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Vgll3 regulates the timing of puberty in farmed Atlantic salmon, but it does not explain family discordance in male maturation following different smolt production regimes

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ABSTRACT

Atlantic salmon (*Salmo salar*) farming is moving towards extended periods of land-based production to minimise the time at sea, but this increases the risk of unwanted male sexual maturation. As the timing of puberty is driven by genetics (e.g. *vgll3*) and the environment, optimising rearing strategies and broodstock management may help alleviate the problem. Subsequently, we used a domesticated strain of *vgll3* heterozygous broodstock to produce three all-male half-sibling families using three different production regimes to assess the timing of first puberty. Firstly, "large smolts" were produced in a flow-through system on 13 °C freshwater and constant light from first feeding (day 0) up to 1 kg (day 354). The mean incidence of pubertal males increased from 2% at 30 g, up to 78% at 1 kg. Genetics explained a significant ($p < 0.05$) amount of the variation, with 93, 71, and 34% of the expected early, intermediate, and late maturing *vgll3* genotypes being pubertal on day 354, respectively. In addition, pubertal males in the early *vgll3* genotype were found at the first sampling at ≈30 g, while it was not observed in the heterozygous and late genotypes until the fish were ≈90 g and ≈280 g, respectively. Secondly, "post-smolts" were produced by switching half the large smolts to 13 °C seawater at 420 g (day 272) and growing them in tanks to 0.95 kg (day 354). This led to a significant 14% reduction in the total incidence of puberty by day 354 compared to the large smolts. However, the regulation of pubertal timing by *vgll3* did not interact with salinity. Thirdly, traditional "under-yearling" smolts were produced using periods of natural or manipulated temperature and photoperiod, followed by transfer to a sea-cage at 150 g in December (day 290) where they stayed for one year until harvest at 4.75 kg (day 648). None of these fish were mature at sea transfer, but 9% were at harvest, with 22, 7, and 2% of the early, intermediate, and late *vgll3* genotypes being mature, respectively. When comparing the regimes, family effects outside of *vgll3* on the prevalence of sexual maturation were significant for the land-based regimes (family means from 46 to 93% on day 354), but not in sea-cages (family means from 7 to 11% on day 648) and evidence for either *vgll3* allele being dominant was mixed. *Vgll3* also had minimal effects on body size/growth in any regime/family. In conclusion, i) selecting for the late *vgll3* allele is an effective method to delay puberty over a range of production regimes, ii) family effects outside of *vgll3* were not consistent across regimes, and iii) using seawater reduced the incidence of precocious puberty during land-based production.

1. Introduction

Atlantic salmon (*Salmo salar*) juveniles have historically been produced in flow-through land-based freshwater facilities until they are ready for seawater 6–12 months after hatching as 30-150 g smolts. At this point they are moved to sea cages for grow-out to market size at 4-5 kg. However, a new trend is to extend the time in land-based facilities by either producing what is commonly called "large smolts" or "post-smolts". These are transferred to sea-cages at sizes of up to 1 kg (Bjørndal and Tusvik, 2020), or may be kept on-land for the entire production

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cycle (Crouse et al., 2021). This switch has significantly improved growth rates due to the manipulation of environmental conditions, as rather than try and replicate seasonal temperature fluctuations and photoperiods, modern recirculation aquaculture systems (RAS) generally keep fish on constant conditions of relatively high temperatures (10–16 °C) and 24 h daylength from first feeding (e.g. Davidson et al., 2016; Fossmark et al., 2021; Pino-Martinez et al., 2021; Ytrestøyl et al., 2023). This ensures high growth rates year-round and leads to fish being up to 1 kg within the first year of hatching. However, it also promotes pre-harvest sexual maturation that has negative effects on growth, health, and flesh quality (Taranger et al., 2010).

Females of domesticated strains of Atlantic salmon tend not to mature until after they have surpassed harvest size, however males do, and they have several problematic precocious phenotypes (Taranger et al., 2010). Traditionally these have been "sneaker" males or "grilse". Sneakers mature before seawater adaptation at a relatively small body size of <30 g and still have the body coloration of immature freshwater juveniles. Grilse are fish that mature after one winter in seawater, they are relatively large being over 1 kg, and they have distinct body coloration and jaw development which makes them easy to distinguish from immature counterparts. However, with the advent of greater environmental manipulation on-land, high percentages of males can start maturing as "jacks" just prior to (smolts) or shortly after (post-smolt) seawater adaptation when they are between 0.1 and 1 kg (e.g. Fjelldal et al., 2011; Fjelldal et al., 2018). In one facility, 70% of males were found to enter puberty before reaching 1 kg (Good et al., 2017) while 100% male maturation has been attained in some smaller scale studies (Fjelldal et al., 2020). High levels of pre-harvest maturation have also been observed in commercial sea farms in New Brunswick (Canada), in that grilsing was as high as 65% in some individual cages, but the median prevalence across 24 farms was 7% (McClure et al., 2007). In contrast, sneakers are relatively non-problematic today as the rapid growth of domestic strains (Harvey et al., 2018) and the use of continuous light during early development (Skilbrei and Heino, 2011) inhibits their occurrence.

One tool to avoid precocious male maturation is genetic selection. The age of puberty is heritable in salmon (Gjerde, 1984) and *vestigial-like protein 3* (*vgll3*) regulates the incidence of parr (Verta et al., 2020), large smolt (Fraser et al., 2023a), post-smolt (Fjelldal et al., 2020), and one and multi-sea-winter maturation (Ayllon et al., 2015, 2019). However, it is still relatively unclear how relevant *vgll3* is for large smolt/post-smolt maturation (jacking). Previously, we assessed large smolt (0.7 kg) maturation in males kept on ambient freshwater which fluctuated with the seasons (Fraser et al., 2023a), but this is irrelevant to RAS systems which use more stable temperatures. In addition, we assessed post-smolts that had been shifted from 6 to 16 °C over a 3-day period in fish around 110 g (Fjelldal et al., 2020). This regime induced a high incidence of maturation, with family averages of 25–97% (Fjelldal et al., 2020), but such rapid rises in temperature are not used by the industry (e.g. Pino-Martinez et al., 2021). It is also unclear whether families that have high incidences of jacking as smolts or post-smolts also have high incidences of grilsing, and/or whether *vgll3* behaves consistently across different environments. For instance, some have suggested the early maturing *vgll3* allele is dominant in one and multi-sea-winter wild salmon (Barson et al., 2015; Czorlich et al., 2018) although this has not been observed by others working on domesticated (Ayllon et al., 2019) or wild (Debes et al., 2021) strains.

Most wild Atlantic salmon populations demonstrate anadromy. Those that reach a minimum size threshold at the end of the summer migrate the following spring after undergoing smoltification; the developmental process which pre-adapts salmon for seawater (Stefansson et al., 2020). Nevertheless, it is feasible to keep anadromous strains in freshwater throughout life without compromising production time to market size (Crouse et al., 2021). This flexibility means salmon can theoretically be moved to seawater at any size above the minimum threshold as there is not expected to be an upper limit. However, the photoperiod cues used to induce smoltification can also trigger puberty in males that are larger than 80 g (Fjelldal et al., 2011; Fjelldal et al., 2018; Fraser et al., 2019, 2023b). To avoid this problem, farmers can smoltify the fish at a small size of 20-70 g before moving them to brackish/seawater (Fossmark et al., 2021). Alternatively, they can use dietary intervention to initiate some indicators of seawater readiness without photoperiod manipulation (Striberny et al., 2021) or simply wait until the fish are large enough to survive sea-transfer without needing to be smoltified (e.g. >200 g, Brown et al., 2018; Ytrestøyl et al., 2023). However, Duston (1994) observed 5, 18, 23, and 27% male maturation in unsmoltified parr (approx. 26 g) which were either maintained on freshwater or moved to 10, 20, or 31 ppt, respectively. Therefore, it would be beneficial to know if salinity exposure also promotes maturity in larger "unsmoltified" salmon transferred from freshwater to seawater without any smoltification protocol and whether this is regulated by *vgll3*.

In the current study, we reared the same 3 half-sibling (common sire) families of all-male Atlantic salmon produced from a domesticated strain of *vgll3* heterozygous broodstock under different production regimes to assess the genetic and environmental regulation of male pre-harvest maturation in land-based large smolts and post-smolts, and in sea-based under-yearlings. Our hypothesises were that i) *vgll3* would consistently regulate the timing of puberty across production regimes, ii) families with high levels of smolt/post-smolt maturation would also have high levels of grilsing, and iii) salinity exposure would trigger puberty. To test these, we used three production regimes; i) constant light, 13 °C, and freshwater from first feeding up to 1 kg to produce "large smolt", ii) constant light and 13 °C from first feeding, but a switch to seawater (35 ppt) without any preparatory environmental cues at 420 g and reared up to 1 kg to produce land-based post-smolts, and iii) the production of traditional under-yearling smolts which were produced on a mixture of heated (range, 12–14 °C) and ambient (range, 5–15 °C) temperatures together with either natural or manipulated photoperiod, and transferred to sea at 150 g in December and grown for one year up to 4.75 kg to produce one sea-winter fish. For the first two regimes, testis development was measured every month from 30 g onwards to assess maturation. For the under-yearlings, testis development was assessed at harvest to quantify grilsing. Length and weight measurements were also taken at various timepoints to determine whether genotype/environment regulates growth rates and/or body condition which often positively correlates with the likelihood of maturation (e.g. Brown et al., 2024).

2. Materials and methods

2.1. Ethics

The experimental work was conducted in accordance with the laws and regulations controlling experiments and procedures on live animals in Norway following the Norwegian Regulation on Animal Experimentation 1996. The experiments were approved by the Norwegian Food Safety Authorities (FOTS #24566 and #25145) and conducted at the Institute of Marine Research (IMR), Matre Research station.

2.2. Fish stock

On the 19th of November 2019, five half-sibling families (designated A-E) of all-male salmon were produced using defrosted (previously cryopreserved) milt of a *vgll3* heterozygous YY male developed at the IMR from the AquaGen strain (Fjelldal et al., 2020) crossed with freshly ovulated eggs from one of five Mowi strain 2 sea-winter females that were also heterozygous for *vgll3*. The fertilised embryos were incubated in darkness at approx. 8 °C (Fig. 1) with each family in its own tray.

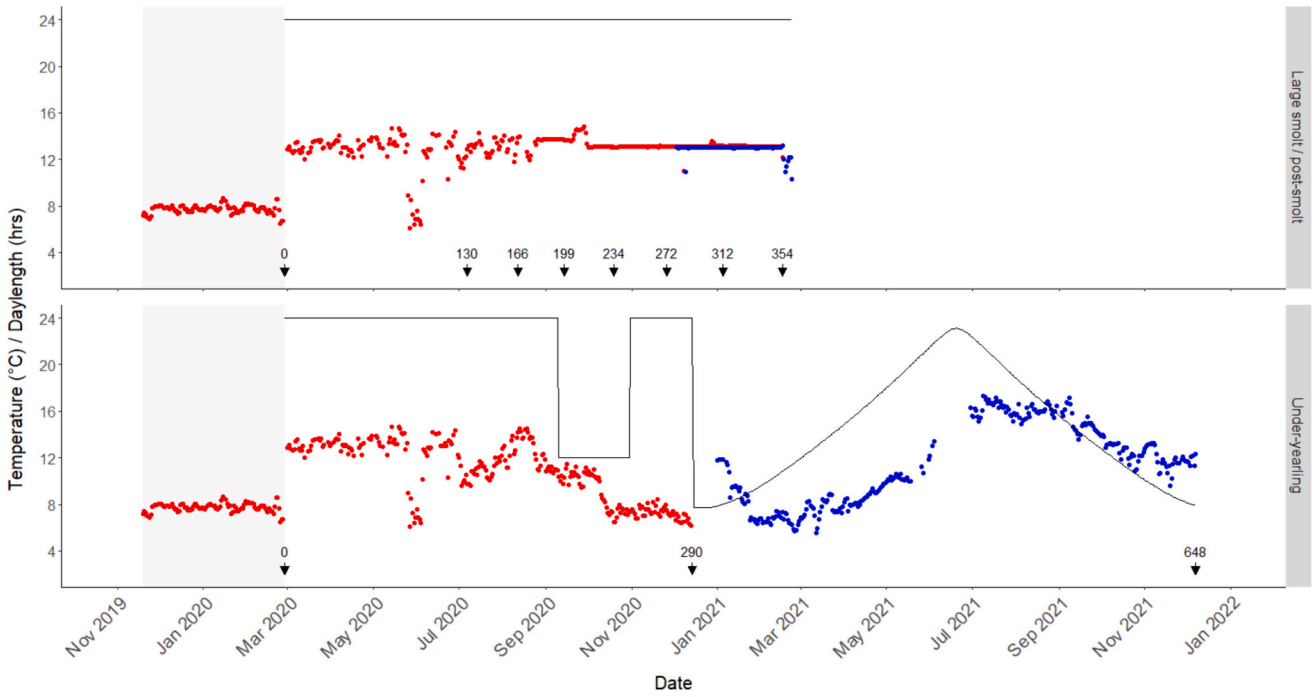

Fig. 1. Rearing conditions for the large smolt, post-smolt, and under-yearling regime. All groups were incubated (shaded area) on a common temperature of approx. 8 °C in darkness. The large smolts remained on freshwater (red points) throughout, whilst the post-smolts were kept on identical conditions as the large smolts until December 2020 whereupon they were switched to seawater (blue points). The under-yearlings were moved from land tanks supplied with freshwater (red points) to sea-cages (blue points) in December 2020. The arrows indicate samplings when the maturity status of the fish was assessed, whilst the numbers refer to the number of days since first feeding. For the under-yearlings, daylength is based on civil twilight (61°N) when in sea-cages and lights on/off when kept on land. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Large smolt/post-smolt production

From incubation until the 14th of September 2020, all families were reared separately, before being mixed into common tanks. Briefly, on the 28th of February 2020 (day 0), approximately 2500 fish/family were moved from hatching trays to five 1 × 1 m (filled with 400 L) tanks (n = 1 tank/family) to begin first feeding. Each family was then divided into two 1 × 1 m tanks/family (1250 fish/tank) on the 21st of April 2020, reduced and split between four 1 × 1 m tanks per/family on the 24th of June 2020 (400 fish/tank), and split between six 400 L tanks/family (250 fish/tank) on the 7th of July 2020. On the 2nd of September 2020, the fish from three (A-C) of the five families were merged into one 3 m ⌀ (filled with 7000 L) tank/family (1500 fish/tank). On the 14th of September 2020, 2550 fish from families A, B, and C (n = 850 fish/family) were implanted with a passive integrated transponder (PIT tag, 2 × 12 mm RFID solutions, Stavanger) for individual recognition, and had a fin clip taken and placed in ethanol for genotyping, before being divided amongst six 3 m ⌀ tanks (n = 141–142 fish/family/tank) for common garden rearing. On the 19th of October 2020, when the fish had all been genotyped (see below), the fish were reduced (n = 250 fish/tank and 21–34 fish/genotype/family/tank) with the excess *vgll3* heterozygous fish primarily being removed. On the 3rd of December 2020, the water in three tanks was gradually switched from freshwater to full-strength seawater (35 ppt) over a 12-day period (15, 25, then 35 ppt on the 3rd, 6th, and 15th of December 2020, respectively). From first feeding until the end of the experiment on the 16th of February 2021, the temperature was maintained at approx. 13 °C and day length was set to continuous light (Fig. 1). Throughout, the fish were fed an appropriate pellet size of a standard commercial diet (Skretting, Stavanger) with 20% excess based on body weights and predicted growth rates.

Information on the exact number of fish sampled for body weight and testes weight within each family and genotype at each timepoint can be found in Table S1 and S2, respectively. In brief, prior to genotyping, sampling was done blind for *vgll3*. Thereafter, we were able to maintain relatively equal sample numbers for each genotype within family and treatment (freshwater vs seawater). On the 7th of July 2020 (day 130), and 12th of August 2020 (day 166), approx. 150 fish from each family were overdosed with anaesthesia (200 mg/L Finquel®), measured for fork length (to the nearest 1 mm) and body weight (to the nearest 1 g), and an adipose fin clip was collected in ethanol for genotyping. Of these, a sub-sample of approx. 40 (day 130) and 60 (days 166) fish/family had their testis weighed (to the nearest 1 mg). On the 14th of September 2020 (day 199), 19th of October 2020 (day 234), 26th of November 2020 (day 272), and the 5th of January 2021 (day 312), all fish were lightly sedated (100 mg/L Finquel®) and measured for fork length and body weight. In addition, a sub-sample of approx. 150 fish/family/time point were overdosed with anaesthesia and had their testis weight recorded. Those sampled on the 14th of September 2020 also had a fin clip taken for genotyping. On the 16th of February (day 354), most of the remaining fish (167–171/family) were killed with an overdose of anaesthetic and their fork length, body weight, and testis weight recorded. An extra 30 fish from the seawater treatment were kept for an additional week before terminal sampling on the 23rd of February 2021 (day 361). In total, 3275 fish were confirmed as phenotypically male and genotyped, and we had the testis and body weight data for 2418 of these.

After tagging, 13 fish died during the experimental period. Only 1 of these was in the fish transferred to seawater. Family A had the most mortalities (n = 8), but there was no association with genotype. Two fish were missing their tag numbers when terminally sampled, whereas sex was not recorded for 5 fish. Two 96 h seawater challenges were conducted with 20 fish each time (10 in freshwater, 10 in seawater), one on the 14th of October 2020 and one on the 28th of November 2020. This was to ensure the fish could tolerate transfer to seawater. At both timepoints, survival was identical between freshwater and seawater treatments (90% in October and 100% in November, in both salinities).

2.4. Under-yearling production

During the reduction on the 24th of June 2020 described above, a subset of each of the five families ($n = 200$/family) were moved to 1 × 1 m tanks ($n = 1$ tank/family) and placed on ambient temperature on the 3rd of July 2020. On the 10th of September 2020, they were moved from continuous light to an artificial winter of 12 h daylength (light/dark [LD]12:12). On the 30th of October, all the fish were vaccinated with a standard six component commercial vaccine and the photoperiod was changed to continuous light to trigger smoltification. On the 14th of December 2020, a total of 840 fish from all five families ($n = 165$–170/family) were lightly sedated (100 mg/L Finquel®), PIT tagged, and reared in common garden in two 3 m ⌀ tanks prior to transfer to sea. Between 77 and 89 fish from each family were included in each tank, except for family C in which an error meant 40 fish were placed in one tank, but 125 in the other. These fish were then moved to one of two sea-cages (5 × 5 m surface area, depth of 7 m) for one year on natural light and temperatures (Fig. 1) before being harvested on the 7th of December 2021.

On the 14th of December 2020 (day 290), a total of 840 fish (n = 165–170/family) were lightly sedated, had their PIT tag recorded, measured for fork length (to 1 mm) and body weight (to 1 g), and assessed externally for signs of maturation (body shape and coloration, running milt). On the 7th of December 2021 (day 648), all the fish were euthanised, the PIT tag recorded, a fin-clip stored in ethanol for genotyping, the fish measured for body weight (to 1 g) and body length (to 0.5 cm), screened for vertebral deformities using palpation, and their external phenotype and gonads inspected for signs of sexual maturation (skin and testis colouration, jaw development, running milt, and subjective assessment of relative testis size) and phenotypic sex determination (the occurrence of testes or ovaries).

At harvest, we retrieved 814 fish of which we attained PIT tag information from 734. The missing PIT tag information disproportionally effected family A in which 34% of the fish were missing, compared to only 7–11% in the other families. Mortality was 3.2% ($n = 26/840$) during the sea-cage period, but as the PIT tag was not recorded for dead fish it was not possible to assign them to certain families. In total, 811 fish were successfully genotyped and 794 fish were identified as male and scored for maturity status. Due to the missing PIT tag information, we ended with genotype and/or family information for 619 males at sea transfer and 704 at harvest (Table S3). Of the latter, 12% were mature while only 6% of the males with genotype but no family information matured (5/82).

2.5. Genotyping

Genotyping of the *vgll3* locus was performed using an allelic discrimination assay for the two missense SNPs in *vgll3* according to Ayllon et al. (2015) and served to distinguish three different genotypes; homozygous early maturing (EE), homozygous late maturing (LL), and heterozygous early/late (EL).

2.6. Calculations

The gonadosomatic index (GSI) was calculated as the % of the total weight accounted for by the testis. Body condition was calculated using Fulton's condition factor (K); K = body weight (g)/ fork length^3 (cm) × 100.

2.7. Occurrence of females

Although we used a YY male, 0.6–3.3% of the offspring within each family had ovaries (In total, 3954 fish were examined). This was expected as we have previously found phenotypic females in our "all-male" lines (Fjelldal et al., 2020). Although we did not assay genotypic sex in this study, we previously found some of these phenotypic females were genetically male (Fjelldal et al., 2020; Ayllon et al., 2020) as have others (Brown et al., 2020). These fish were removed from any further analysis.

2.8. Statistical analysis

The data were transferred to R Statistical software (version 4.0.4, R Core Team, 2021) for all analyses. The packages "nlme" (Pinheiro et al., 2022), "MuMIn" (Bartoń, 2023), "emmeans" (Lenth, 2021), and "ggplot2" (Wickham, 2016) were used for analysis and graphical presentation. Throughout, model diagnostics were assessed via qqplots and standardised versus predicted residual plots. When comparing models, we used AICc to select the model that explains the greatest amount of variation when weighted against complexity (Hurvich and Tsai, 1991). We considered the model with the lowest AICc best described the data. Post hoc tests were carried out using least square means (LSM).

2.8.1. Assigning fish as immature or pubertal

To improve our analytical power, we assigned fish from the large smolt/post-smolt regimes as being either pubertal or immature. As the fish were on constant light for an extended period, there is little synchronisation of the timing of the reproductive process which appears to be "free running" (e.g. Pino-Martinez et al., 2023). The high variation in the GSI was indicative of this, as the values suggested pubertal males were at various stages of development (e.g. Fraser et al., 2023b). In addition, even males with GSI's well within the range of pubertal smolts producing spermatocytes (e.g. >0.5, Fraser et al., 2023b) had no obvious changes in body coloration or jaw development which are often associated with larger reproductive phenotypes (Fig. S1). Therefore, the GSI alone was used to determine the pubertal status as it increases rapidly relatively early in the reproductive process. Previously, we have used a GSI cut-off of 0.07 or 0.10, with those having a lower value considered immature based on combinations of plasma levels of reproductive hormones, histological profiles of the testes, and/or changes in growth rates and body condition (Schulz et al., 2006; Fjelldal et al., 2011; Fjelldal et al., 2018; Fjelldal et al., 2020; Melo et al., 2014, 2015; Kjærner-Semb et al., 2018; Fraser et al., 2019; Fraser et al., 2023a, 2023b). Other independent groups using their own datasets with multiple endpoints have also come to a similar conclusion, with GSI threshold values of 0.05 and 0.06 (Ciani et al., 2021; Pino-Martinez et al., 2023). In the current dataset, we used a cut-off of 0.07 as histograms of GSI demonstrated a relatively clear right skew distribution in each family, genotype, and timepoint (Fig. S2). For the under-yearlings, we sampled fish after they had been on natural light and temperature conditions for one year. This leads to a highly synchronised reproductive process leading males to be fully mature in November/December, which is when we sampled the fish. At this point, mature males are easily identified based on body coloration and jaw development, and/or by visual examination of the testes.

2.8.2. Assessing the prevalence of maturation and its association with body size

Generalized linear models (GLMs) with a binomial distribution were used to analyse the large smolt/post-smolt data for the last four timepoints. We did not analyse earlier timepoints as pubertal fish were too scarce making statistical comparisons difficult. Each timepoint was analysed separately as the seawater treatment was not introduced until the final two time points. The most complex model included fixed effects for family (3 levels, A, B, C), *vgll3* genotype (3 levels, EE, EL, LL), salinity (2 levels where appropriate, freshwater vs seawater), and their 2- and 3-way interactions (Table S4). Tank was not included in the model comparison as the lack of pubertal fish from certain groups within certain tanks prevented various models from converging. To assess whether body size at tagging explained any variation in the likelihood of maturation later in the experiment, we ran a generalized linear mixed-effects models (GLMER) with a binomial distribution with the body metric as a fixed effect (either weight, length, or condition) and genotype (3 levels),

family (3 levels), and salinity group (2 levels) as random effects.

Five GLMs were fit to the under-yearling grilsing data. The most complex model included fixed effects for family (5 levels, A to E) and *vgll3* genotype (3 levels, EE, EL, LL) and their 2-way interaction (Table S5). The cage was not included in the model comparison as the lack of pubertal fish from certain groups within certain cages prevented various models from converging. To assess whether body size at tagging explained any variation in the likelihood of grilsing, we ran a generalized linear mixed-effects models (GLMER) with a binomial distribution with the body metric as a fixed effect (either weight, length, or condition) and genotype (3 levels) and family (5 levels) as random effects.

2.8.3. Assessing maturation status, genotype, and salinity effects on growth/body size

To assess for genotype effects on body size metrics in the large smolt data we fitted linear mixed effect (LME) or linear models (LM's) to the body weight, length, and condition data (Table S6) attained from terminally sampled immature fish. Data from pubertal males was not included as puberty results in an initial growth spurt before a longer-term decline in post-smolts (Fraser et al., 2019). Body condition also increases when the first spermatocytes become present which coincides with a GSI of around >0.5 (Fraser et al., 2023b). The fish switched to seawater (post-smolts) were also not included in the analysis, as this impacted growth (see results). The most complex models included genotype (3 levels) and family (3 levels) as fixed effects and the 2-way interaction. Each timepoint was analysed separately and tank was included as a random effect on the intercept, except for the September timepoint when there was only 1 tank per family. For the under-yearling data, we fitted 19 LME models to the body weight, length, and condition data from the immature fish. The most complex model included genotype (3 levels), family (5 levels), and time (2 levels, days 290 and 648) as fixed effects, the 2- and 3-way interactions, and fish (565 levels) within tank (2 levels) as a random effect on the intercept (Table S7).

We fitted 19 LME models to the body weight, length, and condition data from both pubertal and immature males to assess the impact of seawater exposure in the large smolt and post-smolt data (Table S8). The most complex model included maturity status (2 levels, pubertal or immature), time (3 levels, days 272, 312, and 354) and salinity (2 levels) as fixed effects, all possible 2- and 3-way interactions, and genotype (3 levels), family (3 levels), fish (1427 Levels), and tank (6 levels) as random effects. Here, we only used data from those males terminally sampled on day 354.

2.8.4. Data points removed from the analysis

Those fish with vertebral deformities were removed from the maturation and growth analyses ($n = 44$) as they impact growth (Hansen et al., 2010). Nine fish had a discrepancy between the tank they were reported in at sea-transfer and the cage they were in at harvest ($n = 2$, 2, 3, 2 from families B, C, D, and E, respectively) and were removed from the analysis. In addition, 1 fish from the under-yearling production with a condition factor of 2.4 was removed from the size analysis, as values this high are either errors or from fish with significant skeletal deformities.

3. Results

3.1. Incidence of jacking

The most supported models included the fixed effects of genotype, family, and salinity (when applicable), but there was no evidence for any interactions between them (Table 1).

For the genotype effect, EE fish always had the highest incidences of puberty and LL the lowest (Table 2). This difference was significant for all the timepoints where it could be statistically assessed. The EL genotype had significantly less maturation than the EE, but more than the LL, for the majority of timepoints tested. Independent of family, the first instances of puberty occurred on days 130, 166, and 234 for EE, EL, and LL fish, when the immature males were on average 33, 90, and 283 g, respectively (Table 2). The difference between the EE and EL genotypes was generally larger than between the EL and LL's earlier in the study, but this relationship reversed over time. For the family effect, family B always had the highest incidence of pubertal fish whilst A always had the lowest. This difference was significant at all time points where it was statistically assessed. For the salinity effect, those in freshwater had a higher incidence of pubertal fish compared to those moved to seawater. A comparison of the model R^2 (Table 1), effect size (surmised from Table 2), and odds ratios (Table S9) would suggest genotype generally had the largest outcome on the likelihood of puberty, followed by family, and then salinity.

From the body size data collected at tagging on day 199, there was no evidence that any metric was associated with maturing later in life (GLMER, $p > 0.40$) (Fig. S3A—C).

Table 1
Overview of the model selection process and the results of the highest ranked model for the incidence of puberty. All the models used during the selection process can be found in Table S4.

Smolt regime	Month	Model selection					Results of the top ranked model					
		Description	Rank	Model	Δ AICc	R^2	Fixed effect	R^2	χ^2	df	*p*	
Large smolt	Oct 2020	Highest ranked	1/4	Family + Genotype	0.0	0.51	Genotype	0.04	19	2	<0.001	***
		Most complex	1/4	Family + Genotype	0.0	0.51	Family	0.06	27	2	<0.001	***
	Nov 2020	Highest ranked	1/5	Family + Genotype	0.0	0.43	Genotype	0.24	122	2	<0.001	***
		Most complex	2/5	Family × Genotype	3.0	0.50	Family	0.14	70	2	<0.001	***
	Jan 2020	Highest ranked	1/19	Family + Genotype + Salinity	0.0	0.50	Genotype	0.28	93	2	<0.001	***
		Most complex	10/19	Family × Genotype + Family × Salinity + Genotype × Salinity	6.8	0.90	Family	0.23	121	2	<0.001	***
							Salinity	0.01	3	1	0.065	
	Feb 2021	Highest ranked	1/19	Family + Genotype + Salinity	0.0	0.45	Genotype	0.24	96	2	<0.001	***
		Most complex	11/19	Family × Genotype + Family × Salinity + Genotype × Salinity	12.6	0.92	Family	0.18	125	2	<0.001	***
							Salinity	0.02	9	1	0.004	**
Under-yearlings	Dec 2021	Highest ranked	1/5	Family + Genotype	0.0	0.32	Genotype	0.10	55	2	<0.001	***
		Most complex	2/5	Family × Genotype	9.6	0.82	Family	0.06	32	4	<0.001	***

Table 2
The incidence (%) of puberty related to *vgll3* genotype, family, or salinity. Data are either i) the raw population mean without averaging over genotype or family, or ii) the least square means (LSMs) averaged over family, genotype, and salinity, from the models described in Table 1 together with the 95% confidence levels. Different superscript lowercase letters indicate significant (post-hoc, LSMs, $p < 0.05$) group effects within timepoint (row) for either genotype, family, or salinity.

Regime	Month	Weight (g)*	Genotype**			Family			Salinity	
			EE	EL	LL	A	B	C	Freshwater	Seawater
Large smolt	Jul 2020	33	6.9	0.0	0.0	0.0	2.4	2.3	1.6	–
	Aug 2020	90	1.8	0.5	0.0	0.0	2.1	0.0	0.7	–
	Sept 2020	162	4.1	0.5	0.0	0.7	2.8	0.0	1.2	–
	Oct 2020	283	6.6 (3.0–13.5)^{a}	3.6 (1.6–8.1)^{ab}	0.3 (<0.1–2.6)^{b}	0.4 (<0.1–2.8)^{b}	8.6 (4.3–16.6)^{a}	2.5 (1.0–6.4)^{b}	6.2	–
	Nov 2020	470	80.0 (72.8–85.7)^{a}	42.1 (34.0–50.7)^{b}	15.4 (10.3–22.5)^{c}	24.0 (17.5–32.1)^{b}	74.7 (66.3–81.5)^{a}	36.2 (27.7–45.7)^{ab}	46.3	–
Large smolt/ post-smolt	Jan 2021	821	92.5 (87.5–95.6)^{a}	61.1 (52.4–69.1)^{b}	27.0 (19.3–36.3)^{c}	33.0 (24.5–42.8)^{c}	90.1 (84.5–93.8)^{a}	61.4 (51.6–70.3)^{b}	70.7 (62.6–77.6)^{a}	60.6 (52.3–68.4)^{b}
	Feb 2021	1048	92.7 (88.5–95.4)^{a}	70.7 (62.9–77.5)^{b}	34.1 (26.2–43.1)^{c}	45.5 (36.8–54.4)^{b}	92.8 (88.4–95.5)^{a}	59.8 (50.9–68.1)^{ab}	77.7 (71.2–83.1)^{a}	64.5 (57.1–71.2)^{b}

			Genotype			Family				
			EE	EL	LL	A	B	C	D	E
Under-yearlings	Dec 2021	4743	23.6 (17.6–31.0)^{a}	7.7 (5.3–11.0)^{b}	2.0 (0.8–4.9)^{c}	6.9 (3.5–13.2)^{bc}	6.5 (3.6–11.6)^{bc}	11.1 (6.8–17.7)^{ab}	2.3 (0.9–5.7)^{c}	18.4 (12.4–26.4)^{a}

* Population average based on immature males in freshwater for large smolts/post-smolts or immature fish within the same sea-cage for the under-yearlings.
** *Vgll3* genotype: EE, homozygous early maturing allele; EL, heterozygous; LL, homozygous late maturing allele.

3.2. Incidence of grilsing

The most supported model included the fixed effects of genotype and family, but no interaction (Table 1). The EE genotype and family E had the highest grilsing rates, whereas the LL genotype and family D had the lowest (Table 2). The EL genotype had significantly less grilsing than the EE genotype, but significantly more than the LL. A comparison of the effect size (Table 2) and odds ratios (Table S9) would suggest genotype had a stronger influence on the likelihood of grilsing than family.

From the body size data collected at sea transfer, longer and heavier fish tended to be associated with the likelihood of becoming grilse, but neither were significant (GLMER, $p > 0.10$) (**Fig. S3E—F**).

3.3. The association between post-smolt maturation and grilsing

A comparison of the family effects on the incidence of maturation shows that although family B had significantly more maturation than families A and C in the large smolt regime, there was no family effect between these three families in the under-yearling regime (Table 2).

3.4. Growth in immature males from the freshwater large smolt production

There were no consistent genotype effects on weight or length (Table S10). Genotype was included in two timepoints for both the weight (October 2020 and November 2020) and length (September 2020 and October 2020) models with the results showing the LL fish in family A were transiently longer and heavier than the EL and/or EE genotype or the LL genotype was heavier than the EL genotype irrespective of family (Fig. S4A—D). For body condition, genotype was included in the top ranked model for 4 out of 7 sampling times, 3 as a single variable and once as part of an interaction with family (Table S10). In August 2020, the EE genotype had a higher condition than the LL genotype irrespective of family, and this was still true in family C in September 2020, but in October 2020 and November 2020 the EL genotype had a significantly lower condition than the LL genotype, but the EE genotype was no different to any other, irrespective of family (Fig. S4D—H).

Family effects over time were evident for all aspects of body size (Table S10). Family A was generally lighter, shorter, with a lower body condition than family B, with family C generally intermediate. At the final timepoint, based on their least-square means, family B was on average 12% heavier (0.98 vs 1.10 kg), 2% longer (41.6 vs 42.4 cm), with a 6% higher body condition (1.35 vs 1.43 *K*) than family A.

Of note, body condition fluctuated over time in all families (Fig. 2) with peaks in August 2020 and January 2021, while lows were in September 2020 and October 2020.

3.5. Growth in immature males from the under-yearling production

Genotype was not included in the highest ranked model for condition. It was for the length and weight models, but it was not significant in either (Table S11). There were family × time interactions on weight, length, and condition. Family C was the lightest and shortest throughout, with family E the heaviest and longest at sea transfer and family B the heaviest and longest at harvest. The difference between the largest and smallest value for family means was 8–20% for weight, 3–8% for length, and 5–6% for body condition depending on timepoint.

3.6. Growth following seawater exposure in the large smolt/post-smolt regimes

There was a 3-way interaction between time, salinity, and maturity status on body weight, length, and condition (Table 3). Irrespective of maturity status, the fish switched to seawater on the 26th of November 2020 became shorter, lighter, with a lower condition by the next sampling on the 5th of January 2021 (Fig. 3). Between the 5th of January and the 16th of February 2021, the immature fish in seawater showed the greatest weight and length increase, followed by the immature fish in freshwater. Pubertal fish tended to be heavier with a higher body condition in November but became progressively shorter and lighter as time progressed in both salinity groups. In addition, pubertal fish in seawater failed to show any growth compensation or recovery of body condition between January and February in contrast to the immature fish that regained their condition.

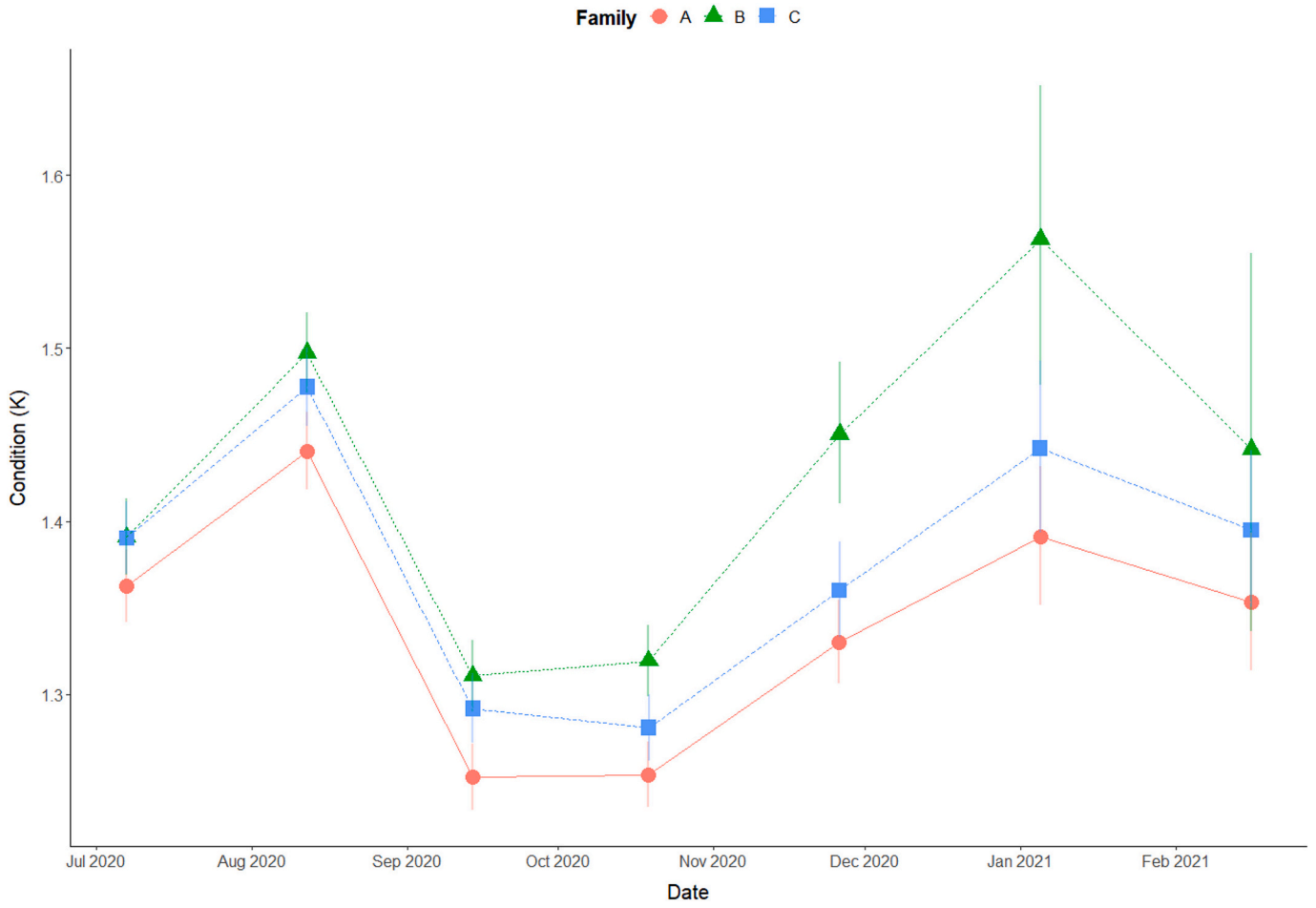

Fig. 2. Body condition over time in immature males from 3 families kept on 13 °C and constant light from first feeding (the 28th of February 2020). The data is displayed as means ±95% CI.

4. Discussion

We found strong support for our first hypothesis that *vgll3* would consistently regulate the timing of puberty across the different production regimes. However, there was no evidence to support our second or third hypothesises that family effects on jacking and grilsing would be correlated or that salinity exposure would trigger puberty. An additional finding was that growth following sea transfer is impaired to a greater extent in pubertal, compared to immature, post-smolts. These results have important implications for Atlantic salmon farmers and broodstock managers.

Previous studies have found *vgll3* to regulate the timing of puberty in domesticated European strains, whereas there is some discrepancy over whether there is any allele dominance. For instance, *vgll3* regulated the incidence of one- and multi-sea-winter maturation in males of the Mowi (Norway) strain (Ayllon et al., 2015, 2019), one-sea-winter maturation in the AquaGen (Norway) strain (Sinclair-Waters et al., 2020), and large smolt (Fraser et al., 2023a) and post-smolt (Fjelldal et al., 2020) maturation in AquaGen males. In contrast, *vgll3* was not found to regulate sea-age at maturation in the domesticated SALTAS (Tasmania) (Mohamed et al., 2019) or Saint John River (North America) (Boulding et al., 2019) strains, although the EE genotype was generally poorly represented (<10%) suggesting strong earlier selection against it in these geographical areas. Regarding allele dominance, the E allele has been reported to be dominant in wild one- and multi-sea-winter males from Norwegian/Finish rivers (Barson et al., 2015; Czorlich et al., 2018; Raunsgard et al., 2024) and in parr in a Finnish (Oulujoki) strain (Verta et al., 2020). However, we found contrasting results with regards to the direction of allele dominance. As time progressed, the results on large smolt/post-smolt maturation went from suggesting the L allele was dominant to suggesting the opposite. In the under-yearling production, grilsing was mainly attributed to the EE genotype suggesting the L allele was dominant. Both Ayllon et al. (2019) and Sinclair-Waters et al. (2020) also found the grilsing rates of EL males to be relatively intermediate between the EE and LL genotypes in the Mowi and AquaGen strains, respectively. In contrast, Debes et al. (2021) suggested the L allele was dominant in an experimental study on male parr maturation from a wild (Neva River in Russia) strain. Therefore, there appears a genotype by environment interaction with regards to whether either *vgll3* allele shows signs of dominance or not.

We found little evidence that *vgll3* regulates body size metrics in immature males. During the large smolt production, there was one time point in which EE males did have a higher body condition than LL males, with the EL intermediate. This matches the results of Debes et al. (2021), who concluded EE fish had marginally higher body conditions than LL fish as EE fish were 1% heavier. However, at other timepoints in the large smolt production, the genotype effect was only evident in some of the families and/or indicated that LL fish had a higher weight/condition than EL fish. In contrast, the family effect on condition was more consistent with a greater effect size. Therefore, further work should determine whether there is a genotype × environment interaction regarding *vgll3* and body size metrics as body size and/or condition are usually positively associated with the likelihood of maturation (Jonsson et al., 2013; Brown et al., 2024).

One of the unanswered questions from the current study is whether *vgll3* regulates the age or size of puberty. The EE fish started maturing

Table 3
Overview of the model selection process and the results of the highest ranked model for body size metrics in males on the large smolt versus post-smolt regimes. All the models used during the selection process can be found in Table S8.

Metric	Model selection						Results of the top ranked model				
	Description	Rank	Model	Δ AICc	R^2m	R^2c	Fixed effect	χ^2	df	*p*	
Weight	Highest ranked and most complex	1/19	Time × Salinity × Pubertal	0	0.64	0.68	Time	8990.4	2	<0.001	***
							Salinity	80.9	1	<0.001	***
							Pubertal	6.9	1	0.009	**
							Time × Salinity	397.4	2	<0.001	***
							Time × Pubertal	314.9	2	<0.001	***
							Salinity × Pubertal	<0.1	1	0.940	
							Time × Salinity × Pubertal	23.9	2	<0.001	***
Length	Highest ranked and most complex	1/19	Time × Salinity × Pubertal	0	0.70	0.72	Time	20,133.1	2	<0.001	***
							Salinity	24.0	1	<0.001	***
							Pubertal	23.0	1	<0.001	***
							Time × Salinity	390.4	2	<0.001	***
							Time × Pubertal	300.9	2	<0.001	***
							Salinity × Pubertal	0.1	1	0.756	
							Time × Salinity × Pubertal	14.7	2	<0.001	***
Condition	Highest ranked and most complex	1/19	Time × Salinity × Pubertal	0	0.19	0.31	Time	165.9	2	<0.001	***
							Salinity	131.3	1	<0.001	***
							Pubertal	87.6	1	<0.001	***
							Time × Salinity	251.2	2	<0.001	***
							Time × Pubertal	142.0	2	<0.001	***
							Salinity × Pubertal	<0.1	1	0.848	
							Time × Salinity × Pubertal	17.6	2	<0.001	***

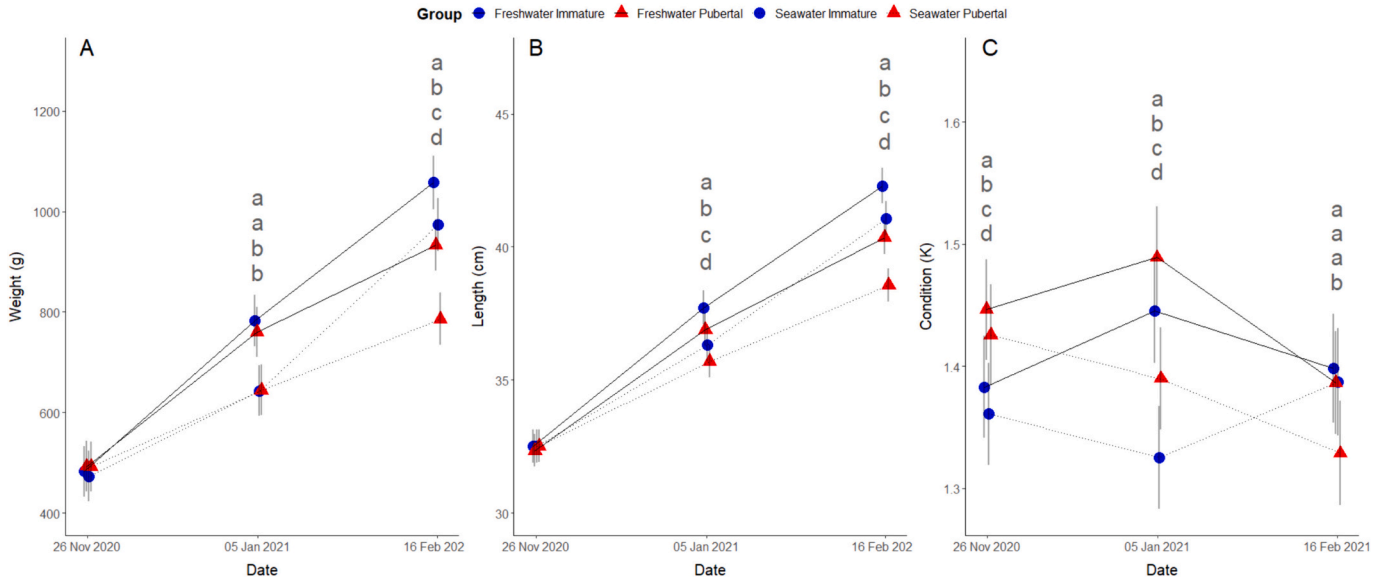

Fig. 3. Least square means (±95% CI) from linearized mixed effect models of body size metrics in male Atlantic salmon reared on-land. All the fish were originally reared in a common environment, but half the fish (post-smolts) were moved to seawater (post-smolts) starting on the 3rd of December 2020 whilst the rest remained on freshwater (large smolts). Different subscript letters indicate significant group effects within timepoint. Note that all the groups were sampled on the same days, but the points in the figure are slightly misaligned within timepoint to more clearly present the error bars.

earlier at smaller body sizes than the EL and LL fish, but there was no alternate group grown at a different rate for a comparison. Nevertheless, size thresholds for developmental processes are well documented in the animal kingdom and are already known to regulate parr maturation (Harvey et al., 2018) and smoltification (Thorpe, 1977) in salmon. In contrast, why fish would need to reach a certain age to mature other than to give a longer opportunity for growth is less clear. Further work should investigate whether *vgll3* has a similar effect on size at first puberty in fish grown at different rates.

Family had a significant outcome on the incidence of precocious maturation although the effect was not consistent across production regimes. This could be problematic for broodstock managers, as when selecting for low grilsing you may enhance post-smolt maturation under different production regimes and vice versa. Therefore, future studies may wish to evaluate other genes that play an important role in the age of puberty such as *six6* (Sinclair-Waters et al., 2020) and *tead3*

(Christensen et al., 2017) for any interaction effects with *vgll3*. In contrast to the effect of *vgll3*, the family effect on maturation was generally positively associated with growth rate and body condition. Fish size is also considered a proxy for energy storage (Shearer et al., 2006), with larger fish more likely to mature early (Jonsson et al., 2012; Fjelldal et al., 2020). However, early life growth within family did not seem to predict the likelihood of jacking or grilsing in our different regimes. This could be because our data did not accurately represent the body size during a theoretical critical decision window (Rowe et al., 1991), although it is currently unclear when this window naturally occurs and when it would occur in either of our regimes that used extensive photoperiod manipulation. In addition, it is noted the different families were not reared in common garden from fertilisation. Therefore, although not expected, it is not possible to determine whether unexpected tank effects during early life rearing may have influenced our results.

The large smolts moved to seawater showed a reduction in the incidence of puberty. This suggests seawater exposure is unlikely to trigger maturation in contrast to what was reported in smaller unsmoltified parr transferred abruptly to seawater (Duston, 1994). At first glance, one would suggest the drop-off in growth rate upon entry into seawater may explain this. However, the immature males showed an element of catch-up growth between the final two timepoints, but no increase in maturation rates. Similarly, de Fonseka et al. (2022) found no effect of salinity on post-smolt male maturation in AquaGen males despite a 30% difference in growth between the seawater and freshwater treatments. Mohamed et al. (2019) also found approx. 55% grilsing in two groups of 22-month-old SALTAS males, one of which had remained in freshwater throughout life whilst the other had been moved to seawater 9 months before assessment. Again, this was despite a 12% difference in growth rates between the two groups. However, female grilsing was slightly lower in those moved to seawater (20 vs 10%, Mohamed et al., 2019). Ytrestøyl et al. (2023) did observe a slight elevation in male maturation in relation to salinity in a RAS. They found 6% male maturation in 600 g SalmoBreed males that had been produced on 12 ppt, continuous light, and a constant temperature of 12.5 °C from 100 g. In contrast, no pubertal males were found in males that had remained on freshwater, continuous light, and 12.5 °C. However, the opposite occurred in two further groups which had been given a square-wave photoperiod at 32 g prior to then being kept on constant light and either freshwater or 12 ppt salinity from 100 up to 600 g. Here, 11% of the males in the square-wave and freshwater treatment were pubertal at 600 g, but there were no signs of male maturation in counterparts on the square-wave and 12 ppt treatment (Ytrestøyl et al., 2023).

We found males maintained on constant light and 13 °C from first feeding grew rapidly, but experienced high levels of puberty. Growth rates were similar to those in the AquaAdvantage salmon, which has been gene edited to enhance growth, kept on almost identical conditions (e.g. just over 1 kg after 12 months when kept on constant light and 13.5 °C freshwater from first feeding, Ignatz et al., 2020). The results from the freshwater large smolt protocol of 40–80% male puberty within each family match data from studies in freshwater RAS. Good et al. (2017) reported approx. 70% of SalmoBreed males grown at 13 °C and given a square-wave photoperiod at 40 g followed by continuous light were pubertal/mature at 12 months post-hatching. Similarly, Crouse et al. (2022) reported 37 and 65% of males from the domesticated Stofnfiskur strain (Iceland) to be pubertal at 1.3 kg when reared on continuous light from first feeding and either 12 or 14 °C, respectively. In flow through systems, Pino-Martinez et al. (2023) reported 25% male maturation in domesticated Salmobreed fish kept on 12.5 °C and constant light from 50 g to 600 g, while Imsland et al. (2014) observed 82% male maturation in post-smolts of the domesticated Bolaks (Norway) strain that had been reared on constant light and 10–13 °C from first feeding up until 330 g. The fish were on freshwater for most of the time but switched to seawater 2 months before sampling without using a preparatory smoltification cue. In contrast, Ytrestøyl et al. (2023) found no signs of puberty in 600 g SalmoBreed males reared on 12.5 °C freshwater and continuous light from 32 g in a RAS. Whether this inconsistency is explained more by genetics, or the environment, is uncertain.

In the large smolt regime, we observed a noticeable reduction in body condition between August and September 2020. Shortly after, there was a large increase in maturation across all the *vgll3* genotypes. The body condition also began to dip again 5 months later when the fish were around 1 kg although the experiment was terminated too early to know when this second dip would peak. Eriksson and Lundqvist (1982) observed body condition to fluctuate on a 10-month cycle in salmon parr kept on constant conditions of LD 12:12 for 14 months at 11 °C. This phenomenon was linked to an endogenous rhythm of smoltification, a process that includes a reduction in body condition (Eriksson and Lundqvist, 1982). Others have reported female rainbow trout (*Oncorhynchus mykiss*) to also have an endogenous rhythm of maturity (Duston and Bromage, 1988). As testes development is usually associated with increasing daylength, similar to smoltification (e.g. Fjelldal et al., 2018; Fraser et al., 2023a, 2023b; Pino-Martinez et al., 2023), it would be interesting to know if this fluctuation in body condition is also linked to an endogenous rhythm of maturity in Atlantic salmon.

Salinity exposure during the post-smolt production had significant effects on growth in the immature fish with an initial reduction, followed by an increase. However, although the body condition of immature males had recovered within 82 days of salinity exposure, body weight had not. As we did not measure feed intake, it is also unclear if this was due to compensatory growth or increased feeding. Ytrestøyl et al. (2023) also found transferring 200 g salmon to seawater without a smoltification protocol resulted in depressed growth during the initial 5 weeks, but with no effect on survival or growth thereafter. In contrast, when transferred at 600 g, the lack of a smoltification protocol had no negative effect on growth during the initial 5 weeks. We have also seen that smolts given a square-wave smoltification protocol show no salinity effect on growth over the initial 42 days when on 12 °C and on constant light, however those fish transferred to seawater (35 ppt) were 30% heavier after 3 months compared to controls that remained in freshwater (de Fonseka et al., 2022). Similarly, in 80-100 g smolts, those given a square-wave grew quicker in seawater following the initial transfer than those previously kept on continuous light and fed smolt feed (Myklatun et al., 2023). As such, although larger salmon can tolerate seawater without any preparatory photoperiodic cues, these cues are expected to aid the transition in terms of maximising growth in smaller (<600 g) fish.

In contrast to the immature males, pubertal post-smolts displayed no recovery of growth compared to freshwater counterparts during the latter part of the study. Similarly, Fjelldal et al. (2022) found 78% of 300 g wild genotype (Etne, Norway) smolts that died following sea transfer were pubertal suggesting it impedes fish welfare and survival during this critical transition. Together, this suggests pubertal post-smolts find seawater more challenging than immature counterparts. Others have also found puberty lowers indicators of hypo-osmoregulatory ability, such as the enzyme Na^+/K^+-ATPase which is involved in ion transport (Fjelldal et al., 2018; Fraser et al., 2023b; Pino-Martinez et al., 2023), and is necessary to perform well in seawater. As such, maturing smolts should be treated with greater care than immature counterparts if they are to be moved from freshwater to seawater, as they appear to be less adapted to perform well in seawater.

In conclusion, male Atlantic salmon produced using modern large smolt/post-smolt protocols show high levels of puberty prior to reaching 1 kg with a rapid increase between 250 and 500 g. The likelihood of entering puberty was regulated by genetics (*vgll3* and family) and the environment (salinity and smolt production regime). Therefore, *vgll3* can be used by broodstock managers to limit precocious sexual maturation. There also appears to be potential to combine *vgll3* with family traits/unknown genes to further prevent precocious puberty in certain production regimes.

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CRediT authorship contribution statement

Thomas W.K. Fraser: Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Aslak Tjølsen:** Writing – review & editing, Investigation, Formal analysis. **Angelico Madaro:** Writing – review & editing, Investigation, Conceptualization. **Tom J. Hansen:** Writing – review & editing, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Per Gunnar Fjelldal:** Writing – review & editing, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

Thomas Fraser reports financial support was provided by the Research Council of Norway. Co-author was an MSc student during the project, but is now employed by Skretting (feed supplier) - A.T. If there are other authors, they 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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.741307.

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