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Atlantic salmon (*Salmo salar*) exposed to different preparatory photoperiods during smoltification show varying responses in gill Na^+/K^+-ATPase, salinity-specific mRNA transcription and ionocyte differentiation

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ABSTRACT

Control of the parr-smolt transformation (or smoltification) is crucial for the husbandry and successful seawater (SW) transfer of Atlantic salmon (*Salmo salar*) reared in freshwater (FW) hatcheries. Photoperiod is an important environmental signal that initiates the complex physiological, morphological and behavioural changes that coincide with marine migration. While the use of long-day photoperiods to initiate smoltification has been well studied, this study investigated how three preparatory photoperiods in FW (LD 08:16, LD 12:12, LD 16:08) preceding exposure to 24-h light (LD 24:0) may influence or enhance smolt performance and growth post-SW transfer. After the photoperiod treatment phase (8 weeks), all groups were exposed to LD 24:0 for 8 weeks (FW) and then transferred to SW for a further 8 weeks. Exposure to LD 16:08 induced rapid development of smolt-related characteristics such as increased gill NKA activity, gill NKAα1b mRNA, and plasma cortisol, and decreased gill NKAα1a mRNA levels and condition factor through the 8-week treatment phase. Subsequent exposure to a LD 24:0 photoperiod resulted in a partial reversal of several of these characteristics, suggesting these fish went through a partial desmoltification. Exposure to LD 12:12 for 8 weeks prior to LD 24:0 elicited an intermediary response in smoltification attributes compared to LD 16:08 and LD 08:16. The LD 12:12 group adapted to SW and showed no negative effects on growth or physiological responses after transfer to SW. Exposure to a shortened photoperiod (LD 08:16) did not elicit any smoltification-related changes prior to LD 24:0, however, exposure to LD 24:0 increased gill NKA activity, plasma cortisol, changes in NKAα1a and NKAα1b mRNA, and the ratio of NKAα1b: NKAα1a. These results were confirmed by the expected changes in NKAα1a and NKAα 1b-positive immuno-reactive gill ionocytes. In summary, after exposure to LD 24:0 fish in the LD 08:16 group showed similar levels of change to those of the LD 16:08 group during the initial FW phase (prior to exposure to LD 24:0). After SW transfer, all groups were able to upregulate SW-specific NKAα1b mRNA and acclimate to SW, even though no increase in cortisol was evident. By the end of the study, there was no difference in SW growth among the groups. Overall, our data indicate that LD 16:08 advanced hypoosmoregulatory characteristics prior to LD 24:0 exposure. In addition, the physiological and molecular indicators measured in this group suggest that fish could have been transferred to SW immediately after 8 weeks in LD 16:08, with no added benefit of successive exposure to LD 24:0, which is typically used by industry to induce smoltification.

1. Introduction

Atlantic salmon (*Salmo salar*) are an anadromous member of the Salmonidae family, and one of the most commonly cultivated aquaculture species around the world. Like several other salmonids, Atlantic salmon have evolved complex physiological adaptations that allow them to survive in both freshwater (FW) and seawater (SW) environments. Atlantic salmon parr that are above a size/growth threshold are sensitive to a seasonal increase in photoperiod typical of spring that cues a suite of complex physiological, morphological, and behavioural changes that ultimately facilitate this species' euryhalinity (Folmar and Dickhoff, 1980; Handeland et al., 2013; Hoar, 1988; McCormick, 2013;

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Strand et al., 2011). This phenomenon is known as the parr-smolt transformation, or 'smoltification' (Hoar, 1988; McCormick, 2013). Prior to this transformation, parr have a poor tolerance to SW and if transferred, are subject to osmotic perturbation, reduced growth and increased mortality (Bendiksen et al., 2003; Duston, 1994; Usher et al., 1991).

Gill Na^+/K^+-ATPase (NKA) facilitates the transfer of ions across the cell membrane via active transport, and is fundamental in maintaining ionic cellular homeostasis (Evans et al., 2005; Skou and Esmann, 1992). Gill NKA is found within gill chloride cells (ionocytes), and exchanges two intracellular Na^+ ions for three extracellular K^+ ions, resulting in a net-negative charge and low-sodium within the cell (McCormick et al., 2009b). The $Na^+/K^+/2Cl^-$ cotransporter (NKCC) then uses the sodium gradient to bring chloride into the cell, and the electrochemical gradient created by NKA allows chloride to passively exit the cell through the apical cystic fibrosis transmembrane regulator (CFTR) (McCormick et al., 2009b). In salmonids, gill NKA activity is tightly linked to development of salinity tolerance of smolts (McCormick, 1993).

The NKA is comprised of 2 essential subunits (α and β), and several different isoforms of these NKAα- and β-subunits have been identified in vertebrates (Richards et al., 2003). The α-subunit has binding sites for Na^+, K^+, ATP and ouabain (an enzyme inhibitor specific to NKA), and is the functional catalytic subunit. The β-subunit is a glycosylated polypeptide that is responsible for the folding and positioning of the NKAα into the plasma membrane (McCormick et al., 2013). The switching of the gill epithelium from an ion-uptake to an ion-secretory organ is key to successful SW transfer or migration into SW, and is driven by the differential expression of key gill NKA genes and their expressed proteins (Christensen et al., 2018; Nilsen et al., 2007; Richards et al., 2003). Richards et al. (2003) identified five different isoforms of NKA α-subunits in gill tissue of rainbow trout (*Oncorhynchus mykiss*), and found that only NKAα1a and NKAα1b were differentially expressed following transfer to SW. NKAα1a exists primarily in the FW gill ionocytes, in which ion-uptake is the primary function (McCormick et al., 2013). On the other hand, NKAα1b is found predominantly in the SW gill ionocytes, and in concert with NKCC and CFTR, facilitates high-capacity ion-secretion in SW (McCormick et al., 2013; Nilsen et al., 2007; Richards et al., 2003). The co-expression of NKAa1b and other ion transporters (NKCC1, CFTR), and a higher abundance in NKA pumps (Karnaky et al., 1976) in the SW ionocytes results in the functional difference between these two isoforms. Several studies have confirmed the salinity-specific transcription of these two isoforms in Atlantic salmon (Bystriansky et al., 2006; Christensen et al., 2018; Madsen et al., 2009; McCormick et al., 2009b), as well as in rainbow trout (Morro et al., 2019) and Arctic charr (Bystriansky et al., 2006).

Under culture conditions, *S. salar* are grown in FW hatcheries, and photoperiod is manipulated in order to initialise smoltification. Exposure to a short-day photoperiod of 1–2 months is necessary for subsequent long day length to stimulate smoltification (Björnsson et al., 1989; Duston and Saunders, 1995), and a 16 h day length is probably the minimum requirement for completed parr-smolt transformation (Strand et al., 2018). A 'square wave' photoperiod (short days followed by exposure to 24 h light) is effective at inducing a parr-smolt transformation comparable to that which occurs under natural spring photoperiod regimes (Duston and Saunders, 1995; Handeland and Stefansson, 2001; Sigholt et al., 1995), and this regime is commonly used by industry to produce smolts year-round (Handeland and Stefansson, 2001). Most previous studies have compared different combinations of short-day and LD 24:0 photoperiods with simulated natural photoperiod (Duston and Saunders, 1990; Handeland and Stefansson, 2001; McCormick et al., 1987; Saunders et al., 1985; Solbakken et al., 1994), however the effects of different preparatory photoperiods before subjection to a square wave LD 24:0 regime have not been well studied. In addition, most previous work has collected samples monthly, with relatively few studies measuring smolt-related attributes more frequently. The objective of this study was to investigate whether three preparatory photoperiods in FW (short day: LD 08:16; medium day: LD 12:12; long day: LD 16:08) preceding exposure to 24-h light could advance the smoltification process and result in improved growth performance after transfer to SW. Morphological, physiological and molecular changes associated with smoltification in FW and after transfer to SW were measured. We examined growth attributes, gill NKA activity, gill NKA mRNA transcription (NKAα1a and NKAα1b), gill NKAα1a- and NKAα1b- immunoreactive ionocytes, plasma osmolality and plasma cortisol levels. All parameters were measured every 15 days over a period of 24 weeks (16 in FW and 8 in SW). We hypothesised that exposure to an increase in photoperiod for 8 weeks prior to LD 24:0 would advance preparedness for transfer to SW, resulting in a reduced FW rearing time.

2. Materials and methods

2.1. Fish rearing and experimental design

Juvenile Atlantic salmon 1+ smolts were supplied by Mountain Fresh Trout & Salmon Hatchery (Harrietville, Victoria, Australia) in June 2016, and transported to the Deakin Aquaculture Futures Facility (Deakin University, Warrnambool Campus, VIC, Australia) and held at 14.0 ± 0.5 °C on a LD 12:12 photoperiod. A total of 65 fish (average weight ~ 60 g) were randomly allocated to each of nine (1000 *l*) experimental tanks in a FW recirculating aquaculture system (RAS). Optimal water quality was maintained by a drum screen, biological and UV filtration and degasser. All fish were acclimated in experimental tanks for 6 weeks under LD 12:12 photoperiod and water was maintained at 14.0 ± 0.5 °C. Fish were fed a commercial smolt diet (Spirit Supreme: Skretting Australia) twice daily to satiation. Each FW tank was equipped with a white block-out canvas hood and a 10 W LED light source (6000 K, approximately 850 LUX at water surface).

After the acclimation period, all experimental tanks were sampled at week 0 in order to obtain baseline data. Three different photoperiod treatments were then abruptly introduced for eight weeks; LD 08:16, LD 12:12, and LD 16:08. Each treatment consisted of 3 replicate tanks (n = 3) and contained 65 fish in each. Following this, LD 24:0 was introduced (abrupt change) to all treatment groups for eight weeks. Fish were then transferred to 1000 *l* tanks in a SW (35‰) RAS equipped with drum screen filtration, bio filtration, degassing, foam fractionator and UV filtration. Fish were then maintained at LD 12:12 photoperiod and 15 °C water temperature for eight weeks. In addition, each treatment group was subjected to a 24-h SW challenge at each sampling time during the LD 24:0 light phase. This was done by taking 4 extra fish per tank at each sampling time and putting them into black 60 *l* tubs filled with SW (35‰) from a marine recirculation aquaculture system (RAS) at approximately 14 °C. Tubs had a mesh lid and an air stone to maintain dissolved O_2. After 24-hours of SW exposure, a blood sample was collected for analysis. There were two moribund fish removed from a LD 12:12 tank at two different times during the SW phase of the experiment, however there was no mortalities otherwise. All experimental procedures conducted during this trial were approved by the Deakin University Animal Ethics Committee (Permit No. B11–2016).

2.2. Sample collection

Each experimental group was sampled at weeks 0, 4 and 8 during the FW treatment phase, weeks 10, 12, 14, and 16 during the FW LD 24:0 phase, and at weeks 18, 20, 22 and 24 during the SW phase. At each sampling time, fish were euthanised using a lethal dose of AQUI-S®. Blood was collected from the caudal vein within 2 min using Na^+-heparinised syringes. Plasma was immediately separated from red blood cells by centrifugation (3000 *g* for 15 min). Blood and plasma were both frozen and kept at −80 °C until further analysis. Plasma osmolality was determined following methods outlined by Schultz et al., 2008. Four to five gill filaments from the right side of each fish

were dissected from the first gill arch and put in ice-cold sodium-EDTA-imidazole (SEI) buffer for NKA activity analysis, and ~ 50 mg was collected for RT-qPCR. These samples were frozen immediately using dry ice and stored at −80 °C. The entire second gill arch was dissected from the left side of the fish and stored in 10% buffered formalin for histology and immunohistochemistry analysis. Total fish mass (± 0.01 g) and fork length (± 1 mm) were measured and recorded.

2.3. Growth and condition

Fulton's condition factor (K) was calculated using the formula $K = 10^n(W/L^3)$ (where: $N = 5$, W = mass in grams and L = fork length in mm). Specific growth rate was calculated using weight (SGR-W) and fork length (SGR-L). SGR-W was expressed as a percentage increase in total mass · day^{-1}, and calculated using the formula *SGR-W = [Ln(final mass) – Ln(initial mass)] / (number of days) x100*. SGR-L was expressed as increase in cm · day $^{-1}$, and calculated as *SGR-L = [Ln(final fork length) – Ln(initial fork length)] / (number of days) x100*.

2.4. Gill Na^+/K^+-ATPase activity

Gill NKA activity was determined following the procedure described by van Rijn et al. (2020), based on McCormick (1993). Gill samples were taken from an −80 °C freezer and thawed on ice. 1 ml of ice-cold 0.15% SEID buffer (SEI buffer with 0.15% deoxycholate) solution was added, and the sample homogenised by hand using a glass tube and Teflon pestle. The supernatant was then centrifuged at 4 °C for 3 min at 5000 x g. Samples were then run in a 96-well microplate in triplicate with two assay solutions: Solution 1 (ouabain negative) contained 4 U lactate dehydrogenase ml^{-1}; 5 U pyruvate kinase ml^{-1}; 2.8 mM phosphoenol pyruvate; 0.7 mM ATP; 0.22 mM NADH; 50 mM imidazole pH 7.5. Solution 2 (ouabain positive) is identical, but also contains 0.5 mM of the NKA inhibiting ouabain. The assay was run for 20 min at 26 °C, and the consumption of NADH as a function of ADP present was measured as a reduction in OD at 340 nm. The final calculation takes into account an ADP standard curve, and results are standardised by protein levels according to the Bradford method (Bradford, 1976). A final value of NKA activity is reported as the ouabain-sensitive production of ADP expressed as μmol ADP · (h)$^{-1}$ · (mg protein)$^{-1}$.

2.5. Real-time quantitative PCR

NKAα1a and NKAα1b mRNA transcripts were quantified using elongation factor 1-α (EF1α) as a reference gene. Primers and assay protocols were designed based on previously published studies with similar experimental conditions (McCormick et al., 2008; Nilsen et al., 2007; Richards et al., 2003). Primers for each gene (Table 1) were manufactured by Integrated DNA Technologies (Baulkham Hills, NSW, Australia). Total RNA was extracted from approximately 10 mg of gill tissue stored in RNA*later*® using an Aurum™ Total RNA Mini Kit (Bio-Rad: 7326820). RNA quality of selected samples was visually assessed on a 1% agarose gel electrophoresis during initial testing stages, and then RNA quality and quantity of every sample was assessed by the ratio of absorbance at 260 nm and 280 nm on a benchtop NanoDrop. RNA was then normalised to 50 ng μl^{-1} using certified DNase/RNase-free H_2O and treated with Turbo DNA-free (Invitrogen) as per manufacturer's instructions to eliminate any residual genomic DNA.

cDNA was synthesised in 0.6 ml PCR tubes by adding 2 μl of RNA to 4 μl of iScript™ Reverse Transcription Super mix (Bio-Rad: 1708840) and 14 μl of RNase-free H_2O. The 20 μl reaction was then cycled in a thermocycler according to the manufacturer's instructions. No-reverse transcriptase (NRT) controls for each sample were also run under the sample conditions.

A qPCR Master Mix was prepared for each gene with 10 μl of SYBR green reagent (Bio-Rad, USA), 0.4 μl of 10 mM forward primer, 0.4 μl of 10 mM reverse primer and 7.2 μl of RNase-free H_2O per well. The total reaction volume of 20 μl results in a 200 nM:200 nM primer concentration ratio once 2 μl of cDNA template are added. Each plate contained duplicate templates for each sample and each gene. NRT controls from each plated sample were pooled and run in duplicate, along with a no-template-control (NTC) in duplicate for each primer set. PCR was started with an incubation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. To assure primer specificity, a melt curve analysis was then conducted with 0.5 °C increments from 65 °C to 95 °C for 5 s per temperature step. Efficiency of each assay was determined by performing ten-fold serial dilutions of pooled cDNA from 10 fish, and was 99.8% 100.2% and 99.0% for EF1α, NKAα1a and NKAα1b, respectively. Fold-expression of target genes (NKAα1a and NKAα1b) was calculated relative to the reference gene (EF1α) and relative to the initial time point (week 1) using the Delta-Delta-C_T ($\Delta\Delta C_T$) method (Livak and Schmittgen, 2001). For $\Delta\Delta C_T$ calculations, an efficiency of 100% was assumed for each primer set.

2.6. Immunohistochemistry

Immunohistochemistry of gills samples was completed using previously published methods (Dymowska et al., 2014; Schultz et al., 2008). In brief, gill samples were dehydrated and paraffin imbedded using an automated tissue processor (Leica ASP200S; Leica Biosystems). Gill sections (5 μm) were cut by a microtome and mounted on IHC-compatible slides by the Biomedical Sciences Histology Facility at the University of Melbourne (School of Biomedical Sciences, Level 2 W238, Medical Building (181), The University of Melbourne, Grattan St, Parkville 3010 Australia. Sections were deparaffinised and rehydrated using the following protocol: 100% Xylene (2 × 3 min), 100% ethanol (2 × 3 min), 99% ethanol (3 min), 95% ethanol (3 min), 70% ethanol (3 min), Milli-Q H_2O (5 min), 1× PBS (5 min). Antigen retrieval was then performed by pre-heating Na-citrate buffer (10 mM, pH 6.5) in a Pyrex® microwave dish, then by laying slides flat in the dish (making sure they remain submerged by buffer), and heating in a microwave on HIGH for 15 min. Slides were then washed in PBS buffer (3 × 3 min). A chicken polyclonal antibody was raised to salmon NKAα1a-specific peptide and a rabbit polyclonal antibody was raised to salmon NKAα1b-specific peptide (McCormick et al., 2013) and sent via airfreight to Deakin University, Geelong.

Sections were circled with a hydrophobic 'DAKO' pen and then exposed to primary antibody mix (anti-NKAα1a,1:500; anti-NKAα1b, 1:800) in antibody dilution buffer (0.01 M PBS, 0.3% Triton X-100, and 0.1% sodium azide, pH 7.1). Sections were then incubated for 16 h at 4 °C. Following this, slides were washed in PBS (3 × 5 min) and then exposed to secondary antibodies (Goat anti-Chicken IgY (H + L) Alexa Fluor 488, 1: 200; Goat anti-Rabbit IgG (H + L) Alexa Fluor 594, 1: 200) (Invitrogen, USA) for 2 h at 4 °C. Finally, slides were washed with 1× PBS (3 × 5 min), and coverslips were added with DAPI mounting

Table 1
Primers used in real-time quantitative PCR.

Gene	Forward Primer	Reverse Primer	Product size
Na^+/K^+-ATPase α1a	GGCCGGCGAGTCCAAT	GAGCAGCTGTCCAGGATCCT	66
Na^+/K^+-ATPase α1b	CTGCTACATCTCAACCAACAACATT	CACCATCACAGTGTTCATTGGAT	81
Elongation factor 1a	GAGACCCATTGAAAAGTTCGAGAAG	GCACCCAGGCATACTTGAAAG	71

media (Sigma Aldrich #10236276001) and sealed with clear nail varnish.

Due to the differences in ionocyte number at the afferent or efferent edges of gill filaments (Wilson and Laurent, 2002), gill ionocyte counts were conducted on sagittal gill sections that were centrally located on the primary filament (Christensen et al., 2012; McCormick et al., 2003), where there is an 'average' number of ionocytes. An LED-powered fully-automatic fluorescent microscope (ZEISS Axio Imager 2; ZEISS Germany) and ZEN Pro imaging software (ZEISS, Germany) was used to acquire the images. ImageJ analysis software (Rueden et al., 2017) was used to count NKAα1a and NKAα1b immuno-reactive ionocytes. In order to eliminate bias as much as possible, 4 sections were tallied separately from each replicate fish, and 6 fish per treatment were analysed, and Images were targeted at these central regions. Count data were presented as cells per millimetre of primary filament. All cell counting was conducted blindly (without knowledge of sample number or treatment) to eliminate experimenter bias.

2.7. Statistical analysis

Statistical analysis was conducted via a linear mixed-model using lme4 (Bates et al., 2015) in RStudio (R Core Team, 2019). Each model investigated the fixed effects of treatment (3 photoperiods) and week, with tank set as a random effect. A one-way ANOVA was also used to investigate differences between FW and SW challenged groups. All data were checked for equal variance and normality of distributions by plotting residuals against predicted values and visualising data distribution boxplots respectively. Log transformations of response variables were applied where appropriate and assumptions re-tested. Any significant effects of treatment within week ($P < .05$) were investigated using Tukey post-hoc multiple pairwise comparisons. All data are summarised as mean ± standard error of the mean (SEM) based on tanks as experimental units. Graphic output was generated using ggplot2 for R (Wickham, 2016).

3. Results

3.1. Growth, condition, and survival

Over the 24-week study period, fish significantly increased in both mass and fork length ($p < .001$). On average, fish mass increased 5-fold over the duration of the experiment. Although there was no significant overall effect of treatment, differences in mass between treatments were evident at week 12 and 14 (Fig. 1). Fish from the LD 08:16 group were significantly lower in mass than fish from the LD 12:12 and LD 16:08 groups at weeks 12 and 14, respectively. There was a significant effect of time (week) ($p < .001$) and treatment ($p = .014$) on fork length. Fish from the LD 16:08 treatment exhibited significantly longer fork length than the LD 08:16 group at weeks 10, 12 and 14 (Fig. 2). Fork length was significantly higher in fish from the LD 12:12 group than in fish from the LD 08:16 group at week 12. By the end of the experiment, there was no significant difference in mass or fork length between the treatment groups.

Although there was no significant effect of photoperiod treatment on specific growth rate based on weight (SGR-W), there was a significant reduction in SGR-W in all groups at the LD 24:0 phase of the experiment when compared to the treatment phase. The SW phase was not significantly different to the other two phases (Fig. 3A). Fish from the LD 16:08 group showed higher Specific growth rate based on fork length (SGR-L) during the treatment phase when compared to the LD 24:0 and SW phase, however there was no significant differences between any other groups (Fig. 3B).

The LD 16:08 group had a significantly lower condition factor (CF) than the LD 08:16 and LD 12:12 groups at weeks 4 and 8. At week 10, the LD 16:08 group had a significantly lower CF than the LD 08:16 group only. Fish in the LD 08:16 and LD 12:12 groups showed a reduction in CF between weeks 8 and 14, however this reduction was only significant for the LD 08:16 group (Supplementary Table 1). There were no significant differences between treatments during the SW phase, with all groups showing an increase in CF after transfer to SW (Fig. 4).

3.2. Physiological attributes

3.2.1. Plasma osmolality

Although there was no significant main effect of treatment, fish from the LD 16:08 and LD 12:12 groups had significantly lower plasma osmolality than fish from the LD 08:16 group at week 10 (Fig. 5). There was no significant difference present in the LD 16:08 group after exposure to LD 24:0, however fish from the LD 08:16 and LD 12:12 groups showed a gradual significant decrease in plasma osmolality between weeks 8 and 14. Fish from all groups showed a significant increase in plasma osmolality after transfer to SW (Fig. 5 and Supplementary Table 2).

SW challenged fish from the LD 16:08 were not significantly different to the FW control at week 10, however, they showed significantly elevated plasma osmolality levels at weeks 12, 14 and 16 (Table 2). SW challenged fish from the LD 12:12 group were significantly higher than FW control at weeks 12 and 14 and showed no significant temporal differences. SW challenged fish from the LD 08:16 group also showed no significant temporal difference but were significantly elevated from

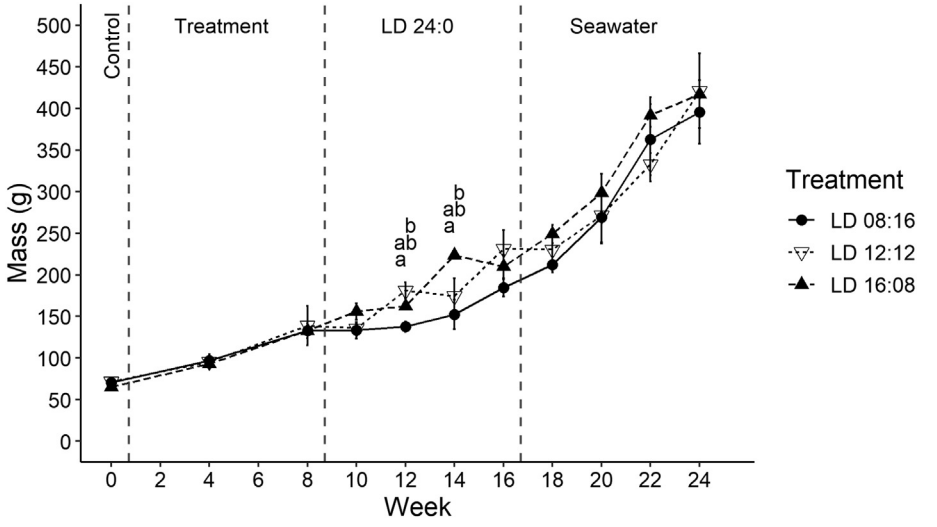

Fig. 1. Mass (g, mean ± SE) of juvenile Atlantic salmon exposed to 3 photoperiod regimes for 8 weeks, followed by 24-h light (8 weeks) and transfer to seawater (SW) (8 weeks). N = 3 replicate tanks in each treatment (4 fish per tank at each timepoint). Change to 24 h light (week 8) and transfer to SW (week 16) are indicated by vertical lines. Significant differences between treatments within week are represented by different lower-case letters ($P < .05$, Tukey-adjusted). The vertical order of the lower-case letters directly relates to the order of the data points. Weeks without annotations had no significant difference between treatments within week.

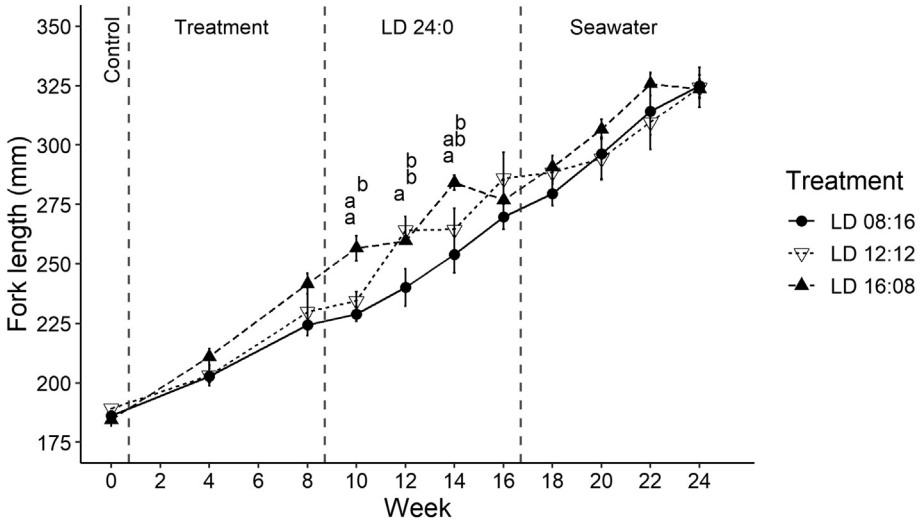

Fig. 2. Fork length (mm, mean ± SE) of juvenile Atlantic salmon exposed to 3 photoperiod regimes for 8 weeks, followed by 24-h light (8 weeks) and transfer to seawater (SW) (8 weeks). $N = 3$ replicate tanks in each treatment (4 fish per tank at each time-point). Change to 24 h light (week 8) and transfer to SW (week 16) are indicated by vertical lines. Significant differences between treatments within week are represented by different lower-case letters ($P < .05$, Tukey-adjusted). The vertical order of the lower-case letters directly relates to the order of the data points. Weeks without annotations had no significant difference between treatments within week.

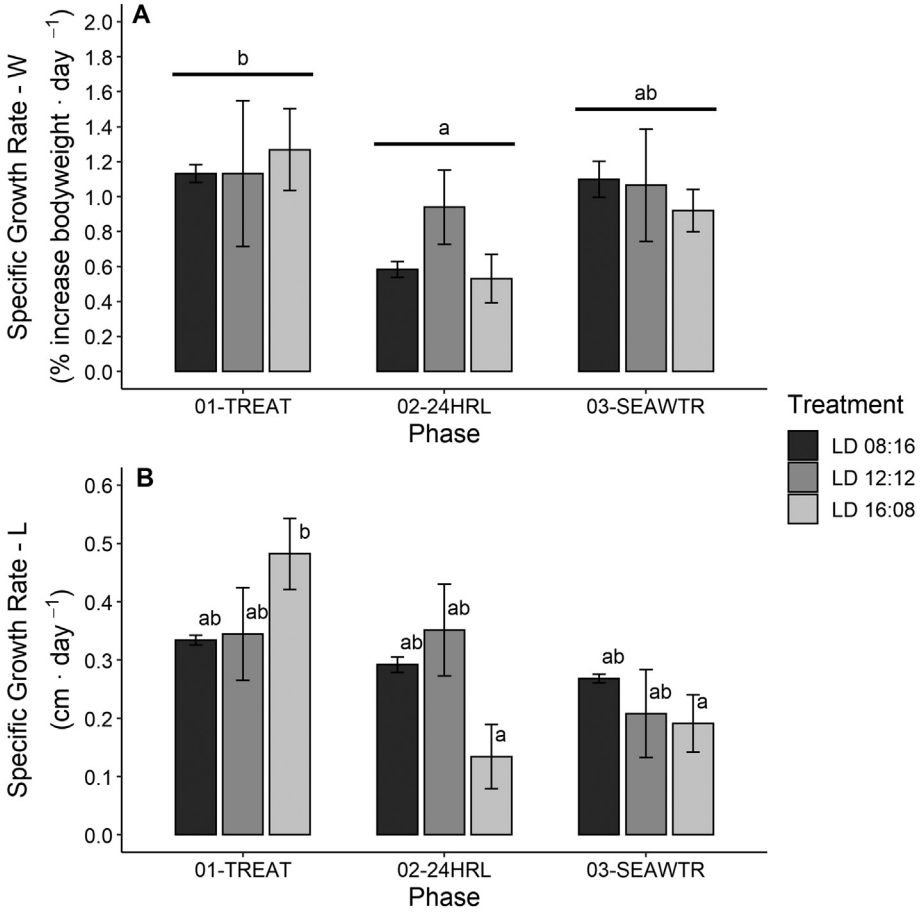

Fig. 3. (A) Specific growth rate – weight (SGR-W) (% increase in bodyweight · day^{-1}, mean ± SE). N = 3 replicate tanks in each treatment (4 fish per tank at each time-point). Treatment groups were pooled as there was no significant effect of treatment. Significant differences between phases are denoted by different lower-case letters (Tukey-adjusted). (B) Specific growth rate – length (SGR-L) (cm · day^{-1}, mean ± SE) of juvenile Atlantic salmon exposed to photoperiod regimes calculated for each phase. N = 3 replicate tanks in each treatment (4 fish per tank at each time-point). Significant differences among phases within photoperiod are denoted by different lower-case letters (Tukey-adjusted).

the corresponding FW control group at weeks 10, 12 and 14 (Fig. 5 inset and Table 2).

3.2.2. Gill Na^+/K^+-ATPase

Fish from the LD 16:08 group exhibited a significant 6-fold increase in NKA activity during the treatment phase of the experiment (Fig. 6), and levels were significantly higher when compared to LD 08:16 and LD 12:12. Four weeks after exposure to LD 24:0, NKA activity in the LD 16:08 group had decreased to nearly basal FW levels. Fish from the LD 08:16 group showed no substantial increase in gill NKA activity prior to exposure to LD 24:0. Fish from the LD 12:12 group showed a non-significant 3-fold increase in NKA activity between weeks 0 and 8, then plateaued at a significantly higher level during the LD 24:0 phase (week 10–16) (Supplementary Table 2). NKA activity was significantly lower in fish from the LD 08:16 group than the LD 16:08 group at week 10 (after 2 weeks of LD 24:0 exposure), however it was significantly higher at weeks 14 and 16 (Fig. 6). There were no significant differences among treatments within week during the SW phase. All treatments showed a 3-fold higher NKA activity at week 24, which was significantly higher than week 0 (Supplementary Table 2).

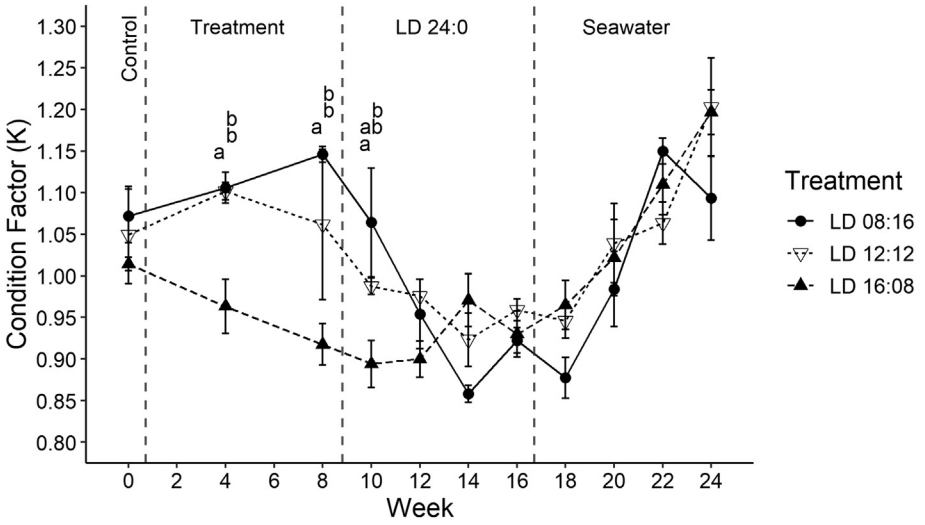

Fig. 4. Condition factor (K, mean ± SE) of juvenile Atlantic salmon exposed to 3 photoperiod regimes for 8 weeks, followed by 24-h light (8 weeks) and transfer to seawater (SW) (8 weeks). N = 3 replicate tanks in each treatment (4 fish per tank at each time-point). Change to 24 h light (week 8) and transfer to SW (week 16) are indicated by vertical lines. Significant differences between treatments within week are represented by different lower-case letters ($P < .05$, Tukey-adjusted). The vertical order of the lower-case letters directly relates to the order of the data points. Weeks without annotations had no significant difference between treatments within week.

3.2.3. Plasma cortisol

Fish from the LD 16:08 group exhibited a significant 17-fold increase in plasma cortisol during the treatment phase, and their plasma cortisol levels were significantly higher at week 8 when compared to fish from the LD 08:16 and LD 12:12 treatments (Fig. 7). Plasma cortisol in the LD 16:08 group decreased significantly after exposure to LD 24:0 (Supplementary Table 2). In the LD 08:16 and LD 12:12 groups, plasma cortisol remained consistently elevated between weeks 8 to 16. There was a significant decreasing trend of plasma cortisol in the LD 16:08 group during the LD 24:0 phase (between weeks 8–16). Fish from the LD 16:08 group presented significantly lower plasma cortisol levels at week 10 compared to the LD 12:12 group, and at weeks 12, 14 and 16 when compared to the LD 08:16 group. There were no significant differences between treatments during the SW phase, with all groups displaying low plasma cortisol similar to initial values at week 0.

3.3. Gill NKA mRNA levels

Fish from the LD 16:08 treatment showed a significant 2.5-fold decrease in gill NKAα1a mRNA levels during the treatment phase (Supplementary Table 3) and had significantly lower gill NKAα1a mRNA levels than fish from the LD 08:16 group at weeks 4 and 8 (Fig. 8A). Fish from the LD 12:12 group showed a significant 1.6-fold decrease in gill NKAα1a mRNA level between weeks 0 and 8. The values at week 8 were also significantly lower than fish from the LD 08:16. Fish from the LD 08:16 group showed no significant change during the treatment phase. During the LD 24:0 phase, fish from the LD 16:08 group did not significantly increase NKAα1a mRNA levels, but at week 16 values were significantly higher when compared to LD 08:16 and LD 12:12 groups. All groups showed a further decrease in gill NKAα1a mRNA levels after transfer to SW to levels significantly lower than week 0 values (Supplementary Table 3). However, there were no significant differences between treatments in the SW phase of the experiment.

Fish from the LD 16:08 group showed a significant increase in gill

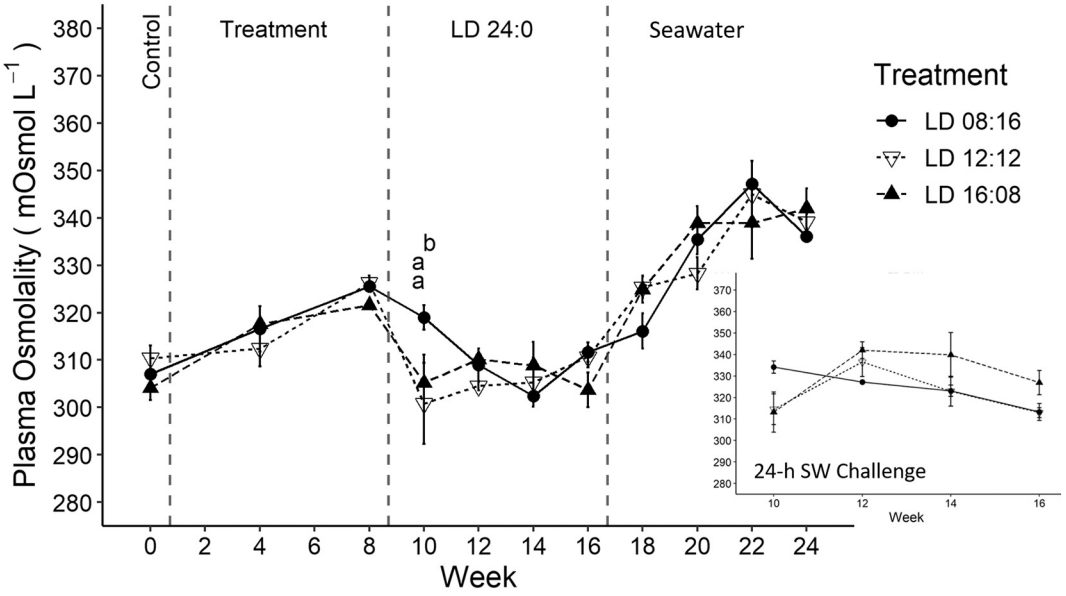

Fig. 5. Plasma osmolality (mOsmol l^{-1}, mean ± SE) of juvenile Atlantic salmon exposed to 3 photoperiod regimes for 8 weeks, followed by 24-h light (8 weeks) and transfer to seawater (SW) (8 weeks). N = 3 replicate tanks in each treatment (4 fish per tank at each time-point). Change to 24 h light (week 8) and transfer to SW (week 16) are indicated by vertical lines. Transfer to seawater tanks was after week 16. Inlayed image represents results from 24-h SW challenged fish. Significant differences between treatments within week are represented by different lower-case letters ($P < .05$, Tukey-adjusted). The vertical order of the lower-case letters directly relates to the order of the data points. Weeks without annotations had no significant difference between treatments within week.

Table 2
Plasma osmolality (mOsmol l^{-1}, mean ± SE) of juvenile Atlantic salmon exposed to freshwater (FW) and seawater (SW) challenge conditions during the 24-h light phase of the experiment.

Treatment	Week	FW control		SW challenge	
		Mean Osmolality	SEM	Mean Osmolality	SEM
LD 08:16	10	319	2.60	334*	2.88
	12	309	3.45	327*	0.84
	14	302	0.87	323*	2.67
	16	312	2.00	313	3.91
LD 12:12	10	301	8.57	315	7.15
	12	305	0.91	336*	6.69
	14	305	5.13	323*	6.93
	16	311	2.22	313	2.28
LD 16:08	10	305	6.01	313	9.34
	12	310	1.17	342*	3.91
	14	309	4.95	340*	10.35
	16	304	3.66	327*	5.66

$N = 3$ replicate tanks in each treatment (4 fish per tank at each time-point). Asterisk denotes that SW challenge group was significantly different to corresponding FW control at that particular week.

NKAα1b mRNA level during the treatment phase (Supplementary Table 3) and were significantly higher than the LD 08:16 and LD 12:12 groups at week 4 (Fig. 8B). Fish from the LD 08:16 and LD 12:12 showed no significant change in gill NKAα1b mRNA levels during the treatment phase (Supplementary Table 3). LD 16:08 and LD 12:12 groups showed a significant increase in gill NKAα1b mRNA levels after transfer to SW. Fish from the LD 08:16 group showed an increase to similar levels seen in the other 2 groups, however this increase was not significant (Supplementary Table 3). A decrease in gill NKAα1b mRNA levels in all groups followed thereafter, however there was no significant difference between treatments. Gill NKAα1b mRNA levels were highly variable in all groups at week 18, with fish from the LD 12:12 group showing the most variation.

Fish from the LD 16:08 group were significantly higher in Log_2 ratio of NKAα1b: NKAα1a mRNA than the LD 08:16 and LD 12:12 groups at week 4 and week 8, however were significantly lower than both groups at week 16, mirroring trends seen in mRNA level, NKA activity and plasma cortisol. Fish from the LD 16:08 group were significantly lower in Log_2 ratio than the LD 08:16 and LD 12:12 groups at week 18 and 20, however there was no significant difference between treatments after 8 weeks in SW (week 24).

3.4. Immunohistochemistry

3.4.1. Gill NKAα1a ionocytes

Gill NKAα1a ionocytes were primarily located on the lamellae (green fluorophore, Fig. 9). All treatment groups exhibited a significant decrease in number of lamellar NKAα1a ionocytes during the treatment phase (Fig. 10B, Supplementary Table 4). Fish from the LD 16:08 treatment had significantly fewer NKAα1a ionocytes (per mm primary filament) on the lamellar than the LD 08:16 and LD 12:12 groups at week 8. During the LD 24:0 phase, fish from the LD 16:08 treatment showed a significantly higher amount of lamellar NKAα1a ionocytes at week 16 than the LD 08:16 and LD 12:12 groups. All treatment groups showed a decrease in number of these cells at week 18 (SW), however this decrease was only significant in fish from the LD 12:12 and LD 16:08 groups, which plateaued thereafter (Supplementary Table 4). There was a significant interaction between treatment and week on number of filamental NKAα1a ionocytes ($p < .001$) (Fig. 10A). Fish from the LD 16:08 group showed significantly fewer filamental NKAα1a ionocytes than fish from the LD 08:16 and LD 12:12 treatment at weeks 4 and 8. During the LD 24:0 phase, both LD 08:16 and LD 12:12 showed a significant decrease in filamental NKAα1a ionocyte number, and showed significantly fewer NKAα1a ionocytes at weeks 12 and 14 when compared to fish from the LD 16:08 group. There were no significant differences between treatment groups in SW, with all treatments exhibiting very low numbers of filamental NKAα1a ionocytes (Fig. 10A).

3.4.2. Gill NKAα1b ionocytes

Gill NKAα1b ionocytes were primarily located on the primary filament (red fluorophore, Fig. 9). Fish from the LD 16:08 had significantly higher amounts of filamental NKAα1b ionocytes than the LD 08:16 and LD 12:12 groups at week 4 and 8 (Fig. 10C). Fish from the LD 08:16 and LD 12:12 groups were only significantly different to each other at week 8. The number of filamental NKAα1b ionocytes was not significantly different between treatments within each week during the LD 24:0 phase, although fish from the LD 08:16 treatment exhibited a significant increase between week 8 and week 12 after exposure to LD 24:0. Fish from the LD 12:12 treatment also showed an increase after LD 24:0 exposure, however this increase was not significant. Fish from the LD 16:08 treatment remained at comparable levels to week 8. Fish from all treatments showed an increase after transfer to SW, however this was only significant in the LD 16:08 and LD 12:12 groups. All treatment groups were showing comparable filamental NKAα1b cell numbers at week 24 (Fig. 10C). Lamellar NKAα1b ionocytes were relatively low in number but showed an overall increasing trend in all treatments throughout the experiment. There was a significant main

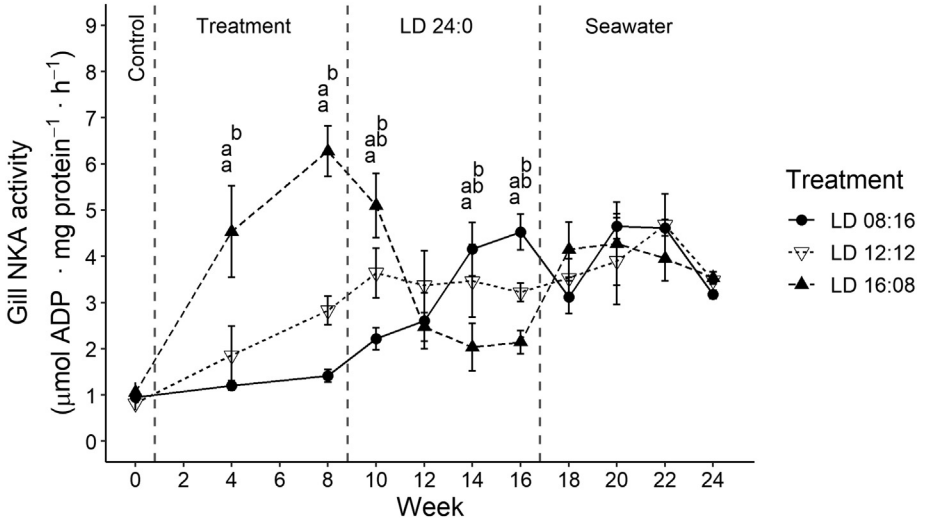

Fig. 6. Gill Na^+/K^+-ATPase (NKA) activity (µmol ADP mg $protein^{-1}$ h^{-1}, mean ± SE) of juvenile Atlantic salmon exposed to 3 photoperiod regimes for 8 weeks, followed by 24-h light (8 weeks) and transfer to seawater (SW) (8 weeks). N = 3 replicate tanks in each treatment (4 fish per tank at each time-point). Change to 24 h light (week 8) and transfer to SW (week 16) are indicated by vertical lines. Significant differences between treatments within week are represented by different lower-case letters ($P < .05$, Tukey-adjusted). The vertical order of the lower-case letters directly relates to the order of the data points. Weeks without annotations had no significant difference between treatments within week.

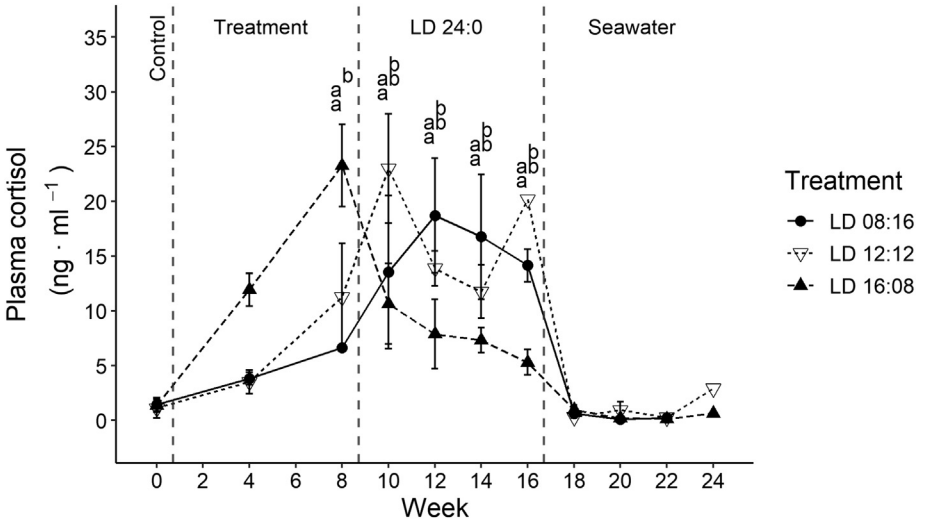

Fig. 7. Plasma cortisol (ng · ml^{-1}, mean ± SE) of juvenile Atlantic salmon exposed to 3 photoperiod regimes for 8 weeks, followed by 24-h light (8 weeks) and transfer to seawater (SW) (8 weeks). N = 3 replicate tanks in each treatment (4 fish per tank at each time-point). Change to 24 h light (week 8) and transfer to SW (week 16) are indicated by vertical lines. Significant differences between treatments within week are represented by different lower-case letters ($P < .05$, Tukey-adjusted). The vertical order of the lower-case letters directly relates to the order of the data points. Weeks without annotations had no significant difference between treatments within week.

effect of time (week) on lamellar NKAα1b ($p < .001$), but no effect of treatment or interaction between treatment and week (Fig. 10D). At week 16, fish from the LD 16:08 treatment were significantly lower in lamellar NKAα1b cell number than the LD 08:16 and LD 12:12 treatments. There were no significant differences between treatments after transfer to SW (Fig. 10D).

4. Discussion

Development of key physiological, morphological and behavioural attributes are fundamental to prepare anadromous salmonids for downstream migration and subsequent habitation of marine environments (Hoar, 1988; McCormick et al., 1987). Exposure to an increased photoperiod is thought to be the most crucial environmental factor to induce these adaptive changes in *S. salar* (Duston and Saunders, 1990; McCormick et al., 2007). Previous studies in Atlantic salmon have shown that square-wave photoperiod regimes, which usually involve a period of short days followed by an abrupt increase to a long day (often LD24:0) for several weeks, induce smolt-related physiological responses that are comparable to those provoked by a naturally increasing photoperiod (Björnsson et al., 1989; Duston and Saunders, 1995; Duston and Saunders, 1990; Handeland et al., 2013; Sigholt et al., 1995). Although smolt-inducing photoperiods have been well described, this study investigated whether different preparatory photoperiod regimes preceding a period of LD 24:0 would affect or improve morphometric, physiological and molecular responses during the induction of smoltification. Our results indicated that the short-day photoperiod protocol of LD 08:16 for 8 weeks enabled fish to respond physiologically to the LD 24:0 photoperiod, and all smoltification indicators changed as expected. The LD 12:12 photoperiod responded similarly to the LD 08:16, but not all indicators changed after exposure to the LD 24:0 photoperiod. The LD 16:08 photoperiod may better prepare Atlantic salmon for osmoregulatory adaptation in SW, as this treatment advanced the physiological and morphological indicators of smoltification within the initial 8-week period compared to LD 08:16 or LD 12:12. These 2 groups showed similar advancements in smoltification parameters only after transfer to LD 24:0. When fish from the LD 16:08 treatment were transferred to the LD 24:0 photoperiod, some of the physiological indicators returned to values observed at week 0, possibly indicating signs of desmoltification. This suggests that fish in this group could have been transferred directly to SW after the 8 weeks on LD 16:08, and that there was no advantage to the subsequent exposure to a LD 24:0 photoperiod.

Total mass and fork length were not significantly different among the groups during the exposure to the short-day photoperiods. The LD 16:08 group were increasing at approximately 0.45 cm · day^{-1} during this period. This was significantly greater than the LD 08:16 group, however this growth rate was not sustained after exposure to LD 24:0. During weeks 10–14, fish from the LD 16:08 group were significantly higher in mass and fork length compared to fish from the LD 08:16 group. Fish from the LD 12:12 only showed significant differences in mass and fork length at week 12. All groups grew in a similar manner after week 14, with no significant difference seen between treatment groups in SW. Any growth advantage that the LD 16:08 had immediately after transfer to the LD 24:0 photoperiod appeared to be compensated for by the other photoperiod groups.

In Atlantic salmon, growth hormone (GH) levels increase following an abrupt change in day length (Björnsson et al., 1989; McCormick, 1995). Although GH levels were not investigated in this study, it is possible that in the LD 16:08 group, GH increased during the short-period exposure only, possibly causing the intial advantage in growth they presented after transfer to the LD 24:0 photoperiod. A decrease in GH during the LD 16:08, and increase in the LD 08:16 and LD 12:12 groups during the LD 24:0 photoperiod would explain the catch up in growth observed at week 16 and thereafter. As GH stimulates hypo osmoregulatory changes, an increase in GH would also explain the osmoregulatory change that occurred in the LD 16:08 group during the short-day photoperiod, and in the LD 08:16 and LD 12:12 after transfer to the LD 24:0 photoperiod. Atlantic salmon exposed to different short day photoperiods significatly increase plasma GH when transferred to the LD 24:0 photoperiod (Björnsson et al., 1989).

In the present study, condition factor was significantly reduced in fish from the LD 16:08 group during the treatment phase. The other two groups only showed a decrease in condition factor after exposure to LD 24:0, with LD 08:16 clearly showing a sharper decrease than LD 12:12 and LD 16:08 groups. Reduction in condition factor is a common characteristic observed during smoltification (Hoar, 1988), characterized by a relative loss in mass, which is associated with metabolic and body composition changes (McCormick and Saunders, 1987), and with lengthening of the caudal peduncle (Winans and Nishioka, 1987). The change in condition factor coupled with the increase in SGR-L in the LD 16:08 group suggests the advancement of smoltification in this group.

Plasma osmolality ranged from ~300–320 mOsmol L^{-1} in all photoperiod treatment groups during the FW phase, and was significantly affected by 24-h SW challenges in all photoperiod treatments. After transfer to SW, plasma osmolality in all treatments increased to values ranging from ~320–340 mOsmol l^{-1}. In the group exposed to LD 16:08, levels plateaued at ~340 mOsmol l^{-1} between weeks 20–24. In the LD 08:16 and LD 12:12 groups, similar levels were observed at weeks 22–24. Levels seen in the present study are in accordance with previous studies in Atlantic salmon (Clarke et al., 1996; McCormick

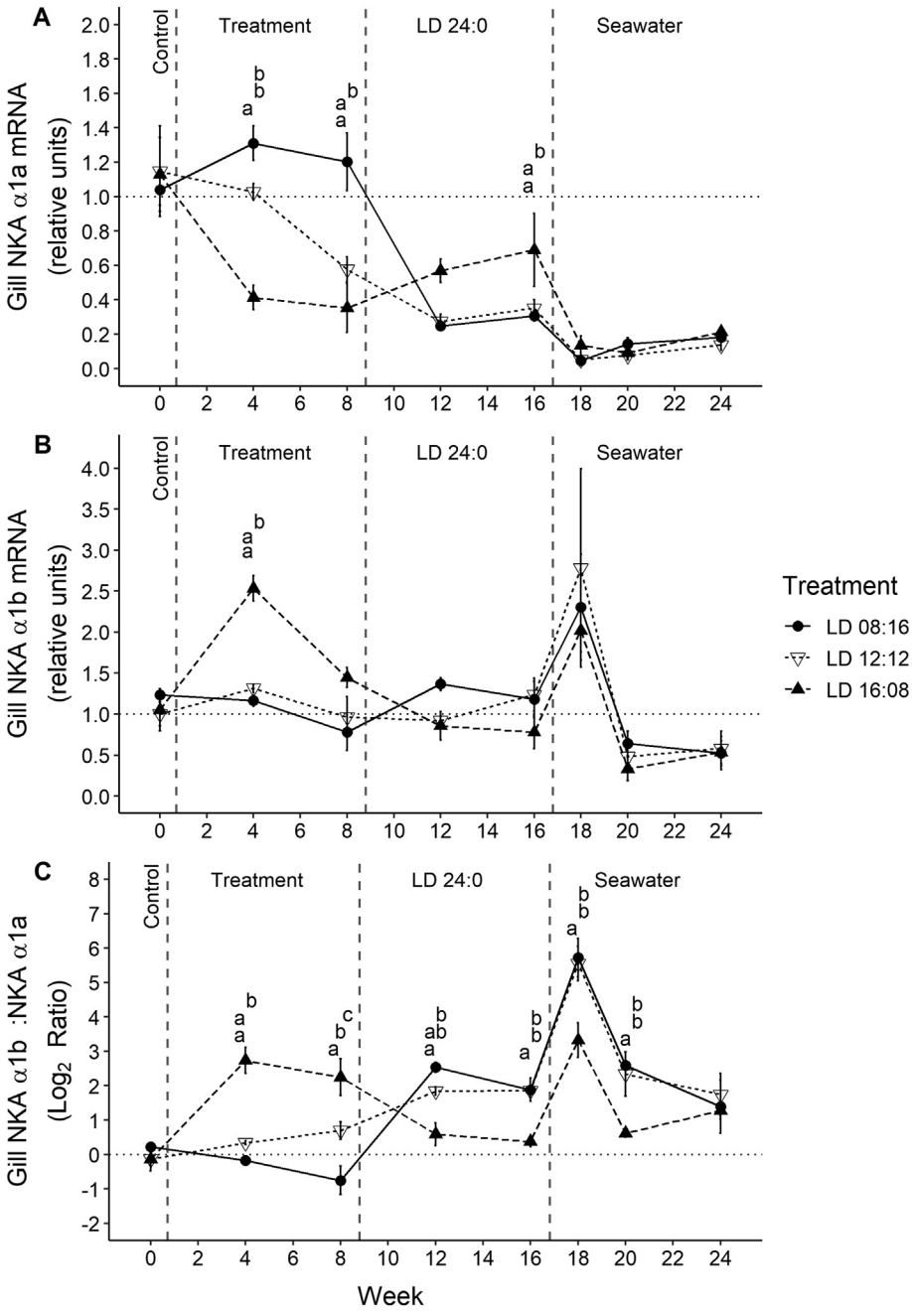

Fig. 8. Gill mRNA levels of (A) NKAα1a (relative units, mean ± SE); (B) NKAα1b (relative units, mean ± SE); and (C) Log_2 ratio of NKAα1b: NKAα1a (mean ± SE) in juvenile Atlantic salmon exposed to 3 photoperiod regimes for 8 weeks, followed by 24-h light (8 weeks) and transfer to seawater (SW) (8 weeks). N = 3 replicate tanks in each treatment (4 fish per tank at each time-point). Change to 24 h light (week 8) and transfer to SW (week 16) are indicated by vertical lines. Significant differences between treatments within week are represented by different lower-case letters ($P < .05$, Tukey-adjusted). The vertical order of the lower-case letters directly relates to the order of the data points. Weeks without annotations had no significant difference between treatments within week.

et al., 2009a; Richards et al., 2003; Zydlewski and Zydlewski, 2012). Although these levels vary somewhat among the literature, ideal plasma osmolality in SW is thought to be between 330 and 340 mOsmol l^{-1} (Clarke et al., 1996). It's common to see a decrease in plasma osmolality in fish that have been exposed to a SW challenge during the smoltification process. As smolts develop mechanisms needed for salt secretion, their ability to osmoregulate in FW can be compromised (McCormick et al., 2009a). The LD 08:16 and LD 12:12 groups maintained significantly higher osmolality after a 24-h SW challenge, during the LD 24:0 phase until week 16, where SW challenge groups showed no significant difference in osmolality relative to FW groups. This indicates that salinity tolerance slowly increased in the LD 08:16 and LD 12:12 groups after exposure to LD 24:0. The LD 16:08 appeared to lose salinity tolerance during the LD 24:0 phase, as SW challenged fish showed similar plasma osmolality levels to the FW group at week 10 but became significantly elevated thereafter during the LD 24:0 phase.

A rapid increase in gill NKA activity was evident in fish exposed to LD 16:08, which subsequently decreased significantly after exposure to LD 24:0 between weeks 12–16. The other two groups only showed significant changes in gill NKA activity after transfer to the LD 24:0 photoperiod regime. Gill NKA activity in fish from the LD 12:12 group appeared to be intermediary between the other 2 groups during the photoperiod treatment phase, but the increase after exposure to LD 24:0 was not as pronounced in fish from the LD 12:12 group compared to the LD 08:16 group. Gill NKA activity in the LD 08:16 and LD 12:12 groups after transfer to SW was similar to the levels observed during the LD 24:0 period. In the LD 16:08 group, gill NKA activity levels rebounded after transfer to SW, and values were very similar among the groups. Studies in Atlantic salmon have shown further increase in NKA activity after transfer to SW, suggesting that this increase is important for long-term adaptation in SW (Handeland et al., 2003; Imsland et al., 2011; McCormick et al., 1989; Stefansson et al., 2012; Stefansson et al., 2003). In this study, only a small rebound in gill NKA activity was seen in the LD 16:08 group. It is possible that an increase in NKA activity

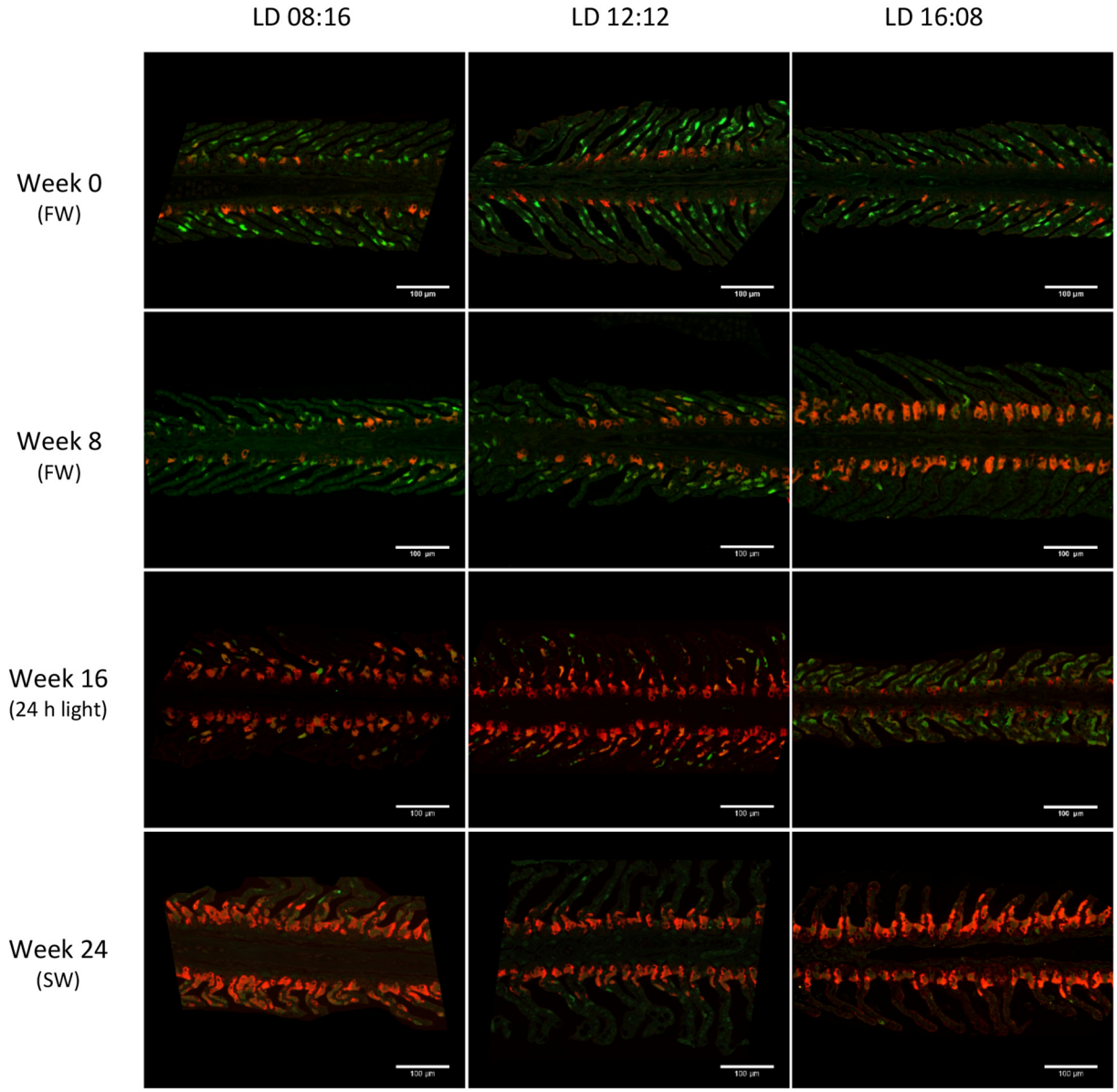

Fig. 9. NKAα1a (green) and NKAα1b (red) immunoreactive ionocytes in gill tissue of Atlantic salmon. Representative samples for Week 0 & 8 (freshwater - FW), week 16 (24 h light) & week 24 (seawater – SW) for each photoperiod treatment are shown. Scale bar represents 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

happened within the first two weeks after transfer, which was not captured due to sampling frequency. Nonetheless, based on the growth and survival data, all groups seemed to be adequately adapted to SW regardless of their initial preparatory photoperiod regime.

Similarly to gill NKA activity, exposure to LD 16:08 for 8 weeks induced an increase in plasma cortisol levels, which decreased significantly after exposure to LD 24:0 between weeks 10–16. The other two groups showed increased in plasma cortisol levels only after exposure to LD 24:0, but the profile of increase was different between the LD 08:16 and LD 12:12. Peak gill NKA activity in FW in the LD 08:16 (during the LD 24:0 phase) and LD 16:08 (during exposure to LD 16:08) groups coincided with increased levels of plasma cortisol, corroborating with previous studies (Madsen, 1990; McCormick et al., 2002; Shrimpton and McCormick, 1998). Cortisol typically increases 10-fold as part of the smoltification process (McCormick et al., 2007), and is known to be involved in increased salinity tolerance, NKAα transcription, and salinity-specific ionocyte differentiation (Kiilerich et al., 2007; McCormick, 2001; Pelis and McCormick, 2001). Cortisol dropped to very low levels after transfer to SW in all treatment groups. The first cortisol response was measured 15 days after SW transfer, and it is possible that increased levels occurred within the first 2 weeks. A previous study comparing anadromous and landlocked Atlantic salmon showed that plasma cortisol levels increased 4 days after transfer to SW in the anadromous population (Nilsen et al., 2008). Elevated levels of cortisol within the first 3 days after transfer from FW to SW have also been documented in rainbow trout (Taylor et al., 2007) and coho salmon (Redding et al., 1984). It is possible that cortisol may have a short-term role in acclimation to SW and may not be necessary for long-term adaptation or increase in gill NKA activity.

Measuring the transcription of NKAα1a, NKAα1b, and the ratio of NKAα1b: NKAα1a mRNA transcripts provided further insight about their roles during exposure to different preparatory photoperiods. During exposure to the treatment photoperiods, there was a significant drop in the transcription of NKAα1a in fish from both the LD 12:12 and LD 16:08 groups. This was accompanied by a concurrent increase in NKA activity and plasma cortisol levels in fish from the LD 16:08 group.

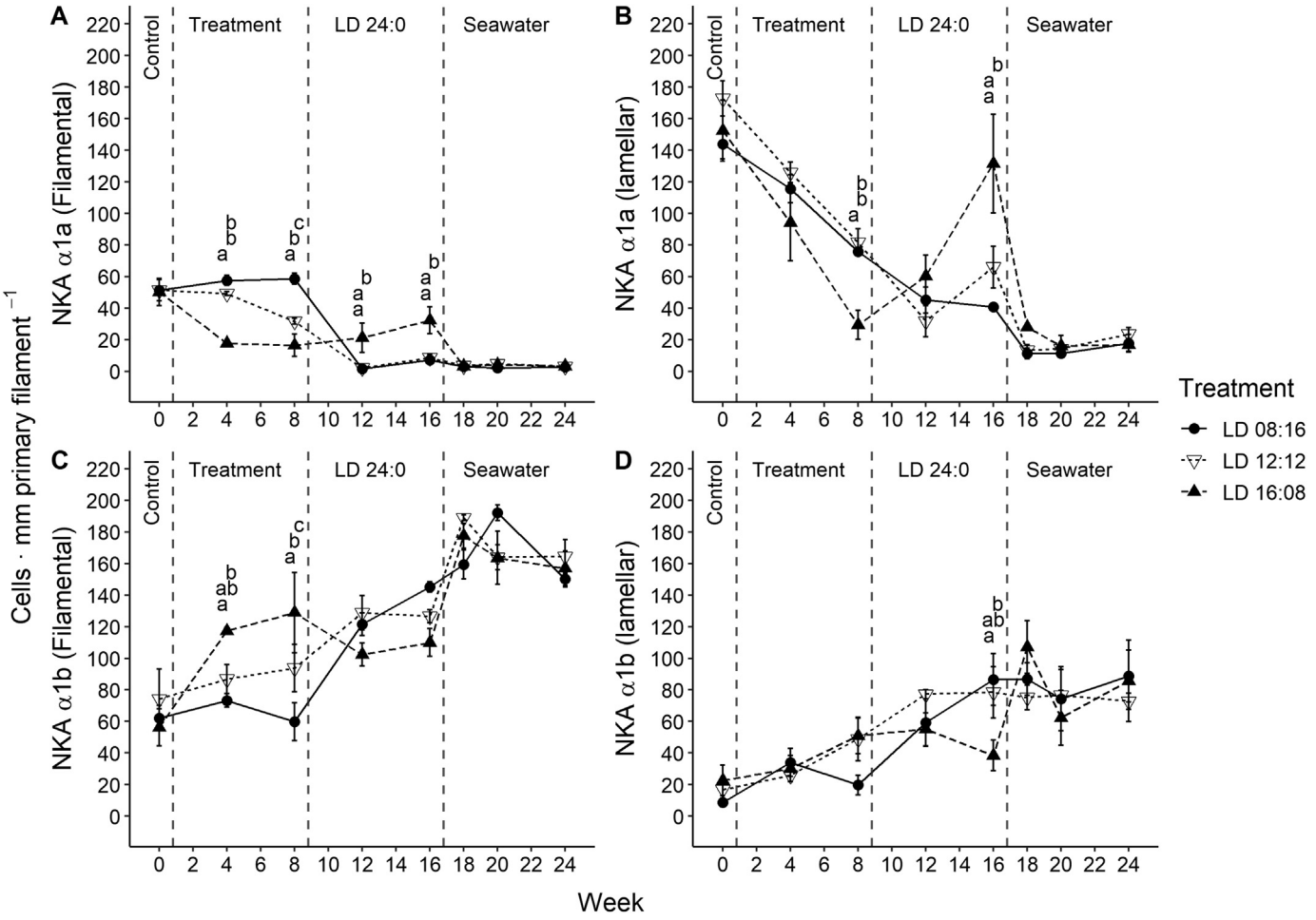

Fig. 10. Total counts (cell number per mm primary filament, mean ± SE) of gill NKAα1a ionocytes (green) localised on the filament (A), gill NKAα1a ionocytes (green) localised on the lamellae (B), gill NKAα1b ionocytes (red) localised on the filament (C), gill NKAα1b ionocytes (green) localised on the lamellae (D). Two fish per replicate tank in each treatment were processed (6 fish per treatment at each time-point). Four sections from each sample were mounted on slides, with 4 images of each fish taken. Change to 24 h light (week 8) and transfer to seawater (SW) (week 16) are indicated by vertical lines. Significant differences between treatments within week are represented by different lower-case letters ($P < .05$, Tukey-adjusted). The vertical order of the lower-case letters directly relates to the order of the data points. Weeks without annotations had no significant difference between treatments within week. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The LD 12:12 group also showed an increasing trend in NKA and cortisol, however these increases were not significant. After transfer to LD 24:0, NKAα1a transcription increased in the LD 16:08 and decreased in the LD 08:16 and LD 12:12 groups. The profile of these mRNA transcript levels and the coinciding levels of gill NKA activity and plasma cortisol further supports the premise that LD 12:12 photoperiod elicited an intermediary response in important smolt-related attributes when compared to the LD 08:16 and LD 16:08 groups. After transfer to SW, NKAα1a mRNA transcription levels were low in all groups. There was a transient increase in NKAα1b mRNA transcription in fish from the LD 16:08 group during exposure to the preparatory photoperiod, which decreased during the LD 24:0 period. This peak in NKAα1b transcription at week 4, preceding peaks in gill NKA and plasma cortisol by approximately one month is consistent with previous studies (Christensen et al., 2018; Nilsen et al., 2007). All groups showed increased NKAα1b transcription levels 2 weeks after transfer to SW. This suggests salinity has an important role in the regulation of these genes and likely the differentiation of the ionocyte isoforms (Madsen et al., 2009; Madsen et al., 1995; Pelis and McCormick, 2001).

The ratio between NKAα1b and NKAα1a mRNA transcripts during the exposure to the preparatory photoperiods showed a photoperiod dependent response, with the highest ratio seen in the LD 16:08 group followed by LD 12:12. After transfer to LD 24:0 this was inverted, as the ratio increased in the LD 08:16 and LD 12:12. NKAα1b:NKAα1a mRNA ratio significantly increased in all treatments after transfer to SW, however the peak at week 18 was significantly lower in the LD 16:08 group than the other two treatments. This suggests that fish from the LD16:08 group may retain some smolt-related characteristics even though they appeared to go through a partial desmoltification after exposure to LD 24:0. Overall, transcription changes in fish from the LD 12:12 group during the FW phase did not correspond with the changes in plasma cortisol and NKA activity. It is possible that these physiological differences may affect the osmoregulatory capacity of the fish (Morro et al., 2019). This response could be linked to the fact that during the acclimation phase (prior to the beginning of the treatment period), all groups were on a LD 12:12 for 6 weeks. Thus, this group was exposed to LD 12:12 for 14 weeks prior to transfer to LD 24:0. Nonetheless, the ratio of NKAα1b:NKAα1a seemed to have represented more adequately what happened with the LD 12:12 group during the FW and SW phase. The ratio of NKAα1b:NKAα1a highly correlates with salinity tolerance (Christensen et al., 2018), and levels reported in the present study are in line with what has been previously described (Christensen et al., 2018; Madsen et al., 2009).

To elucidate whether the changes in protein production, hormone production and mRNA level were having cellular effects, we examined immuno-reactive ionocytes with specific NKAα antibodies (Fig. 9). Our

results support the model proposed by McCormick et al. (2013): that during the FW development of smolt characteristics, inactive NKAα1b-positive ionocytes proliferate in the gill epithelium, and that these are then activated upon transfer to SW. We could clearly see in the LD 16:08 group that the number of NKAα1a-positive ionocytes (lamellar and filamental) and NKA NKAα1b-positive ionocytes had significantly changed by week 8 (lower NKAα1a and higher NKAα1b), which corresponded with peaks NKAα1b-mRNA levels, NKA activity and plasma cortisol levels. In addition, the number of NKAα1b-positive ionocytes increased after transfer to SW. These results are in accordance with previous work, and indicates these two isoforms are photoperiod-sensitive and salinity-specific (Bystriansky et al., 2006; Nilsen et al., 2007; Richards et al., 2003). Although signs of a partial desmoltification in this group occurred (decreased NKA activity, decreased NKAα1b-positive ionocytes, increase in NKAα1a-positive ionocytes, etc.) after the introduction of 24-h light, the abrupt transfer to SW resulted in the activation of NKAα1b-positive ionocytes that were presumably inactive, and fish were able to adapt to the SW environment.

Overall, this study demonstrated that the photoperiod regimes affected smoltification differently not only during the treatment phase (weeks 0 to 8), but also during the LD 24:0 photoperiod phase (weeks 10 to 16). The LD 16:08 group appeared to be the most prepared for SW transfer up until week 8–10. Once transferred to LD 24:0, fish in the LD 16:08 group showed signs of desmoltification, including decreased branchial NKA activity, plasma cortisol, increase in NKAα1a mRNA level and decreased in NKAα1b:NKAα1a ratio, and increase in the total counts of gill NKAα1a ionocytes. These are all physiological indicators that fish are changing from a migratory profile to a re-adaptation to remain in FW (Morro et al., 2019). Fish were transferred to SW at a time when they were presenting signs of desmoltification (8 weeks after transfer to LD 24:0.). Based on their growth, survival, and comparison with the other groups, fish from the LD 16:08 group seemed to have adapted well in SW, despite showing signs of desmoltification. Arnesen et al. (2003) also demonstrated that juvenile Atlantic salmon showing signs of desmoltification in FW still retain a high capacity to adapt to SW. Another previous study indicated large juvenile Atlantic salmon retained in FW outside of the smolt window are able to adapt to SW in the absence of preparatory photoperiod manipulation (Brown et al., 2018). In terms of practical application, the results of the LD 16:08 group suggest that these fish could have been transferred to SW after week 8, potentially resulting in a significant performance advantage when compared to the other groups. It seems that this photoperiod provided the adequate *zeitgeber* for Atlantic salmon smoltification (Björnsson et al., 1989), while the LD 12:12 only provided some development after transfer to 24 h-light. The LD 08:16 treatment repeated the physiological profiles seen in the LD 16:08, but only after transfer to continuing 24 h-light for 8 weeks.

The profile of physiological indicators of smoltification following exposure to varying preparatory photoperiods suggest that an 8 week period of LD 16:08 prepares the fish for SW transfer without compromising growth performance, and advanced the onset of smoltification when compared to the use of LD 12:12 and LD 8:16 followed by LD 24:0. Higher peak levels of NKA activity and NKA mRNA transcript levels in FW suggest that LD 16:08 may be a more suitable photoperiod than LD 24:0 for the initiation of smoltification, and showed that there was no added benefit of a subsequent LD 24:0 photoperiod. In fact, these fish began to present signs of desmoltification. Furthermore, the partially desmolted fish still showed high hypo-osmoregulatory ability after transfer to SW. In terms of growth, all treatments had similar growth rates by the end of the study, even though the LD 16:08 group had an advantage after transfer to the LD 24:0 photoperiod. This suggests that fish in LD 16:08 may have performed better in SW if they had been transferred directly to SW without exposure to LD 24:0. Overall, all photoperiod treatments resulted in fish that were able to adapt to SW. Further studies should be designed to test whether the use of LD 16:08 as a smoltification stimulus would show growth or health advantages compared to fish transferred after exposure to LD 24:0.

Declaration of Competing Interest

None.

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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.2020.735744.

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