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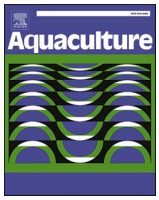

Interaction of temperature and feed ration on male postsmolt maturation of Atlantic salmon (*Salmo salar* L.)

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ABSTRACT

Early maturation of Atlantic salmon male postsmolts is undesirable in aquaculture due to its negative impact on growth, welfare and seawater adaptation, however it is an increasing problem under intensive rearing conditions. The effect of temperature and feeding ration on sexual maturation during the induction of smoltification was tested with a 3 × 2 factorial design including three temperatures (8, 12.5 and 18 °C) and two feeding rations (100% and 67%), from 28 October 2018 to 30 May 2019. Fish in the six resulting treatments (1800 parr, initial mean weight 23.1 ± 7.2 g) were reared in flow-through under continuous light (LD24:0) except for a 5-week winter signal (LD12:12) introduced on 4 February 2019 to induce smoltification. A major effect of temperature determining the life history pattern of each group was observed. At 18 °C, a majority of the males were maturing in May under both rations (100% males in 18–100% and 88.9% males in 18–67%). Fish at 18 °C experienced an early activation of the BPG (Brain-Pituitary-Gonad) axis and displayed very poor signs of smoltification. At 12.5 °C, early maturation was also present, although at lower proportion than at 18 °C and more dependent upon feed ration (40% of males maturing in 12.5–100%, 8.6% in 12.5–67%). Salmon at 12.5 °C displayed the best growth within each feed regime, some early signs of smolting but subsequent low Na^+, K^+-ATPase activity after the winter signal. At 8 °C, salmon showed best signs of smoltification and lowest growth, while no males matured. The feed restriction had a minor effect on maturation at 18 °C but a greater impact at 12.5 °C, evidenced by significant differences between 12.5–100% and 12.5–67% in gonadosomatic index, plasma 11-ketotestosterone, insulin-like growth factor-I, and pituitary *fshb* (follicle stimulating hormone β-subunit gene) transcription. Onset of maturation was associated with the photoperiod change from short to long daylength, which probably also induced a slight activation of the BPG axis in non-maturing groups. Our results suggest that postsmolt producers must be cautious when using sustained high water temperatures, or the risk of early maturation and poor hypo-osmoregulatory performance will increase significantly. In addition, reducing the feeding ration will not help decrease incidence of maturation without significantly affecting growth.

1. Introduction

Early maturation of male Atlantic salmon postsmolts or "jacking" has become a growing concern in aquaculture due to its increasing occurrence under intensive rearing conditions (Fjelldal et al., 2020; Fraser et al., 2019; Good and Davidson, 2016). Other salmon early maturing phenotypes, like 0+ parr or 1 sea-winter grilse, have also been considered a challenge in the sector for many years (Berrill et al., 2003; Davidson et al., 2016; Maugars and Schmitz, 2008; McClure et al., 2007; Rowe et al., 1991; Skilbrei and Heino, 2011). In all cases, sexual maturation is initiated with the activation of the Brain-Pituitary-Gonad (BPG) axis (Schulz et al., 2010; Taranger et al., 2010) in response to several environmental and internal factors (Good and Davidson, 2016). This activation is characterized by increases in gonadotropin production in the pituitary, first of Fsh (Follicle-stimulating hormone) and later of Lh (Luteinizing hormone), as well as by increases in sex steroids like 11-

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KT (11-ketotestoterone) (Maugars and Schmitz, 2008; Melo et al., 2014; Schulz et al., 2010). Other hormones related with the growth axis such as Igf-I (Insulin-growth factor-I) also seem to stimulate the activation of the BPG axis (Baker et al., 2000) and to support 11-KT production during maturation (Campbell et al., 2003; Le Gac et al., 1996). Together, gonadotropins, steroids and Igf-I regulate the process of testis maturation or spermatogenesis (Schulz et al., 2010). Early maturation can seriously impact the economy of production and fish welfare, due to the associated reduction in growth rates (Fraser et al., 2019; Taranger et al., 2010; Thorpe, 2007), negative effects on immunity (Harris and Bird, 2000; Hou et al., 1999; Taranger et al., 2010) and on hypo-osmoregulatory abilities (Lundqvist et al., 1989; McCormick et al., 2005; Shrimpton, 2013).

In nature, the occurrence of early maturation depends on the assessment of genetically determined size/growth/energy thresholds during specific time windows defined by photoperiod cues (Bromage et al., 2001; Thorpe, 2007; Thorpe, 1994; Thorpe et al., 1998). Since salmon capacity for growth in nature is limited by seasonal variations in temperature, light and access to feed among others, only a percentage of males normally undergo maturation early (Thorpe, 1994). In contrast, in intensive aquaculture facilities, growth of juvenile salmon is maximized by exposure to continuous light from first feeding, constant high temperature, and unlimited access to high energy feed. These conditions represent a highly stimulatory environment for growth and development, allowing salmon to mature earlier. The only photoperiodic cue that salmon under those conditions experience is a "winter signal" (some weeks under reduced photoperiod LD12:12) introduced to induce smoltification (Björnsson et al., 2000; Fjelldal et al., 2011). It has been shown that fast-growing male salmon reared in that type of stimulatory environments and subjected to winter signals or equivalent photoperiod cues, can have a strong tendency to mature early during or after smoltification (Fjelldal et al., 2018; Fjelldal et al., 2011). Maturation and smoltification, although generally considered in developmental conflict (Good and Davidson, 2016; Thorpe, 1994b), have been observed simultaneously in response to the same photoperiod cue in salmon reared under intensive conditions (Fjelldal et al., 2018, Fjelldal et al., 2011).

Studies using intensive rearing environments and feeding to satiation have reported high proportion of postsmolt maturation. For example, Imsland et al. (2014) observed highest growth and incidence of male maturation (82%) in postsmolts reared at 12.7 °C and continuous light, in contrast to those reared at 8.3 °C and simulated natural photoperiod (8% maturation). Fjelldal et al. (2011) subjected parr to three temperatures (5, 10 and 16 °C) and two photoperiod regimes (either LD12:12 followed by LD18:6, or LD12:12 followed by LD24:0), observing early maturation (47%) only in males at 16 °C that experienced an increase in daylength from LD12:12 to LD24:0. They also reported that maturing postsmolts developed seawater tolerance, indicating a simultaneous onset of smoltification and maturation. Other studies have used "maturation regimes", consisting of increasing temperature up to 16 °C and daylength to LD24:0 after the winter signal (or natural photoperiod), maintained for 2–3 months (Fjelldal et al., 2018; Melo et al., 2014). These two studies reported high proportion of maturing males (~90% and 70–90% respectively), evidencing a link between high temperature, increasing daylength and high maturation. In addition, Fjelldal et al. (2018) concluded that smoltification and maturation commenced simultaneously after observing, in immature and maturing postsmolts, similar increased gill Na^+, K^+-ATPase activity (NKA) which is a standard smoltification indicator (Björnsson et al., 2011; McCormick et al., 1995; Stefansson et al., 2007).

The link between early puberty and growth has been explored by using ration as an experimental factor (Berglund, 1995; Berglund, 1992; Herbinger and Friars, 1992; Rowe et al., 1991), or by assessing variables concomitant with diet such as size, energy stores, or condition factor (Kadri et al., 1996; Rowe et al., 1991; Simpson, 1992). These studies have generally reported higher percentages of maturation in fast-growing groups or those fed high-energy diets. More recently, Trombley et al. (2014) subjected parr kept under natural photoperiod and temperature to 100% and 50% feed rations. Restricted feeding resulted in lower somatic growth, proportion of male maturation (62% vs 29% respectively), gonadosomatic index and gonadotropin transcription. Similarly, Crespo et al. (2019) provided either a 43% or a 100% ration to larger salmon (initial body weight 1750 ± 42.4 g) kept in sea cages under ambient light and temperature for several months. The group fed a 43% ration displayed lower body weight, condition factor, and incidence of grilse maturation. However, in both studies maturing males were still present at remarkable proportions in the restricted feeding groups, suggesting that caloric restriction might be unsuitable to control early male maturation (Crespo et al., 2019).

According to the studies reviewed, the importance of both temperature and feed regime for early maturation seems clear. However, we are not aware of previous studies specifically investigating postsmolt maturation in response to different combinations of temperatures and rations. Thus, the present study was designed with the intention to induce diverting life-history strategies that would help reveal differences in maturation-associated endocrine processes in response to intensive rearing conditions. High temperature can induce maturation (Fjelldal et al., 2011; Imsland et al., 2014; Melo et al., 2014), as well as impair smoltification (McCormick et al., 1999; Stefansson et al., 2008). Considering this, temperatures were selected aiming to induce a clear tendency to sexually mature (18 °C), to smoltify (8 °C), and to include a standard temperature used in Norwegian intensive aquaculture (12.5 °C). In regard to feed rations, a 100% ration was provided as a standard used in industry. The 67% ration was selected to introduce a caloric restriction that may reduce proportion of maturation, but minimizing the impact on growth observed with more severe restrictions (Crespo et al., 2019; Trombley et al., 2014). Various morphometric, endocrine and physiological markers were used to assess onset, development and interaction of maturation and smoltification.

2. Materials and methods

2.1. Ethic statement

The authors confirm that ethical policies of the journal, as per the journal's author guidelines page, have been adhered to. The study was approved by the local representative of Animal Welfare at the Department of Biological Sciences, University of Bergen, Norway (FOTS application ID7183), and samplings were performed as established by the Norwegian Animal Research Authority.

2.2. Experimental setup

This experiment was carried out from October 2018 to May 2019 in a flow-through system at the Department of Biological Sciences (BIO, Bergen, Norway). A 3 × 2 factorial design was used, combining three water temperatures (8, 12.5 and 18 °C) and two feeding rations (100% and 67%), producing six experimental groups (Fig. 1) reared in duplicate in freshwater.

On 27 September 2018, 1800 Atlantic salmon parr (mean weight 23.1 ± 7.2 g, mean condition factor 1.11 ± 0.07) of Salmobreed strain (Erfjord Stamfisk AS) were transferred from a commercial Recirculation Aquaculture System (RAS) (Bremnes Seashore AS, Trovåg, Rogaland, Norway) and randomly distributed among 12 tanks of 0.5 m^3 ($n = 150$ fish/tank). This batch had hatched in mid-April 2018, and from first feeding (mid-May 2018) had been kept at 15.5 ± 2.6 °C and LD24:0. After transfer, fish were acclimated for one week at 12.5 °C, specific flow rate 0.5 $L.kg^{-1}.min^{-1}$, 100% feed ration and LD24:0 photoperiod. Temperatures were then gradually adjusted in the respective treatments from 12.5 °C to 8 °C in two days, and to 18 °C in three days. The feed ration was reduced in six tanks (2 replicates of each temperature) to 67% by introducing a fasting every third day, while the remaining six tanks

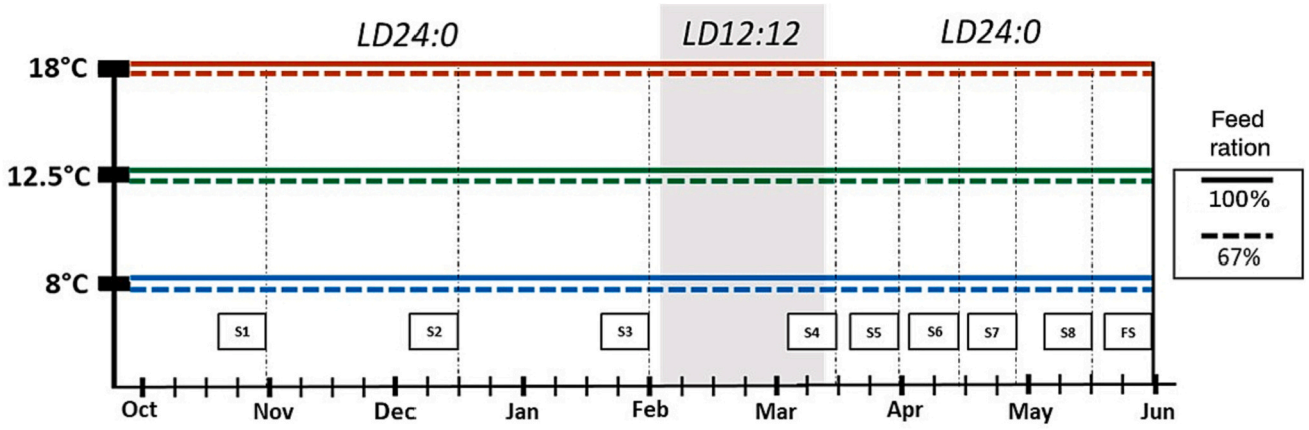

Fig. 1. Diagram displaying experimental conditions for each of the six groups, photoperiods applied and timing of the samplings performed. The experiment started in October 2018 when fish mean weight was 23.1 ± 7.2 g, and finished on 30 May 2019 when we assessed final percentage of male maturity in each of the groups. The six experimental groups were run in duplicate. Samplings carried out are serially labeled from S1 until the final sampling (FS) in late May. In such final sampling, only percentage of maturation was assessed.

were maintained at a 100% ration. To calculate the feed rations, we used data on Specific Growth Rate (%) per day for different body weights and temperatures published by Skretting, mean body weight and biomass in the tanks. Feed rations were re-adjusted for each tank after all samplings. Fish were fed Biomar CPK 40® (23–26% fat, 45–48% protein) and CPK 100® (24–27% fat, 43–46% protein) pellets, provided by Bremnes Seashore AS. Feed was supplied over a 24 h cycle using conveyor-belt feeders working continuously. LD24:0 photoperiod was maintained to ensure maximum growth until 4 February 2019, when photoperiod was changed to LD12:12 to provide a winter signal to induce smoltification. Daily dawn and dusk consisted on 30 min of increasing or decreasing light that was equally detracted from the dark and light periods. During the winter signal, feeding time was reduced to 12 h, starting 15 min after dawn. After 5 weeks in LD12:12, on 11 March all groups were returned to LD24:0 until the end of the experiment on 30 May 2019.

2.3. Sampling regime

Eight main samplings were performed, plus a final sampling on 30 May 2019 in which we only assessed final proportion of male maturation in the remaining individuals. At each of the eight samplings, at least $n = 6$ males per tank ($n = 12$ per experimental group) were collected. Fish were euthanized with an overdose of benzocaine by bath (>50 mg/L Benzoak vet.® 20%, ACD Pharma AS, Norway). Blood was collected from the caudal vein with heparinized syringes, and centrifuged for 4 min at 5000 rpm. Plasma was divided in two aliquots for Igf-I and 11-KT analyses, and immediately frozen on dry ice. Fork length and body weight were measured to the nearest 0.1 cm and 0.1 g respectively. Gonads were examined to determine gender and degree of maturation, keeping only the males. Testes were excised and weighed to the nearest 0.001 g. The first gill arch from the right side was frozen in SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) for subsequent measurement of Na^+, K^+, ATPase activity (NKA). Finally, the pituitary gland was excised, placed in RNAlater® and incubated overnight at 4 °C. All samples were stored at −80 °C until analysis. Condition factor (K) was calculated as $K = 100 \times W/L^3$ where W is fish weight in g and L fork length in cm. Gonadosomatic index was calculated as GSI (%) $= W_{gonad} \times 100/W_{body}$ (both in g) and used as an index of maturity status. A subsample of 408 fish were randomly selected for 11-KT and gene expression analyses, that included at least 6 individuals (for the first three samplings) and 10 individuals (for each of the five remaining samplings) per experimental group and sampling. Igf-I and NKA activity analyses were performed for the last six samplings. Morphometric analyses (body weight, condition factor, GSI) were performed on all individuals from the eight main samplings.

2.4. Lab analyses

2.4.1. Gene transcription analyses of pituitary fshb and lhb

Real-time PCR (RT-PCR) was used to analyze transcription of the genes *fshb and lhb*, responsible for production of the beta subunits of the two gonadotropins Fsh and Lh. Pituitary total RNA was extracted with the RNeasy® Plus Micro Kit (Quiagen, Germany), using 2-mercaptoethanol (Bio-Rad, USA) according to manufacturer's instructions. Total RNA concentration was measured in a Qubit® 3.0 Fluorometer with the Qubit® RNA BR (Broad Range) Assay Kit (Invitrogen™, ThermoFisher Scientific Inc., USA). RNA purity was checked with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and confirmed by 260/280 and 260/230 ratios above 1.8. Total RNA (150 ng) was reverse-transcribed to cDNA using Superscript™ III Reverse Transcriptase and Oligo(dT)20 Primer (Invitrogen™, ThermoFisher Scientific Inc., USA) as per manufacturer's instructions. Gene transcription was analyzed in a Bio-Rad CFX96 Touch Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA, USA) using iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA) in a total reaction volume of 12.5 μL per well, including 2.5 μL of 1:40 diluted cDNA and 0.2 μM of each primer. The RT-PCR protocol consisted of 3 min at 95 °C followed by 35 cycles at 95 °C for 15 s and 60 °C for 1 min. Samples were run in duplicate with the oligos listed in Table 1. Duplicates with a CV > 1.5% were not considered for analysis. Before RT-PCR, a standard curve was built for each pair of primers to calculate primer efficiency, using seven 2-fold dilutions obtained from pooled samples. The efficiency (E) was estimated with the formula $E = 10^{(-1/slope)}$, obtaining the slope from the plot of log (RNA concentration of the pool) versus threshold cycle (Ct) values. Specificity of the amplicon was confirmed with a melting curve. After RT-PCR, relative transcription of the genes was calculated with the efficiency-corrected method using *ef1a* as reference gene (Pfaffl, 2001).

2.4.2. Plasma 11-ketotestosterone analysis

Sex steroids were extracted from 100 μL plasma in two steps with ethyl acetate and subsequently dissolved in 1 mL buffer (0.1 M phosphate, 0.4 M NaCl, 1 mM EDTA), following a methodology modified from Pankhurst and Carragher (1992). Extracted samples were stored at −20 °C until analysis by enzyme-linked immunosorbent assay (ELISA) (Cuisset et al., 1994), validated for Atlantic salmon (Vikingstad et al., 2016). ED80 and ED20 standard values were 0.04 $ng.mL^{-1}$ and 1.00 $ng.mL^{-1}$ respectively, while the detection limit of the assay was 0.005 ng.

Table 1
Oligo sequences used for RT-PCR.

Gene	Primer	Sequence (5′ > 3′)	GenBank accession number	Reference	Efficiency
fshb	ss_fshb-F	GCGAAACGACGGATCTGAA	–	Maugars and Schmitz, 2008	96.6%
	ss_fshb-R	GGCAACGGGTATGAAGAAGG			
lhb	ss_lhb-F	CCCAACGTGCTTAGTCATTC	NM_001173671.1	Pino Martinez et al., 2021	95.4%
	ss_lhb-R	AAACCGGCTCCTTGGTG			
ef1a	ss_ef1α-F	CCCCTCCAGGACGTTTACAAA	AF321836	Olsvik et al., 2005	100.0%
	ss_ef1α-R	CACACGGCCCACAGGTACA			

mL^{-1}. Samples with concentrations above the highest values of the standard curve were diluted and re-run until they fitted within the straight line. Internal standards for 11-KT were prepared from mature male Atlantic salmon. Individual samples with an interassay CV > 10% were repeated. Acetylcholine esterase-labeled tracers and microplates precoated with monoclonal mouse antirabbit IgG were obtained from Cayman Chemicals (USA), while standard steroids were purchased from Sigma Aldrich®.

2.4.3. Plasma insulin-like growth factor-I analysis

Plasma Igf-I concentration was quantified by time-resolved fluoroimmunoassay (TR-FIA) as in Small and Peterson (2005), using recombinant salmon/trout Igf-I (GroPep Bioreagents Pty Ltd., Adelaide, Australia) as a standard. Plasma (10 μL) was first extracted with an acid-ethanol extraction (Shimizu et al., 2000). Igf-I from the extraction was bound with rabbit anti-barramundi Igf-I (GroPep), which was bound in a goat anti-rabbit IgG pre-coated plate (DELFIA® yellow plate; PerkinElmer, Turku, Finland). Time-resolved fluorescence was measured in a Tecan Spark® multimode microplate reader (Tecan Group Ltd., Switzerland) at 340 nm for excitation and at 615 nm for reading. ED80 and ED20 standard values were 2.08 $ng.mL^{-1}$ and 0.29 $ng.mL^{-1}$ respectively, while the detection limit of the assay was 0.06 $ng.mL^{-1}$. Internal standards for Igf-I were prepared from pooled plasma of Atlantic salmon. Parallelism between Atlantic salmon plasma and a standard has been previously confirmed for this method (Balseiro et al., 2018; Hevrøy et al., 2015). In addition, TR-FIA for Igf-I has showed similar sensitivity to an ELISA assay (FIA) for Igf-I determination (Andoh, 2005).

2.4.4. Na^+, K^+, ATPase activity in gills

Na^+, K^+, ATPase activity was measured as described by McCormick (1993). NKA activity was estimated in an ouabain-sensitive protein fraction obtained from gills based on the hydrolysis of ATP to ADP, a reaction that is enzymatically linked to the oxidation of NADH to NAD^+ by pyruvate kinase and lactic dehydrogenase. This reaction was performed with and without ouabain, a specific NKA inhibitor. The difference in ATP hydrolysis was measured for 10 min at 25 °C and 340 nm in a Tecan Spark® multimode microplate reader, and expressed as μmol ADP × mg $protein^{-1}$ × $hour^{-1}$.

2.5. Statistical analyses

Fisher's Exact Test for count data was used to compare the incidence of male maturation between temperatures, feed rations and between pooled replicate tanks of each treatment at each sampling point. For statistical models, distribution of response variables was initially checked for normality with Shapiro-Wilk's test, and outliers explored using boxplots. Generalized Linear Mixed Models (GLMM) or Linear Mixed Effects models (LME) were fitted between response variables and the predictors "temperature", "feed regime", "time" and their two-way interactions as fixed effects, and "tank" as random effect. When the random variance caused by tank replicate was below 5% of the total model variance, and depending on the distribution of the response variable, Generalized Linear Models (GLM) with Gamma distribution and "log" link, or three-way ANOVA with best-fitting transformation of the response variable, were applied. When necessary, model residuals were checked graphically to assess model assumptions such as normality (with q-q plots), non-linear patterns (residuals vs fitted plots), homogeneity of variance (scale-location plots) or influential outliers (residuals vs Leverage plots with Cook's distance). Homogeneity of variance was also checked with Levene's test. For each response, the mixed model used, random tank variance, final model fitted, distribution family and link or transformation applied, are detailed in supplementary Table 1. Significant models were followed by Tukey HSD post-hoc tests to find significant differences in the response variable at each temperature, feeding regime and time point. All plots of response variables over time for the different temperatures and feed regimes represent mean ± standard error of mean. A significance level $\alpha = 0.05$ was used. All statistical analyses were performed in R and Rstudio, using the packages "car" (Fox and Weisberg, 2019), "ggplot2" (Wickham, 2016), "ggpubr" (Kassambara, 2017), "Rmisc" (Hope, 2013), "emmeans" (Lenth et al., 2018), "lme4" (Bates et al., 2015) and "nlme" (Pinheiro et al., 2017).

The GSI threshold defining onset of maturation was set at GSI > 0.06%. In the past, different GSI values have been used as cut-off for onset of maturation. For example, Peterson and Harmon (2005) concluded that a GSI > 0.3% was indicative of early maturation in grilse, while Good et al. (2017) used GSI > 0.1% as threshold value for post-smolts based on experience and visual inspection of gonads. Recent studies have reduced the GSI cut-off value for maturation by comparing GSI with other indicators of sexual development. For example, Melo et al. (2014) stated that GSI > 0.05% was indicative of sexual maturation in postsmolts based on gonadotropin expression and plasma androgen levels, while Ciani et al. (2021) concluded on a similar threshold value for parr (GSI > 0.05%) based on evidence of testis development after histological inspection. Based on this literature, on visual inspection of maturing and immature testes, and on distribution and outliers of the variable GSI (Fig. 2), we decided to use GSI > 0.06% as indicative of engagement in sexual maturation. Ilustration of various males found at different stages of maturation is presented in Fig. 3A.

3. Results

3.1. Proportion of male maturation

Fig. 4 displays the percentage of maturing males (GSI > 0.06%) over time in the six experimental groups. Maturation was first observed in April, therefore only results from late March onwards are presented. Incidence of maturation was dependent upon temperature and feed regime (both $p < 0.001$). Significant differences in proportion of maturing males between treatments occurred from early April (see Fig. 4), with higher percentage in 18–100% (78.6%) and 18–67% (47.7%) than in the rest (all $p < 0.05$). From April onwards, the percentage of maturing males continued to increase at 18 °C up to almost 100% of the individuals at the end of the experiment in late May, independent of ration. Sexual maturation was also present at 12.5 °C, although at lower prevalence and delayed in time with respect to both 18 °C groups. At this temperature, the effects of the feed restriction were evident, with lower incidence in 12.5–67% (8.6% males) and a delay in sexual maturation at the end of the experiment compared to the group

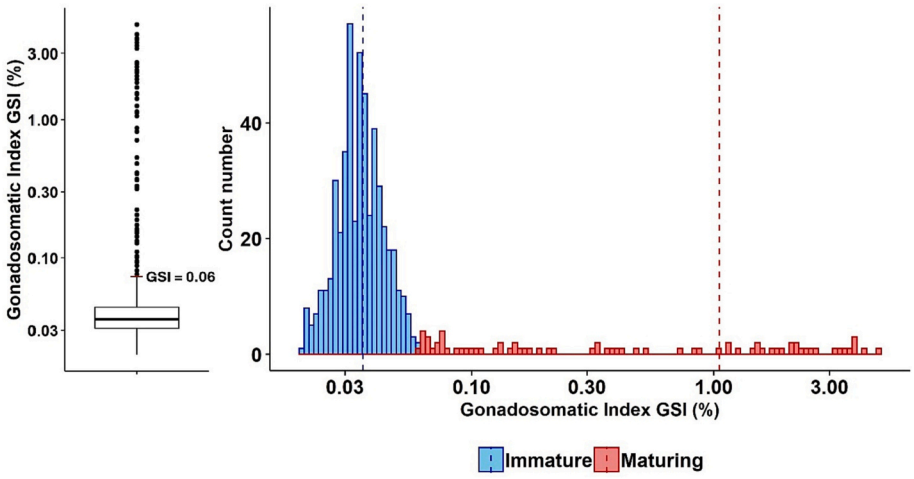

Fig. 2. Boxplot and histogram of the variable GSI in our experiment, indicating the threshold value used to differentiate between immature and maturing males. The dashed lines in the histogram represent the mean GSI value of each maturity category. Note that GSI in the "Y" axis of the boxplot and the "X" axis of the histogram are displayed in logarithmic scale. In immature male salmon, GSI is normally distributed, and as maturation proceeds, GSI starts increasing and this distribution changes from normal to highly right-skewed. Boxplot and histogram showed that values of GSI up to 0.06% followed a normal distribution with mean ± sd of 0.036 ± 0.008% (those considered immature fish), while values over 0.06% appeared as outliers (fish that were considered maturing).

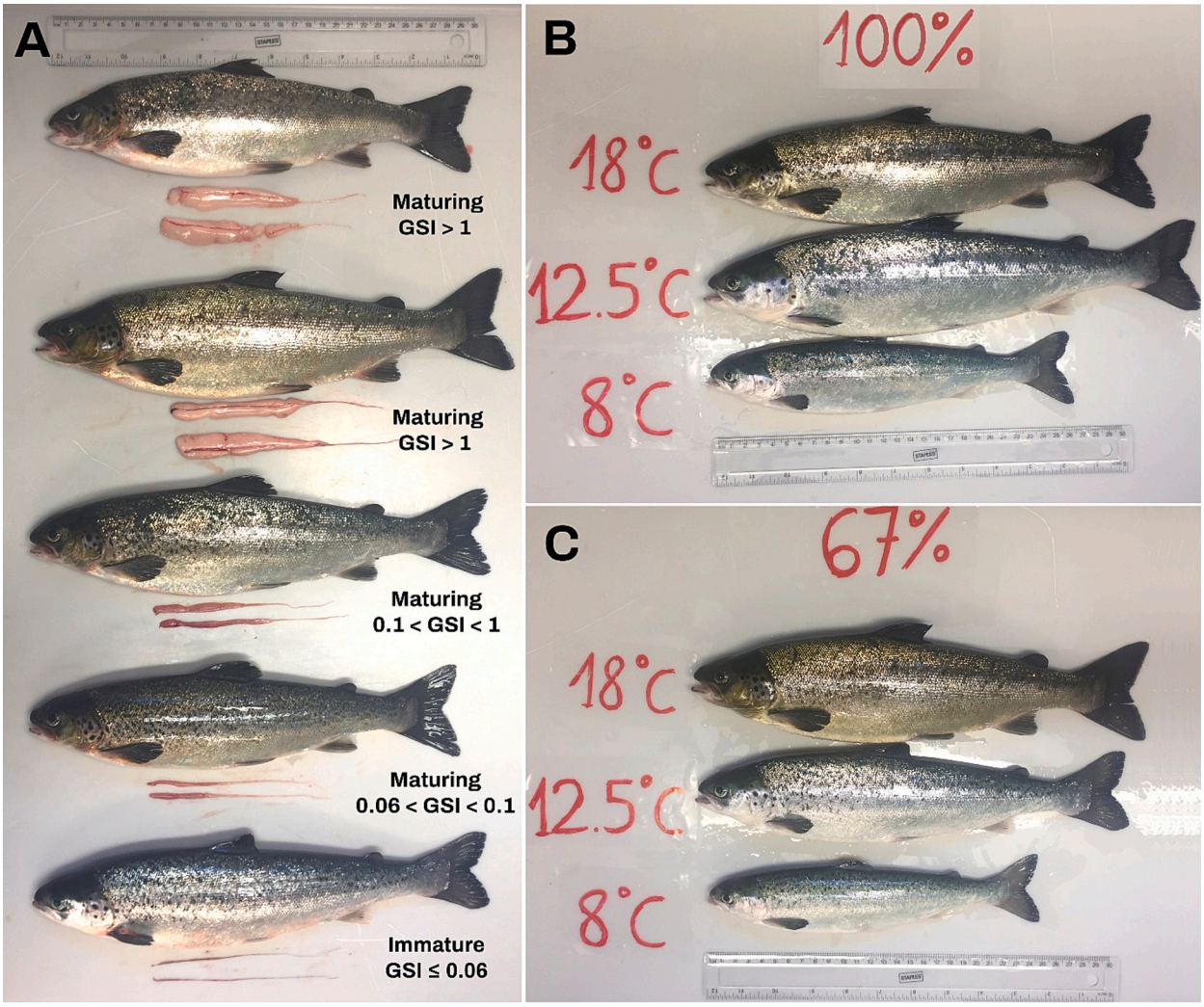

Fig. 3. Ilustration of various males found at different stages of maturation and their dissected gonads (A), and representative morphology of individuals from the six treatments at the end of the experiment in late May (B and C).

12.5–100% (40% males). No maturation occurred at 8 °C. Irrespective of the feeding group, early maturation at 18 °C was observed in males from 200 g, while at 12.5 °C most maturing individuals were over 400 g (Supplementary fig. 1). Maturation only occurred after returning to LD24:0 from the 5-week LD12:12 winter signal. By the end of the experiment, no males had completed maturation since none was found releasing milt. Out of all individuals dissected in the trial, 47.9% were males (725) and 52.1% females (787).

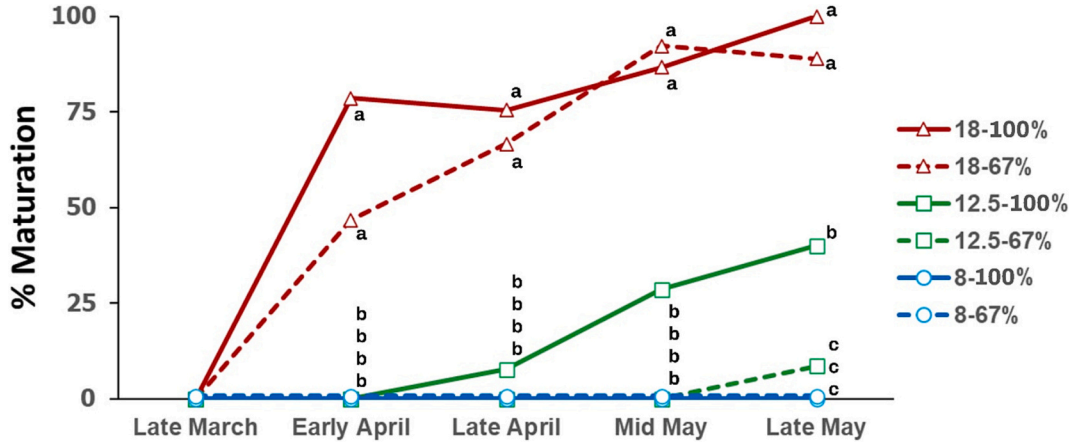

Fig. 4. Percentages of male sexual maturation over time in each experimental group ($n \geq 12$ Atlantic salmon males per sampling, pooled from two replicate tanks). GSI = 0.06% was set as threshold value for maturation, with fish showing GSI ≤ 0.06 considered immature and fish with GSI > 0.06 considered maturing. Letters "a", "b" and "c" indicate significant differences ($p < 0.05$) between experimental groups for each sampling after Fisher's Exact Tests for count data.

3.2. Morphometrics and GSI

Body weight was significantly dependent on all model terms and on interactions temperature×feed ration and temperature×time (all $p <$ 0.005). Within each feeding ration, differences in body weight between 18 °C and 12.5 °C were not significant (Fig. 5A), apart from the last sampling in mid-May in the 100% ration when mean weight of the group at 12.5 °C was greater ($p < 0.001$). Large differences in body weight within feed rations were however observed between both 12.5 °C and 18 °C versus 8 °C, with those at 8 °C displaying significantly lower weight than the other two in all samplings (Fig. 5A). Groups held at the same temperature but different feeding regimes also displayed significant differences in weight. Fish in 18–100% were heavier than those in 18–67% in most of the samplings (all $p < 0.05$), except in December, early March and mid-May. Salmon in 12.5–100% grew more than those in 12.5–67% from early April (all $p < 0.001$). No significant differences in body weight were observed between 8–100% and 8–67%.

Condition factor (K) was significantly dependent upon all model terms and interactions (all $p < 0.001$). Within each feeding ration, K was higher in 18 °C than in the other two temperatures for most of the experiment (all $p < 0.05$) except from late October in 100% ration and late March in 67% ration (Fig. 5B). Between feeding regimes, differences occurred primarily between 12.5–100% and 12.5–67% after the winter signal when K was higher in 12.5–100% (all $p < 0.05$), and between the 18 °C groups only in late March ($p < 0.001$). No significant differences occurred between 8 °C groups. Over time, there were no significant changes in 12.5–100%, while in 18–100% there was only an early increase from late October to mid-December ($p < 0.001$). In 18–67%, K remained high and stable apart from a drop in late March (both $p <$ 0.05). In 12.5–67% a decrease in K occurred from mid-December to late March ($p < 0.001$), followed by an increase until the end of the experiment ($p < 0.001$). Finally, in 8–100%, a significant decrease in K occurred after the winter signal ($p < 0.001$), while a similar but not significant pattern occurred in 8–67%.

GSI was significantly dependent on temperature ($p < 0.001$), feed ration ($p < 0.05$), time ($p < 0.001$), and interaction temperature×time ($p < 0.001$). Within each feeding ration, GSI at 18 °C was significantly higher than at 8 °C from December, and higher than at 12.5 °C from February (Fig. 5C). Between feeding regimes, GSI was higher in 18–100% than in 18–67% in early April and mid-May (both $p < 0.05$), and higher in 12.5–100% than in 12.5–67% in mid-May ($p < 0.05$). Over time, significant increases in GSI occurred in both 18 °C groups in two steps. In 18–67% there was an initial rise from October to December ($p < 0.05$) followed by an abrupt increase from late March to early April ($p < 0.01$). In 18–100% the early rise was observed from December to March ($p < 0.05$) followed by a dramatic increase from late March to early April ($p < 0.001$). In 12.5–100%, GSI rose from late March to mid-May ($p < 0.05$), while no significant change occurred in 12.5–67%. Slight peaks in GSI were present in both 8 °C groups in early April after the winter signal, simultaneous to those observed at 18 °C. In 8–100%, this peak increased from February until early April ($p < 0.05$), decreasing (not significantly) from early April to early May ($p = 0.070$). In 8–67%, the increase occurred from February to April ($p < 0.05$), followed by a decrease until early May ($p < 0.05$).

External appearance of males from the six treatments at the end of the experiment in late May is presented in Fig. 3B and C. Generally fish in 12.5 °C reached the largest size within each feeding regime. Fish in both 18 °C groups displayed the largest K from the beginning of the experiment and early acquired bronze coloration. Fish in both 8 °C groups grew the least, had the lowest K and were most silvered.

3.3. Plasma 11-ketotestosterone concentration and gene expression of pituitary *fshb* and *lhb*

Plasma concentration of 11-KT was significantly dependent on all model terms (all $p < 0.001$), and on interactions temperature×feed regime ($p < 0.05$) and temperature×time ($p < 0.001$). In each feeding regime, mean plasma levels of 11-KT were significantly higher at 18 °C than at the other two temperatures, from late March in 18–100% and from early April in 18–67% (Fig. 6A). Between feeding regimes, males in 18–100% displayed a significant peak in 11-KT in late April, that was not present in 18–67% ($p < 0.05$). Between 12.5–100% and 12.5–67%, males in the 100% ration group had higher 11-KT levels in early April and late May (both $p < 0.005$). Over time, levels of 11-KT increased in all groups after the winter signal, but the intensity of this response was dependent on temperature. In both 18 °C groups, plasma 11-KT increased significantly after the winter signal, between early March and early April (both $p < 0.005$). In 12.5–100%, an increase occurred from late March to early May ($p < 0.005$), with high variability due to the lower proportion of maturation in this group. Finally, the three groups 12.5–67%, 8–100% and 8–67% displayed minor but significant increases in plasma 11-KT after the winter signal, from March to May (all $p < 0.01$).

Pituitary transcription of *fshb* was significantly dependent on all model terms and interactions (all $p < 0.01$). In each feeding regime, *fshb* levels were significantly larger in both 18 °C groups, apart from December and mid-March in 100% ration, and late October and mid-March in 67% ration (Fig. 6B). Between feeding regimes, significant differences occurred only between the 12.5 °C groups, with higher levels of *fshb* transcripts in the 100% ration from early April until the end of the

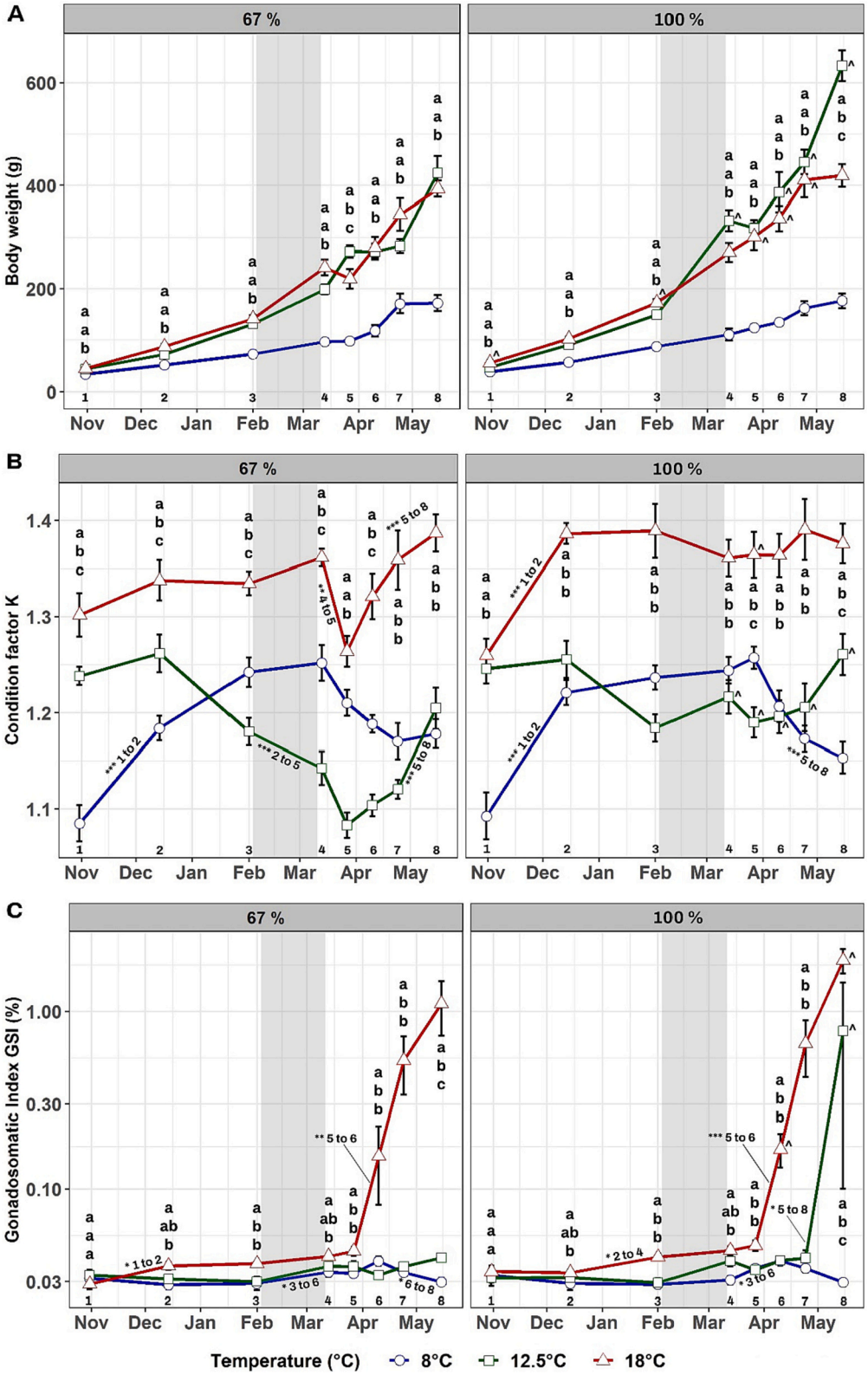

Fig. 5. Mean body weight (A), condition factor K (B) and gonadosomatic index (C) of Atlantic salmon males over time in the six experimental groups. Treatment means at each time point are calculated including all males ($n \geq 12$ per treatment), immature or maturing, and bars display standard error (SE). In each of the plots, the feeding regimes are displayed separately, with the 67% groups shown on the left and the 100% ration groups on the right. The "Y" axis in the GSI fig. (C) is displayed in logarithmic scale for visualization purpose. The grey-shaded area shows the duration of the LD12:12 winter signal. Letters "a", "b" and "c" indicate significant differences ($p < 0.05$) between groups within a feeding regime for a given time point. Signs "^" indicate significant differences ($p < 0.05$) between groups reared at the same temperature but at different feeding regimes at a given time, and they are located next to the largest value of the pair. Numbers from 1 to 8 at the bottom of the graphs represent the sampling number and aid with explanation of significant differences over time within each group. These are displayed with asterisks as follows: (*) *p*-value<0.05, (**) p-value <0.01, (***) p- value<0.001. Asterisks are located next to the corresponding line and are followed by the sampling numbers between which such significant difference occurred.

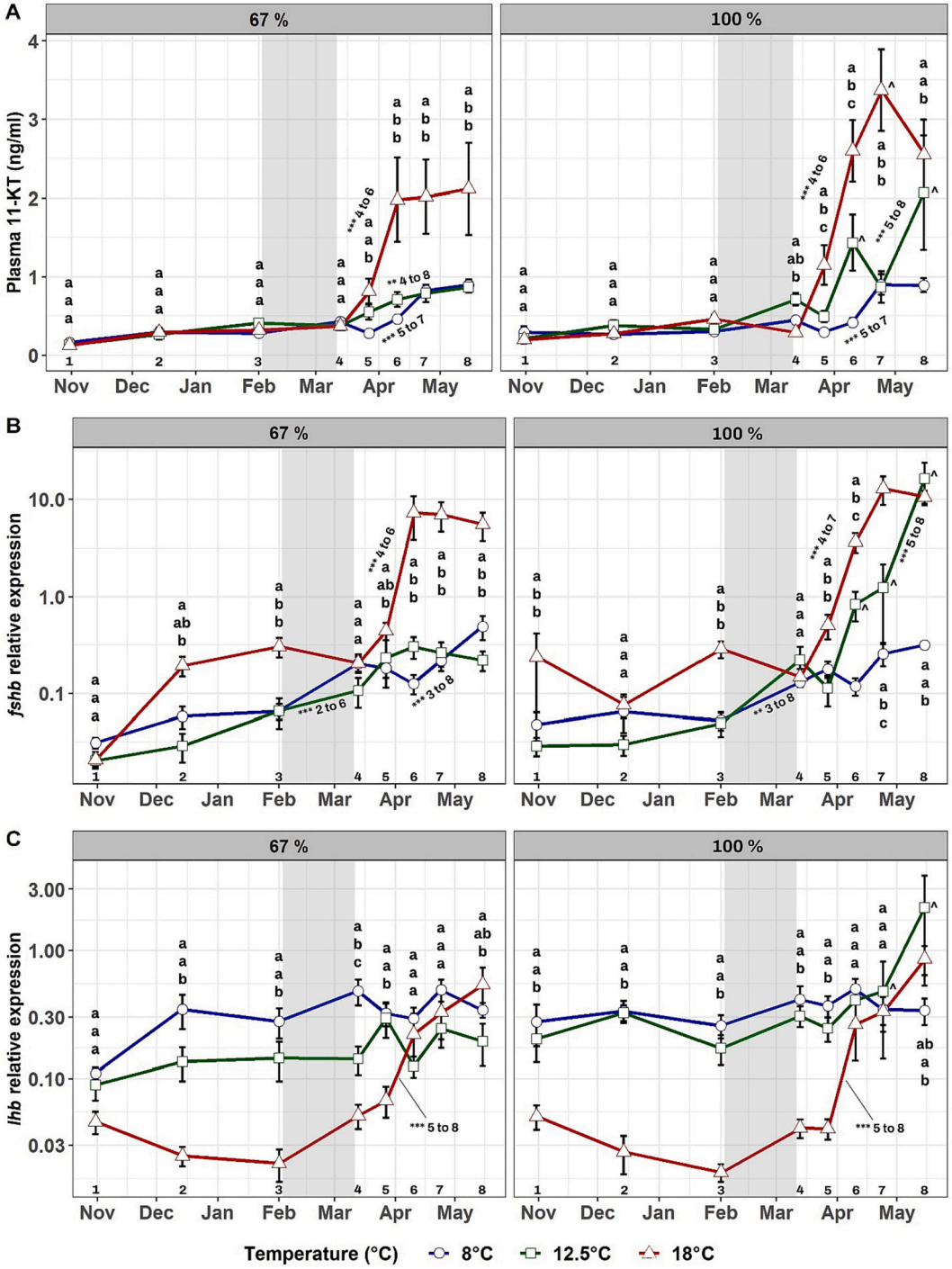

Fig. 6. Mean plasma 11-KT concentration (A), pituitary *fshb* relative expression (B) and *lhb* relative expression (C) of Atlantic salmon males over time in the six experimental groups. Treatment means at each time point are calculated including all males ($n = 6$ per treatment and sampling before the WS, 10 after), immature or maturing, and bars display standard error (SE). In each of the plots, feeding regimes are displayed separately, with the 67% groups on the left and the 100% ration groups on the right. The "Y" axes in the *fshb* (B) and *lhb* (C) figures are displayed in logarithmic scale for better visualization. The grey-shaded area shows the duration of the LD12:12 winter signal. Letters "a", "b" and "c" indicate significant differences ($p < 0.05$) between groups within a feeding regime for a given time point. Other significant differences are explained in the text. Signs "^" indicate significant differences ($p < 0.05$) between groups reared at the same temperature but at different feeding regimes at a given time, and they are located next to the largest value of the pair. Numbers from 1 to 8 at the bottom of the graphs represent the sampling number and aid with explanation of significant differences over time within each group. These are displayed with asterisks as follows: (*) p-value<0.05, (**) p-value <0.01, (***) p- value<0.001. Asterisks are located next to the corresponding line and are followed by the sampling numbers between which such significant difference occurred.

experiment (all $p < 0.01$). Finally, over time increases in *fshb* transcription occurred in 18–100% and 18–67% from early March to early-late April (both $p < 0.001$), immediately after the winter signal. An increase in *fshb* transcription was also observed in 12.5–100% but delayed in respect to the 18 °C groups and with higher variability (late March to mid-May, $p < 0.001$). Slight but significant increases were present in 12.5–67% (from February to early April, $p < 0.05$), 8–100%, and 8–67% (in both cases significant from February to mid-May, both $p < 0.01$).

Relative transcription of pituitary *lhb* was significantly dependent on temperature, feed regime, time (all $p < 0.01$), and on interactions temperature×feed regime and temperature×time (both $p < 0.01$). In each feeding regime, *lhb* transcript levels were consistently and significantly lower in the 18 °C groups than in the other two temperature groups from the beginning of the experiment until late March (Fig. 6C). After this, transcript levels of this gene rose in both 18 °C groups. Between feeding regimes, significant differences occurred only between the 12.5 °C groups, with higher levels of *lhb* in 12.5–100% compared to 12.5–67% in early April and mid-May (both $p < 0.05$). Finally, over time increases in *lhb* transcript levels occurred in 18–100% and 18–67% after the winter signal, until the end of the experiment (both $p < 0.001$). Although in 12.5–100% in the last sampling one male displayed the highest relative transcription of *lhb* in the whole experiment, the rest of individuals in this group had lower transcript levels, and therefore the model did not show any significant difference over time in this group. None of the remaining groups (12.5–67%, 8–100%, 8–67%) displayed any differences in *lhb* transcript levels over time.

3.4. Plasma Igf-I concentration and gill NKA activity

Plasma Igf-I concentration was significantly dependent upon temperature, feed ration, time, and interactions temperature×time and feed regime×time (all $p < 0.001$). Within feeding regimes, Igf-I levels were generally higher at 18 °C than in the rest of the groups and lowest in 8 °C. However, significant differences between 18 °C and 12.5 °C occurred primarily in the 100% ration towards the end of the experiment, but not in the 67% ration (Fig. 7). Between feeding rations, higher Igf-I levels were present in the groups receiving a 100% ration compared with groups held at 67% ration, from early April until the end of the experiment (all $p < 0.01$ except from 18 °C in May). Over time, a similar pattern of variation in plasma Igf-I was observed in the six groups, but with different intensity depending on temperature and feed regime. In the 67% group at 12.5°C, a significant increase in Igf-I concentration occurred from early April until mid-May ($p < 0.01$). In all 100% ration groups, a rise in plasma Igf-I concentrations occurred in parallel from early to late April (all $p < 0.001$), followed by a decrease in Igf-I which was only significant in 18 °C ($p < 0.001$).

NKA activity was significantly dependent upon temperature ($p < 0.001$), time ($p < 0.05$), and the interaction temperature×time ($p < 0.001$). In both feeding regimes, NKA was consistently the lowest at 18 °C across the whole experiment. The highest enzyme activity was observed from April onwards in the two 8 °C groups, and in some individuals from 12.5–67% before February and from 12.5–100% in mid-March (Fig. 8A). Between feeding regimes, significant differences in NKA occurred between 12.5 °C groups in mid-March and between 8 °C groups in late March (both $p < 0.05$). Over time, no significant variation in NKA was present in any of the 18 °C or 12.5 °C groups, while in both 8 °C groups a significant increase in NKA occurred from late March until late May (both $p < 0.05$).

NKA activity was also studied in relation to the maturity status of the fish (maturing vs immature), time and their interaction (Fig. 8B), by fitting a square root-transformed two-way ANOVA. This model showed that NKA activity was significantly dependent on maturity, time and their interaction (all $p < 0.001$). NKA was significantly higher in immature than in maturing salmon all April and May (all $p < 0.01$). In immature salmon, NKA activity increased significantly from late March until May ($p < 0.001$), whereas no significant differences occurred over time in maturing males. Maturing individuals (GSI > 0.06%) had consistently low NKA activity (supplementary fig. 2, GSI (%) vs NKA activity).

4. Discussion

Sustained high temperature (18 °C) induced an early activation of the BPG axis in males irrespective of the feed ration, resulting in almost 100% of them maturing in May as postsmolts and displaying very poor signs of smoltification. At 12.5 °C the proportion of maturing males at the end was significantly lower than at 18 °C, and more dependent upon feed regime. Finally, at 8 °C, fish did not mature and displayed best signs of smoltification irrespective of the ration. This reveals that water temperature was the main determinant of life history pattern of each group, which is very relevant for salmon producers in the current context of intensive aquaculture. Our results show that maintaining water temperatures over 12.5 °C in postsmolt production facilities can significantly increase the risk of early male maturation and poor smoltification.

4.1. High temperature was the main driver for initiation of sexual maturation

Water temperature acted as the main factor determining the life history strategy choice. At 18 °C, the proportion of maturing males in May was virtually 100% in both full and restricted rations, evidencing a great stimulatory effect of constant high temperature on maturation that overrode the potential detrimental effects of the caloric restriction. This

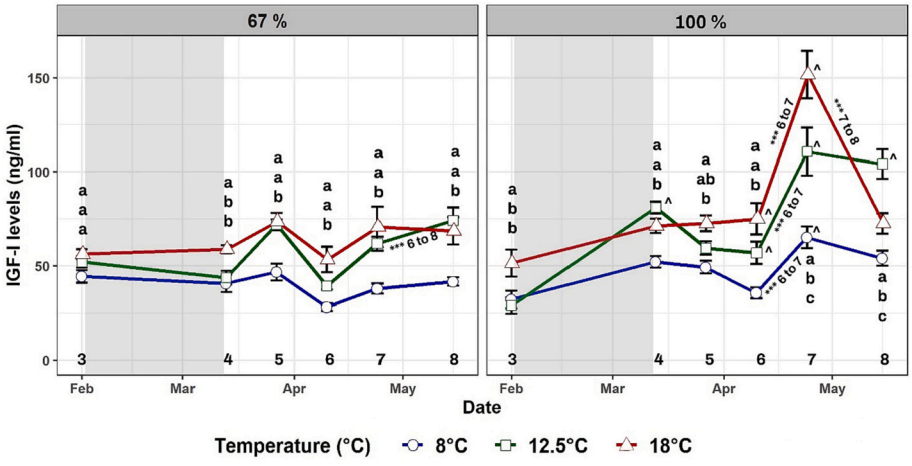

Fig. 7. Mean plasma Igf-I concentration of Atlantic salmon males over time in the six experimental groups. Treatment means at each time point are calculated including all males (n = 6 per treatment and sampling before the WS, 10 after), immature or maturing, and bars display standard error (SE). Feeding regimes are displayed separately, with the 67% groups on the left and the 100% ration groups on the right. The grey-shaded area shows the duration of the LD12:12 winter signal. Letters "a", "b" and "c" indicate significant differences ($p < 0.05$) between groups within a feeding regime for a given time point. Other significant differences are explained in the text. Signs "^" indicate significant differences ($p < 0.05$) between groups reared at the same temperature but at different feeding regimes at a given time, and they are located next to the largest value of the pair. Numbers from 3 to 8 at the bottom of the graphs represent the sampling number and aid with explanation of significant differences over time within each group. These are displayed with asterisks as follows: (*) p-value<0.05, (**) p-value <0.01, (***) p- value<0.001. Asterisks are located next to the corresponding line and are followed by the sampling numbers between which such significant difference occurred.

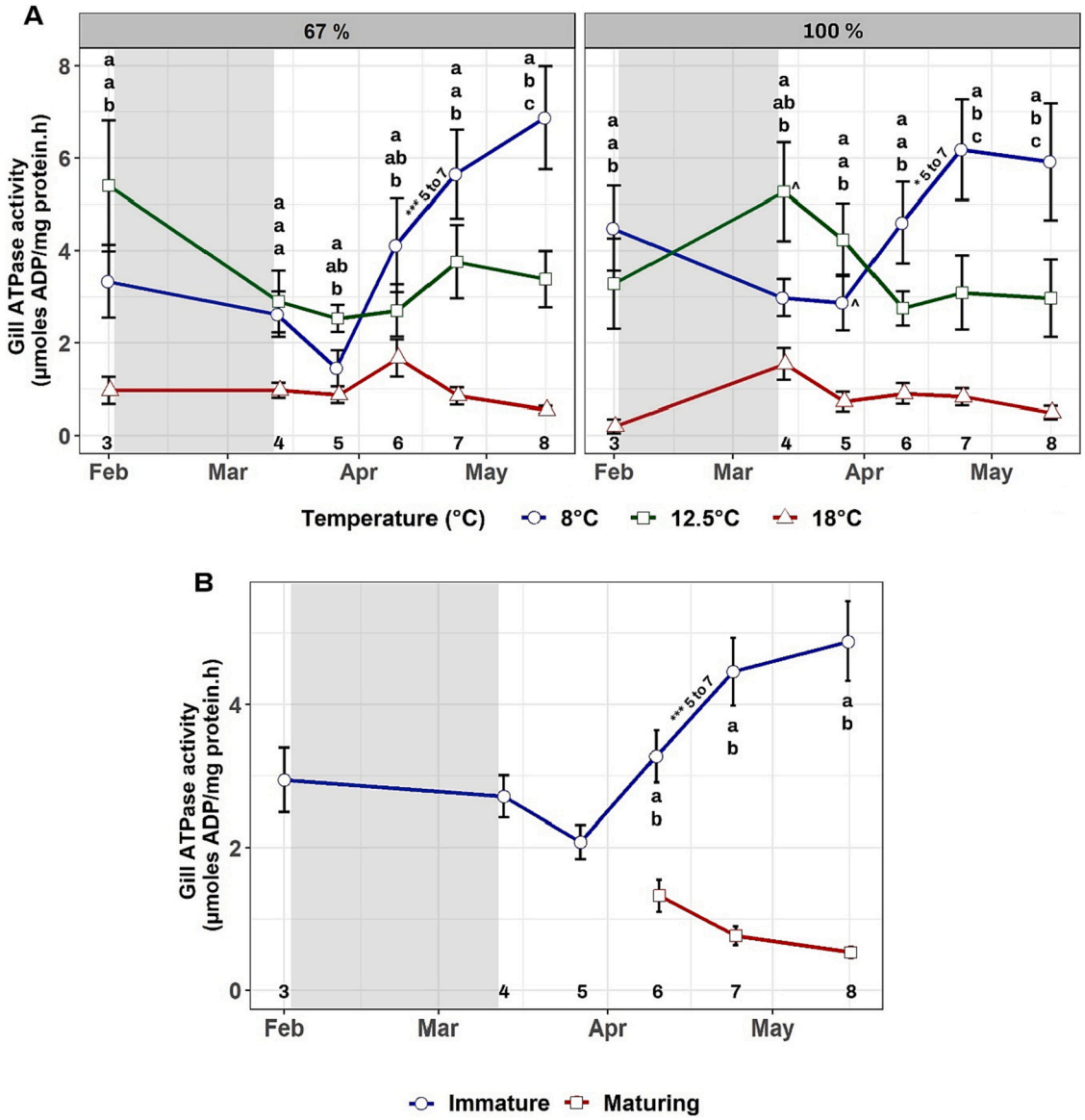

Fig. 8. Mean gill NKA activity in Atlantic salmon males over time in the six experimental groups (A) and comparison between immature and maturing (B). Treatment means at each time point in (A) are calculated including all males (n = 6 per treatment and sampling before the WS, 10 after), immature or maturing, and bars in all graphs display standard error (SE). In (A), feeding regimes are displayed separately, with the 67% groups on the left and the 100% ration groups on the right. The grey-shaded area shows the duration of the LD12:12 winter signal. In both figures, letters "a", "b" and "c" indicate significant differences ($p < 0.05$) between groups for a given time point. Signs "^" indicate significant differences ($p < 0.05$) between groups reared at the same temperature but at different feeding regimes at a given time, and they are located next to the largest value of the pair. Numbers from 3 to 8 at the bottom of the graphs represent the sampling number and aid with explanation of significant differences over time within each group. These are displayed with asterisks as follows: (*) p-value<0.05, (**) p-value <0.01, (***) p-value<0.001. Asterisks are located next to the corresponding line and are followed by the sampling numbers between which such significant difference occurred.

is consistent with previous studies that also highlighted a strong stimulatory effect of high temperature on jacking after observing highest proportion of maturing males at temperatures between 12 and 16 °C (Fjelldal et al., 2011; Imsland et al., 2014; Melo et al., 2014). In contrast, at moderate temperature (12.5 °C) this stimulatory effect was not as prevalent, resulting in a significantly lower proportion of maturing males and the greater relative influence of the feed ration evidenced by clear differences in percentage of maturation, growth, and most physiological markers between 12.5–100% and 12.5–67%. Finally, at low temperature (8 °C) the maturation process was totally inhibited, thus allowing smoltification to proceed (Thorpe, 1994). The lack of differences in any variable between 8–100% and 8–67% evidences the restrained metabolism and lack of sexual development as a result of low temperature.

The physiological changes by which exposure to constant high temperature may stimulate sexual maturation could be related to two main processes. First, high temperature increases appetite and growth in juveniles (Handeland et al., 2008), leading to higher availability of energy stores necessary for maturation (Jonsson et al., 2013; Rowe et al., 1991). Second, high temperature may directly stimulate reproductive investment (Adams and Thorpe, 1989), causing an early activation of the BPG axis. Brief discussion of these two mechanisms is attempted in following lines.

Salmon at 18 °C and 12.5 °C within each feeding regime displayed similar body weight throughout the experiment, while groups at 18 °C had higher condition factor. This entails differences in feed intake, lipid metabolism and energy acquisition induced by different temperatures, which probably affected the proportion of maturing males. For example, Handeland et al. (2008) suggested that smolts up to 120 g display higher appetite at temperatures from 14 to 18 °C, which could contribute to

increase the availability of surplus resources for early maturation. Imsland et al. (2014) also found higher feed intake in salmon groups reared at higher temperature, which grew best and later matured at higher proportion. It has also been suggested that at higher temperature salmon can use surplus energy more efficiently, surplus energy that can be used later for sexual maturation (Jonsson et al., 2013). Thus, higher appetite and higher availability of energy could explain the higher condition factor observed at 18 °C from the beginning of the experiment, since high condition factor has often been linked to high energy levels (Herbinger and Friars, 1991). Consequently, salmon at 18 °C may have soon acquired energy requirements for sexual development (Thorpe, 2007) and be ready for maturation early.

However, if temperature effects on early maturation were mediated only through fast growth, then similar proportion of maturation could be expected at 12.5 °C and 18 °C within each feed regime, since mean body weight was similar. However, proportion of maturation at 18 °C was higher than at 12.5 °C. This entails that another mechanism, induced by high temperature to some extent independently of growth and energy acquisition, must have also contributed to early maturation. By this mechanism, high temperature might have promoted physiological differences linked to sexual development in contrast to moderate temperature, despite both temperatures apparently inducing similar growth. Support for this argument comes from the significantly higher GSI and *fshb* transcript levels observed in both 18 °C groups already from December–January, compared to groups at 12.5 °C. Fsh is the gonadotropin that first orchestrates initiation of sexual maturation and spermatogenesis in teleosts (Nóbrega et al., 2015; Schulz et al., 2019; Schulz et al., 2010). Thus, such higher *fshb* and GSI at 18 °C may reveal an early sign of activation of the BPG axis before introduction of any photoperiod cue, as a result of the stimulatory effect of high temperature alone. Further support to this argument may come from the significantly lower baseline transcript levels of *lhb* observed at 18 °C for most of the experiment, simultaneously to the higher *fshb* and GSI. According to Schulz et al. (2010), plasma Lh levels are maintained low or undetectable at onset of spermatogonial proliferation, become detectable at meiotic phase and clearly increase at spermiation. In our study, individuals at 18 °C may be actively keeping *lhb* transcription low because high temperature had stimulated early proliferative processes in the testes and therefore Lh was not necessary at this stage. Overall, the elevated *fshb* and GSI simultaneous to the low baseline transcription of *lhb* at 18 °C could be revealing an early commitment to maturation before any photoperiod cue in response to sustained exposure to high water temperature. This stimulatory effect on the BPG axis at 18 °C is worth further investigation to better understand how the use of constant high temperature in aquaculture promotes early maturation.

4.2. Temperature-dependent endocrine changes related with sexual maturation

Endocrine markers of sexual maturation revealed an earlier and more pronounced activation of the BPG axis at 18 °C, that was similar under both feed rations. Onset of testis growth was linked to increases in pituitary *fshb* transcript levels and plasma 11-KT concentrations occurring right after the winter signal, from mid-March until late April, when groups at 18 °C entered early stages of maturation. This is consistent with previous authors who stated that pituitary Fsh and 11-KT increase dramatically at initiation of sexual maturation and during the proliferative phase of spermatogenesis (Maugars and Schmitz, 2008; Melo et al., 2014; Schulz et al., 2010; Taranger et al., 2010). Our results suggest that Fsh is the main endocrine factor orchestrating onset of sexual maturation in fish, which is aligned with studies on Atlantic salmon (Schulz et al., 2019) or zebrafish *Danio rerio* (Nóbrega et al., 2015). Our findings also reveal how exposure of salmon to constant 18 °C can induce an earlier and more intense activation of gonadotropin production that results in a very high proportion of males engaging in early maturation. A similar endocrine response was observed also in 12.5–100%, but this was delayed in time and had higher individual variability due to the lower proportion of males initiating maturation at 12.5 °C. The fact that mean pituitary *fshb* transcription was not affected by the feed restriction at 18 °C, but it was at 12.5 °C, reveals the crucial stimulatory role of high temperature on the BPG axis overriding any influence of energy resources. Furthermore, the early *fshb* transcription pattern observed pre-winter signal suggests that 18 °C could have caused a majority of males to commit to sexual maturation early. If this is the case, according to Thorpe (2007, 1994) energy resources would be allocated primarily to maturation at the expense of other processes, and therefore the influence of a caloric restriction on the physiology of maturation could be to some extent reduced. This would explain why males in 18–100% were heavier than those in 18–67% throughout the experiment, but both had similar pituitary *fshb* transcription pre- and post-winter signal, as well as similar proportion of maturation. This overall indicates that rearing salmon under constant high temperature may promote early physiological commitment to sexual maturation and stimulate its initiation.

However, despite this early stimulatory effect, high temperature could also be responsible for an endocrine disruption impairing final completion of maturation (Taranger et al., 2003; Vikingstad et al., 2016). The absence of males producing milt and the few differences in *lhb* transcription between treatments after the winter signal suggest that maturing males did not reach late spermatogenesis. This is because a peak in Lh production after those of Fsh and 11-KT is necessary for salmon to complete spermatogenesis and produce mature spermatozoa (Maugars and Schmitz, 2008; Schulz et al., 2010; Taranger et al., 2010). A simple explanation for the lack of complete maturation might be that salmon required more time to complete the process. Maturing groups had roughly 2 months from onset of maturation to the end of the experiment, while in nature this process can take from spring to October/November. However, some authors (Taranger et al., 2003; Vikingstad et al., 2016) have reported that warm water affects testicular function and impairs milt production in salmon, either inhibiting pituitary secretion of Lh, or disrupting the correct synthesis of 17α,20β-dihydroxy-4- pregnen-3-one (17,20βP, a sex steroid stimulating final gamete maturation in salmonids). Similar findings have been reported for rainbow trout (Pankhurst and Thomas, 1998). It is likely that this effect was present at a relevant degree at an extreme temperature as 18 °C. This entails that using constant high temperature in aquaculture may stimulate the initiation of maturation, but negatively affect its completion, which is consistent with seasonal patterns of temperature variation occurring during initiation and completion of salmon maturation in nature. However, even if not completed, initiation and development of maturation can still have negative implications for salmon producers. Maturing postsmolts have shown reduced growth compared to immature males, which can represent a cost at harvest size (Fraser et al., 2019). Furthermore, postsmolts that may terminate maturation due to unsuitable rearing conditions seem prone to mature at the next opportunity (Fraser et al., 2019).

Circulating Igf-I, produced in the liver in response to pituitary Gh, is primarily involved in processes like somatic growth and smoltification (Beckman, 2011; Reinecke, 2010; Shimizu et al., 2006). Igf-I can also play a role in onset of maturation in fish (Campbell et al., 2003) by supporting 11-KT on induction and progression of spermatogenesis (Schulz et al., 2010). However, our results show no clear evidence of the role of Igf-I in this process. This is because early maturation occurred similarly in groups with presence (18–100%) and absence (18–67%) of peaks in plasma Igf-I. Moreover, the group 12–67% (40% maturation), displayed a peak in Igf-I that was not present in 18–67% (~100% maturation). Consequently, based on our data, linking Igf-I and onset of maturation is difficult. It is known that Igf-I production responds to all factors we manipulated, such as temperature, photoperiod or nutritional status (Beckman, 2011; Dickhoff et al., 1997; Reinecke, 2010). Thus, increases in plasma Igf-I in 18–100% and 12.5–100% in late April might simply result from the return to LD24:0 and the better nutritional condition after receiving a 100% ration, entailing that rearing conditions

could be masking the potential link between Igf-I and maturation. In addition, part of the action that Igf-I exerts on onset of spermatogenesis may occur from paracrine/autocrine Igf-I produced in the testis as observed in rainbow trout *Oncorhynchus mykiss* (Le Gac et al., 1996) or Nile tilapia *Oreochromis niloticus* (Berishvili et al., 2006). Furthermore, specific regulation of spermatogonial proliferation could rather be controlled by local testis factors responsive to gonadotropins such as Anti-Müllerian Hormone (Amh) or Igf3 (Crespo et al., 2016; Nóbrega et al., 2015; Schulz et al., 2019).

4.3. Feed restriction only modulated the effect of temperature on early maturation

A 67% feed ration was used to reduce growth and energy levels in salmon reared at high (18 °C), moderate (12.5 °C) and low (8 °C) temperatures, aiming to test if the resulting caloric restriction would reduce the incidence of early maturation. At 18 °C, the 67% ration significantly reduced growth, as well as plasma 11-KT and Igf-I in late April, but it did not alter the proportion of maturing males nor their gonadotropin transcription. This entails that the 67% ration indeed diminished nutritional status and energy availability, but that the effect on maturation of this energy restriction was diluted by the remarkable stimulation of high temperature on the BPG axis. On the contrary, at 12.5 °C the relative effect of restricted feeding on growth, body condition, proportion of maturation and most physiological markers was much greater than between the 18 °C groups. This suggests that in absence of the highly stimulatory effect of high temperature, the influence of energy availability becomes more relevant for the activation of the BPG axis, and only those individuals clearly exceeding the required thresholds (Thorpe, 2007) may commence maturation. This is consistent with Jonsson et al. (2013), who stated that exothermic organisms like Atlantic salmon can be compared with an engine that uses food as fuel and temperature acts as accelerator. Thus, at higher temperature salmon can utilize the feed more efficiently and obtain more surplus resources for early maturation, while at lower temperature a feed restriction can have larger impact on caloric availability and consequently on early maturation.

However, early maturation still commenced in a small proportion of males (~8%) in 12.5–67% at the end of the experiment, in individuals that were significantly smaller than those in 12.5–100%. These results are aligned to previous studies that tested the effect of feed restrictions on early maturation. For example, Trombley et al. (2014) fed salmon with 100% and 50% rations, observing lower body weight, GSI, gonadotropin transcription and proportion of maturing males in the group fed the 50% ration. However early maturation still occurred in a remarkable proportion in the 50% ration (29% of males), a percentage that might be unacceptable for industry. Similarly, Crespo et al. (2019) subjected salmon to 100% and 43% rations, and observed a reduction in growth and proportion of maturation in the restricted feeding group. However, maturation still occurred in males fed the 43% ration, which made these authors conclude that a caloric restriction seems unsuitable to control early maturation in salmon aquaculture. Our results also support the idea that restricting the feed ration may not be the best option to control early maturation in aquaculture if producers are not willing to accept a significant reduction in growth performance, which would probably entail economic and welfare impacts. The use of restricted rations can lead to establishment of dominance hierarchies, which may result in high growth variability (Juell, 1995) and aggression (Cañon Jones et al., 2010). This aggressive behavior can lead to poor welfare indicators, including high presence of fin damage, reduced body condition and growth in submissive fish (Cañon Jones et al., 2017; Cañon Jones et al., 2010), increasing their susceptibility to disease and risk of mortality.

4.4. Maturation and smoltification: mutually exclusive or in developmental conflict?

The 5-week LD12:12 winter signal was introduced in all groups to induce smoltification, thus allowing to explore the interaction between this process and early maturation. In our study, the two processes appeared mutually exclusive, with smolting individuals remaining immature, and maturing fish displaying very low NKA activity, high condition factor and bronze coloration. These signs suggest that maturing fish developed poor hypo-osmoregulatory abilities and seawater adaptation (Handeland et al., 2003; McCormick et al., 1987; Stefansson et al., 1991) but this was not tested since the whole experiment took place in freshwater. Apart from a potential hypo-osmoregulatory disruption caused by ongoing maturation (Schulz et al., 2006; Taranger et al., 2010), we observed a clear effect of temperature on NKA activity, with consistently low activity of this enzyme at 18 °C throughout the experiment, signs of higher NKA activity at 12.5 °C, and clear increases after the winter signal only at 8 °C. This implies that disruptive effects that maturation may have on development of seawater tolerance were masked by the effect of temperature.

Temperature is not a *zeitgeber* for smoltification as photoperiod is (McCormick et al., 2002), but it determines the time needed to reach the smolt window (Handeland et al., 2004). Moderate high temperatures advance this optimum period of seawater adaptations, but also advance the subsequent loss of hypo-osmoregulatory abilities and de-smoltification (Handeland et al., 2004; McCormick et al., 1999; Stefansson et al., 1998). Very high temperatures close to thermal tolerance levels can instead completely disrupt the development of seawater tolerance (Stefansson et al., 2008). This considered, it is likely that different temperatures determined different mechanisms affecting changes in NKA activity. At 12.5 °C, some individuals displayed relatively higher NKA before the winter signal possibly as a result of reaching 150–200 g (Imsland et al., 2014), but afterwards NKA activity did not increase. This poor NKA activity after the winter signal may result from the long period at that temperature in freshwater (Stefansson et al., 1998) and from the large size attained before the winter signal (Imsland et al., 2014). Both factors probably caused fish to miss the smolt window and de-smoltify (Stefansson et al., 2008). At 18 °C however, the consistently low levels of NKA activity throughout the experiment regardless of size and maturity status suggest a direct impairing or inhibiting effect of high temperature on smoltification (Stefansson et al., 2008). It is likely that exposure to constant 18 °C is outside thermal limits for smoltification in Atlantic salmon, as it has been shown for other salmonids like Chinook salmon *Oncorhynchus tshawytscha* above 17 °C (Marine and Cech, 2004) or rainbow trout above 13–15 °C (Zaugg, 1981). Causes for this low NKA activity at high temperature could include a reduction in number of gill ionocytes as a result of higher cell death or poor renewal, or changes in endocrine systems regulating increase of NKA during smoltification (McCormick et al., 1999). Finally, the trend in NKA activity at 8 °C suggests that fish smoltified, which reflects a different choice of life strategy in response to the same photoperiod cue depending on temperature. These findings have clear implications for aquaculture producers, especially considering the current trend to grow larger smolts in RAS at higher temperatures. The resulting suboptimal development of osmoregulatory abilities at high temperature can increase the risk of poor performance, susceptibility to disease and mortality after transfer to seawater.

In addition to the previous temperature effect, ongoing endocrine regulation of sexual maturation probably affected smoltification negatively (Schulz et al., 2006; Taranger et al., 2010). Previous studies have shown that increasing sex steroids during maturation can disrupt development of hypo-osmoregulatory abilities (Lundqvist et al., 1990; Lundqvist et al., 1989; Madsen et al., 2004; McCormick et al., 2005) and these negative effects can extend long after sexual maturation (Lundqvist et al., 1990; Saunders et al., 1994). Despite this, smoltification and maturation are not considered mutually exclusive but rather in

developmental conflict (Thorpe, 1986), and various authors have reported they can occur sequentially (Berrill et al., 2003; Saunders et al., 1994) or to some extent simultaneously (Fjelldal et al., 2018; Fjelldal et al., 2011). In our study these two processes appeared mutually exclusive at high and low temperature. This probably occurred due to an early "choice" of maturation at 18 °C as life history strategy, in contrast to a total inhibition of sexual development at 8 °C that allowed smoltification to proceed after the winter signal (Thorpe, 1994, 2004; Thorpe et al., 1998). At 12.5 °C conditions did not stimulate an early commitment to maturation and consequently salmon could smoltify early, but the large size attained and a moderate stimulatory effect of temperature induced some males to mature after the winter signal. Results from Fjelldal et al. (2018), who observed simultaneous smoltification and maturation, may be also aligned with this view. Fjelldal et al. (2018) reared salmon at lower temperature (~5 °C and 12 °C) during the presmolt stage and during their "winter" photoperiod, after which they increased photoperiod (LD24:0) and temperature (16 °C). This entails that fish in Fjelldal et al. (2018) did not experience the same early stimulatory conditions as our 18 °C groups, and in terms of life strategy choice before the photoperiod switch, their fish may be similar to our 12.5 °C or 8 °C groups.

4.5. Photoperiod seemed to trigger the onset of maturation

The photoperiod switch from winter to spring is considered the entraining cue that triggers onset of maturation in salmon with sufficient energy stores (Bromage et al., 2001; Taranger et al., 2010; Thorpe, 1994). In our study, the 5-week winter signal was introduced in all groups and consequently we had no contrasting photoperiod group. Despite this, our data seemed to confirm the role of photoperiod as *zeitgeber* for maturation, with an immediate and intense activation of the BPG axis in 18–100% and 18–67% after returning to LD24:0. In addition, some signs of reproductive activation were present during the same period in non-maturing groups (significant increases in 11-KT linked to slight but significant variations in GSI in the groups at 8 °C). This suggests that non maturing males may also experience a weak but relevant activation of the physiology of reproduction in response to photoperiod, which has been sometimes referred to as "dummy run" (Fraser et al., 2019; Okuzawa, 2002). Dummy runs have been observed in salmonids (Amano et al., 1992; Prat et al., 1996), and early signs of sexual development (increased *fshb* expression and GSI) have been reported in non-maturing Atlantic salmon in response to spring-summer environmental conditions (Maugars and Schmitz, 2008; Pino Martinez et al., 2021). This is consistent with recurring seasonal cycles of testis growth-shrinkage observed in various fish species (Schulz et al., 2010), and with the fact that early stages of spermatogonial proliferation are considered reversible (Schulz et al., 2010). This evidence suggests that in aquaculture settings, a photoperiod cue can generally stimulate an activation of the BPG axis, but this activation would only result in sexual maturation if individuals had before experienced conditions permitting physiological commitment to the process (Thorpe, 2007; Thorpe, 1994).

5. Conclusion

Water temperature determined the life history strategy choice of male salmon. Thus, males at 18 °C only early matured, those at 8 °C only smolted, and those at 12.5 °C showed best growth and early signs of smoltification, although later some matured and most de-smolted. The restricted feed ration decreased growth at 18 °C and 12.5 °C, but its effect on maturation was temperature dependent. At 18 °C, the restricted ration did not affect the proportion of maturing males, while at 12.5 °C, it had a greater impact on proportion and physiological markers of maturation, although not arresting the process in all individuals. These results have clear implications for aquaculture producers within the current context of intensive postsmolt production in RAS. The constant high temperatures used can increase the risk of early maturation and poor hypo-osmoregulatory performance, leading to economic losses. The use of a restricted ration to control early maturation does not seem a good alternative in industry since it will entail assuming reduced growth.

Author contributions

SH and AKDI established the projects and gathered the funding. EPM and SH designed the study. EPM, PB, and SH performed samplings. EPM performed gene expression and NKA analyses, while PB revised and guaranteed quality of results. NK and BN contributed with Igf-I and 11-KT analyses respectively. EPM carried out data analysis, drafted and wrote the manuscript. MSF, SOS and AKDI provided editorial assistance and helped writing the document. All authors critically revised the manuscript and approved the final version.

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Data availability statement

The data supporting the findings of this study are available from the corresponding author, upon reasonable request.

CRediT authorship contribution statement

Enrique Pino Martinez: Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Pablo Balseiro:** Conceptualization, Methodology, Validation, Writing – review & editing. **Sigurd O. Stefansson:** Conceptualization, Writing – review & editing, Resources. **Nobuto Kaneko:** Formal analysis, Resources, Writing – review & editing. **Birgitta Norberg:** Formal analysis, Resources, Writing – review & editing. **Mitchell S. Fleming:** Conceptualization, Writing – review & editing. **Albert K.D. Imsland:** Conceptualization, Writing – review & editing, Project administration, Funding acquisition. **Sigurd O. Handeland:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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

Data will be made available on request.

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Appendix A. Supplementary data

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