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Atlantic salmon male post-smolt maturation can be reduced by using a 3-hour scotophase when inducing smoltification

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ABSTRACT

Photoperiod regulates the occurrence of unwanted male post-smolt maturation during the production of large (>100 g) Atlantic salmon (*Salmo salar*) smolts. However, the optimal daylength for triggering smoltification, but not male puberty, has yet to be established. We used either continuous light (24:0 light/dark) or long days (18:6 and 21:3) after a six week "winter" zeitgeber (12:12) to induce smoltification in fish of around 120 g reared at 16 °C. The fish were sampled 1, 2, 3, and 6 weeks after the initiation of the three different photoperiod treatments (n = 153 males in total with 9–18 males/photoperiod/time point). As expected, the smoltification indicator gill Na^+/K^+-ATPase (NKA) was significantly ($p < 0.05$) elevated and peaked 2 to 3 weeks after the initiation of the different photoperiods. Pubertal males were identified in all treatments via the combined use of relative testis size and histology, plasma 11-ketotestosterone, changes in body condition, and growth rate. The total incidence of puberty was significantly higher among males on continuous light at 33% (n = 16/49) compared to 10% (6/61) and 12% (5/43) in 21:3 and 18:6, respectively. The incidence of puberty increased over time in all photoperiods, with 62% (8/13), 19% (3/16), and 38% (3/8) of the males from 24:0, 21:3, and 18:6 pubertal at week 6, respectively. The mean weight of males that went on to initiate puberty was significantly higher (13%) at the beginning of the trial compared to those that remained immature (mean weight, 127 vs 112 g, respectively), but there was no initial difference in body condition. Puberty significantly reduced gill NKA by 35% compared to immature males at week six but had no effect at earlier time-points. Photoperiod had no effect on the female GSI, and they were all considered immature. In conclusion, the incidence of male puberty during smoltification is regulated by photoperiod and leads to an earlier decline in a key indicator of seawater readiness. As such, photoperiods with a short scotophase (21:3 or 18:6) following the winter zeitgeber in a square-wave (long-short-long day) smolt regime are recommended to limit the incidence of male puberty.

1. Introduction

Atlantic salmon (*Salmo salar*) display a large degree of phenotypic plasticity for the age at maturation. Wild males typically mature during the early freshwater life stages as parr or following one (grilse) or more sea-winters (Fleming, 1996). Domestic salmon show a similar phenotypic plasticity, but may also mature either directly before, or shortly after, sea transfer (Fjelldal et al., 2011). These males are called "mature post-smolts" or "jacks". The phenotype is rarely observed in wild Atlantic salmon (Klemetsen et al., 2003). It is generally un-wanted in salmon farming as it reduces hypo-osmoregulatory ability (Fjelldal et al., 2018) and long-term growth (Fraser et al., 2019) in seawater. In contrast, females rarely mature before their first sea-winter in the wild (Klemetsen et al., 2003) and domesticated females generally require even longer (≥2 sea-winters) and normally reach harvest size before then (Taranger et al., 2010).

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Post-smolt maturation was evident in domestic stocks of Atlantic salmon in the early 1990s but has become more prevalent recently due to changes in technology that allow for the greater control of environmental conditions. For instance, approx. 15% male post-smolt maturation was observed in fish smoltified on a simulated natural photoperiod at 8–11 °C and transferred to sea at approx. 100 g in May (Stefansson et al., 1993). In Scotland, 24% post-smolt maturation (the sex was not stated) was observed in fish that had been kept on a 10-month condensed natural cycle and moved to seawater 2 months early in March (Thrush et al., 1994). More recently, 50–80% of males matured at <1 kg when kept in modern environments characteristic of those found in recirculating aquaculture systems (RAS) (Good et al., 2017; Imsland et al., 2014). These systems are increasingly being used to produce large (0.1 to 1.0 kg) fast growing smolts/post-smolts using heated water (10 to 18 °C, Fossmark et al., 2021; Roalkvam et al., 2021; Ytrestøyl et al., 2022) combined with continuous light (Qui et al., 2015; Good et al., 2017; Ytrestøyl et al., 2022). These fish may then be switched/transferred to seawater (Fang et al., 2021; Ytrestøyl et al., 2022), or maintained in freshwater up until harvest (Qui et al., 2015; Crouse et al., 2022). Notably, the temperatures and daylengths used in commercial RAS are generally the same as those used by researchers to induce post-smolt maturation (16 °C and constant light, Fjelldal et al., 2011, 2018; 12 °C and constant light, Fraser et al., 2019). Post-smolt maturation has also been observed when producing smolts at 10 °C (17% of males were pubertal in smolts of approx. 200 g. Sambraus, 2016).

High levels of maturation in RAS may be the result of the high water temperatures, photoperiod manipulation, and fast growth, which are all risk factors for the more thoroughly researched grilsing phenotype (McClure et al., 2007; Taranger et al., 2010). The exact mechanisms that lead to the initiation and timing of puberty in salmon are currently unknown, but it is hypothesised that a developmental threshold must first be met (Thorpe et al., 1998) and photoperiods are important zeitgebers (Duston and Bromage, 1986). The exact time of the year when males make the decision to mature is unclear, with males showing the first signs of puberty anywhere between the winter or summer preceding the autumn spawning season (Hunt et al., 1982; Youngson and McLay, 1985; Youngson et al., 1988). The first signs of puberty are generally considered to be elevations in plasma 11-ketotestosterone (11-KT), relative testis size (i.e. the gonadosomatic index), and molecular markers of gonadotroph activity in the pituitary (Fjelldal et al., 2018; Kjærner-Semb et al., 2018; Crespo et al., 2022). Relative testis size also correlates well with the various stages of testis development in wild and domestic salmon (Idler et al., 1981 and Fjelldal et al., 2018, respectively).

Previously, Fjelldal et al. (2011) observed 47% of males entered puberty when exposed to continuous light (24:0 light/dark) after a "winter" zeitgeber (12:12) in Atlantic salmon kept on 16 °C, compared to 0% in those exposed to a short day (18:6). The winter zeitgeber is one step in the "square-wave" photoperiod (long-short-long days) used to induce smoltification in farmed salmon (Thrush et al., 1994); smoltification being the developmental process by which salmon go from a freshwater to seawater phenotype. Therefore, farmers may unintentionally trigger puberty at the same time as smoltification under certain conditions. However, the full extent to which daylength after the winter zeitgeber regulates the timing of post-smolt maturation and the development of smoltification is unknown. This is important, as longer days improve growth rates (Imsland et al., 2014; Ytrestøyl et al., 2022) and can have practical connotations in RAS such as allowing feeding to be spread out over the whole day to reduce loads on filters. In addition, Duston and Bromage (1987) concluded that the change in photoperiod direction (from short to long, or long to short), rather than the magnitude of change in daylength in hours, is more important for the timing of ovulation/spermiation in maturing rainbow trout (*Oncorhynchus mykiss*). However, it is unclear whether the same principle governs smoltification. Therefore, our objective was to determine whether it is possible to use a relatively long day with a shorter scotophase than used in Fjelldal et al. (2011) to reduce post-smolt maturation, but still initiate smoltification. As such, we compared 24:0 and 18:6 to 21:3, assessed a key smoltification indicator (gill Na^+/K^+- ATPase [NKA]), and included a more thorough assessment of puberty development over time by measuring growth rate, body condition, testis size, testis histology, and plasma 11-KT. Our hypothesis was that a longer daylength will induce a greater incidence of post-smolt maturation, and puberty will impair markers of hypo-osmoregulatory ability (i.e. reduce gill NKA).

2. Material and methods

2.1. Ethics

The present experiment was approved by the Norwegian Animal Research Authority (FOTS ID 8521) and performed according to prevailing animal welfare regulations.

2.2. Fish stock and rearing conditions

Eyed eggs at 350° days were provided by Aquagen AS (QTL InnOva IPN) on the 4th of December 2014. Hatching occurred on the 9th of January 2015, and first feeding began on the 10th of March 2015 under conditions of continuous light and 12 °C water temperature. Heated water was provided until the 21st June, thereafter natural temperatures (range 4–14 °C) were used (Fig. 1). Continuous light was provided until the 1st of October 2015, whereupon simulated natural photoperiod was used (based on civil twilight, 61° N). The fish were kept in a flow-through system supplied by filtered and ozone treated local freshwater mixed with a small quantity of filtered and UV treated local seawater to maintain salinity within 0.8 ± 0.1 ppt and pH within 6.4 ± 0.2 throughout the experiment.

A total of 380 fish were individually marked with passive integrated transponder (PIT) tags on the 12th of January 2016 and moved to the experimental facility on the 28th of January 2016 when they were divided equally between six 1 × 1 × 0.43 m tanks (n = 62–64 fish/tank, approx. 15 kg/m^3). On the 11th of February 2016, the photoperiod was shifted from simulated natural to either continuous light, 21:3, or 18:6, with two tanks per photoperiod. Simultaneously, the temperature in all tanks was gradually increased from 4.7 °C (natural) to 16 °C over a 4-day period (Fig. 1).

The fish were fed continuously for 18 h per day, coinciding with the light period of the 18:6 group, by automatic feeders (ARVO-TEC T Drum 2000, Arvotec, Huutokoski, Finland). For illumination, two 18 W fluorescent day light tubes (OSRAM L 18 W/840 LUMILUX, OSRAM GmbH, Ausburg, Germany) were used. Photoperiod and feeding were controlled automatically by a computer system (Normatic AS, Norfjordeid, Norway). There was no alteration in light intensity to mimic dawn or dusk, the lights controlling photoperiod were maintained at the same intensity whenever they were on.

2.3. Sampling protocol

On the 11th of February 2016 (week 0), 30 fish (n = 16 males) were sampled for gonad weight, testes histology, and plasma 11-ketotestosterone (11-KT). These fish were netted out of the tank, anesthetized in 100 mg/L Finquel® (MS222), a blood sample taken from the caudal vein, and euthanized by decapitation. The testes were weighed to 0.01 g and then preserved in Bouin's fixative. Following this, 10–15 fish/tank (n = 2–10 males/tank/time point, n = 9–18 males/photoperiod/time point, see Table S1 for exact sample numbers per time point) were sampled in the same way on the 18th and 24th of February and the 3rd and 31st of March 2016 (weeks 1, 2, 3, and 6, respectively). The variation in the n between tanks was due to the sex of the fish being unknown prior to sampling, and the need to maintain stocking densities while ensuring enough fish were available for the last sampling time. In total, 170 males were sampled, 16 prior to the experiment and between 44 and 56 for

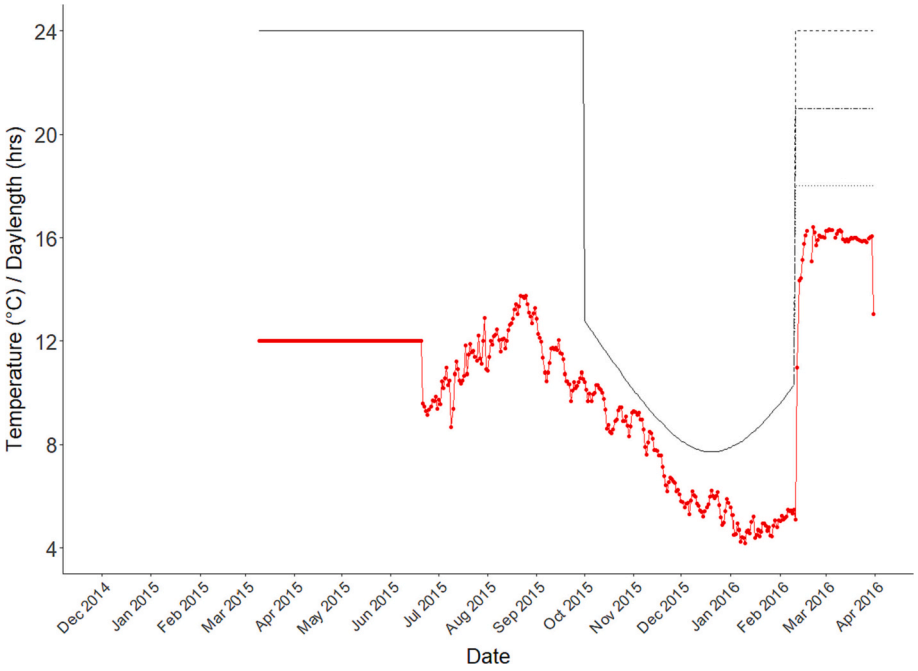

Fig. 1. The temperatures (red points and line) and daylengths (solid line = pre-experiment; dashed = 24:0 [light/dark]; two-dash = 21:3; dotted = 18:6) used from first feeding (March 10th 2015) until the end of the experiment. The experiment began on the 11th of February 2016 when duplicate tanks of fish were moved to 16 °C and one of 3 photoperiod regimes. The experiment ended on the 31st of March 2016. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

each photoperiod, and 199 females. For 9 fish, sex was not recorded. For each fish, the second gill arch on the left side of the body was sampled and stored on dry ice before moving to −80 °C for analysis of NKA enzyme activity. The body weight data was used to calculate specific growth rate (SGR, %/day); $(e^{q} - 1)100$ (Houde and Scheckter, 1981) where $q = [\ln(W_2) - \ln(W_1)]\ (t_2 - t_1)^{-1}$ (Bagenal and Tesch, 1978), and W_2 and W_1 are average body mass at times t_1 and t_2, respectively. Body condition was calculated using Fulton's condition factor (*K*); K = body weight (g)/ fork length^3 (cm) × 100.

2.4. 11-ketotestosterone

Steroids were extracted from blood plasma by a method modified from Pankhurst and Carragher (1992). Briefly, plasma samples (100 μL) were mixed with 1 mL ethyl acetate, vortexed for 20 s and centrifuged for 3 min at 1800 rpm and 4 °C. The organic phase was collected by a Pasteur pipette and the hydrophilic phase was extracted once more with 1 mL of ethyl acetate. The extracts were evaporated in a Speed Vac centrifuge (Savant 1000, USA), and dissolved in 1 mL buffer (phosphate 0.1 M, pH 7.4, 0.4 M NaCl, 1 mM EDTA) by heating (60 °C for 10 min). The extracted and dissolved steroids were stored at −20 °C until analysis by enzyme-linked immunosorbent assay (ELISA, Cuisset et al., 1994). The ED80 and ED20 were 0.04 ng/mL and 1.00 ng/mL, respectively, and the detection limit of the assay was 0.005 ng/mL. Internal standards were prepared from mature male (11-KT) Atlantic salmon plasma extracted with ethyl acetate as described above. The accepted inter-assay coefficient of variation was 10% and those assays with a higher deviation of the internal standard were re-run. The intra-assay coefficient of variation was 6.2% for 11-KT (n = 10). Acetylcholine esterase-labelled tracers and microplates pre-coated with monoclonal mouse anti-rabbit IgG were supplied by Cayman Chemicals (USA). Anti-11-KT was a kind gift from Dr. David E. Kime, Sheffield University, UK, with details on cross-reactivity given by Cuisset et al. (1994). Standard steroids were purchased from Sigma Aldrich (Sigma reference standards).

2.5. Gill Na^+/K^+-ATPase (NKA)

The gill NKA enzyme activity was assessed using the method of McCormick (1993). Briefly, the assay measured the hydrolysis of ATP based on the two enzymatic reactions that convert NADH into NAD+ by pyruvate kinase and lactic dehydrogenase in the presence or absence of Ouabain, an inhibitor and the baseline indicator of NKA. The readings were taken from a Tecan SPARK® (BERGMAN DIAGNOSTIKA) microplate reader at 25 °C and 340 nm wavelength. The protein concentration within homogenized samples was determined by Bicinchoninic acid (BCA) protein analysis (Smith et al., 1985). Only the males were analysed as none of the females matured.

2.6. Histology

After dissection and weighing, a testis tissue fragment was fixed in Bouin's fixative, dehydrated in graded alcohol, and embedded in paraffin according to conventional techniques. Testes sections of 3 μm thickness were stained with 1% hematoxilin-eosin and analysed qualitatively by light microscopy. Proliferation of spermatogonia and Sertoli cells was assessed by immunocytochemical localization of the proliferation marker phosphorylated histone H3 (pH 3. Hendzel et al., 1997; Cobb et al., 1999). Due to cost limitations, testis histology was undertaken on only 78 of the 170 males (see Table S2 for sample sizes/ photoperiod/week). We ensured the selection covered a range of males from each photoperiod and week, but tended to focus on fish that scored ≤2 (see paragraph below), as these had GSI or 11-KT values within the crossover range for puberty (e.g. a GSI of between 0.04 and 0.13% and 11-KT between 1 and 3 ng/mL) (e.g. Kjærner-Semb et al., 2018). The sections were scored for the presence of the most advanced stage of spermatogenesis with the following stages observed: (0) germ cell free, (1) type A spermatogonia, (2) type B spermatogonia, (3) spermatocytes, (4) spermatids, and (5) spermatozoa (Fig. S1). The identification was mainly based on the size, shape, and staining of the germ cell nuclei and followed the description given earlier in Melo et al. (2014). In addition, proliferation was scored on the scale 1, 1.5, or 2, indicating either uniformly low, areas with low and high, or uniformly high proliferation, respectively, based on a qualitative assessment by a single researcher (Fig. S1). One male was found to have testis showing a similar histology profile to a germ cell free individual (e.g. a *dnd* knockout male, see Wargelius et al., 2016). This male was from the 18:6 treatment sampled at week 6, presumed sterile, and removed from further analysis.

2.7. Assigning males as immature or pubertal

To improve the analytical ability, we identified males as either being pubertal (i.e. which will become a mature post-smolt) or immature. Initially, we determined that GSI, plasma 11-KT, SGR, Δ body condition, and testis proliferation were all positively associated with testis staging (Fig. S2). As we did not have histology data for all fish, we subsequently looked for bimodality in the GSI, 11-KT, SGR, and Δ body condition data to determine whether males could be assigned as either pubertal or immature based on certain threshold values. Such bimodality was expected, as immature post-smolts have a GSI of ≤0.06% (Schulz et al., 2006; Fjelldal et al., 2011; Melo et al., 2014, 2015) whereas plasma 11-KT is generally above 1-2 ng/mL in pubertal fish (Kjærner-Semb et al., 2018; Fjelldal et al., 2018). In addition, males undergo a growth spurt during the initial stages of post-smolt maturation leading to elevated SGR and an increase in body condition (Fraser et al., 2019; Fjelldal et al., 2020). Bimodality was observed in all four parameters, although it was less convincing in the SGR data (Fig. S3). We determined threshold values of 0.08%, 1.3 ng/mL, 1.1%, and 0.98 for GSI, 11-KT, SGR, and Δ body condition, respectively, with those ≥ the threshold being considered pubertal. Every individual was then awarded a score of 1 for each parameter in which it had attained the threshold. Therefore, each male could theoretically be given a score of between 0 and 4, with 0 indicating it was considered immature for all four indicators and 4 indicating it was considered pubertal for all four indicators. Males with a score of ≥3 were considered pubertal and all these fish showed testis development with the presence of spermatocytes and a high score for the proliferation marker pH 3. Males with a score of 2 (for which we had histology data for all fish) were assigned as pubertal if germ cell proliferation had been given a value of 2, whereas those with a value of ≤1.5 were considered immature. Subsequently, pubertal fish were clustered separately from immatures when using Principal Component Analysis (PCA) within each time point (Fig. S4) indicating our assignment was robust. Finally, GSI, 11-KT, SGR, and Δ body condition were compared between immature and pubertal males. For all parameters, there was no overlap between immature and pubertal males at week 6, although at earlier timepoints GSI and 11-KT appeared the most robust markers of puberty (Fig. S5). For 6 males ($n = 5$ from the 21:3 and $n = 1$ from the 24:0 fish all at week 1) there was no weight data available at the time of sampling, so the GSI, SGR, and Δ body condition could not be calculated. Based on the threshold value determined above, as all these males had 11-KT values of ≤0.41 ng/mL they were subsequently all considered immature. For 2 females the body weight data was missing (from week 1, 18:6) and for 1 female the gonad data was missing (week 3, 18:6).

2.8. Statistical analysis

The data were transferred to R Statistical software (version 4.0.4, R Core Team, 2021) and we used the "nlme" (Pinheiro et al., 2022), "emmeans" (Lenth, 2021), "ggplot2" (Wickham, 2016), and "ggbiplot" (Vu, 2011) libraries for analysis and data presentation. Significance was assigned at $p < 0.05$. The Akaike Information Criteria with a correction for small sample sizes (AICc, Hurvich and Tsai, 1991) was used when comparing models (see below). AICc compares the amount of variation explained by a model weighted against its complexity, with a lower value indicating a better model fit. Models with a Δ AICc score within 2 of the lowest AICc can also be considered to have substantial support (Burnham and Anderson, 2002).

As the data was non-parametric, we used Kruskal-Wallis to compare GSI and 11-KT between photoperiods (3 levels; 18:6, 21:3, and 24:0) based on data pooled for week. Subsequently, we used Kruskal-Wallis to analyse each week separately to look for photoperiod effects within week. When the model was significant, we used Dunn's test for post-hoc analysis. To assess the incidence of post-smolt maturation, we used a generalised linear model (GLM) with a binomial distribution with photoperiod as fixed effects. Initially, we analysed the data pooled for all time points, and then ran separate analyses for weeks 3 and 6. Due to the low incidence of puberty, weeks 0, 1, and 2 were not analysed and tank was not included in the analysis. In contrast, the GSI data for females fitted a linear mixed effect (LME) model based on residuals being both linear and normally distributed. The initial LME model included time (5 levels; week 0, 1, 2, 3, and 6) and photoperiod (3 levels) as fixed effects, their interaction, and tank as a random effect. A model without the interaction was compared to the initial model using the AICc (Table S3).

We compared body weight between phenotype (3 levels; immature females, immature males, and those males that became pubertal), at weeks 0 and 6, using LME models. Here, weeks 1, 2, and 3 were not included due to the low number of pubertal males, and photoperiod was included in the model as a random effect. The same approach was used to assess body condition and SGR between phenotypes. Finally, we assessed whether photoperiod affected growth rates in immature fish. Here, the data from week 6 only was included in an LME model with SGR as the dependent variable, photoperiod as a fixed effect (3 levels), and tank was included as a random effect.

We initially used a GLM to assess gill NKA (natural log) over time with week (5 levels) as a fixed effect. Tank was not included as a random effect as the pre-trial fish came from only 1 tank. Next, we used LME models to assess gill NKA enzyme (natural log) activity over time with photoperiod (3 levels) and week (4 levels) as fixed effects, their interaction, and maturity status (2 levels; immature and pubertal) and tank as random effects. We then used AICc to determine which model had the best fit (Table S4). This model was then repeated but only using the data from the immature males (Table S5). Finally, to determine whether puberty affected NKA, we re-ran this model but set photoperiod as a random effect, maturity status as a fixed effect, and only used data from weeks 3 and 6 due to the small number of pubertal males at earlier timepoints. Although AICc suggested there was no general effect of puberty, or any interaction between week and maturity status (Table S6), we ran an independent LME model for week 6 as we have seen previously mature males suffer from higher mortality if transferred to full strength seawater at this time (Fjelldal et al., 2022).

The "Anova" command within the "car" library was used to extract the results for the main effects of the model. When models were significant, least square (LS) means were used for post-hoc analyses. The raw data (Fraser et al.xlsx) and the R script (Fraser et al.R) used to analyse the data can be found in the supplementary material.

3. Results

3.1. Incidence of male puberty

When all the data was pooled for week, there was a significant effect of photoperiod on 11-KT and the incidence of pubertal males (11-KT, Kruskal-Wallis; $\chi^2 = 12$, df = 2, $p = 0.003$: Incidence of puberty, GLM; $\chi^2 = 11$, df = 2, $p = 0.005$) (Fig. 2). The incidence of puberty was 33%, 10%, and 12% in the 24:0, 21:3, and 18:6 treatments, respectively. For both parameters, 24:0 had significantly higher values than 21:3 and 18:6, but there was no difference between 21:3 and 18:6. For GSI, the model was close to significant (Kruskal-Wallis; $\chi^2 = 6$, df = 2, $p = 0.062$) and the trends followed those for 11-KT and the incidence of puberty. Within week effects were transiently significant for GSI (week 6), 11-KT (weeks 1 and 3), and the incidence of puberty (week 3) and matched the general model that 24:0 had the highest values.

3.2. Incidence of female maturation

No females were judged to have entered puberty (max GSI value <0.3). Model selection provided no support for an effect of week or the interaction with photoperiod. Although photoperiod alone provided the model with the best data fit, it was not significant (LME, $\chi^2 = 4.4$, df = 2, $p = 0.112$, Fig. S6).

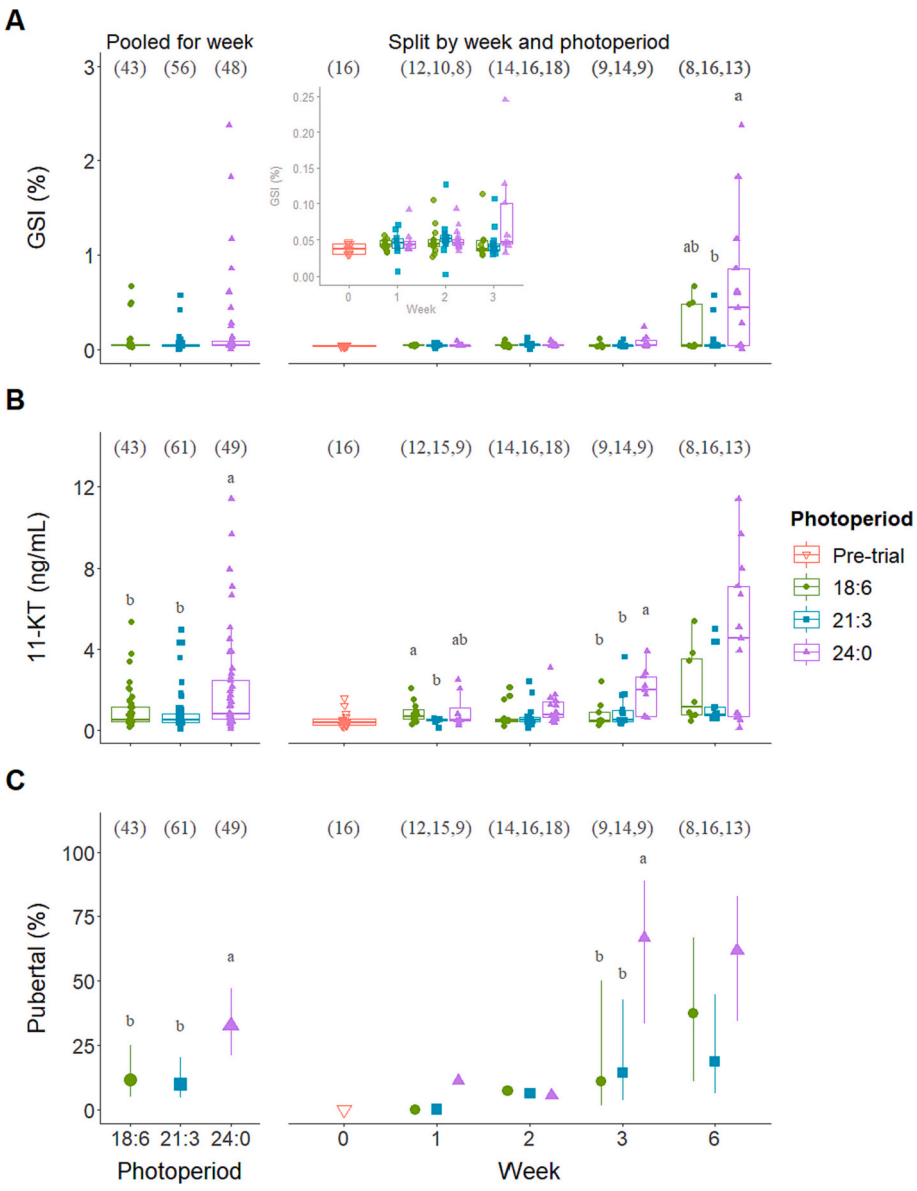

Fig. 2. The incidence of puberty pooled or split over time in response to the daylength (photoperiod) after a winter signal. The (A) gonadosomatic index (GSI), (B) plasma 11-ketotestosterone (11-KT), and (C) incidence of pubertal fish. In (AB), all datapoints are presented together with a boxplot identifying the medians (central line), 25th and 75th percentiles (box margins), and the largest and smallest values within ×1.5 the 75th and 25th interquartile ranges (arms). In (C), the data are means ±95% CI. Note the incidence of puberty was too low to statistically analyse weeks 0–2, therefore only the means are presented. Different lowercase letters indicate significant photoperiod effects ([AB] Dunn's post-hoc, [C] LS means, $p < 0.05$) either within pooled data (left side), or within week (right side). The number in brackets shows the *n* for (left side) each photoperiod in total or (right side) each photoperiod within week. (A) contains a magnified figure of the data from weeks 0–3.

3.3. Body size and growth

There was no effect of sex on body weight among immature fish at either week 0 or week 6, but those fish which became pubertal were significantly larger than those that remained immature at both time points (Table 1). Pubertal males also displayed significantly higher growth rates throughout the experiment compared to immature fish, and had significantly higher body condition at week 6, but not at week 0. Although there was a slight positive trend between SGR and daylength, the effect was not significant in immature fish (LME; $\chi^2 = 5$, df = 2, $p = 0.098$).

3.4. Gill NKA activity

When all the data was pooled for maturity status and photoperiod, there was a significant effect of time (GLM; $\chi^2 = 37$, df = 3, $p < 0.001$)

Table 1
Body weight, condition, and specific growth rate (SGR) in Atlantic salmon at the beginning (week 0, 11th February) and end (week 6, 31st March) of the study. Data are pooled for photoperiod. Results from linear mixed effect models are included. Different subscript letters within a row indicate a significant (post-hoc; least square means, $p < 0.01$) group effect within week. Data are means (95% CI, n = 27–184/phenotype at week 0 and 14–38/phenotype at week 6).

Parameter	Week	Immature		Pubertal males	Model results		
		Females	Males		χ^2	df	p
Weight (g)	0	113 (106–120)^b	112 (103–121)^b	127 (108–145)^a	11	2	0.005
	6	196 (163–229)^b	186 (141–231)^b	279 (224–333)^a	40	2	<0.001
Condition (*K* factor)	0	1.20 (1.17–1.23)	1.19 (1.16–1.23)	1.20 (1.13–1.26)	1	2	0.675
	6	1.17 (1.09–1.24)^b	1.15 (1.05–1.25)^b	1.40 (1.28–1.51)^a	62	2	<0.001
SGR (%/day)	6	2.04 (1.54–2.53)^b	1.91 (1.28–2.54)^b	3.03 (2.28–3.78)^a	34	2	<0.001

with a significant increase in NKA activity between weeks 0 and 3 (from a mean value of 1.7 to 3.3 μmol ADP/mg protein/h), before a decline from weeks 3 to 6 (Fig. 3A). The initial model comparison including pubertal males provided no support for an effect of photoperiod on NKA (Table S4, Fig. 3B). When removing the pubertal males from the analysis, there was a significant effect of time with week 1 being lower than weeks 2, 3 and 6, but again the best model suggested no effect of photoperiod on NKA over time (Table S5, Fig. S7).

There was a trend for pubertal males to have lower NKA activity at week 6, which was significant based on an analysis of that time point only (LME; $\chi^2 = 4$, df = 1, $p = 0.049$). However, we found no general effect of puberty or an interaction with time when data from week 3 was included (Table S6, Fig. 3C).

4. Discussion

A short scotophase reduced unwanted male maturation during smoltification. Pubertal males also showed an earlier reduction in gill NKA enzyme activity, which may have implications on their performance when transitioning to seawater. This improves our understanding of the environmental factors that trigger puberty and has important implications on their manipulation during salmon farming.

As found in Fjelldal et al. (2011), a short scotophase after the winter zeitgeber reduced the likelihood of triggering post-smolt maturation in males. Here, we show this scotophase can be at least as short as 3 h. As we only kept the fish on these conditions for six weeks it is unclear if the maturation rates would even out over time if the fish had been kept on their respective photoperiods for longer. However, this was not the case in a long-term study in a RAS facility in China. In Oriental Ocean Company (China) salmon that were initially 850 g in size, approx. 48%, 20% and 10% of females and 36%, 15%, and 10% of males, had reached advanced stages of maturation when reared for 6 months from August on 16 °C and either LD 24:0, 12:12, or 8:16, respectively, after initially been on a natural photoperiod (Qui et al., 2015). Therefore, the current work and that of Fjelldal et al. (2011) and Qui et al. (2015) all suggest longer daylengths can promote maturation when combined with high temperature.

We found some mature males in the 18:6 treatment which was not the case in Fjelldal et al. (2011). We also found the 24:0 treatment had 62% male maturation at the final timepoint compared to only 47% in Fjelldal et al. (2011). This could be due to the use of 120 g parr in the current work compared to 83 g in Fjelldal et al. (2011). Current theory is

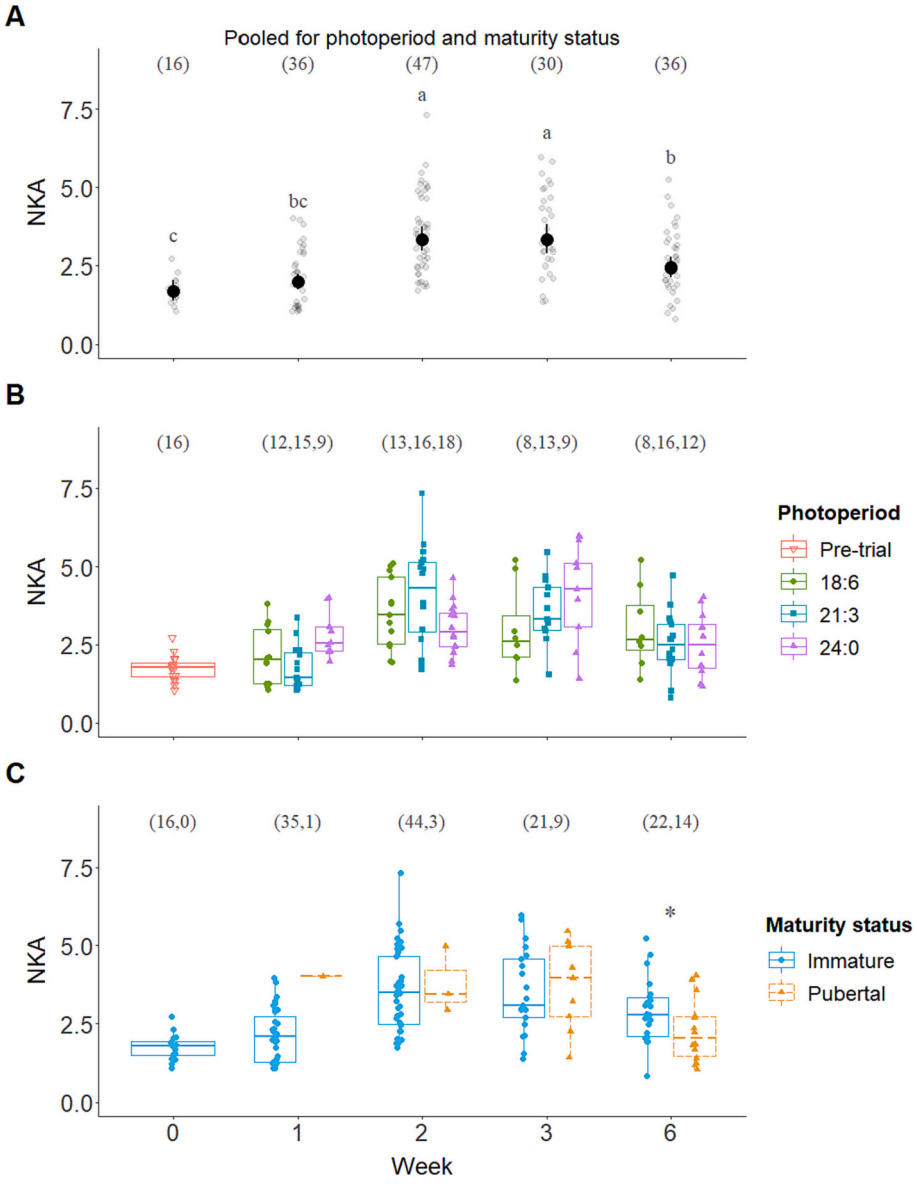

Fig. 3. Gill Na^+/K^+-ATPase (NKA, μmol ADP/mg protein/h) enzyme activity over time. (A) Predicted means ±95% CI with the raw datapoints overlaid from a linear mixed effect (LME) model. (B) Raw data split by photoperiod over time. (C) Raw data split by maturity status over time. In (A), different lowercase letters indicate significant week effects (Post-hoc, least-square means, $p < 0.05$) whereas in (C) the asterisk represents a significant effect of maturation status within week (LME, $p = 0.036$). Boxplots show the medians (central line), 25th and 75th percentiles (box margins), and the largest and smallest values within ×1.5 the 75th and 25th interquartile ranges (arms). The numbers in brackets are the *n* for (A) each week or (B, C) the different groups within week.

that a developmental threshold must be reached, possibly linked to energy reserves (Rowe and Thorpe, 1990a), growth rates (Jonsson and Jonsson, 2007), and/or body size (McClure et al., 2007), prior to initiating puberty. We found evidence for this in that those which became pubertal were on average 13% heavier at the start of the experiment than those that remained immature, but there was considerable overlap. Whether this is a cause or effect of deciding to mature is unknown. In contrast, body condition was not predictive in terms of identifying those fish that would go on to enter puberty at the start of the study. However, it was rapidly elevated once the decision to mature had been made as found previously (Rowe and Thorpe, 1990b; Fraser et al., 2019; Fjelldal et al., 2020). This could be because body size is a better indicator of stored energy than proximate composition (Shearer et al., 2006). In addition, our experience from developing models to induce post-smolt maturation via a square-wave photoperiod (>12 h day to 12:12 to 24:0) is that the incidence is increased when using larger fish (Fjelldal et al., 2018). Indeed, 98% male maturation was achieved when introducing approx. 300 g post-smolts to a square-wave photoperiod at 16 °C (Fjelldal et al., 2022).

Zeitgebers regulate the timing of spawning in salmonids and are manipulated to inhibit parr maturation (Skilbrei and Heino, 2011) and grilsing (Taranger et al., 1998). To inhibit parr maturation, salmon can be kept on continuous light until they have reached the size threshold for smoltification, which is then prioritised over maturation in the spring (Duston, 1994). To inhibit grilsing, salmon can be exposed to continuous light during the preceding winter (Taranger et al., 1998). This may ensure the decision to mature coincides with poor growing conditions (Adams and Thorpe, 1989; Rowe and Thorpe, 1990a) meaning these fish may not attain the size/growth threshold required for maturation (Thorpe et al., 1990). However, in our study constant light promotes maturation. Randall et al. (1998) also demonstrated that continuous light could advance or delay maturation timing in rainbow trout depending on the season in which it was introduced. This is expected to be due to the existence of an endogenous rhythm regulating the timing of maturation which is entrained by photoperiod cues (Randall et al., 1998). Alternatively, temperature regulates the size at first maturation in fishes (Kuparinen et al., 2011), the physiological response to changes in day length (McCormick et al., 2000), and the biological systems that integrate the photoperiod signal into the central nervous system (photoreceptors and melatonin) (Nisembaum et al., 2015). Therefore, it is unknown how our use of 16 °C (a relatively high temperature for salmon) may be affecting the integration of the photoperiod signal into the brain-pituitary-gonad (BPG) axis which regulates the timing of maturation (Taranger et al., 2010).

How photoperiod controls the incidence of post-smolt maturation following continuous light vs short day is unclear. Thorpe (2007) proposed that puberty is regulated by inhibition as the decision is based on biochemical conditions (e.g. size, growth rate, and/or energy stores) during a critical window in the spring. In other words, a mechanism exists that prevents maturation outside of the window even if the growth or size thresholds are met. As there was no photoperiod effect on body size, condition, or growth, we assume our treatments had no impact on biochemical factors. Alternatively, photoperiod is known to regulate both the "time-keeping" hormone melatonin (Randall et al., 1995) and pituitary thyroid stimulating hormone (Irachi et al., 2021) in salmonids. Both are involved in triggering a hormonal cascade along the BPG axis leading to seasonal testis development in mammals (Ono et al., 2008) and would be an interesting avenue of future research.

Daylength had no effect on gill NKA enzyme activity. We are unaware of any studies that have identified a minimal constant daylength and/or increase in daylength, required to initiate smoltification after the winter zeitgeber in a square-wave regime. The current data would suggest 18:6, 21:3 and/or an increase of ≥6 h, was sufficient. It may also be that, as with the timing of ovulation/spermiation in rainbow trout (Randal et al., 1998), the direction of change is more relevant than the actual difference in daylength although further larger studies using more natural conditions (i.e. not 16 °C) are recommended. The NKA values we attained were relatively low but match values from other recent studies during smoltification in our lab (de Fonseka et al., 2022) and elsewhere (Bernard et al., 2020; van Rijn et al., 2020). It is unclear why such low values are reported but could be related to the use of high water temperature (Bernard et al., 2020, but see Fjelldal et al., 2018 who also used 16 °C and attained NKA values ~10 after using a square-wave photoperiod). It is accepted that we did not complete seawater challenges for a more complete analysis. However, we regularly use continuous light at 16 °C to produce smolts that transition to seawater without mortalities or stunting (e.g. Fjelldal et al., 2011, 2018; Melo et al., 2014; Fraser et al., 2020).

We found tentative evidence of impaired hypo-osmoregulatory development in pubertal males as they had lower values than immatures at week six. This could support the idea that smoltification and maturation are in developmental conflict. However, when using 16 °C, peak smolt is expected after 22 days (week 3), based on the idea that it is reached 350° days after the initiation of the spring zeitgeber (Handeland et al., 2004), but there was no effect of puberty at this time. Nevertheless, the earlier decline in gill NKA in pubertal fish may be linked to elevations in sex steroid hormones (Lundqvist et al., 1989; Hirano et al., 1990) and/or early initiation of "desmoltification" (Handeland et al., 2004), as mature fish will need to return to the river to spawn. Alternatively, these fish may not migrate all the way out to sea, but instead remain closer to land where salinity levels are lower. Therefore, they may not need to develop the same level of hypo-osmoregulatory as one or more sea-winter fish. Indeed, wild Atlantic salmon populations that mature during their first season "at sea" (as post-smolts) are found in North America and are referred to as "estuarine" salmon (Klemetsen et al., 2003).

The size of the fish used in the current study may reduce the likelihood of detecting a conflict between maturation and smoltification. Smoltification allows salmon to transition to seawater at small sizes with few perturbations to growth and survival. However, pre-elevated gill NKA is not a pre-requisite for parr to survive in seawater. For instance, just over 50% of 26 g parr survived for 4 months in 31 ppt salinity albeit with stunted growth (Duston, 1994). Nevertheless, body size is positively associated with growth and survival following seawater transition (Duston, 1994). The size at which salmon do not need to be in the smolt window to survive and grow in seawater is not well established. No negative effect on survival, albeit a small transient reduction in growth, was reported in approx. 200 g salmon transferred directly to seawater without receiving a winter zeitgeber to induce smoltification (Ytrestøyl et al., 2022). Imsland et al. (2014) also observed a peak in gill NKA in fish kept on continuous light from approx. 16 g when they reached 200 g. As our pubertal fish were already >200 g at peak smolt, it is likely they could rapidly adapt to seawater due to their body size alone.

In conclusion, using only three hours of darkness reduces the incidence of male puberty during smoltification compared to continuous light. The mechanism remains unclear, but from a management perspective, using shorter days to produce large smolts reduces the problem of post-smolt maturation.

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CRediT authorship contribution statement

Thomas W.K. Fraser: Investigation, Data curation, Formal analysis, Writing – original draft. **Tom J. Hansen:** Funding acquisition, Conceptualization, Resources, Investigation, Writing – review & editing. **Birgitta Norberg:** Investigation, Writing – review & editing. **Tom Ole Nilsen:** Resources, Investigation, Writing – review & editing. **Rüdiger W. Schulz:** Investigation, Writing – review & editing. **Per Gunnar**

Fjelldal: Funding acquisition, Conceptualization, Resources, Investigation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data is attached as an excel file in the supplementary material.

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Appendix A. Supplementary data

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