquaculture management in a variety of farmed and potential farmed fish species (Allan and Burnell, 2013; Deacon and Hecht, 1996). In the case of salmonids, the artificial manipulation of these environmental factors has been shown to be a useful strategy for the production of out-of-season smolts, as well as for optimizing the induction of in-season smoltification (Handeland et al., 2013; Jørgensen et al., 2007; Solbakken et al., 1994). Consequently, photoperiod is routinely manipulated in order to obtain maximum smoltification rates in Atlantic salmon (*Salmo salar*) and Arctic char (*Salvelinus alpinus*) production (Johnston, 2008; Stead and Laird, 2002) while temperature is less often controlled due to heating costs.

In recent years, there has been an increase in the aquaculture production of the sea-run phenotype of rainbow trout (*Oncorhynchus mykiss*), which reached over 87,000 tonnes in Norway alone in 2016 (Food and Agriculture Organization of the United Nations, 2016). Industrial interest on this phenotype arises from the need to diversify production and the possibility to expand on-growing production sites to low salinity locations, which are more suitable for rainbow trout smolts than

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for Atlantic salmon (Altinok and Grizzle, 2001) and are less prone to salmon louse (*Lepeophtheirus salmonis*) infestations. However, our understanding of the environmental control of rainbow trout smoltification is insufficient and there is a need for appropriate tools to assess rainbow trout seawater-readiness. In the absence of a rainbow trout specific all-season production protocol, most hatcheries are rearing rainbow trout at continuous light (LL) and natural temperature while seawater-readiness is regarded exclusively as size-dependent. Under these conditions, high mortalities and high numbers of fish that experience sub-optimal growth (growth-stunted phenotype) upon seawater transfer, especially in summer post-smolts, have been reported by the industry. While LL has been compared to other photoperiod regimes and has been shown to be an appropriate option for rainbow trout smoltification (Morro et al., 2019), the effect of temperature and its interaction with photoperiod remain untested.

In order to reduce the incidence of growth-stunted fish, optimizing rearing conditions for rainbow trout smoltification, as well as identifying and implementing biomarkers of seawater-readiness and biomarkers for the early detection of growth-stunted fish are all crucial. In this sense, insulin-like growth factor (IGF)-I is a peptide hormone that promotes growth of fish (Wood et al., 2005). The activity of circulating IGF-I is stabilized and regulated by the presence of multiple IGF-binding proteins (IGFBPs). In salmonids and other teleost species, plasma IGF-I is positively correlated with growth rate and can be used as a positive marker of growth (Beckman, 2011; Picha et al., 2008). On the other hand, IGFBP-1b, one of three major circulating forms of IGFBP, is negatively correlated with growth rate and may be useful as a negative marker of growth in salmonids (Kaneko et al., 2019; Kawaguchi et al., 2013; Shimizu et al., 2006).

Another factor to take into consideration during production is disease, which remains as one of the main threats (Asche et al., 2009; Dale et al., 2009; Kristoffersen et al., 2009). Therefore, immune capacity is a key factor to take into consideration when optimising new production protocols. Since blood cells constitute the main line of defence against pathogens and non-self particles, haematopoiesis has been used as a proxy of immune capacity (Martin et al., 2012). A number of transcription factors related to haematopoiesis control such as the transcription factor Pu.1 (also known as SPI1) are well known. Low concentrations of Pu.1 are essential for the differentiation of granulocyte-monocyte progenitor (GMP) myeloid cells (i.e. mast cells, eosinophils, neutrophils, macrophages and dendritic cells) (Orkin and Zon, 2008; Ribas et al., 2008). In turn, cells from this myeloid lineage produce more Pu.1, increasing its abundance and shifting the haematopoiesis towards the productions of lymphoid cells. Interleukin 1β (IL1β) is a pro-inflammatory cytokine produced by immune cells like lymphocytes, monocytes and macrophages upon detection of signs of an infection or injury. IL1β enhances cell-mediated immunity by promoting the proliferation and maturation of lymphocytes (Pleguezuelos et al., 2000; Reis et al., 2012). IL-4/13 is the ancestral family cytokine related to both mammalian IL-4 and IL-13, involved in the stimulation of B lymphocyte proliferation and activation of macrophages (Martinez et al., 2009; Sequeida et al., 2017; Takizawa et al., 2011). Finally, the transcription factor GATA3 is essential for the development of the T lymphocyte lineage and differentiation of T helper type 2 cells (Kumari et al., 2009). Understanding the interplay of these key transcription factors and expression of cytokines in response to any changes in environmental conditions during smoltification is critical to assess the impact on the immune-competence of fish.

The objectives of this study were (1) to test the effect of different combinations of photoperiod and temperature regimes on the smoltification of rainbow trout through the measurement of the gill NKA activity, and gill *nkaα1a*, *nkaα1b*, and *nkcc1a* transcription over a three month period in freshwater, (2) to identify the optimal regime in terms of growth and its regulation (circulating IGF1 levels) and (3) to evaluate the effect of such regimes on the immune capacity of the fish as a function of the transcription of haematopoiesis related genes *pu.1*, *il1β*, *il4/13* and *gata3* in head kidney.

2. Materials and methods

2.1. Animals

Juvenile rainbow trout (*Oncorhynchus mykiss*) of 1+ years with an initial weight of 75.0 ± 15.1 g were used in this experiment (n = 716). Fish were fed *ad libitum* using a standard commercial dry diet (Skretting AS) from automatic feeders according to 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 environmental temperature except for the high temperature group, where it was raised for a period as part of the experimental setup, water flow of 0.4 L/kg/min and O_2 was above 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 the 8th of March 2017, n = 160 fish were individually Carlin tagged for recording of 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 67 untagged fish per tank. After two weeks of acclimation, the photoperiod and temperature regimes were initiated. The experimental design consisted in the combination of two temperature treatments (high temperature (HT) or natural temperature (NT)) and two photoperiod treatments (LL or simulated natural photoperiod (SNP)) resulting in a total of four treatments: HT + LL, HT + SNP, NT + LL and NT + SNP. High temperature water was achieved using heat interchange with fjord seawater and homogenised in a series of head tanks with degassers before pumping into the experimental tanks. Therefore, maximum temperature achievable for HT and the time frame during which HT could be higher than NT was conditioned by fjord water temperature. Temperature of the HT group was set to 8 °C with minor deviations from the 8th of March until the natural water temperature converged at 8 °C (temperature convergence, TC) on the 8th of May (Fig. 1a). Fish at NT received water at ambient temperature for the duration of the experiment (8th of March to 13th of September 2017). After TC point, both NT and HT groups received water at natural temperature.

LL photoperiod was constant light for the duration of the experiment, while SNP started at LD12:12 and increased light time by 45 min every week (Fig. 1b). During the freshwater phase (8th of March to 2nd of June), weight and length were recorded in tagged fish on 8th March, 5th May and 2nd June. In June, 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 allow for the study of their growth during the seawater phase. Weight and length were recorded once more at the end-point sampling on the 13th of September.

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

Lethal samplings of six fish per tank (n = 12 per group) were conducted every two weeks during the freshwater phase. Samplings took place on 23rd of March, 6th of April, 20th of April, 4th of May, 18th of May and 31st of May. A final lethal sampling was done 15 weeks after seawater transfer (13th of September).

Fish were quickly dip-netted out of the tanks and euthanized by a

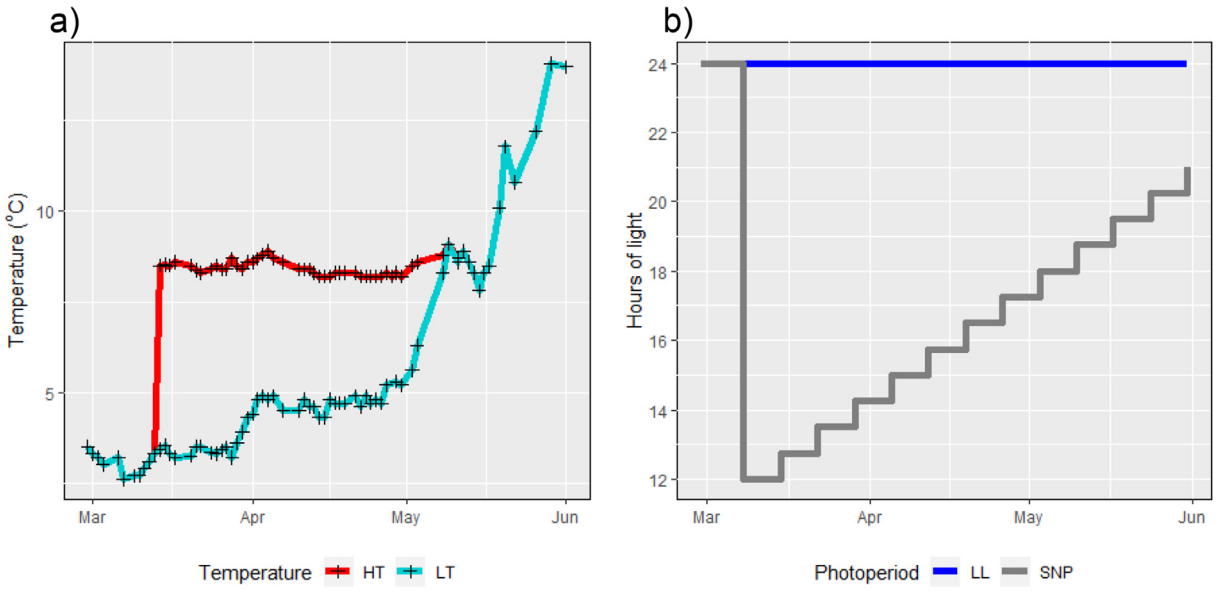

Fig. 1. Representation of the temperature and light regimes during the freshwater experimentation phase. Mean temperature of high temperature tanks and natural temperature tanks (a) and number of hours of light for each of the four different photoperiod treatments (b). Symbol + indicates when the temperature was measured. HT = high temperature, NT = natural temperature, LL = continuous light, SNP = simulated natural photoperiod.

lethal overdose (over 250 ng/ml) of isoeugenol (AQUI-S), following Directive 2010/63/EU guidelines. For each fish, weight and length were recorded. Blood was extracted immediately after animals were euthanized using heparinised syringes and centrifuged at 3,000 × *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 250mM, Na_2EDTA 10mM, Imidazole 50mM (all Sigma-Aldrich)) and the other one in RNAlater (ThermoFisher Scientific). Head kidney 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

Gill NKA activity of all freshwater fish (n = 12 per group) was analysed. For the seawater phase (final sampling), only the 50 fish above the third quartile in length (34.0 cm) with the highest condition factor and the 50 fish below the first quartile in length (31.5 cm) with the lowest condition factor were analysed.

NKA activity was measured according to McCormick's (McCormick, 1993), which couples the hydrolysis of ATP to the enzymatic production of NAD^+ through the activity of the enzymes pyruvate kinase and lactate dehydrogenase, and uses the NKA inhibitor ouabain to trace the baseline. Briefly, gill filaments were thawed before the assay, homogenised in 100 µl of SEI buffer plus 25 µl of SEID buffer (SEI buffer plus 0.1% deoxycholic acid (Calbiochem)) and centrifuged at 5,000g for 60 s. Then, 10 µl of supernatant was added in quadruplicates to a 96-well UV microplate (ThermoFisher Scientific). Finally, 200 µl of assay medium without inhibitor was added to half of the replicates, while the other half received assay medium with NKA inhibitor (0.5 mM ouabain (Sigma-Aldrich)). Kinetic assay readings were carried out at 340 nm for 10 min (60 cycles) at 25 °C in a Sunrise-basic spectrophotometer (Tecan). 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. RNA isolation and cDNA synthesis

RNA isolation was carried out on gill and head kidney freshwater samples. Before total RNA isolation of samples, 20–25 mg of tissue was homogenized in RLT Plus buffer (Qiagen) with stainless steel beads (5 mm) (Qiagen) 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 2.5 µg (head kidney) of total RNA using oligo (dT_{20}) primer and the Superscript III kit (Fisher Scientific) using a MicrolabSTARlet Liquid Handling Workstation (Hamilton Robotics).

2.6. Transcription assay

Osmoregulation related gene transcription (*nkaa1a*, *nkaa1b* and *nkcc1a*) was measured in gill samples and haematopoiesis related gene transcription (*pu.1*, *il1β*, *il4/13* and *gata3*) in head kidney samples, using real-time polymerase chain reaction (RT-PCR) with minor differences between gene sets. 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 both amplification efficiencies for each oligo pair and optimal dilution for cDNA template. Samples were run in 12.5 µl duplicates using iTaq universal SYBR green supermix (Bio-Rad), 0.20 µM of each primer and 2.5 µl of diluted cDNA (dilution 1:50 for gill and 1:75 for head kidney). Each plate included a negative control as well as a common sample (pooled sampled) used for the intercalibration of assays among plates. The relative transcription levels of the genes were normalized following the efficiency corrected method (Pfaffl et al., 2004) using *ef1α* as an endogenous reference gene (Olsvik et al., 2005). Primers used in this study are summarized in the Table 1.

Table 1
RT-PCR oligos used in present work.

Gene name	Primer sequence (5′ > 3′)	Accession number	Reference
nkaa1a	CCAGGATCACTCAATGTCACTCT CAAAGGCAAATGGGTTTAATATCAT	CK878443	(Morro et al., 2019; Nilsen et al., 2007);
nkaa1b	GCTACATCTCAACCAACAACATTACAC TGCAGCTGAGTGCACCAT	CK879688	(Morro et al., 2019; Nilsen et al., 2007);
nkcc1a	GATGATCTGCGGCCATGTTC CTGGTCATTGGACAGTTCTTTG	AJ417890	(Morro et al., 2019; Nilsen et al., 2007);
pu.1	GTCTGAGAGACCACATTGC TCTTGTTGCCCAATTCTCC	NM_001124513	Present work
il1β	CGTCACTGACTCTGAGAACAAGT TGGCGTGCAGCTCCATAG	AJ223954	Løvoll et al. (2007)
il4/13	ATCCTTCTCCTCTCTGTTGC GAGTGTGTGTGTATTGTCCTG	AB574337	Deshmukh et al. (2013)
gata3	GCGCACAAACAGAGATTTGA TCCAAGGTCGTATCCAGTCC	NM_001195792	Martin et al. (2012)
efa1	CCCCTCCAGGATGTCTACAAA CACACGGCCCACGGGTACT	AF498320	Genge et al. (2013)

2.7. Time-resolved fluoroimmunoassay for plasma IGF-I and IGFBP-1b

Circulating IGF-I and IGFBP-1b levels were measured in plasma collected from 32 randomly selected tagged fish (n = 8) at the beginning (June) 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, serum 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 (PerkinElmer) 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 IGF-I (GroPep) or 20 µl plasma (Cleveland et al., 2018). Standards and samples were diluted in standard assay buffer. Plates were incubated overnight with shaking (600 rpm at 4 °C). After centrifugation (1 min at 3,000 × *g*), europium labelled (0.05 ng µl^{-1}) IGF-I was added to each well and the plate incubated overnight under agitation (600 rpm at 4 °C). Plates were washed six times with 200 µl washing buffer 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 excitation, and emission and read wavelengths at 340 and 615 nm, respectively.

Serum IGFBP-1b levels were quantified by TR-FIA, which has been validated for rainbow trout, as described in Fukuda et al. (2015). Briefly, a competitive method was employed by following a procedure for DELFIA immunoassays (PerkinElmer). Serum samples were first incubated overnight at 4 °C with antiserum against purified salmon IGFBP-1b (Shimizu et al., 2006), in a 96-well microtiter plate coated with goat anti-rabbit IgG (PerkinElmer). Biotinylated salmon IGFBP-1b was added to each well and incubated overnight at 4 °C. After washing with DELFIA Wash Buffer (PerkinElmer), each well received Eu-labelled streptavidin (PerkinElmer) followed by DELFIA Enhancement Solution (PerkinElmer). Time-resolved fluorescence was measured using a SPARK multimode microplate reader (Tecan).

2.8. Growth calculations

Specific growth rate in length (SGR-L) was calculated using the formula and results are expressed in % day^{-1}:

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

Similarly for specific growth rate in weight (SGR-W):

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

Thermal growth coefficient in length (TGC-L) was calculated using the formula (Iwama and Tautz, 1981; Jobling, 2003):

$$1000 \times \left\{ \frac{(\sqrt[3]{Length_{Final}} - \sqrt[3]{Length_{Final}})}{Degree\ days} \right\}$$

Similarly for thermal growth coefficient in weight (TGC-W):

$$1000 \times \left\{ \frac{(\sqrt[3]{Weight_{Final}} - \sqrt[3]{Weight_{Final}})}{Degree\ days} \right\}$$

The condition factor was calculated with Fulton's formula (Ricker, 1975):

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

2.9. Data analysis and representation

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

For statistical significance testing, data from duplicate tanks per treatment was pooled. Two-way ANOVA was performed at each time point to test for differences among treatments (effect of temperature and photoperiod). One-way ANOVA was used for each treatment on the whole time-series to find differences among time points (effect of time). Similarly, one-way ANOVA was used to test differences among treatments for IGF-I and IGFBP-1b plasma levels. 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 ($p < 0.05$) were followed by Tukey's posthoc test to confirm which treatments or time-points produced significantly different results.

3. Results

3.1. Mortality and fish growth

Overall mortality during the 15 weeks in seawater was of 6.74% (25 fish. HT + LL: 7, HT + SNP: 6, NT + LL: 6, NT + SNP: 6).

When analysing tagged fish, it is possible to visualize the growth trajectory of each fish and to calculate thermal growth coefficient (TGC-L and TGC-W) and specific growth rate (i.e. SGR-L and SGR-W). Fish at HT became significantly larger in length by the time the second

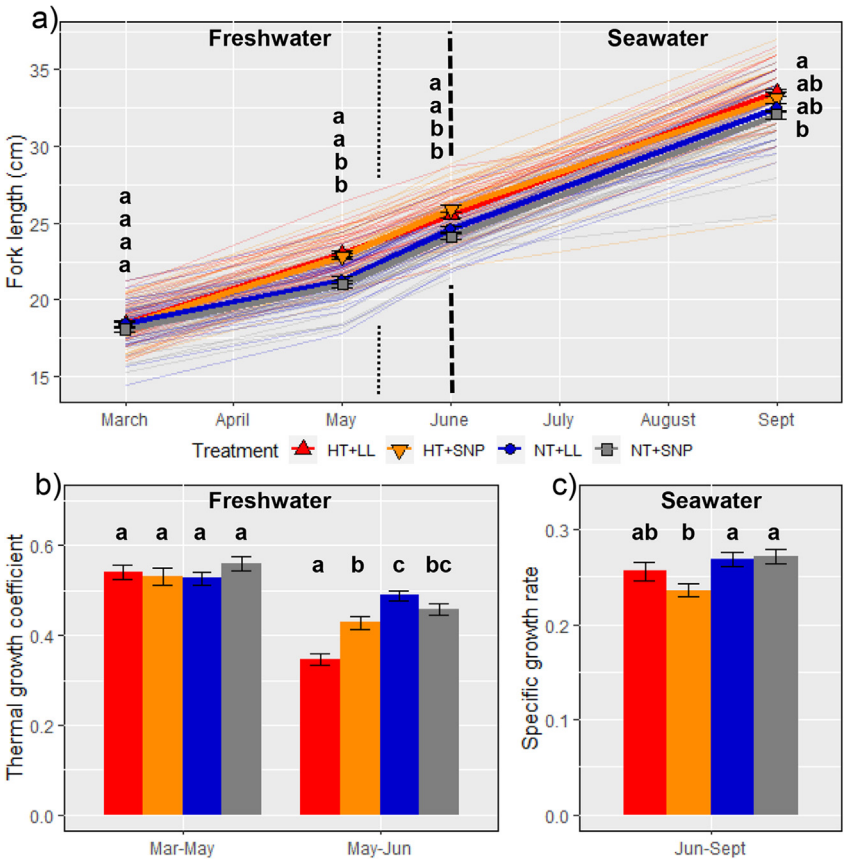

Fig. 2. Fork length (cm, ± 1 s.e.) of juvenile rainbow trout reared in freshwater under four different photoperiod and temperature treatments and transferred to seawater on the 2nd of June. Mean fork length (points and thick lines) and individual growth trajectory (thin lines) of each tagged fish (a), mean thermal growth coefficient in length between measurements in freshwater (b), and mean specific growth rate in length between measurements in seawater (c). Measurements took place on 8th March, 5th May, 2nd June and 13th of September. Error bars indicate standard error. Dashed line indicates seawater transfer. Dotted line indicates temperature convergence point. Different letters indicate statistical differences ($p < 0.05$) in a time point. Lack of letters indicates lack of significant differences. HT = high temperature, NT = natural temperature, LL = continuous light, SNP = simulated natural photoperiod.

sampling took place, in May (Fig. 2a, temperature effect, p: $6.6*10^{-15}$, df: 1, F: 74.90), even though similar TGR were observed for all groups during the March–May period (Fig. 2b). The differences in length stayed significant until the next sampling in June (temperature effect, p: $1.6*10^{-9}$, df: 1, F: 41.32, interaction, p: $2.8*10^{-2}$, df: 1, F: 4.9). However, after May, an increase in the TGC of the NT groups was observed which progressively reduced the length difference between HT and NT groups. This compensatory growth of NT was maintained during the three months in seawater (Fig. 2c), after which the differences in length between HT and NT groups had been reduced. Despite the fish kept at HT still being larger, this difference in length was only significant between fish kept at NT + SNP and at HT + LL (temperature effect, p: $9.0*10^{-4}$, df: 1, F: 11.50; Tukey test, p: $5.8*10^{-3}$).

Very similar results were obtained when performing the same tests on fish weights (Supp. Fig. 1).

3.2. Osmoregulation

The measurement of NKA activity was used to assess how the different treatments affected osmoregulatory capacity during the experiment (March to June in freshwater and a final assessment in seawater in September). No clear increase of NKA activity during the freshwater stage was detected for either of the NT treatments, both lacking significant differences among sampling time points during the whole freshwater stage (Fig. 3; Supp. Table 1). HT + LL peaked in May before decreasing again but this treatment too had no significant differences during the freshwater stage. The treatment with the clearest increase in NKA activity was HT + SNP, which was highest in mid-April and was significantly different from the values of the same treatment in June (p: $3.0*10^{-6}$, df: 6, F: 7.48; Tukey test, p: $6.8*10^{-5}$). NKA activity in seawater was consistently lower than in freshwater. However, no significant differences at any specific sampling were found among treatments, neither in freshwater (Fig. 3a) or seawater (HT + LL: 2.79 ± 0.36, HT + SNP: 2.37 ± 0.46, NT + LL: 2.43 ± 0.25, NT + SNP: 2.09 ± 0.22 μmol ADP mg protein^{-1} h^{-1}) (Supp. Table 1). In seawater, no significant differences related to fish length were found either (50 fish above the third quartile in length with the highest condition factor and 50 fish below the first quartile in length with the lowest condition).

While the transcription of the freshwater isoform *nkaα1a* remained relatively stable until May for NT groups, fish kept at HT decreased *nkaα1a* transcription after mid-March and remained low until May, thus originating significant differences between HT and NT groups in April (temperature effect, p: $5.0*10^{-8}$, df: 1, F: 47.71), mid-April (temperature effect, p: $2.0*10^{-6}$, df: 1, F: 31.96 and interaction: p: $4.4*10^{-2}$, df: 1, F: 4.34), and in May (temperature effect, p: 2.1*10-5, df: 1, F: 23.98). Afterwards, in mid-May, the transcription for both temperature groups converged at similar levels, coinciding with TC. Then, in June, new differences originated, this time driven by photoperiod, with HT + LL (Tukey test, p: $1.1*10^{-3}$) and NT + LL (Tukey test, p: $3.5*10^{-3}$) being significantly higher than NT + SNP (photoperiod effect, p: $3.8*10^{-4}$, df: 1, F: 15.33) (Fig. 3b).

The transcription of the seawater isoform *nkaα1b* seemed to be mainly driven by photoperiod. LL groups experienced an increase in mid-April and then quickly decreased, while in SNP groups *nkaα1b* experienced a slower increase, peaking in mid-May. All groups were at similar transcription levels before seawater transfer in June. Significant differences were present during the LL peak in mid-April (photoperiod effect, p: $2.6*10^{-4}$, df: 1, F: 16.35) and during the SNP peak in mid-May (photoperiod effect, p: $1.4*10^{-2}$, df: 1, F: 6.73) (Fig. 3c).

The transcription of *nkcc1a* was overall comparable between groups during the whole freshwater period, with the exception of samples taken in April, when groups at NT presented higher levels, this difference being significant between NT + LL and both HT + LL (Tukey test, p: $1.7*10^{-2}$) and HT + SNP (Tukey test, p: $3.4*10^{-2}$) (temperature effect, p: $1.0*10^{-3}$, df: 1, F: 12.83). As a general trend, the transcription

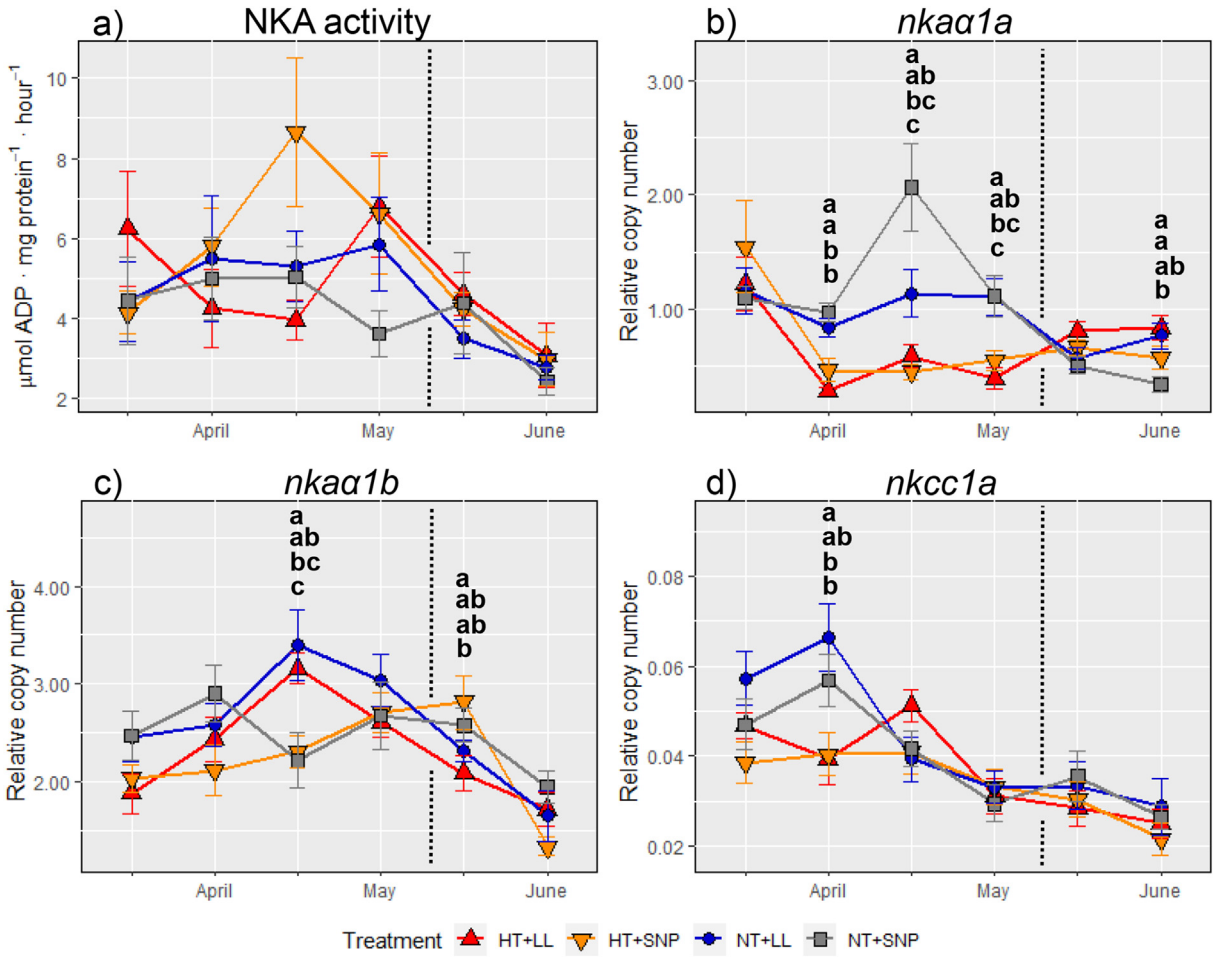

Fig. 3. NKA activity (a) and transcription of related genes, *nkaα1a* (b), *nkaα1b* (c) and *nkcc1a* (d) in gill of juvenile rainbow trout in freshwater under four photoperiod and temperature treatments. Error bars indicate standard error. Different letters indicate statistical differences ($p < 0.05$). Samplings took place on 23rd of March, 6th of April, 20th of April, 4th of May, 18th of May and 31st of May. Dotted line indicates temperature convergence point. Different letters indicate statistical differences ($p < 0.05$) in a time point. Lack of letters indicates lack of significant differences. HT = high temperature, NT = natural temperature, LL = continuous light, SNP = simulated natural photoperiod, NKA = Na^+,K^+–ATPase, *nkaα1a = nka α subunit 1a*, *nkaα1b = nka α subunit 1b*, *nkcc1a* = Na^+-K^+-$2Cl^-$ *cotransporter*.

of this gene tended to decrease progressively with time (Fig. 3d).

3.3. Plasma IGF-I and IGFBP-1b abundance

Neither the IGF-I levels measured prior seawater transfer nor after 15 weeks in seawater showed significant differences among fish reared in the different freshwater treatments (Fig. 4a). But there was an overall effect of seawater transfer on plasma IGF-I levels, which were lower in fish in seawater (p: $7.8*10^{-6}$, df: 1, F: 23.81). No significant correlation was found between plasma IGF-I level in September and SGR during June–September (SGR-L: p: 0.41, r = −0.15, slope = 64.58; SGR-W: p: 0.12, r = −0.28, slope = 51.90).

Similarly for IGFBP-1b levels, there were no significant differences among fish reared in the different freshwater treatments, either in June or September (Fig. 4b). However, plasma IGFBP-1b levels were significantly higher in fish in seawater than in freshwater (p: $1.1*10^{-9}$, df: 1, F: 51.52). Moreover, there was a negative correlation between plasma IGFBP-1b level in September and SGR-L during June–September (SGR-L: p: $1.1*10^{-2}$, r = −0.45, slope = −12.80; SGR-W: p: $1.8*10^{-2}$, r = −0.42, slope = −5.20).

3.4. Haematopoiesis

Overall, temperature strongly affected the transcription of the tested haematopoiesis genes in head kidney, especially for *pu.1* and *il4/13*. With few exceptions towards the end of the freshwater phase, after the TC point, NT treatments presented invariably the highest transcription levels, often finding significant differences between HT and NT treatments. As a general trend, transcription levels decreased as the temperature increased and the differences between HT and NT treatments became progressively smaller as their temperature drew nearer (Fig. 5).

The transcription of *pu.1* was clearly affected by temperature, being higher at lower temperatures. Significant differences due to temperature were present in mid-March (temperature effect, p: $6.5*10^{-10}$, df: 1, F: 70.65), April (temperature effect, p: $3.8*10^{-7}$, df: 1, F: 38.87), and mid-April (temperature effect, p: $8.7*10^{-5}$, df: 1, F: 20.15; photoperiod effect, p: $4.6*10^{-2}$, df: 1, F: 4.33), each progressively smaller as they approached the TC point. In mid-May, with the temperature treatment effectively removed, treatments at LL showed the highest transcription values (photoperiod effect, p: $7.9*10^{-3}$, df: 1, F: 7.96), finding significant differences between HT + LL and HT + SNP (Tukey test, p: $2.8*10^{-2}$) (Fig. 5a).

The transcription of *il1β* was for the most part stable in time and with small differences due to treatment. Only the April sampling showed significant differences, with treatments at NT having the highest values (temperature effect, p: $2.0*10^{-4}$, df: 1, F: 17.30) (Fig. 5b).

The transcription of *il4/13* was similar to that of *pu.1*, showing

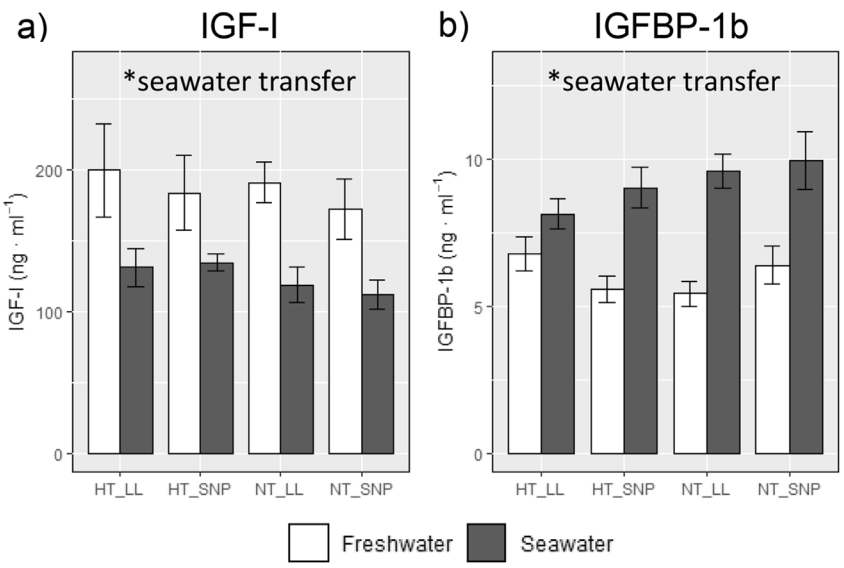

Fig. 4. Plasma IGF-I (a) and IGFBP-1b (b) levels of juvenile rainbow trout reared in freshwater under four photoperiod and temperature treatments. Error bars indicate standard error. Asterisks (*) indicate an overall (i.e. treatment-independent) effect of seawater transfer. Samplings took place on May 31st (white bars) and September 13th (black bars). HT = high temperature, NT = natural temperature, LL = continuous light, SNP = simulated natural photoperiod.

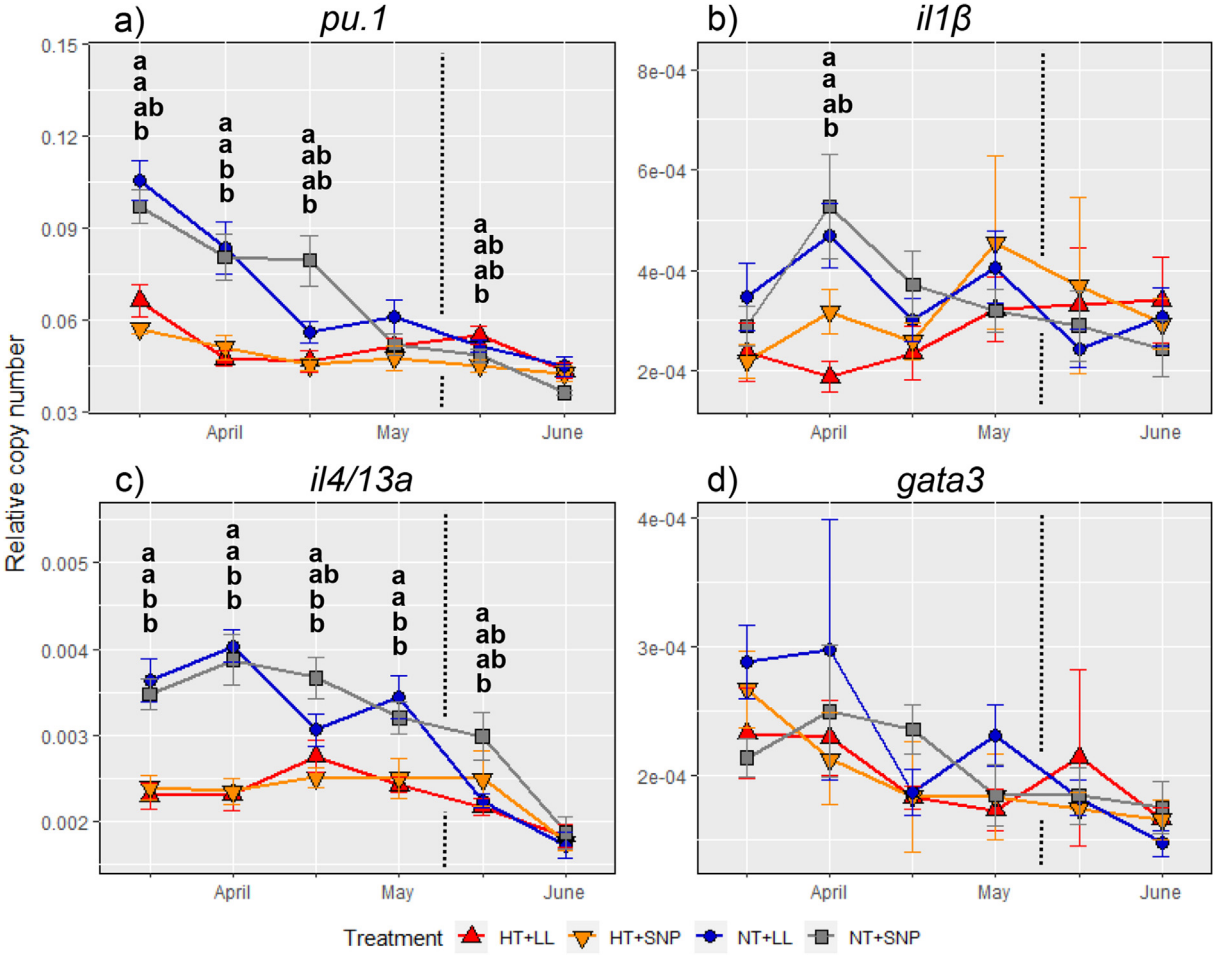

Fig. 5. Liver transcription of genes of the related to haematopoiesis, *pu.1* (a), *il1β* (b), *il4/13* (c), and *gata3* (d), of juvenile rainbow trout reared in freshwater under four photoperiod and temperature treatments. Error bars indicate standard error. Samplings took place on 23rd of March, 6th of April, 20th of April, 4th of May, 18th of May and 31st of May. Dotted line indicates temperature convergence point. Different letters indicate statistical differences ($p < 0.05$) in a time point. Lack of letters indicates lack of significant differences. No letters indicate lack of significant differences. HT = high temperature, NT = natural temperature, LL = continuous light, SNP = simulated natural photoperiod, *il1β* = interleukin 1 β, *il4/13* = interleukin 4/13.

higher values in NT when the temperature treatment was in effect and reducing the differences between HT and NT as TC approached. Significant differences due to temperature were present in mid-March (temperature effect, p: $6.4*10^{-8}$, df: 1, F: 46.61) April (temperature effect, p: $5.9*10^{-9}$, df: 1, F: 59.27), mid-April (temperature effect, p: $3.6*10^{-4}$, df: 1, F: 15.78, photoperiod effect, p: $3.6*10^{-2}$, df: 1, F: 4.79), and in May (temperature effect, p: $1.0*10^{-4}$, df: 1, F: 19.12). Then, in mid-May after TC, treatments at SNP registered the highest transcription values (photoperiod effect, p: $2.8*10^{-2}$, df: 1, F: 5.26), with NT + SNP being significantly higher than HT + LL (Tukey test, p: $4.3*10^{-2}$) (Fig. 5c).

Finally, no significant differences among treatments were found for the transcription of *gata3*. However, a downwards trend was observed in all treatments, with the highest values found in mid-March and April and the lowest in June (Fig. 5d).

4. Discussion

In Atlantic salmon aquaculture, it is common practice to manipulate the photoperiod to match circannual rhythms. This allows the production of smolts throughout the year (Handeland et al., 2013; Johnston, 2008; Stead and Laird, 2002). Manipulation of temperature while is possible it is rarely done due to the obvious high costs of heating large volumes of water. This study aimed at evaluating the effect of different temperature and photoperiod combinations on the induction of smoltification in rainbow trout using both traditional and well-established smolt assessment tools, such as NKA activity measurement, and less established molecular tools, like the transcription of osmoregulatory genes *nkaα1a*, *nkaα1b* and *nkcc1a*. Moreover, the transcription of haematopoiesis related genes *pu.1*, *il1β*, *il4/13* and *gata3* in head kidney was measured to anticipate how, in the event of an infection during the freshwater phase, the fish would be prepared to respond. This experiment was performed on the winter to summer period, since the majority of the problems encountered by seawater-transferred rainbow trout producers occur when transferring 1 + smolts to seawater in summer.

As expected fish size (length and weight) was increased by HT (Ege and Krogh, 1914; Pauly, 1979). However, when comparing TGC to avoid the effect of temperature on growth, HT seemed less advisable, as no differences between groups were found while the temperature treatment was in effect (March to May period). Then, in early May (8th of May), when natural water temperature reached 8 °C NT experienced a strong compensatory growth, growing faster than those fish in HT groups during the May to June period and slightly faster during the June to September period in seawater (similar to Solbakken et al., 1994). As a result, the length and weight differences that the fish at HT had achieved in May were reduced 4 months later (1 more month in freshwater, 3 in seawater), although the fish at HT were still slightly bigger (3.17% more length, 8.48% more weight) and fish kept at NT + SNP were still significantly smaller than those kept at HT + LL. The effects of the phenomenon of compensatory growth in fish, mainly following a fasting period but also due to suboptimal temperature, crowding, or other stressful conditions like hypoxia, have been well-studied (Remen et al., 2014; Ribeiro and Tsuzuki, 2010; Won and Borski, 2013). In fact, its effects can even be positive, with fish that underwent compensatory growth reaching a bigger final size than the control group (Hayward et al., 1997), thus making it a very interesting phenomenon from a production perspective. Nonetheless, the mechanism by which this compensatory growth in response to temperature occurred remains unknown, as no changes in circulating IGF-I levels were found. This is consistent with the IGF-I abundance in plasma, abundance in white muscle and liver transcription during refeeding experiments (Montserrat et al., 2007a,b) but also unlike muscle transcription and plasma abundance in other refeeding experiments (Chauvigné et al., 2003; Picha et al., 2006), making the role of this hormone in compensatory growth unclear. Temperature was in fact not the only driver of growth. The interaction between temperature and photoperiod also had a slight effect on growth, as seen on TGC-L in May–June (HT + SNP higher than HT + LL), and on TGC-W (NT + LL higher than NT + SNP). It is relevant to note that a winter photoperiod signal was tested previously and deemed detrimental for rainbow trout growth and development of smolt traits in spring-summer (Morro et al., 2019). Therefore, no winter signal was incorporated into the design of the present study. It is also remarkable to see the spread of individual growth trajectories for length did not dramatically increase after seawater transfer and that no fish grew less than 0.10 $cm*day^{-1}$, unlike what was seen in a previous study (Morro et al., 2019), likely because in that case the fish had clearly desmolted. However, while seawater mortality was negligible in the previous study, 6.93% of the fish transferred to seawater died in the present study which, had they survived, might have become growth-stunted. Although harvest size was not reached in the present experiment, size at the end-point sampling provides an indication about possible differences among treatments regarding size at harvest. However, it is unclear whether LL or SNP would be better on either the scenario of typical winter-spring temperatures of a northern region or in the scenario of a warmer year, a warmer region or artificially increased temperature.

NKA activity in fish kept at treatments presented peaks in activity at different times, which are normally used to pinpoint the smolt window. Fish at NT did not seem to have a clear smolt window, while according to this test the most suitable seawater transfer time for HT + SNP would be in mid-April and for HT + LL in May. However, the activity levels in the first sampling date were already within smoltification levels (Ewing et al., 1994; Madsen and Naamansen, 1989), pointing at the possibility that the increase in NKA activity took place previously. It is interesting to note that the NKA activity levels in seawater were lower than in freshwater showing that the levels in freshwater are above those needed for efficient hypo-osmoregulatory activity (Morro et al., 2019). Regardless, the data shows that a timely seawater transfer was possible until mid-May. When it actually took place, two weeks later in June, the fish appeared to have just started their desmoltification, which might explain the elevated mortality in seawater (6.93%). In fact, finalization of freshwater treatment and seawater transfer should have occurred before TC. As for the effect of the treatments, no differences were found for NKA at any point, unlike what has been observed for Atlantic salmon (Handeland et al., 1998; Solbakken et al., 1994), showing again the challenges associated with dysregulating the biological clock of this species by manipulating environmental factors (Morro et al., 2019). Hence, since the lack of significant differences in NKA activity does not allow for the identification of the most suited treatment, attention needs to be shifted to the transcription of NKA related genes. Interestingly, the data shows that they are regulated by different environmental factors: while the transcription of *nkaα1a* is strongly regulated by temperature and *nkcc1a* is mildly responsive to it, *nkaα1b* seems to be driven mainly by photoperiod. According to this, high temperature treatment decreased the osmoregulatory capacity of the fish in both freshwater and seawater, as they negatively affected the transcription of both a freshwater and a seawater osmoregulatory gene. In any case, the downregulation of *nkcc1a* was only transient, whereas that of *nkaα1a* lasted for months, hinting that the fish would be more likely to migrate at HT (Sogard et al., 2012). On the other hand, looking at *nkaα1b*, a long day photoperiod in the absence of a winter signal stimulates hypo-osmoregulatory capacity in mid-April, while an increasing photoperiod after a strong winter signal would stimulate it one month later, in a smaller magnitude, and is consistent with the lower hyper-osmoregulatory capacity of the same groups (HT + SNP ad NT + SNP) in June, after the increased temperature treatment was over. Admittedly, this effect of photoperiod on *nkaα1b* was not seen in a previous study (Morro et al., 2019), which started earlier on in the year. Further experimentation involving seawater transfer at different times, when significant differences for the transcription of these genes are present, followed by short and long term reassessment of the

performance of the fish in seawater are needed to validate gene transcription of NKA related genes as seawater-readiness markers. It is hard to identify the most suitable treatment to induce hypo-osmoregulatory competence in rainbow trout. However, the elevated transcription of *nkaα1b* in mid-April and *nkcc1a* in April seems to suggest that the seawater transfer during the first half of April of fish kept at LL would be the best strategy at NT, while at HT it would also be LL in mid-April.

No differences in plasma IGF-I levels were detected among treatments, either before or after seawater transfer. Similarly, in a previous study an advanced photoperiod increased plasma IGF-I levels but LL and SNP had similar levels (Morro et al., 2019). Water temperature is known to affect circulating IGF-I levels (Beckman, 2011; Gabillard et al., 2005) but its effect might not last for several weeks. Beckman et al. (2004) reported in post-smolt coho salmon (*O. kisutch*) that a rapid drop in water temperature affected plasma IGF-I levels for four weeks but fish appeared to be acclimatized after six. In the present study, fish were first reared in different water temperatures for about six weeks, with temperature differences being progressively smaller until TC, followed by nearly four weeks of no temperature differences among treatments. It is possible that an effect, if any, of water temperature disappeared during that time. Furthermore, a lack of correlation between plasma IGF-I and growth rate was unexpected (Beckman et al., 2004; Kaneko et al., 2019; Kawaguchi et al., 2013). One possible reason for this is the time interval to calculate growth rate. Most studies reported positive correlations between plasma IGF-I level and growth rate for one to two weeks (Beckman, 2011). In the present study, on the other hand, growth rate was calculated during 15 weeks. Moreover, decreased IGF-I levels after 15 weeks in seawater contrasted with the results in the previous study, where plasma IGF-I levels were higher in fish in seawater than in freshwater. However, growth rates of fish during this phase in the present study (approximately 0.25%/day) were comparable or even higher than those in the previous study (approximately 0.20%/day). The reason for the opposite response of plasma IGF-I is currently unknown but the result suggests that plasma IGF-I levels did not affect overall growth performance. In regards to IGFBP-1b, the negative correlation between plasma levels and SGR-L in rainbow trout during their 15-week seawater period is consistent with previous literature (Kaneko et al., 2019; Kawaguchi et al., 2013; Shimizu et al., 2006). Although more frequent sampling is necessary to validate the utility of plasma IGFBP-1b as an index of growth repression, this is the first report on a significant negative relationship between plasma IGFBP-1b and SGR in rainbow trout.

Before TC, the transcription of haematopoiesis related genes was independent of the photoperiod treatment and unequivocally affected by temperature, with higher temperatures causing lower transcription levels of both adaptive and innate immune related genes. The exception was *gata3*, involved in the regulation of T lymphocytes, stimulation of phagocytosis and antibody production by B cells (Kumari et al., 2009; Tort et al., 2003), which did not respond to temperature variations. On the other hand, the key regulator of the development of the GMP myeloid cell lineage (i.e. mast cells, eosinophils, neutrophils, macrophages and dendritic cells, Orkin and Zon, 2008), *pu.1*, was strongly affected by temperature. Macrophages and dendritic cells play critical roles in antigen presentation during inflammatory responses, while eosinophils, basophils, and mast cells are involved in a variety of allergic and innate immune responses (Galli, 2000; Iwasaki and Akashi, 2007). However, as the temperature of fish kept at NT and at HT drew nearer, the differences decreased and did not seem to have further effects beyond the TC point. A pattern that can also be seen for the transcription of *il4/13*, which was the most affected gene by temperature. Through a lower *il4/13* transcription, high temperatures would result in less proliferation of B lymphocyte and immunoglobulins and lower activation of macrophages (Martinez et al., 2009; Sequeida et al., 2017; Takizawa et al., 2011). Therefore, this could potentially affect the core components of the adaptive immune system: antibodies and memory B cells (Nutt and Kee, 2007), as well as the function associated to macrophages. Finally, *il1β* was slightly higher at NT during the first part of the freshwater experiment, being associated with a very mild and transient difference of both B and T lymphocyte abundance and activity between temperature groups (Pleguezuelos et al., 2000; Reis et al., 2012). More importantly, the general lack of big fluctuations over time in *il1β* transcription confirms that no infections occurred during the freshwater experimentation phase. It is interesting to see how, although the photoperiod treatment could have an effect on *pu.1* and *il4/13,* as seen in mid-May, and even on *nkaα1a* as seen in June, the effect of temperature before TC overrides any possible effects of photoperiod. While a higher transcription of haematopoiesis related genes is likely to be indicative of more robust fish against disease because they would be able to quickly produce large amounts of defence molecules (Martin et al., 2012), thus making NT more desirable than HT for rainbow trout production in this sense. Whether this higher resource investment in immune cells at NT is good for the fish is still a matter of debate.

In conclusion, while a larger length and heavier weight were achieved in freshwater by fish kept at HT, soon after TC this size advantage was quickly lost due to the compensatory growth that fish kept at NT experienced. While the temperature treatment could continue during more months, requiring temperatures above 8 °C and potentially maintaining the length and weight differences of fish at HT for longer, high temperatures can negatively affect NKA activity (Handeland et al., 2000). In fact, it has been suggested that smolt development is inhibited in the migrating strain of rainbow trout (steelhead trout) at a temperature between 10 °C and 15 °C (Adams et al., 1973). In this sense, data suggests LL photoperiod regimes would be more suitable than SNP for both NT and HT scenarios in terms of the development of osmocompetence in seawater if the fish were to be transferred in April. In terms of transcription of haematopoiesis genes, HT resulted in lower transcription levels, especially for *pu.1* and *il4/13*, thus decreasing the proliferation of lymphoid cells and macrophages (Iwasaki and Akashi, 2007; Pleguezuelos et al., 2000; Sequeida et al., 2017). This is likely to affect the immune response of fish kept in these conditions, especially the adaptive immune response, potentially resulting in a lower abundance of crucial immune cells, reducing the value of prophylactic measures, and at the same time providing more suitable conditions for the proliferation of pathogens (Austin and Austin, 2012). Overall, the apparent disadvantages of using increased temperature treatments to induce smoltification on rainbow trout surpass the unclear advantages of it, therefore discouraging the use of water heating strategies and alerting farms found in warmer climates of the possible implications that their conditions might have on rainbow trout.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2019.734711.

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