# Photoperiod revisited: is there a critical day length for triggering a complete parr–smolt transformation in Atlantic salmon *Salmo salar*?

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The present study investigated whether there is a critical length of photoperiod needed to stimulate a completed parr–smolt transformation (PST) in Atlantic salmon *Salmo salar*. In two experiments, *S. salar* parr of the Norwegian aquaculture strain held on continuous light were exposed to a short photoperiod (6L:18D) followed by exposure to 8L:16D, 12L:12D, 16L:8D, 20L:4D and 24L:0D in experiment 1 or to 6L:18D followed by maintenance 6L:18D or exposure to 12L:12D and 24L:0D photoperiods in experiment 2. All groups, irrespective of photoperiod

treatment, developed improved hypo-osmoregulatory ability. However, the development was greatest in the groups exposed to 20L:4D and 24L:0D in experiment 1 and 24L:0D in experiment 2. In experiment 2, gill $Na^+$– $K^+$-ATPase activity increased in the group exposed to 24L:0D, but not in the groups exposed to 12L:12D and 6L:18D. The groups exposed to 20L:4D and 24L:0D in experiment 1 and 24L:0D in experiment 2 also grew better than fish exposed to shorter photoperiods. In experiment 2 only the group exposed to 24L:0D showed a decrease in condition factor and increases in plasma growth hormone and brain type 2 deiodinase mRNA abundance. Hence, only the groups exposed to photoperiods above 16L:8D developed classical smolt indices in the present experiment, leading us to conclude that the photoperiod increase needs to exceed 16 h daylight for stimulating a complete PST in the *S. salar* used in the present study.

1 | INTRODUCTION

A multitude of developmental changes constitute parr–smolt transformation (PST) in Atlantic salmon *Salmo salar* L. 1758 , including increased salinity tolerance, silvering, increased growth in length and a slimmer body shape, metabolic preparations and migratory behaviour (McCormick, 2013). These are regulated by systemic and paracrine hormone actions and, in natural systems, the timing of PST is commonly accepted to be controlled by increasing photoperiod in spring. In captivity, PST is achieved by mimicking the natural photoperiodic cue, either by exposing pre-smolts to a simulated, natural increase in photoperiod or by exposure to a summer–winter–summer photoperiod sequence, effectively compressing the duration of the

winter phase (Duston & Saunders, 1990; Thrush *et al*., 1999). Previous studies have shown that a minimum period of exposure to a short (winter) photoperiod (*i.e.* 6 weeks), followed by an increase in photoperiod, is necessary for the pre-smolt Atlantic salmon to achieve a completed PST (Handeland & Stefansson, 2001; Berge *et al*., 1995; Ebbesson *et al*., 2007). Whereas little or nothing is known about the mechanisms initiated by the short photoperiod, the responses to the increase in photoperiod are well described. This phase (hereafter termed final smolting) is characterized by a decrease in condition factor and an increase in salinity tolerance, growth rate, gill $Na^+$–$K^+$-ATPase (NKA) activity and plasma growth hormone (GH) concentration (McCormick, 2013). Recently, it was shown that the increase in photoperiod stimulates expression of type 2 thyroid hormone deiodinase (*dio2b*) in smolting salmon (Lorgen *et al*., 2015), suggesting that increased brain levels of tri-iodothyronine (T3) contribute to final smolting.

PST includes development of migratory behaviour and, in natural systems, completion of PST is considered to coincide with the time of seawater (SW) entry (McCormick, 2013). PST completion and SW entry must be timed to take place during spring–summer when conditions in the sea are favourable (the "ecological smolt window"; McCormick *et al*., 1998). In *S. salar* populations living at high latitudes in Norway, SW entry occurs in late June–mid July (Orell *et al*., 2007) (*i.e.* after 6 months of increasing photoperiod following the winter solstice). Based on the 350 degree-days (cumulative, average daily temperature; $D^o C$) needed for completing the final smolting in Norwegian aquaculture strains of *S. salar* (Handeland *et al*., 2004), it is unlikely that the final smolting starts at winter solstice in pre-smolts at high latitudes. Rather, triggering must occur sometime in late winter. This led us to wonder whether final smolting is triggered when the increase in photoperiod exceeds a threshold, or critical photoperiod and if so, what the value of a critical photoperiod might be.

To this address these questions, we performed two PST experiments in groups of *S. salar* pre-smolts that were transferred from a winter photoperiod of 6 or 8 h photoperiod to increased photoperiods up to 24 h. We assessed smoltification through a range of parameters reflecting osmoregulatory ability (SW tolerance and gill NKA activity), morphology (condition factor based on length and mass) and endocrine status (plasma growth hormone (GH) concentration and brain expression of thyroid hormone deiodinase *dio2b)*.

## 2 | MATERIALS AND METHODS

### 2.1 | Fish material and experimental set-up

The experiments were carried out at the Aquaculture Research Station in Tromsø, northern Norway (69° N) using *S. salar*, of the AquaGen strain (AquaGen; www.aquagen.no) derived from eggs hatched in January 2015. After start-feeding in May, the fish used in experiment 1 were held at continuous light (24L:0D; L = light and D = darkness) and a water temperature of 10° C until August 11, when the photoperiod was altered to 6L:18D in order to give the fish a winter signal. After 50 days of the winter regime, the fish were transferred to 5 different light regimes (Figure 1), at which they were held for 15 weeks. For each light regime, there were duplicate tanks of fish. Before transfer, all fish were individually tagged with Floy FFT-69 fingerling tags (www.floytag.com). The tags had one colour for each light regime and for monitoring individual growth rate another 10 fish in each tank was marked with white Floy tags. After start-feeding, the fish to be used in experiment 2 were held at 8° C and 24L:0D until August. From August to January, water temperature was gradually decreased from 8 to 4° C. In January the photoperiod and temperature were altered to 6L:18D and 7°C, respectively. After 50

days (on March 15), the fish were transferred to three different, duplicated light regimes and a water temperature of $10^{\circ}$ C (Figure 1) for 9 weeks. Before transfer, 50% of the fish had been individually tagged with Floy tags of different colours, one colour for each light regime and distributed to replicate tanks. Throughout both experiments, the fish were fed commercial feed (Skretting; www.skretting.com) according to manufacturer's recommendations. The duration of feeding was the same for all treatment groups; 8 hours during the light period of the short-day group in experiment 1 and 6 h during the light period of the short-day group in experiment 2 (Figure 1).

The experiments were performed in accordance with the ethical guidelines included in the block permission for smolt experiments obtained by the Aquaculture Research Station from the Norwegian Food Safety Authority.

## 2.2 | Sampling from freshwater

At all sampling dates during the photoperiodic treatment of experiment 1 (30 September, 26 October, 16 November, 16 December and 16 January, *i.e.* at day 1, 26, 47, 78 and 109 after the end of the winter period), the fish tagged with white Floy tags in all treatment groups were anesthetized in benzocaine (60 mg $l^{-1}$) and body mass ($M$, 0.1 g) and fork length ($L_F$, 0.1 cm) measured for calculating development of condition factor and specific growth rate. In experiment 2, 10 (2 x 5) fish without Floy tags from each treatment group were sampled on 16 March, 30 March, 13 April, 3 May and 18 May (*i.e.* on day 1, 15, 29, 49, 64 after the end of the winter period) and killed in a lethal dose of benzocaine (160 mg $l^{-1}$). $M$ and $L_F$ were measured and a small biopsy of gill tissue was sampled from the second gill arch on the left side of the fish by fine-tip forceps. The biopsy was placed in a plastic tube containing 100 µl SEI solution (0.3 M

sucrose, 0.02 M $Na_2$-EDTA and 0.1 M imidazole) and subsequently frozen at $-80°$ C until analysis of NKA activity. Blood samples were drawn from the caudal vessels with 1 ml Li-heparinized vacutainers, centrifuged at 2780 $g$ and $1°$ C for 8 min. Plasma was then removed and stored at $-18°$ C until analysed. Finally, the whole brain (pituitary removed) was dissected out and stored in 1 ml RNAlater (Thermo Fisher Scientific; www.thermofisher.com) at $4^o$ C for 24 h and then at $-20^o$C until analysed for *dio2b* messenger (m)RNA abundance.

## 2.3 | Seawater-challenge

A standardized SW challenge test (Blackburn & Clarke, 1987) was initiated on each of the sampling dates in fresh water in both experiments. At the start of each test, 16 (2 x 8) fish (experiment 1) and 10 (2 x 5) fish (experiment 2) from each light regime were randomly netted (only fish tagged with coloured Floy tags) from each tank and transferred directly to a common test tank supplied with SW ($7°$ C, salinity33). After 24 h, the fish were killed with a lethal dose of benzocaine. Blood samples were drawn from the caudal vessels with 1 ml Li-heparinized vacutainers, centrifuged at 2780 $g$ and $1°$ C for 8 min. Plasma was then removed and stored at $-18°$ C until analysed.

## 2.4 | Analyses

The hypo-osmoregulatory ability of the sampled fish was assessed by measuring plasma chloride concentration (Corning 925 chloride titrator, CIBA Corning Diagnostics, Essex, England) and plasma osmolality (FiskeOne-Ten Osmometer, Fiske Associates, MA, USA). Gill NKA activity

was measured as μmol ADP mg protein$^{-1}$ hour$^{-1}$, using the enzyme assay described by

McCormick (1993).  To determine GH concentrations in the plasma samples we used a *S. salar*

somatotropin ELISA Kit (Catalog no MBS288370_DATA; MyBioSource;

www.mybioscience.com). Briefly, plasma samples were diluted 1:5 to bring GH levels within the

standard-curve range. Further analyses were done in accordance with the manufacturer's

instructions. Concentrations determined in the diluted samples were corrected for the diluted

factor.

For analysing brain *dio2b* mRNA abundance, brains (around 30 mg) were first

homogenized using a Qiagen TissueLyser II (Qiagen; www.qiagen.com). Total RNA was then

extracted using the RNeasy Plus Universal Mini Kit (Qiagen). This kit included an initial step of

genomic (g)DNA removal and a reverse-transcription (RT) test on a selection of samples to

ensure that the removal was effective. Total RNA was subjected to additional DNase treatment

(Turbo DNase; Ambion Inc.; www.thermofisher.com) and reverse transcription of total RNA

(0.5μg) was performed using iScriptTM advanced cDNA synthesis kit for quantitative (q)RT-

PCR (Bio-Rad Laboratories; www.biorad.com) per 20 μl cDNA reaction according to the

manufacturer's instructions. cDNA was then diluted ten-fold. The mRNA abundance of *dio2b*

(forward, GGATGTGAGGCAGTATCTGGAACAG; reverse, GCCTGTCATTTGTGGTCAGA;

Lorgen *et al*., 2015) and EF-1 (forward, GAGAACCATTGAGAAGTTCGAGAAG; reverse,

CACCCAGGCATACTTGAAAG; Murashita *et al*., 2009) were analysed by performing RT-

PCR. The amplification steps were as follows: 50°C for 10 min, 95°C for 5 min, (95°C for 10 s,

60°C for 30 s) × 40, 95°C for 10 s followed by melt-curve analysis. Standard curves were

generated with two-fold dilutions to check the efficiency of the primers. All qPCR analyses were

run with CFX96 real-time (rt)-PCR detection system (Bio-Rad) and the software CFXManager

3.0 (Bio-Rad).  Relative-fold change of gene expression was calculated using the ΔΔCt method

(Livak & Schmittgen, 2011) with Elongation factor 1$\alpha$ (*ef1$\alpha$*), a stable reference gene in *S. salar* (Olsvik *et al*., 2001) was used to normalize the Ct values of the target gene.

## 2.5 | Data treatment and statistics

Condition factor ($K$) was calculated by the formula ($ML_F^{-3}$)100, where $M$ is body mass in g and $L_F$ is fork length in cm. Specific growth rate ($R_{SG}$) was calculated by the formula [(ln$M_F$ − In$M_t$)100]($t_1 − t_0$)$^{-1}$, where $M_F$ and $M_t$ are body mass at the start and the end of the sampling period, respectively and $t_1 − t_0$ is the number of days between measurements. With very few exceptions, the data were normally distributed (Lilliefors test, Systat 13.1; www.systatsoftware.com) and reported values are arithmetic means and S.E. For each light-regime dataset on $M$, $K$, plasma osmolality, plasma chloride, gill NKA activity, plasma GH and normalized brain Dio2b mRNA abundance were analysed using a nested general linear model analysis of variance to investigate the effects of time, light regime and tank (replicate). A Tukey honest significant difference (HSD) *post hoc* test was used to identify when significant differences occurred. No significant effect of tank (replicate) was found and duplicates were therefore pooled in the results presented here. Possible differences in specific growth rates were analysed using a one-way ANOVA. A probability level of $P \leq 0.05$ was considered significant. All statistical computations were performed with Systat 13.1.

## 3 | Results

### 3.1 | Hypoosmoregulation and gill NKA activity

All groups displayed plasma chloride concentrations above 165 mmol l$^{-1}$ after the SWT at the two first sampling dates, except for fish that had been held at 24L:0D in experiment 1. In this group plasma chloride concentration had decreased to an average of 160 mmol l$^{-1}$ at the second sampling date, when the concentration was significantly lower than in the other groups (Fig 2(a)). Later in the sampling period, all groups showed significant decreases in plasma chloride concentrations after SWTs. The fish held at 24L:0D and 20L:4D in experiment 1 reached levels lower than 150 mmol l$^{-1}$ faster than those held at shorter photoperiods. Also in experiment 2 all groups showed significant decreases in plasma chloride concentrations after SWTs test, but in April and May the fish from the 24L:0D group had significantly lower plasma chloride levels than the those held on shorter photoperiods and at the last sampling in mid-May plasma chloride level in the 24L:0D group was still significantly lower than that in the 6L:18D group.

Plasma osmolality changed in a similar pattern as the plasma chloride concentrations. All groups displayed high concentrations at the end of the winter treatment (> 395 mOsm kg$^{-1}$) in October (experiment 1) and in March (experiment 2). Following transfer to the treatment photoperiods, plasma osmolality decreased significantly in all groups within the first 6 weeks, but to significantly lower levels in the group held at 24L:0D than in the groups held at 12L:12D and 8L:16D in experiment 1 (Figure 3(a)). In experiment 2, plasma osmolality decreased significantly in all groups until the end of the experiment but at a significantly faster rate and to a lower level in the 24L:0D group than in the other two groups (Figure 3(b)).

Only fish in the 24L:0D group had a significant increase in gill NKA activity during the course of the study and in May the level in the 24L:0D groups was significantly higher than in the 6L:18D and 12L:12D groups (Figure 4).

### 3.2 | Growth, specific growth rate and condition factor

In both experiment, there was a significant and positive, effect of photoperiod on growth (Figure 5(a),(b)). More specifically, this effect was manifest as a significantly higher growth rates of fish held at 20L:4D and 24:0D in experiment 1, compared with fish held at other photoperiods (Figure 6(a)). Specific growth rate data in experiment 2 was obtained without individually tagged fish, rendering statistical analyses impossible, but the result confirmed that in experiment 1; a markedly higher $R_{SG}$ in the 24L:0D group than in the other groups (Figure 6(b)).

In experiment 1 there was a significant decrease in condition factor only for the fish in the 20L:4D and 24L:0D groups during the course of the experiment. Condition factor was significantly lower in these groups than in the other groups on the sampling in December (Figure 7(a)). In experiment 2, $K$ decreased significantly in the 24L:0D group, but not in the two other groups and was significantly lower in the 24L:0D group than in the 6L:16D and 12L:12D groups throughout (Figure 7(b)).

Plasma GH concentration and brain *dio2b* mRNA abundance in experiment 2. The abundances of brain *dio2b* mRNA were not measured at the first sampling date (15 March), but for the rest of the sampling dates there was a significantly higher abundance in the 24L:0D group than in the other groups throughout the experiment (Figure 8). Only fish in the 24L:0D group had a significant increase in plasma GH concentration, which in May were significantly higher in the 24L:0D group than in the fish held at 6L:18D and 12L:12D (Figure 9)

## 4 | DISCUSSION

It is generally accepted that *S. salar* smolting is stimulated by the increase in photoperiod in spring. However, photoperiod increases continuously from the winter solstice to the summer

solstice and more specific information about when during this period smolting is triggered is lacking. A previous study in *S. salar* reported that progressively increasing photoperiod, culminating in exposure to constant light only induces expression of smolt characteristics in pre-smolts that had previously been exposed to a photoperiod of 13 h or less (Berge *et al*., 1995). This suggests that a period of exposure to photoperiods short enough to be perceived as a winter regime is necessary for longer photoperiods to trigger smolting. Correspondingly, the present study showed that the amplitude of photoperiodic change clearly affected smolt development and that, for a winter (short-day) acclimated, high-latitude *S. salar* parr, an increase in photoperiod to above 16 h is necessary for triggering PST.

In the present study salinity tolerance increased in all treatment groups, irrespective of photoperiodic treatment (Figures 2 and 3). This was also the case in the group maintained at 6 h photoperiod in experiment 2. Increases in salinity tolerance, independent of a photoperiodic cue, have been seen in many previous studies (Duston & Saunders, 1990; Sigholt *et al.*, 1995; Handeland *et al*., 2013). Very little is known about the triggering mechanism, but there is evidence for the presence of a size-dependent smolting window during which the pre-smolt may develop smolt characters without apparent external cues (Handeland *et al*., 2013; Imsland *et al*., 2014). The temporal synchrony in the increase in salinity tolerance between groups in the present study indicates that there may have been a triggering cue. This may have been the 2 h increase in photoperiod in the short-day group in experiment 1 and the increase in water temperature (Figure 1) preceding the increase in salinity tolerance in the fish in experiment 2 that did not experience an increase in photoperiod (Figure 1). Although temperature *per se* is not considered to be a zeitgeber of smolting, temperature has been shown before to trigger an increase in salinity tolerance in winter-acclimated *S. salar* parr ready to smoltify (McCormick *et al*., 2002). Whether

temperature may be a trigger for a complete PST is, however, disputed (McCormick *et al*., 2002; Stefansson *et al*., 2007; Handeland *et al*., 2013).

Although salinity tolerance increased in all groups in the present study (Figures 2 and 3), the increase was stronger in the groups exposed to the longest photoperiods. This is best illustrated by the results in experiment 2; plasma chloride concentration and osmolality following SWTs were significantly lower in the fish exposed to 24 h daylight compared with those that were maintained at 6 or transferred to 12 h daylight (Figures 2(b) and 3(b)). Importantly, there was no difference between the 6 and 12 h daylight groups in plasma chloride concentration and osmolality following SWTs, indicating that the increase in salinity tolerance in these groups did not reflect a completed PST. This assumption is further supported by the fact that only the group transferred to 24 h light in experiment 2 showed an increase in gill NKA activity concomitant with the increase in salinity tolerance (Figure 4). It is generally accepted that an elevated gill NKA activity is necessary for the ability of salmonids to maintain ion and water balance in SW (Evans *et al*., 2005) and it is a paradox that the 6 and 12 h photoperiod groups in experiment 2 developed a markedly better hypo-osmoregulatory ability without any changes in gill NKA activity (Figures 2(b), 3(b) and 4). This could, however, be related to the increase in size and a more favourable surface-area-to-volume ratio in these fish, as shown previously (Duston & Saunders, 1990; Arnesen *et al*., 1992). The increase in gill NKA activity in the fish exposed to constant light in experiment 2 concurred with an increase in plasma GH levels, confirming the stimulatory role of GH (partly *via* insulin-like growth factor) on gill NKA activity in euryhaline fish species (Takei & McCormick, 2013). The lack of increases in plasma GH levels in the 6 and 12 h photoperiod groups is in correspondence with the lack of increases in gill NKA activity in these groups and a further evidence of an incomplete PST in these groups. A similar difference between the groups in experiment 2 was seen for brain expression of *dio2b*, which was only

upregulated in the group exposed to constant light. This gene has recently been shown to be responsive to increasing photoperiods in smolting *S. salar* (Lorgen *et al*., 2015) and has also been implicated in the timing of seasonal life-history transitions in mammals and birds (Hazlerigg & Simonneaux, 2014).

The increase in growth rate with increasing photoperiod seen in the present study (Figure 6(a),(b) correspond to the findings in numerous previous studies with *S. salar* (Boeuf & Bail, 1999). It is believed that the increase in growth rate is caused by an increasing level of circulating GH, in turn caused by the extended photoperiod (Björnsson *et al*., 1997). The results in the present study support this, since in experiment 2 the fish in the group transferred to 24 h photoperiod had higher plasma GH levels and higher growth rates than the fish in the other two groups (Figure 9). Furthermore, this group was also the only group in experiment 2 showing a decrease in *K* (Figure 7), which was to be expected due to the stimulatory effect of GH on skeletal (length) growth (Björnsson *et al*., 1997). The increase in growth rate in the groups that were exposed to long days occurred even though all groups were fed only during the 8 or 6 h of daylight experienced by the short-day groups in experiment 1 and 2, respectively. This finding must be interpreted as a stimulation of appetite by the long day and associated neuroendocrine responses, rather than by length of the period during which feed is available. The latter has been considered important for food intake and growth since the *S. salar* is a visual feeder and consequently, that it's feeding opportunity depends on photoperiod (Ali, 1959; Thorpe *et al*., 1990).

It is commonly said that the role of photoperiod in the stimulation and timing of smolting is through entrainment of a circannual rhythm of smolting. However, a complete PST is considered a "once in a life-time" life-history transition (Björnsson & Bradley, 2007), which then, by definition, is not a circannual rhythm *per se* but a transition that is governed by a

circannual timer. It is possible that components of the PST follow a circannual rhythm in *S. salar* juveniles deprived from photoperiodic cues, since Eriksson & Lundquist (1982) showed repeated circannual (10 months) changes in silvering and *K* in juveniles held on a 12L:12D photoperiod regime. Timing and completion of a true PST seems, on the other hand, to rely on exposure to a winter-summer photoperiodic cue soon after the parr has reached a stage by which they are ready to smoltify. Within this context and all data taken together, the results from the present study show that the photoperiod increase needs to exceed 16 h for stimulating a complete PST in the *S. salar* parr used in the present study. This finding translates to *S. salar* living at high latitudes has, however, not been shown. Triggering of smolting under a gradually increasing daylength in natural system is not necessarily like that evoked under the experimental, photoperiod regime used here.

Surprisingly, no information seems to exist on photoperiod conditions needed for *S. salar* smolting in relation to latitude. *Salmo salar* smolts in the sub-arctic River Tana ($70^{\circ}$ N) in northern Norway enter sea during late June and early July (Orell *et al*., 2007). Based on data from the present experiment, it took between 330 and 380 D$^{\circ}$C from the abrupt change from short to long days until gill NKA activity and salinity tolerance reached peak levels, *i.e.* comparable with the 350 D$^{\circ}$C reported by Handeland *et al*. (2004). Assuming that the *S. salar* in River Tana have completed PST at the time of sea entry and that the river temperature is on the average $4^{\circ}$C during the smolting period, it can be calculated that the start of the development of gill NKA activity and salinity tolerance must have been in April, *i.e.* at a time when photoperiod is longer than 16 h. In contrast, an increase from 10 to 15 h photoperiod increased plasma GH levels and gill NKA activity and decreased *K*, in a more southern distributed ($43^{\circ}$ N) *S. salar* in the U.S.A. (McCormick *et al*., 1995). Further studies on the chronobiology of *S. salar* smolting in relation to latitude is warranted.

In a very interesting, early study, Thorarensen & Clarke (1989) demonstrated that coho or Chinook salmon *Oncorhynchus tshawytscha* (Walbaum 1792) parr maintained on short day (6 h) and a 1 h light pulse in the middle of the night developed a significantly greater growth rate and SW adaptability than those moved from 6 to 10 h photoperiod and a similar increase in growth rate and SW adaptability as those moved from 6 to 16 h photoperiod. Likewise, it was shown that a skeleton photoperiod treatment also governed spawning time in rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) (Duston & Bromage, 1986). Surprisingly, no later reports from experiments with salmonids using such skeleton photoperiod regimes can be found. The finding tempted the authors to conclude that the timing of the day when light is experienced, rather than the accumulated number of hours of exposure to light, initiate PST in *S. salar*. This points further to an "external coincidence" mechanism (*sensu* Bünning, 1936) underpinning the photoperiodic stimulation of PST. The mechanism implies that the prevailing photoperiod entrains an endogenous, circadian rhythm of photosensitivity and that the initiation of long day phenotype relies upon the occurrence of daylight during the photosensitive period. Further studies are needed to test this hypothesis.

In summary the present study revealed development of hypo-osmoregulatory ability in all treatment groups, irrespective of photoperiodic treatment. However, this development does not indicate a completed PST, since only the groups exposed to photoperiods exceeding 16 h daylight developed a full suite of smolt indices, including increases in gill NKA activity, plasma GH concentration and brain *dio2b* mRNA abundance and decreased *K*. It is concluded that the photoperiod increase needs to exceed 16 h for stimulating a complete PST in *S. salar* parr living in northern habitats.

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Figure captions

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**FIGURE 1** Light- and temperature regimes experienced by fish used in experiment 1 and 2. □, The length of day; ▮, the length of night.

**FIGURE 2** Plasma chloride concentrations (mean ± S.E.) following seawater treatment of fish kept at 8L:18D (●), 12L:12D (○), 16L:8D (▼), 20L:4D (Δ) and 24L:0D (■) in experiment 1 and 6L:16D(X), 12L:12D (○), and 24L:0D (■) in experiment 2. *,Significantly different plasma chloride concentration in the 24L:0D compared with other groups ($P < 0.05$).

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2    Change Chloride to chloride and delete repeat label and numerals from RH y-axis

FIGURE3. Plasma osmolality (mean ± S.E.) following seawater treatment of fish kept at 8L:16D (●), 12L:12D (○), 16L:8D (▼), 20L:4D (Δ) and 24L:0D (■) in experiment 1 and 6L:18D (X), 12L:12D (○), and 24L:0D (■) in experiment 2.*(1), Significantly lower levels in the 24L:0D group than in the 8L:16D, 12L:12D and 16L:8D groups. *(2), Significantly lower level in the 20L:4D group than in the 8L:16D and 12L:12D groups. *(3), Significantly higher level in the 8L:16D group than in the rest of the groups  and *(4) , Significantly lower level in the 24L:0D group than in the 6L:18D and 12L:12D groups ($P < 0.05$).

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2          Change Osmolality to osmolality and delete repeat label and numerals from RH y-axis

FIGURE 4. Gill $Na^+$–$K^+$-ATPase activity (mean ± S.E.) in the 6L:16D (X), 12L:12D (○) and 24L:0D (■) groups. * Significantly higher gill $Na^+$–$K^+$-ATPase activity in the 24L:0D group than in the other groups at the same sampling date ($P < 0.05$).

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FIGURE 5. Changes in body mass ($M$, mean ± S.E.) of fish kept at 8L:16D (●), 12L:12D (○), 16L:8D (▼), 20L:4D (Δ) and 24L:0D (■) in experiment 1 and 6L:18D (X), 12L:12D (○), and 24L:0D (■) in experiment 2. 1(*), Significant higher body weight of the fish held at 24L:0D at the last sampling date in experiment 1 compared to the body weight of the group held at 8L:16D; 2(*), significantly higher body weight of the fish held at 24L:0D than of those held at 6L:18D and 12L:12D ($P < 0.05$).

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1          Change a), b) to (a), (b)

2          Change Body mass to $M$ and delete repeat label and numerals from RH y-axis

FIGURE 6. Specific growth rate ($R_{SG}$; mean ± S.E.) of fish kept at different light regimes during the photoperiodic treatment period.  *, Significantly higher $R_{SG}$ than groups without asterix in experiment 1 ($P < 0.05$).

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3      Change SGR mass to $R_{SG}$ and delete repeat label and numerals from RH y-axis

FIGURE 7. Condition factor ($K$; mean ± S.E.) of fish kept at 8L:16D (X), 12L:12D (○), 16L:8D (▼), 20L:4D (Δ) and 24L:0D (■) in experiment 1 and 6L:16D (●), 12L:12D (○), and 24L:0D (■) in experiment 2. 1(*), Significantly lower condition factor in the 24L:0D and 20L:4D groups than in the 8L:16D, 12L:12D and 16D:8L groups; 2(*), significantly lower condition factor in the 20L:4D group than in the 8L:16D and 12L:12D groups in experiment 1; 3(*), significantly lower condition factor in the 24L:0D group than in the 6L:18D and 12L:12D groups; 4(*), significantly lower condition factor in the 24L:0D groups than in the 6L:18D groups ($P < 0.05$).

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1      Change a), b) to (a), (b)

2      Change Condition factor to $K$ and delete repeat label and numerals from RH y-axis

FIGURE 8. Normalized brain *dio2b* mRNA abundance (mean ± S.E.) in the 6L:18D (X), 12L:12D (○) and 24L:0D (■) groups. Significantly higher abundance in the 24L:0D group than in the: *(1), 6L:18D and 12L:12D groups in March; *(2), 6L:18D group in April; *(3) 12L:12D group in May ($P < 0.05$).

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FIGURE 9. Changes in plasma GH concentration (mean ± S.E.) in the 6L:18D (X), 12L:12D (○) and 24L:0D (■) groups. *, Significantly higher plasma GH concentration in the 24L:0D group than in the other groups at the same sampling date ($P < 0.05$).
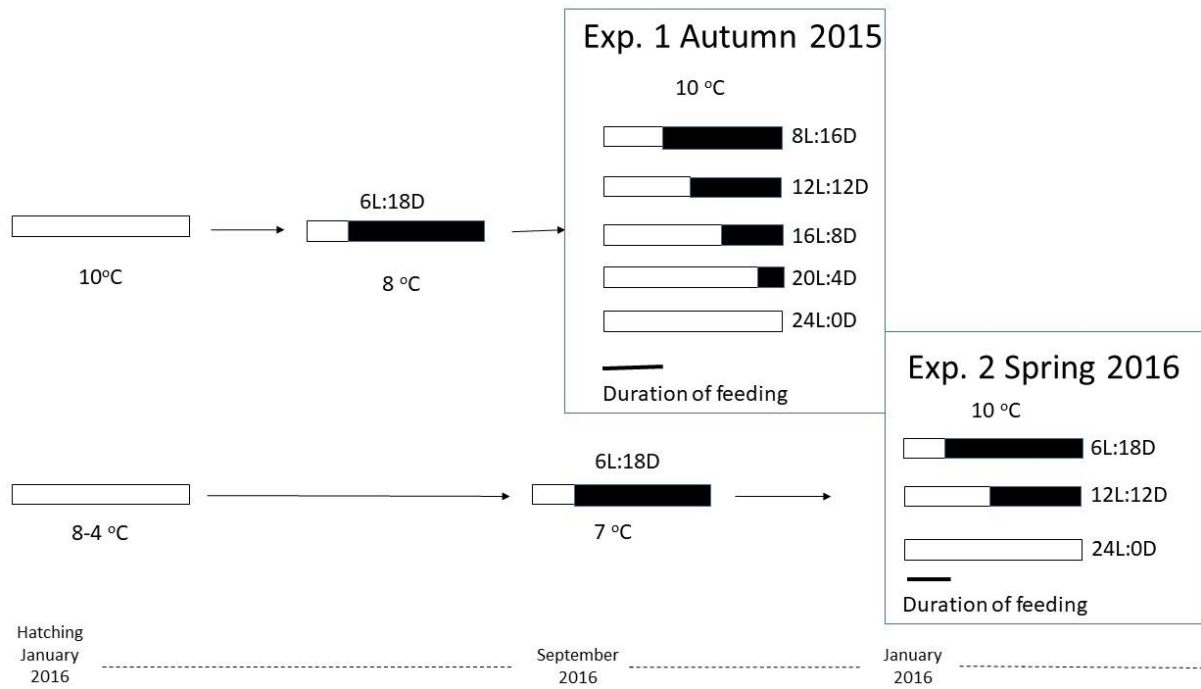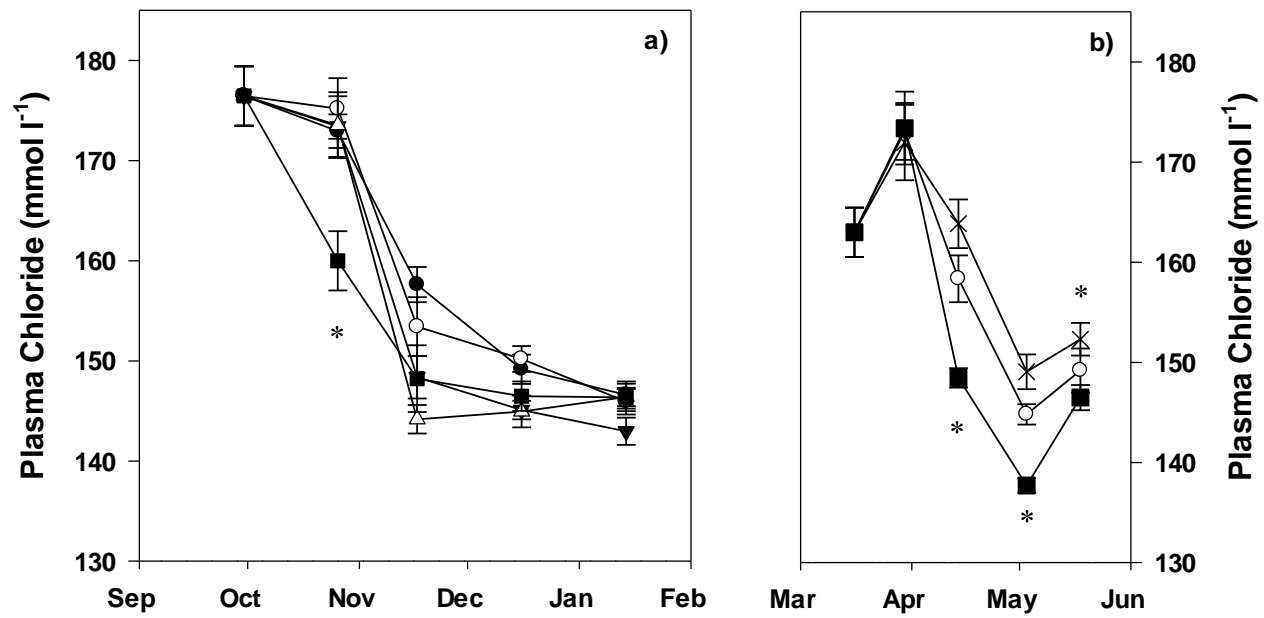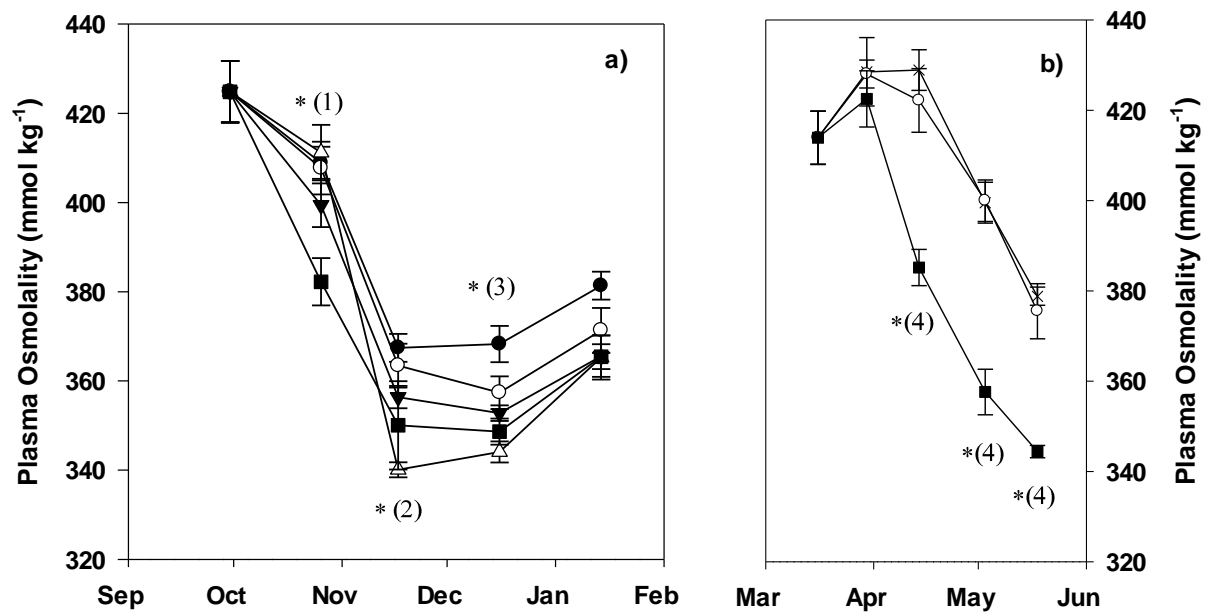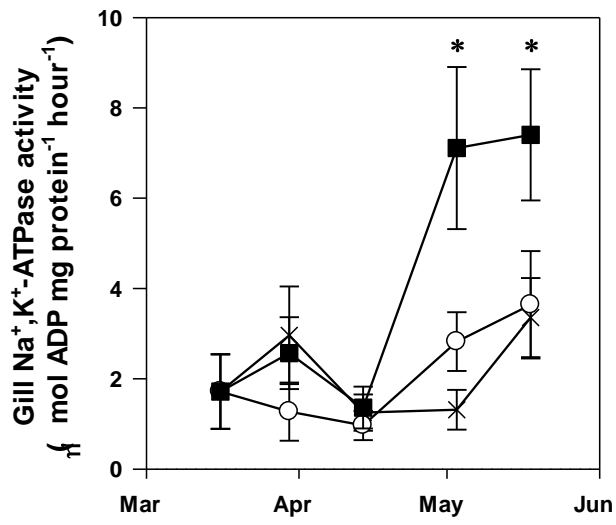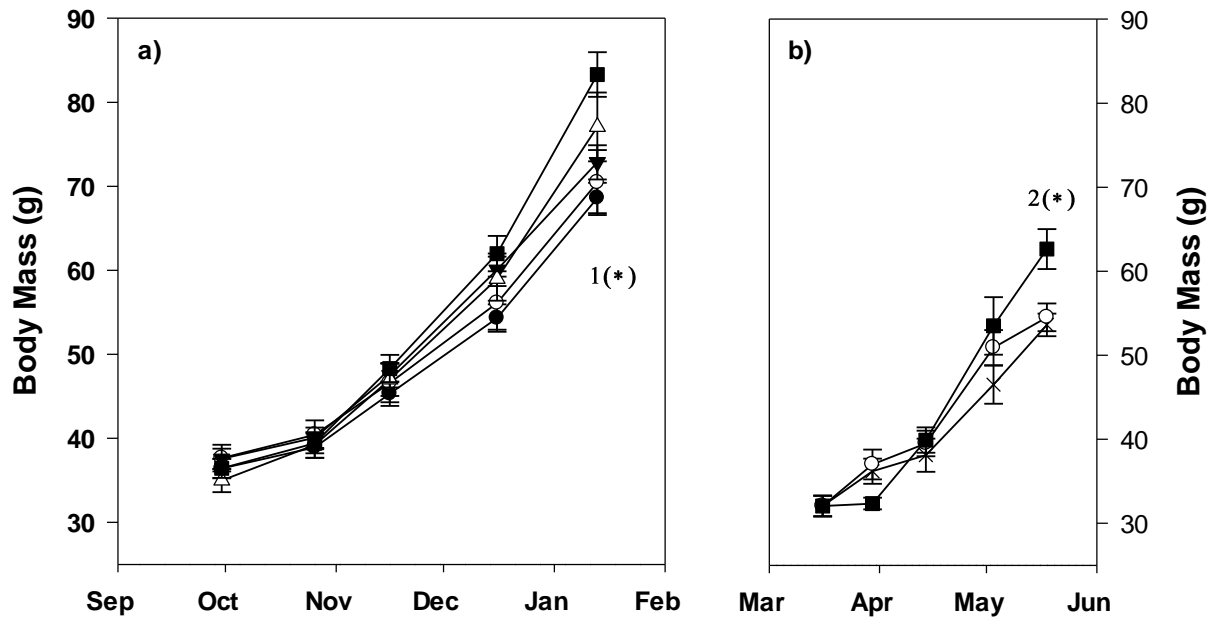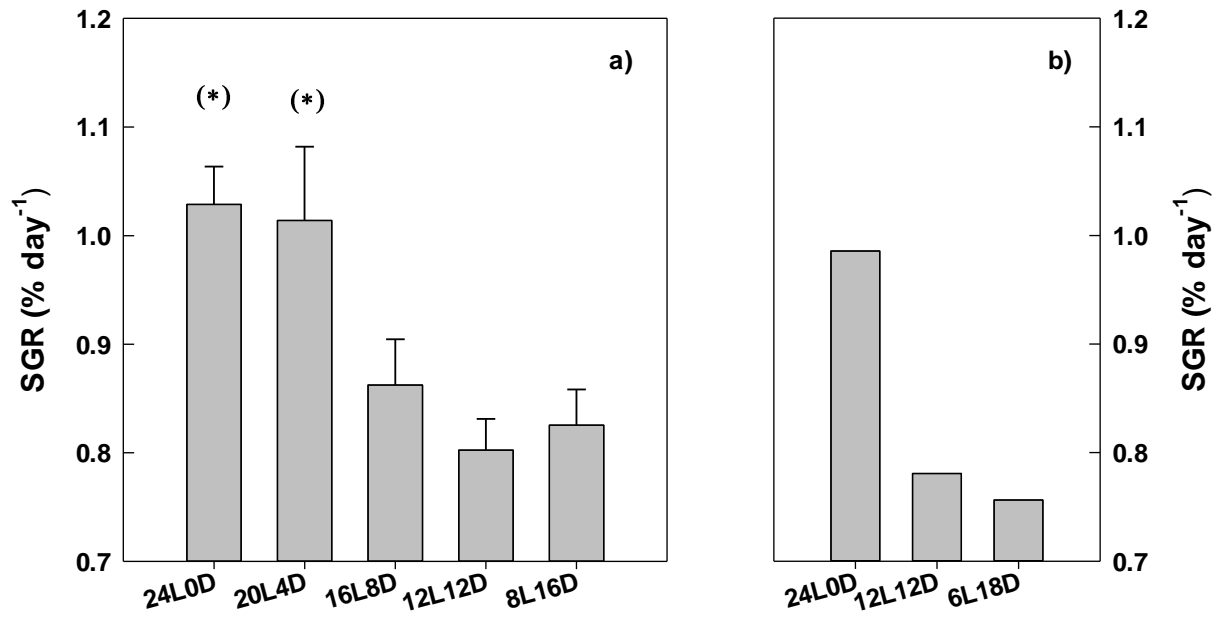
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