Altered spawning seasons of Atlantic salmon broodstock genetically and epigenetically influence cell cycle and lipid-mediated regulations in their offspring

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Abstract

Manipulating spawning seasons of Atlantic salmon (*Salmo salar*) is a common practice to facilitate year-round harvesting in salmon aquaculture. This process involves adjusting water temperature and light regime to control female broodstock maturation. However, recent studies have indicated that altered spawning seasons can significantly affect the nutritional status and growth performance of the offspring. Therefore, gaining a deeper understanding of

the biological regulations influenced by these alterations is crucial to enhance the growth performance of fish over multiple generations.

In this study, we investigated four different spawning seasons achieved through recirculating aquaculture systems (RAS) and sea-pen-based approaches. In addition to the normal spawning season in November (sea-pen), three altered seasons were designated as off-season (five-month advance, RAS), early season (two-month advance, sea-pen), and late season (two-month delay, sea-pen). We conducted comprehensive gene expression and DNA methylation analysis on liver samples collected from the start-feeding larvae of the next generation.

Our results revealed distinct gene expression and DNA methylation patterns associated with the altered spawning seasons. Offspring from RAS-based off-season spawning exhibited lower larval weights and showed altered lipid-mediated regulations, which may potentially hinder growth performance. In contrast, early and late seasons affected cellular processes, particularly cell cycle regulation, with implications for potential negative impacts on future growth potential. The implications of our findings are significant for aquaculture and nutritional studies, providing valuable tools for assessing growth potential and optimizing production strategies.

Keywords

Broodstodk, Spawning season, Intergenerational, Nutrition, Omics, DNA methylation, Cell cycle, Lipid metabolism

Introduction

The Atlantic salmon (*Salmo salar*) follows an anadromous life cycle, beginning in freshwater, migrating to seawater for most of its life, and returning to freshwater to spawn. In salmon aquaculture, the timing of spawning is adjusted to ensure a year-round availability for harvesting. This manipulation involves controlling abiotic factors such as water temperature and photoperiod to influence the spawning timing [1, 2]. Additionally, apart from the traditional open sea-based cages, a land-based approach utilising recirculating aquaculture systems (RAS) is employed to replicate the salmon's natural lifecycle more accurately, encompassing both saltwater gonad maturation and final freshwater maturation [3]. Despite the ongoing and successful practices of spawning season alterations in salmon aquaculture, recent studies have reported that these practices can impact the nutritional status of broodstock, leading to negative effects on the growth performance in the next generation [4, 5].

Nutritional programming, in the context of fish nutrition, refers to strategically manipulating the feeding regime of fish during critical developmental stages to enhance their long-term growth performance [6-8]. It can be expanded into intergenerational programming by providing specific nutrients or dietary components to the parents before reproduction [8, 9]. Hence, spawning season alterations of salmon broodstock can be seen as an example of intergenerational nutritional programming even though the nutritional manipulation occurs indirectly through abiotic factors. The principal target of the programming is to ensure the production of healthy and robust offspring in this context.

While various nutrients influence the health condition and growth performance of fish, including Atlantic salmon [10-12], altered spawning seasons have appeared to affect the status of multiple metabolites and nutrients including micronutrients, amino acids, and lipid

classes [4, 5]. These nutrients are also closely associated with vital biological functions essential for growth, such as one carbon (1C) metabolism, the citric acid cycle, and the Cahill cycle. Key micronutrients like vitamin B12, folate, vitamin B6, and methionine are integrated with the 1C metabolism [13, 14], which also regulates cellular methylation potential through S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH), critical for various cellular processes [15]. The citric acid cycle enables the catabolism of carbohydrates, fats, and amino acids, releasing stored energy crucial for growth and development [16], while the Cahill cycle facilitates the transport of amino groups and carbons between muscle and liver [17], both playing essential roles in various developmental processes. Amino acids are essential for growth and tissue development, in addition to being the building blocks of proteins [18], while lipids serve as major energy reserves and contribute to the formation of cell membranes, also supporting various developmental processes [19].

Beyond the importance of nutrients, understanding genetic influences within the context of nutritional programming provides invaluable insights from a molecular biology perspective. Recent studies have revealed that alterations in micronutrient levels within the feed can have genetic effects on various biological pathways in salmon, including lipid metabolism, protein synthesis, and post-transcriptional regulation [20, 21]. Moreover, an additional layer of information comes from the analysis of DNA methylation, which offers valuable epigenetic perspectives. DNA methylation, being an epigenetic regulatory process, responds to environmental stimuli, governing cell stability, differentiation, and development [22, 23]. As DNA methylation is inherited during cell replication, it can lead to changes in epigenetic regulation over the long term [22, 24].

The present study investigates the genetic and epigenetic effects resulting from four different spawning seasons. These seasons include the natural sea-pen-based spawning in November, an accelerated RAS-based spawning occurring five months earlier, and two

variations of sea-pen-based spawning: one with a two-month advance and another with a two-month delay relative to the natural spawning season. The main analysis of the study focuses on liver samples collected from start-feeding larvae at the end of the endogenous feeding phase. The liver serves as a key target organ for various biological reactions crucial to growth, and using start-feeding larvae helps eliminate the influences of exogenous feeding. To uncover both genetic and epigenetic impacts comprehensively, we employed RNA-sequencing (RNA-seq) for gene expression and reduced-representation bisulfite sequencing (RRBS) for DNA methylation analysis. Our findings provide valuable insights into the significance of the nutritional state in broodstock, as it can affect the growth of their offspring. Additionally, our study offers potential usages of gene expression and DNA methylation data as powerful tools for assessing current and future growth performance, which can significantly benefit a wide range of nutritional programming studies.

Methods and Materials

Ethical considerations

The experiment adhered to the ARRIVE guidelines for design and reporting. Broodstock and embryos used in the study were obtained from AquaGen's commercial production facility of Atlantic salmon at their breeding station in Kyrksæterøra, Norway.

The conditions and protocols applied in this experiment were identical to those used in commercial production, including certain confidential information protected under commercial interests. Throughout the sampling procedures for broodstock and larvae, anesthetics were administered by the supplier's instructions and compliance with both Norwegian and European legislation on animal research.

Formal approval from the Norwegian Animal Research Authority (NARA) was not required for this experiment. This exemption is because the experimental conditions fall under practices recognized as commercial animal husbandry, thereby exempting them from the European Convention on the protection of animals used for scientific purposes (2010/63/EU), specifically under Article 5d. Additionally, the Norwegian ethics board also approved the experiment by the Norwegian regulation on animal experimentation, § 2, 5a, d, for activities classified as "non-experimental husbandry (agriculture or aquaculture)" and "procedures in normal/common breeding and husbandry".

Experimental design and sampling

As the details of experimental design and sampling methods have been described elsewhere [4, 5], we only present summarized descriptions here. The study utilized broodstock and embryos obtained from AquaGen's breeding station in Kyrksæterøra, Norway. The female broodstock, sourced from the 15th generation, covered four spawning seasons: off-season, early, normal, and late seasons.

The off-season broodstock were reared in a land-based recirculating aquaculture system (RAS) in saltwater (12% salinity), while the rest of the broodstock were raised in sea-based net pens. All broodstock were fed to satiation with broodstock feed (EWOS Breed 3500) until transferred to freshwater. The normal season broodstock matured naturally under ambient photoperiod and temperatures, whereas artificial controls were applied to regulate maturation for the other spawning seasons. Specifically, the off-season and early season broodstock were subjected to five- and two-month advance maturation, respectively, while the late season broodstock were subjected to a two-month delay. The fish received no further feeding after the transfer to freshwater. The maturation process in freshwater lasted for 109,

140 163, 120, and 166 days for off-season, early, normal, and late season broodstock,
141 respectively, before fertilization to obtain the next generation (Supplementary Table S1).

142 From each spawning group, five females from separate tanks were sacrificed using
143 Benzoak (200 mg/L, ACD Pharmaceuticals AS). After stripping, each female's growth
144 measures, including body weights (n=5 per spawning season), were recorded. Liver samples
145 were dissected and flash frozen in liquid N2 for nutrient analysis. All oocytes were fertilised
146 with cryopreserved sperm from two pooled males. Newly fertilized eggs (2 d°) were flash-
147 frozen in liquid N2 for nutrient analysis (Supplementary Table S1).

148 At the end of the endogenous feeding phase (start-feeding larvae 979–994 d°;
149 Supplementary Table S1), 36 larvae from each broodstock were randomly selected,
150 euthanised with an overdose of buffered tricane methanesulfonate (MS222, Pharmaq,
151 Norway), and their growth measures, including body weights (n=180 per spawning season),
152 were recorded. Additionally, liver samples were dissected from larvae and flash frozen in
153 liquid N2 for RNA and DNA analysis.

154 Growth measure and nutrient analysis

155 We collected the data of body weights for broodstock and larvae from previous studies [4, 5].
156 In addition, we obtained the data on selected nutrients and metabolites from the same studies,
157 specifically vitamin B6 & B12, folate, S-adenosylmethionine (SAM), S-
158 adenosylhomocysteine (SAH), cholesterol, the sum of six different lipids, glutamate, L-
159 serine, L-lysine, L-alanine, L-glutamine, urea, and B-alanine [4, 5]. To analyse the data from
160 all four spawning seasons, we performed ANOVA followed by Tukey's HSD test.

161 ## DNA and RNA extraction

162 RNA extraction was carried out using the BioRobot EZ1 and EZ1 RNA Universal Tissue kit
163 (Qiagen), followed by DNase treatment using the Ambion DNA-free DNA removal kit
164 (Invitrogen, USA) according to their respective protocols. The RNA quantity was assessed
165 using the NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies) and the Agilent
166 2100 Bioanalyzer with the RNA 6000 Nano LabChip kit (Agilent Technologies). For DNA
167 isolation, the DNeasy Blood & Tissue Kit (Qiagen, Cat. No. #69506) was used as per the
168 manufacturer's protocol. Liver samples underwent RNase A treatment (provided by the
169 Qiagen kit, 50ng/µL, 10 min at room temperature) followed by proteinase K treatment (New
170 England Biolabs, #8102S 20µg/µL, 1.5 h at 55°C). DNA was eluted in Milli Q water. DNA
171 quantification was performed using the Qubit High Sensitivity Assay (Life Technologies
172 #Q32854). Detailed methods for both DNA and RNA extraction were described elsewhere
173 [20].

174 ## Library preparation of high throughput sequencing

175 Liver samples were sequenced at two different sequencing facilities for specific analyses. For
176 RNA-seq, the liver samples were processed at the DeepSeq sequencing facility at Nord
177 University, Bodø, Norway, and the library preparation was conducted using the NEBNext
178 Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs). Sequencing
179 was performed on the NextSeq500 machine (Illumina). For RRBS, the liver samples were
180 processed at the CeMM Biomedical Sequencing Facility, Vienna, Austria. The genomic DNA
181 was extracted, digested by MspI, and bisulfite-converted before library preparation. The
182 RRBS libraries were sequenced on Illumina HiSeq 3000/4000 instruments. Detailed methods
183 of the library preparation for RNA-seq and RRBS were described elsewhere [20].

Atlantic salmon genome and genomic annotation

The reference genome (ICSASG version 2) and RefSeq data (version 100) for gene annotation were downloaded from the NCBI web site (https://www.ncbi.nlm.nih.gov/assembly/GCF_000233375.1). In cases where gene symbols were either outdated or unavailable from RefSeq data (version 100), gene symbols from a newer version of RefSeq data (version 102) and UniProt [25] were used. The genome regions were annotated into intron, exon, three promoter regions, and flanking regions. Promoter regions were divided into three categories based on their distance from transcription start sites: P250 (1-250 bp), P1K (251-1000 bp), and P5K (1001-5000 bp). Flanking regions were defined as 5000 upstream from P5K and 10000 downstream from the 5' end of the gene.

Pre-processing of high throughput sequencing

For both RNA-seq and RRBS data, the same pre-processing procedures were followed as described in a previous study [20]. This involved trimming the reads using Cutadapt [26] and Trim Galore! (Barbraham Institute), aligning the trimmed reads to the reference genome using STAR [27] for RNA-seq and Bismark/Bowtie 1 [28, 29] for RRBS with their default parameters. The mapped RNA-seq reads were quantified using featureCounts [30], while the mapped RRBS reads were processed by Bismark [28] for methylation calling and CpG site extraction. The samples were divided into four spawning season groups for further analysis: off-season, early, normal, and late seasons, and clustering analysis was performed using the factoextra package (https://CRAN.R-project.org/package=factoextra). RNA-seq counts were subjected to a variance stabilizing transformation (VST) using DESeq2 [31] before principal component analysis (PCA).

206 Raw reading data were uploaded to the repository of the Sequence Read Archive
207 (SRA) on the NCBI web site and available under the accession numbers PRJNA680425 and
208 PRJNA642998 for RNA-seq and RRBS, respectively.

209 Differential gene expression analysis

210 Differentially expressed genes (DEGs) were identified using DESeq2 [31] with an adjusted p-
211 value cut-off of less than 0.05. P-values were adjusted by the Benjamini-Hochberg procedure
212 [32]. From the candidate DEGs, those with absolute log fold changes (LFCs) greater than or
213 equal to 1.2 were selected as the final set of DEGs by using the lfcThreshold argument of the
214 results function of DESeq2 [31]. Shrunken LFCs values, calculated by the normal shrinkage
215 method provided by DESeq2 [31], were used for both heatmaps showing functional
216 annotation results and scatter plots showing merged results between DNA methylation and
217 gene expression differences.

218 Functional annotation with KEGG

219 Over-representation analysis (ORA) was performed on the Kyoto Encyclopedia of Genes and
220 Genomes (KEGG) database [33] using clusterProfiler [34]. Gene lists of DEGs identified
221 from three pair-wise comparisons were used as input, and enriched pathways were
222 determined based on adjusted p-values less than 0.05. P-values were adjusted by the
223 Benjamini-Hochberg procedure [32].

224 Methylation rate analysis

225 Mapped RRBS reads were filtered by methylKit [35], discarding reads with coverage less
226 than or equal to 10 and above the 99.9th percentile. Additional RRBS data from two other
227 studies were obtained for post-smolt and harvesting stages [20, 21] and underwent the same
228 pre-processing procedures to compare the distributions of methylation rates. Raw data of

229 these RRBS sequencing data can be found in SRA on the NCBI site under the accession
230 numbers of PRJNA680423 and PRJNA628740 for post-smolt and harvesting stages,
231 respectively. Ridge plots were generated using the ggridges R library (https://CRAN.R-
232 project.org/package=ggridges).

233 **Differential methylation analysis**

234 Differentially methylated CpG sites (DMCs) were identified by methylKit [35], and Q-values
235 were calculated using the sliding linear model (SLIM) method [36]. CpGs were identified as
236 DMCs when Q-values were less than 0.01 and methylation differences were greater than or
237 equal to 15%.

238 To filter out DMCs that were strongly affected by altered spawning seasons, we used
239 the counts of DMCs per gene in two promoter regions: P250 and P1K. Genes were sorted by
240 the count of DMCs in a descendant order, separately for P250 and P1K, and genes that
241 contained at least three DMCs were selected for further analysis.

242 **Bioinformatics analysis**

243 In-house R and Python scripts with Snakemake [37] were utilized for high-throughput
244 sequence analysis, basic statistical analysis, and figure generation.
245

246 # Results

247 **Altered spawning seasons impacted the weight and nutritional status of**
248 **offspring**

249 The present study examines the effects on gene expression and DNA methylation patterns in
250 the liver of offspring hatched from four different spawning seasons: an off-season based on a

recirculating aquaculture system (RAS) and three sea-pen based seasons (early, normal, and late) (Fig. 1a). Off-season spawning occurred in June, early season in September, and late season in January, relative to the normal spawning months of November. (see detailed dates and degree days in Supplementary Table S1). As the growth performance and nutritional status of the broodstock and offspring have already been assessed and discussed elsewhere [4, 5], this section only provides a summary emphasizing the weight and the status of selected nutrients and metabolites.

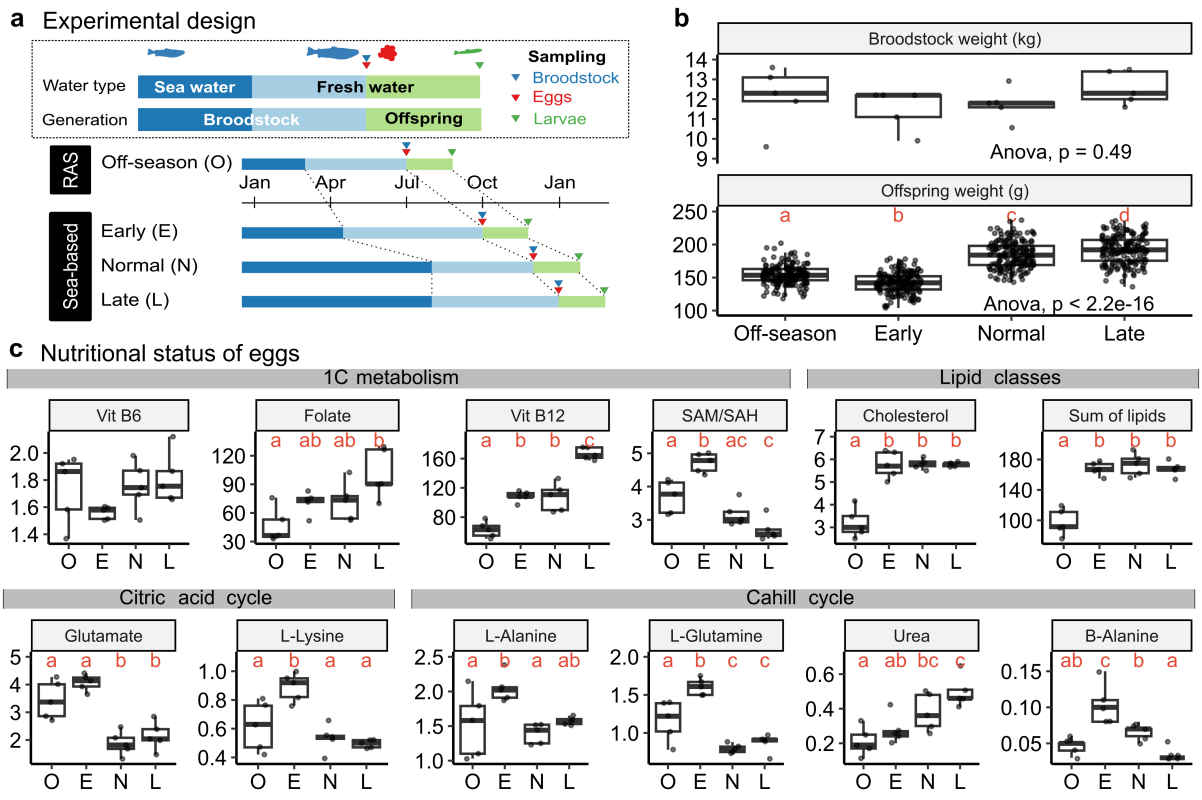

Fig. 1. Experimental design, body weights, and nutritional status across spawning seasons.

a) Schematic diagram illustrating four distinct spawning seasons: off-season (O), early (E), normal (N), and late (L). Off-season was conducted in RAS, while others were in a sea-based environment. Broodstock were transferred from seawater (blue bars) to freshwater (light blue bars) before spawning. Offspring were raised in freshwater until sampling (green bars). Triangles indicate sampling points: broodstock (blue), eggs (red), and larvae (green). **b)** Boxplots showing body weights of broodstock (n=5) and larvae (n=180). Red letters represent Tukey's HSD compact letter display after ANOVA tests ($p<0.05$). **c)** Boxplots of 12 nutrients and metabolites in egg samples. Nutrients and metabolites are categorised into four groups: 1C metabolism, lipid classes, citric acid cycle, and Cahill cycle. Units: 1C metabolism group (Vitamin B6: mg/kg ww, Folate: µg/100 g ww, Vitamin B12: µg/kg ww); remaining groups: lipid classes (mg/g ww), citric acid cycle (µmol/g ww), Cahill cycle (µmol/g ww). Red letters represent Tukey's HSD compact letter display after ANOVA tests ($p<0.05$).

271 Broodstock weights showed no significant differences at spawning (upper part of Fig.
272 1b), but offspring weights at the larvae stage (979 and 994 degree days) showed noticeable
273 variation (lower part of Fig. 1b). Specifically, off-season and early season larvae had lower
274 weights compared to normal and late seasons. This trend was consistent with the observations
275 in egg sizes, where eggs from off-season and early-season exhibited smaller sizes compared
276 to the other two seasons (Supplementary Table S2).

277 Altered spawning seasons widely impacted the nutritional status of offspring eggs,
278 particularly regarding 1C metabolism, lipid classes, citric acid cycle, and Cahill cycle [4, 5],
279 but with inconsistent patterns across nutrients and metabolites (summarised in Fig. 1c and
280 Supplementary Tables S3-S6). Previous studies also reported that altered spawning seasons
281 affected the nutritional status of the broodstock [4, 5]. Thus, altered spawning seasons
282 significantly influenced the nutritional status of broodstock without impacting their weights,
283 subsequently affecting offspring nutritional status and growth potential of their offspring.

284 To investigate the underlying genomic and epigenetic regulations associated with the
285 observed differences in nutritional status and growth performance, we utilised RNA-seq for
286 gene expression analysis and RRBS for DNA methylation analysis on liver samples collected
287 from larvae at the end of the endogenous feeding phase.

288 Early and late spawning seasons exhibited similar gene expression patterns 289 when compared to the normal season

290 We performed gene expression analysis on 20 liver samples divided into four groups (n=5) to
291 investigate the impact of different spawning seasons to the offspring when compared to the
292 normal season (detailed statistics for each sample are provided in Supplementary Table S7).

Principal component analysis (PCA) differentiated the off-season from the normal season when considering all mapped genes (left part of Fig. 2a). However, when focusing on the top 500 genes with high variances, the early season moderately deviated from the normal season (right part of Fig. 2a). In both cases, the late season fell between the normal and early seasons.

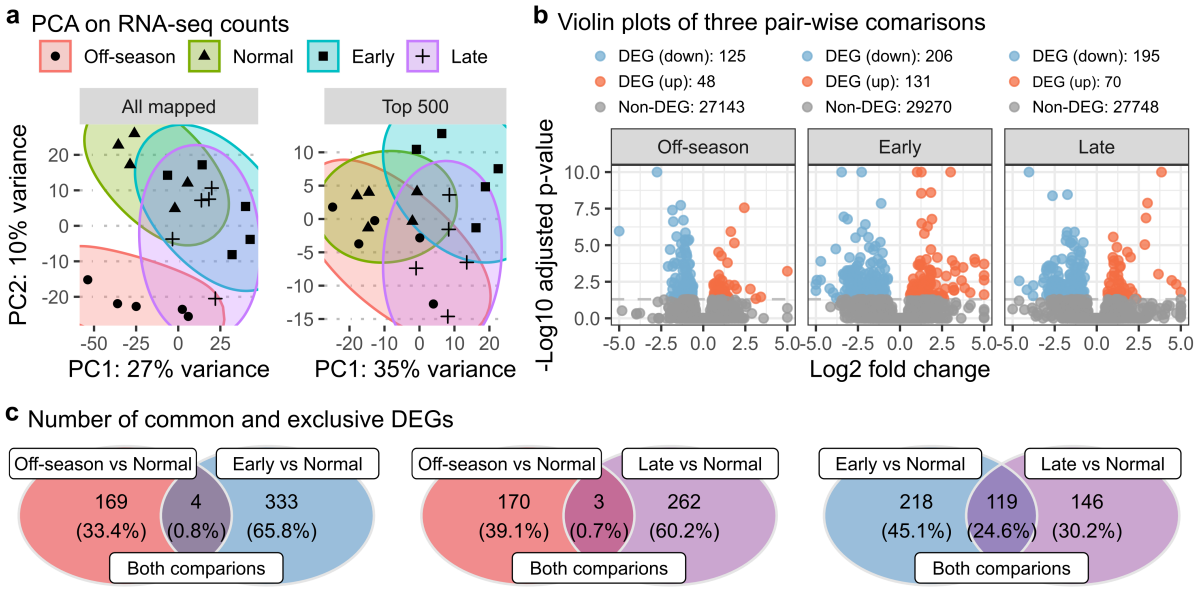

Fig. 2. Gene expression analysis comparing altered spawning seasons to the normal season.

a) PCA plots displaying clustering patterns of four spawning seasons - off-season (red), normal (green), early (blue), and late (purple) - using 20 RNA-seq samples. "All mapped" plot includes 45,153 genes, while "Top 500" includes top 500 genes with high variance. **b)** Volcano plots showing differential expression analysis results for three pairwise comparisons: off-season vs. normal (Off-season), early vs. normal (Early), and late vs. normal (Late). Dots represent down-regulated DEGs (blue), up-regulated DEGs (red), and non-DEGs (grey). Counts of DEG and non-DEGs were provided in the legend. **c)** Venn diagrams illustrating overlaps of DEGs identified between two comparisons, indicating common DEGs as well as those exclusive to a single comparison.

In three pairwise comparisons for differential expression analysis (off-season vs. normal, early vs. normal, and late vs. normal), we identified 173, 337, and 265 differentially expressed genes (DEGs), respectively (Fig. 2b). All comparisons revealed a higher number of down-regulated genes than up-regulated genes. Additionally, Venn diagrams indicated that the DEGs from the off-season vs. normal comparison had very few overlapping genes (<1%) with the other two comparisons (depicted in the first and second diagrams in Fig. 2c). On the

other hand, early vs. normal and late vs. normal comparisons shared approximately 25% of the DEGs (depicted in the third diagram in Fig. 2c).

The results from PCA and differential expression analysis suggest that the expression pattern of the off-season differed from both the early and late seasons when compared to the normal season. Furthermore, the early and late seasons exhibited similar gene expression patterns, with the late seasons showing more resemblance to the normal seasons.

Altered spawning seasons affected various biological pathways, including those related to metabolism, cellular processes, and organismal systems

To explore the impact of altered spawning seasons on biological pathways, we conducted functional analysis on DEGs using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Over-representation analysis (ORA) identified a total of 11 enriched KEGG pathways, which were classified into three functional categories: metabolism, cellular processes, and organismal systems (Fig. 3).

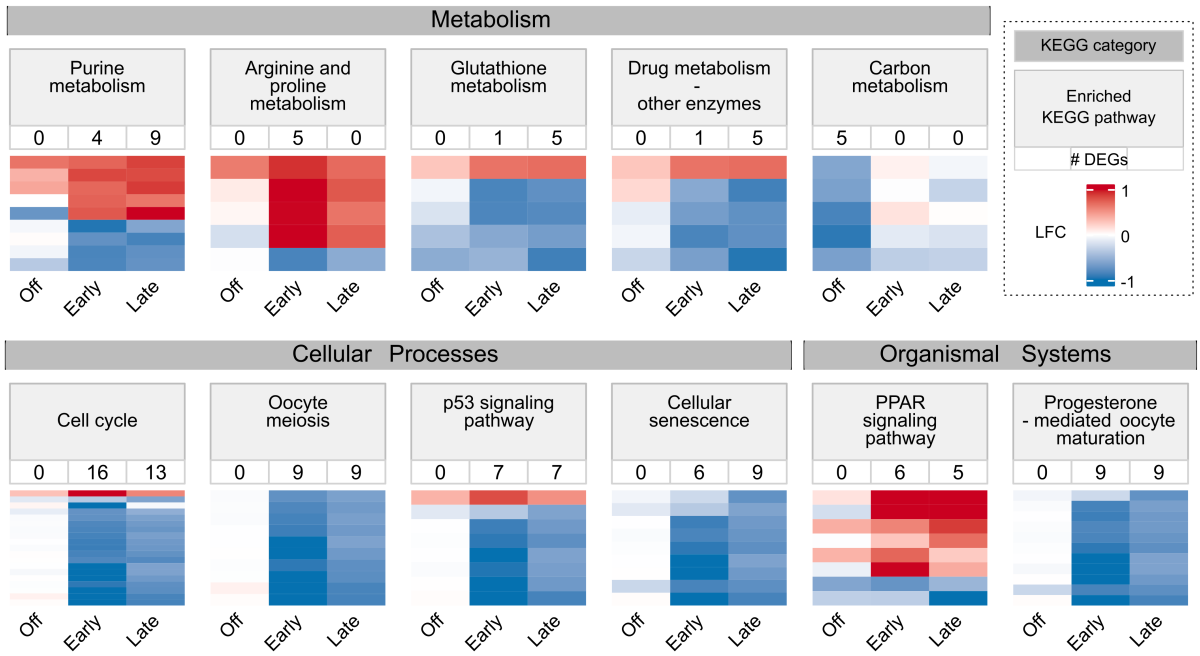

Fig. 3. Enriched KEGG pathways for DEGs identified in three pair-wise comparisons.

Heatmaps showing log fold changes (LFCs) of genes associated with 11 KEGG pathways enriched through over-representation analysis. LFCs underwent normal shrinkage transformation. Genes were included if identified as DEGs in at least one of the three comparisons: off-season vs normal (Off), early vs normal (Early), and late vs normal (Late). Number below each pathway represents DEG count. KEGG pathways are categorized into metabolism, cellular processes, and organismal systems groups.

In the off-season vs. normal comparison, ORA identified only one enriched pathway, carbon metabolism (Supplementary Table S8). All five DEGs associated with carbon metabolism showed down-regulation (Fig. 3).

For the early vs. normal comparison, ORA revealed six enriched pathways: one in metabolism, three in cellular processes, and two in organismal systems (Supplementary Table S9). Metabolism-related pathways exhibited both up- and down-regulation, while most genes in cellular processes showed significant down-regulation. In organismal systems, PPAR signalling showed up-regulation, while progesterone-mediated oocyte maturation showed down-regulation (Fig. 3).

The late vs. normal comparison identified nine enriched pathways: three in metabolism, four in cellular processes, and two in organismal systems (Supplementary Table S10). Remarkably, this comparison shared five enriched pathways with the early vs. normal comparison, indicating similar impacts on biological pathways between them. Moreover, these pathways displayed consistent expression patterns in terms of up- and down-regulation between early and late seasons. Notably, genes associated with cellular processes exhibited strong down-regulation for both seasons (Fig. 3).

Altered spawning seasons affected multiple pathways in metabolism, cellular processes, and organismal systems. The off-season appeared to down-regulate carbon metabolism, while the early and late seasons primarily had strong suppressive effects on pathways associated with cellular processes. These findings may be linked to the reduced

356 weight of off-season and early season larvae, as well as variations in the nutritional status of
357 the eggs.

358 Altered spawning seasons had an impact on the expression of genes in a 359 similar manner

360 To further investigate the genes commonly affected by multiple spawning seasons, we used
361 overlapping DEGs (represented as intersections of Venn diagrams in Fig. 2c) by applying two
362 filtering steps: selecting DEGs found in at least two comparisons and choosing the top five
363 DEGs from each comparison based on adjusted p-values (Supplementary Table S11). This
364 filtering approach revealed a total of 13 genes (Table 1). Among them, two genes (gene IDs:
365 106612264 and 106601246) lacked corresponding gene names and symbols. All genes,
366 except *pc* and *pitpnm2*, consistently displayed up- or down-regulation across all spawning
367 seasons, suggesting that all altered spawning seasons had a similar effect on this specific set
368 of genes (Table 1).

369
370 **Table 1**. List of top five DEGs identified in at least two pairwise comparisons.
371

Pattern[1]	Gene ID	Symbol	LFC[2]			Gene name[3]	Function
			Off-season	Early	Late		
All Down	106563634	*pcyt1b*	-1.9 *	-1.1	-1.6 *	choline-phosphate cytidylyltransferase B	Metabolism
	106612672	*bmp5*	-1.2 *	-1.2 *	-0.7	bone morphogenetic protein 5-like	Signalling
	106585803	*rhobtb4*	-0.3	-1.1 *	-0.9 *	Rho related BTB domain containing 4	Ubiquitin mediated proteolysis
	106608456	*prox3*	-0.2	-2.3 *	-1.7 *	prospero homeobox 3	Lymphangiogenesis
	106584736	*tcf12*	-0.7	-3.3 *	-2.3 *	transcription factor 12	Myogenic regulatory
Up & Down	106592454	*pc*	-1.7 *	1.5 *	0.9	pyruvate carboxylase, mitochondrial-like	Pyruvate metabolism, TCA cycle, redox
	106568312	*pitpnm2*	0.6	-2.1 *	-2.6 *	membrane-associated phosphatidylinositol transfer protein 2-like	Cell signalling
All Up	106612264	-	5.2 *	4.1	6.1 *	Uncharacterized	-
	106601246	-	1.8 *	2.2 *	1.5	Uncharacterized	-

	106613828	*crim1*	0.3	3.0 *	3.0 *	cysteine-rich motor neuron 1 protein-like	CNS development,Bone morphogenic and growth factor
	106566973	*krt8*	0.1	1.3 *	1.0 *	keratin, type II cytoskeletal 8	Liver development
	106583637	*klf15*	2.3	4.5 *	3.8 *	Krueppel-like factor 15	Metabolism
	106569008	*cyp7a1*	1.4	3.1 *	2.9 *	cytochrome P450 7A1	Metabolism

[1]All Down: All three comparisons showed down-regulation, Down+Up: Comparisons showed both down- and up-regulation. All Up: All three comparisons showed up-regulation. [2]Log fold changes of pairwise comparison against the normal season. *: significant LFC (adjusted p-value<0.05). [3]When gene symbols were unavailable, we utilised the gene symbols from either the latest NCBI version or UniProt as substitutes.

These genes can be classified into three categories by relevant biological functions: metabolism (*pcyt1b*, *pc*, *klf15*, and *cyp7a1*) [38-40], tissue development (*prox3*, *crim1*, and *krt8*) [41-44], and information processing, which can be further classified into three sub categories as signalling (*bmp5* and *pitpnm2*) [38, 45], transcription (*tcf12*) [46], and post-translation (*rhobtb4*) [38]. As these categories are typically associated with growth, all three altered spawning seasons affected a certain set of genes associated with growth regulation, despite PCA plots (Fig. 2a) and Venn diagrams (Fig. 2c) showing a distinct gene expression pattern of the off-season.

Overall methylation rates increased until the harvesting stage, except around transcription start sites

We conducted DNA methylation analysis on 20 liver samples from larvae across four different spawning seasons (n=5; detailed statistics in Supplementary Table S12) to investigate the epigenetic effects of altered spawning seasons on DNA methylation. To examine regional DNA methylation patterns, CpG sites were categorised into three genomic regions based on their positions relative to closely located genes: gene body (GB; including exons and introns), promoter (P; including P250, P1K, and P5K), and flanking regions (Fig. 4a; see Methods and Materials for definitions).

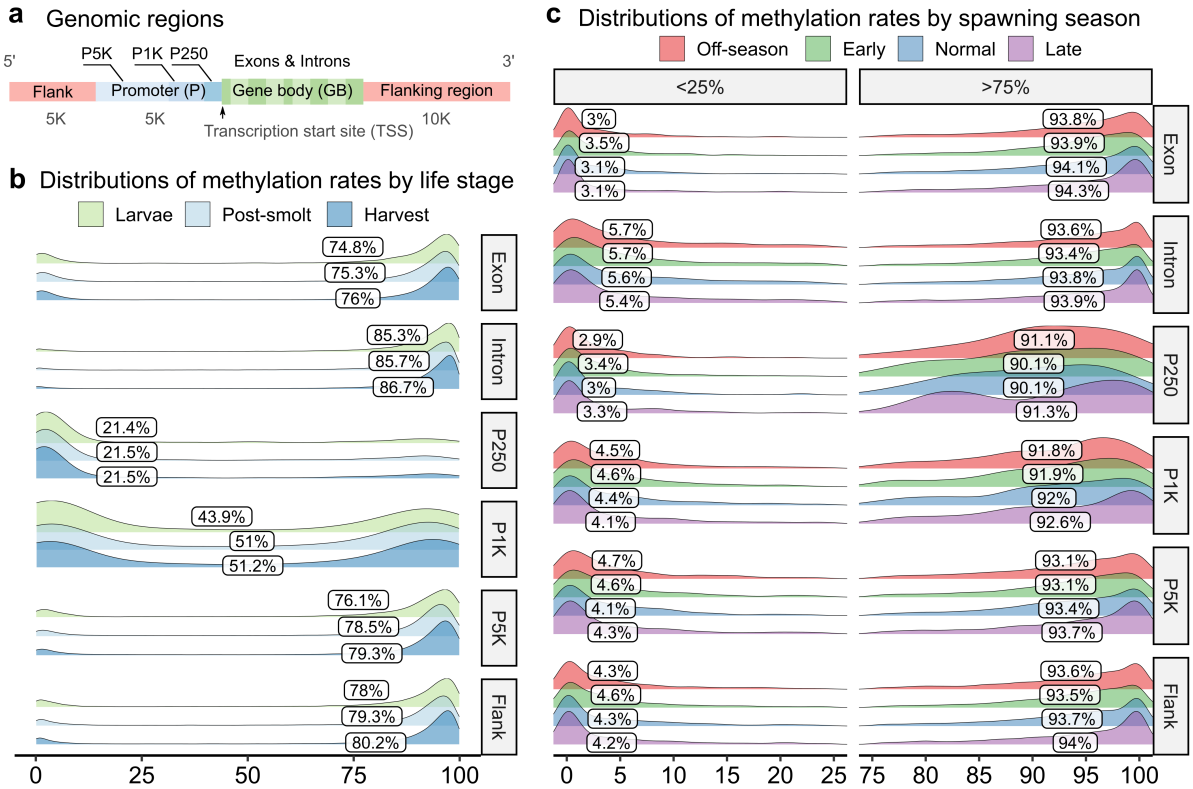

Fig. 4. Distribution of methylated CpG sites across genomic regions.

a) Genomic region diagram illustrating three regions: flanking regions (Flank, red), promoters (P, blue), and gene bodies (GB, green). Promoter region is divided into three subregions: P250, P1K, and P5K. Gene body includes exons and introns. **b)** Ridge density plots showing distribution patterns of methylated CpG sites in percentage across six genomic regions. Plot includes methylation data from the present study (larvae, green) and two other studies: post-smolt (light blue) and harvest (blue) for comparison. **c)** Ridge density plots showing distribution patterns of methylated CpG sites for four spawning seasons: off-season (red), early (green), normal (blue), and late (purple). Plot is divided into two ranges: 0-25% and 75%-100%.

Before analysing the effects of altered spawning seasons, we compared RRBS data from two other studies focusing on liver samples from different developmental stages: post-smolt (~31 weeks) and harvest (~45 weeks) [20, 21]. Distributions of methylation rates showed significant differences among life stages, except for P250 (Kolmogorov–Smirnov test; Supplementary Table S13). Ridge density plots displayed peak values ranging from 75% to 100%, excluding P250 and P1K (Fig. 4b). Notably, the peaks of the harvesting stage shifted closer to 100% compared to the larvae and post-smolt stages, supporting the trend of average methylation rates: larvae < post-smolt < harvest (average methylation rates are shown in text labels of Fig. 4b). This comparison suggests that methylation rates generally

increase as salmon grow, at least until the harvesting stage, while methylation rates near transcription start sites (TSSs) remain lower and more stable than in other genomic regions.

Altered spawning seasons contributed to similar shifts in DNA methylation rates as observed in growth performance

We analysed distributions of methylation rates across spawning seasons and observed significant differences among them, except for P250 and P1K (Kolmogorov–Smirnov test; Supplementary Table S14 and Supplementary Fig. S1). To highlight peaks across seasons, we divided the ridge density plot into 0-25% and 75-100% sections (Fig. 4c).

Even though distributions in the 0-25% range were similar, significant differences were evident in multiple comparison to some degree (Kolmogorov–Smirnov test; Supplementary Table S15). Notably, the comparison between early and late seasons showed significant differences in all regions except for P250. Moreover, peaks in the early season were further from 0% compared to other seasons, supporting the trend of average methylation rates in the 0-25% range (average methylation rates are shown as text labels in the left side of Fig. 4c).

In the 75-100% range, distributions significantly differed among spawning seasons, excluding P250 and P1K (Kolmogorov–Smirnov test; Supplementary Table S16). Notably, peaks in normal and late seasons shifted closer to 100% compared to the off-season and early season, except for P250, supporting the trend of average methylation rates: early ≈ off-season < normal < late (average methylation rates are shown as text labels in the right side of Fig. 4c). This order closely aligns with weight measurements observed at the larvae stage (Fig. 1b, Supplementary Table S2). Considering both these comparisons and those across different life stages, methylation rates are linked to growth with region-specific patterns of methylation shifts.

Differential methylation patterns indicated higher dynamic regulation in promoter regions compared to other regions

We conducted six pairwise comparisons to identify CpG sites with differential methylation (Fig. 5a). Our objective was to capture a comprehensive pattern of methylation differences by considering all possible pair-wise combinations. Remarkably, all comparisons consistently demonstrated similar characteristics, for both the number of differentially methylated CpGs (DMCs) and the balanced ratio of hypo- and hyper-methylation (Fig. 5a).

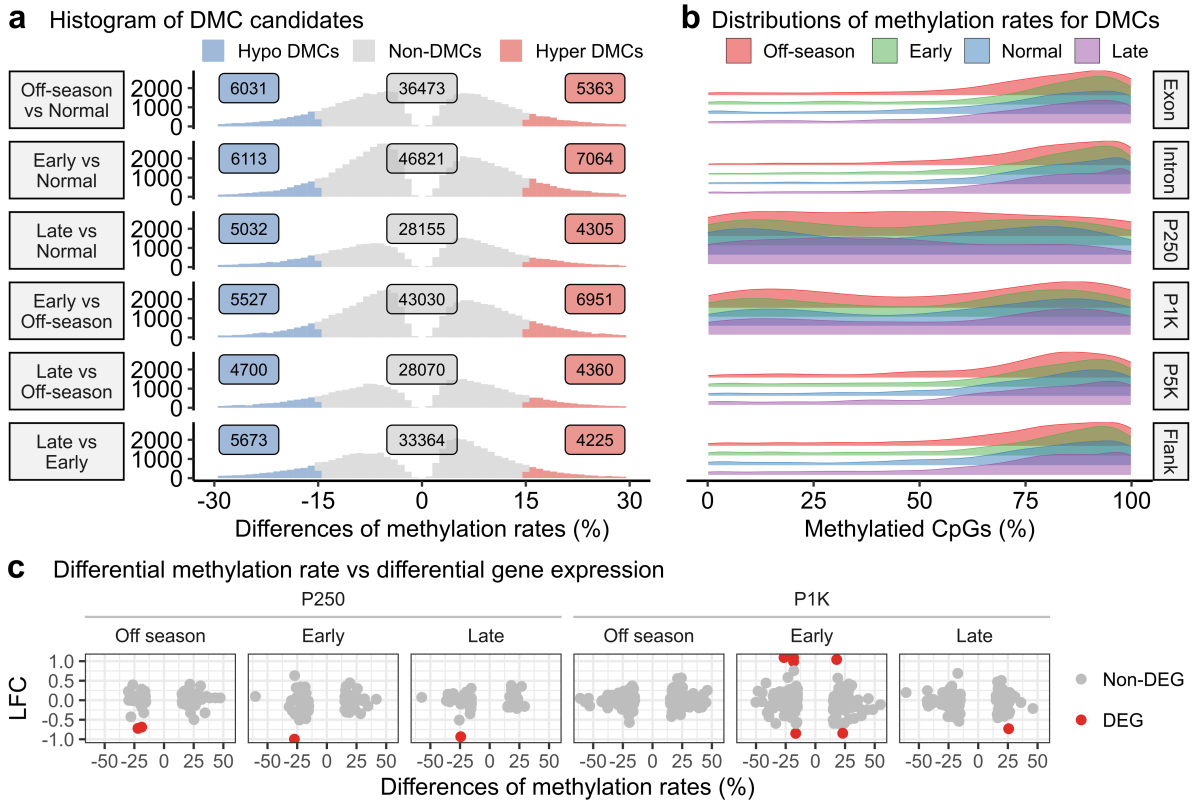

Fig. 5. DNA methylation differences and correlations with gene expression.

a) Histograms displaying distributions of methylation rate differences in six pairwise comparisons. Hypo-methylated DMCs in blue, hyper-methylated DMCs in red, non-DMCs in grey. All CpG sites in plots showed significant methylation differences. **b)** Ridge density plots illustrating distribution patterns of methylated CpG sites across four spawning seasons: off-season (red), early (green), normal (blue), and late (purple). CpG sites were selected if at least one site identified as DMC in any of six comparisons. **c)** Scatter plots showing relationship between methylation rate differences and log fold changes (LFCs) of gene expression. DMCs were selected from three comparisons: off-season vs. normal (Off season), early vs. normal (Early), and late vs. normal (Late), and also selected based on two regions, P250 and P1K. Corresponding genes were linked to these DMCs chosen, and their LFCs were transformed by normal shrinkage. Data points are color-coded to indicate

456 DEGs in red and non-DEGs in grey.
457

458 Further investigation focused on a set of 10,308 common DMCs, identified in at least
459 one comparison. Distributions of methylation rates for these common DMCs exhibited a
460 distinct left-skewed pattern across the exon, intron, P5K, and flanking regions (Fig. 5b). This
461 observation strongly suggested that methylation differences resulting from altered spawning
462 seasons primarily occurred within regions that were already highly methylated. In contrast,
463 the P250 and P1K regions displayed nearly uniform methylation rates across a range of 0-
464 100%, indicating significant methylation differences occurred irrespective of the underlying
465 methylation rates. These findings imply a potentially greater level of dynamic regulation of
466 DNA methylation in P250 and P1K compared to the other regions.

467 Filtering multiple DMCs identified key genes affected by spawning season 468 alterations

469 We examined genes with multiple DMCs in three pairwise comparisons against the normal
470 season. Since most genes had only a single DMC, especially in promoters (~75% in P250 and
471 ~70% in P1K, Supplementary Fig. S2), we avoided identifying differentially methylated
472 regions (DMRs) using a sliding window approach. Instead, we filtered out genes strongly
473 supported by multiple DMCs based on specific criteria (see Methods and Materials), resulting
474 in a total of 11 genes (Table 2), including six genes for off-season vs. normal and three genes
475 each for the other two comparisons (Supplementary Table S17).

476
477 **Table 2**. List of genes with multiple DMCs in their promoter region.
478

Pattern[1]	Gene ID	Symbol	DMC[2]			Gene name	Function
			Off-season	Early	Late		
All hypo	106563027	*slc31a1*	Hypo (3) *	-	Hypo (3) *	solute carrier family 31 member 1	Transporter

Hyper & Hypo	106567512	*stk32a*	-	Hypo (2)	Hypo (2), * Hyper (1)	serine/threonine-protein kinase 32A	Signalling
	106607889	*inf2*	Hypo (1)	Hyper (3) *	-	inverted formin-2-like	Actin filament polymerizatio n
	106584059	*scp-2*	Hyper * (4)	Hypo (1)	Hyper (1)	sterol carrier protein 2-like	Lipid transfer, transporter
	106579168	*klhl11*	Hypo (1), * Hyper (2)	-	-	kelch-like protein 11	Ubiquitination
All hyper	100196480	*ctl2b*	Hyper (3)	Hyper (7) *	Hyper (2)	CTLA-2-beta	Unknown
	106573279	-	-	Hyper (1)	Hyper (3) *	Uncharacterized	-
	106583651	*helz2*	Hyper * (4)	-	-	helicase with zinc finger domain 2-like	Transcriptiona l coactivator
	106581656	*lrrfip2*	Hyper * (3)	-	-	leucine-rich repeat flightless-interacting protein 2-like	Signalling
	106609256	*prrx2*	Hyper * (3)	-	-	paired mesoderm homeobox protein 2-like	Transcription
	106576160	*ttll12*	-	Hyper (3) *	-	tubulin tyrosine ligase-like family, member 12	Post-translational modification

[1]All hypo: All three comparisons showed hypo-methylated DMCs, Hyper & Hypo: Comparisons showed both hypo- and hyper-methylated DMCs. All hyper: All three comparisons showed hyper-methylated DMCs. [2]Either hyper or hypo-methylated DMC against the normal season. The number in brackets represent the number of DMCs. *: genes selected by filtering of multiple DMCs.

All 11 genes were identified by a single comparison except for *slc31a1* identified in two comparisons (off-season vs. normal and late vs. normal). Most genes (9 out of 11) exhibited either hypo-methylated or hyper-methylated DMCs, but not both, within a single comparison (Table 2). Furthermore, approximately 60% of the genes (7 out of 11) consistently displayed either hypo-methylation or hyper-methylation even across multiple comparisons ('All hypo' and 'All hyper' in the pattern column of Table 2, respectively).

Literature analysis associated these genes with two biological functions: development (*inf2*) and information processing, which can be further classified into four sub categories as transporter (*slc31a1* and *scp-2*) [47, 48], signalling (*stk32a* and *lrrfip2*) [25, 49], post-

494 translation (*klhl11* and *ttll12*) [25, 50], and transcription (*helz2* and *prrx2*) [51, 52]. However,
495 *ctl2b* and an uncharacterised gene (Gene ID: 106573279) had no clear associations with
496 specific biological functions. Although these genes were potentially linked to growth
497 performance, none of the 11 genes were identified as DEGs in our gene expression analysis.

498 Altered spawning seasons influenced gene expression and DNA methylation 499 of cell cycle-related genes

500 To investigate potential associations between DNA methylation and gene expression, we
501 focused on DMCs identified in three pairwise comparisons against the normal season (off-
502 season vs. normal, early vs. normal, and late vs. normal) in P250 and P1K. By correlating
503 their methylation differences with corresponding log-fold changes (LFCs), we found a total
504 of 11 DMCs: four DMCs in P250 and seven DMCs in P1K associated with DEGs (Fig. 5c,
505 Supplementary Table S18). Surprisingly, CpG sites with methylation differences greater than
506 30% lacked clear association with differentially expressed genes (light grey dots labelled as
507 non-DEGs in Fig. 5c), suggesting that differentially methylated sites showed inconsistent
508 links with differential gene expression.

509 These 11 DMCs were associated with seven genes that were DEGs in the
510 corresponding comparisons (Table 3). We expanded this list of seven genes with additional
511 information from all pair-wise comparisons (additional entries were marked with parentheses
512 in Table 3). Among them, three genes (*caprin-1*, *kifc1*, and *aurkb*) exhibited consistent
513 regulations of DMCs and DEGs compared to the normal season. Specifically, *caprin-1* and
514 *kifc1* had hypo-methylated CpG sites, while *aurkb* had hyper-methylated CpG sites; all three
515 genes showed down-regulated gene expression (Table 3). Notably, all three genes (*caprin-1*,
516 *kifc1*, and *aurkb*) were associated with cell cycle regulation [53-55], while the remaining
517 genes were linked to metabolism (*cyp8b1*, *adrenodoxin*, and *lpin1*) [33, 56, 57] and transport

518 (*slc43a1a*) [58]. These findings indicate that altered spawning seasons had genetic and

519 epigenetic effects on several genes associated with cell cycle regulation.

520
521 **Table 3**. List of DEGs that contain DMCs in their promoter region.
522

Gene ID	Symbol	DMC[1]			DEG[2]			Gene name	Function
		Off-season	Early	Late	Off-season	Early	Late		
106562317	*caprin-1*	Hypo	(Hypo)	(Hypo)	Down	(Down)	(Down)	caprin-1	Cell cycle
106599887	*cyp8b1*	Hypo	-	-	Down	-	-	5-beta-cholestane-3-alpha,7-alpha-diol 12-alpha-hydroxylase	Metabolism, cytochrome P450
106588407	*kifc1*	-	Hypo	Hypo	-	Down	Down	carboxy-terminal kinesin 2	Cell cycle, Meiosis
106582038	*adrenodoxin*	-	Hypo		-	Down	-	adrenodoxin	Metabolism, cytochrome P450
106604665	*slc43a1a*	-	Hypo	-	-	Up	(Up)	solute carrier family 43 member 1a	Transporter
106561604	*aurkb*	(Hyper)	Hyper	Hyper	-	Down	Down	aurora kinase B	Cell cycle
106570052	*lpin1*	-	Hyper	-	-	Up	-	phosphatidate phosphatase LPIN1	Metabolism

523
524 [1]Either hypo- or hyper-methylated DMC against the normal season. Parentheses indicate that the site was
525 identified as DMC, but the corresponding genes was not identified as stringent DEG.[2]Either up- or down-
526 regulation against the normal season. Parentheses indicate that down- or up-regulation was determined by non-
527 stringent DEGs.
528

529 # Discussion

530 In the present study, we investigated the influence of altered spawning seasons on gene

531 expression and DNA methylation in offspring. Previous studies emphasized the significant

532 impacts of these changes on the nutritional status of both broodstock and their offspring, with

533 no observed effects on the body weights of broodstock [4, 5]. Our primary aim was to reveal

534 the genetic and epigenetic effects resulting from observed variations by utilizing liver
535 samples from larvae at the first feeding stage.

536 What insights do our omics analyses provide?

537 Gene expression analysis revealed distinct patterns associated with altered spawning seasons.
538 Specifically, early and late seasons exhibited similar gene expression patterns when
539 compared to the normal season. The differential expression of genes associated with
540 metabolism, cellular processes, and organismal systems indicates that spawning seasons can
541 have wide-ranging effects on biological pathways related to growth and development.
542 Furthermore, the analysis with overlapped DEGs revealed that all altered spawning seasons
543 affected genes associated with metabolism, tissue development, and information processing
544 similarly. Hence, while early and late seasons influenced a wide range of biological pathways
545 in a similar manner, all three seasons, including the off-season, also similarly affected a
546 certain set of genes, especially those strongly differentiated from the normal season.

547 DNA methylation analysis provided valuable insights into the epigenetic effects of
548 different spawning seasons. Altered spawning seasons led to increased methylation rates,
549 which appeared to be linked to higher body weights. Notably, these shifts primarily occurred
550 in regions that were already highly methylated, but the rates were altered independently of
551 the underlying methylation rates in promoter regions around transcription start sites. Our
552 findings imply that methylation rates could serve as a measure of the current growth levels,
553 even when comparing different treatment groups at the same developmental stage.

554 Several genes with multiple DMCs exhibited consistent hypo- or hyper-methylation
555 patterns across multiple spawning seasons in promoter regions, suggesting a common
556 epigenetic response to altered spawning seasons. These genes were associated with key
557 biological functions, such as development and information processing, further supporting

558 their role in influencing growth performance. Although both gene expression and DNA
559 methylation analyses resulted in similar biological functions impacted by altered spawning
560 seasons, they showed little overlap between DEGs and genes with DMCs. This could be
561 attributed to regulatory differences between gene expression and DNA methylation within the
562 same biological pathway, or it might be a limitation caused by the restriction enzyme used in
563 RRBS.

564 The observed correlations between DNA methylation and gene expression were
565 weaker than anticipated in our liver samples. Notably, CpG sites with methylation differences
566 exceeding 30% lacked a clear link to differential gene expression. Additionally, our findings
567 revealed inconsistent correspondence between hypo-methylation and active gene expression
568 and between hyper-methylation and gene suppression. These results suggest that the
569 regulation of DNA methylation is more intricate than commonly assumed as it is known that
570 high promoter CpG methylation reduces gene expression, while low promoter CpG
571 methylation allows for active gene expression [59]. Within promoter regions, several factors,
572 including underlying gene expression levels, transcription factor binding sites, and chromatin
573 remodelling, likely play a role in regulating actual gene expression.

574 What are the significant biological differences observed in offspring?

575 Off-season spawning in June, facilitated through a RAS, resulted in Atlantic salmon offspring
576 with lower larval weights compared to normal and late seasons. Offspring eggs also exhibited
577 reduced levels of vitamin B12 and lipid classes relative to normal and late seasons. Gene
578 expression analysis indicated significant down-regulation of genes associated with central
579 carbon metabolism (CCM). CCM involves several biological pathways, including the citric
580 acid cycle, and acts as a major source of energy for growth and development. Moreover,
581 lipids are interconnected with CCM through the utilisation of acetyl-CoA as a central

582 metabolite [60]. Among the six genes with multiple DMCs from the off-season vs. normal
583 comparison, sterol carrier protein2-like (*scp-2*) contained four hyper-methylated DMCs in its
584 promoter. The *scp-2* gene encodes a transfer protein that plays a key role in intracellular lipid
585 transport [48]. Overall, RAS-based off-season spawning appeared to impact lipid-mediated
586 regulations both genetically and epigenetically. This negative impact on lipid-related
587 mechanisms potentially accounted for impaired growth performance observed in body
588 weight.

589 Offspring resulting from early season spawning in September also exhibited lower
590 weights at the larval stage compared to those from normal and late seasons. However, the
591 nutritional status of offspring eggs displayed higher levels of SAM/SAH, lysine, glutamine,
592 and alanine. Gene expression analysis revealed significant down-regulation of genes
593 associated with cell cycle regulation, while genes involved in metabolism showed both down-
594 and up-regulation. Among the five genes that exhibited differential expression and had at
595 least one DMC in their promoters, two of them were associated with cell-cycle regulation.
596 Specifically, the carboxy-terminal kinesin 2 (*kifc1*) gene promotes mitotic spindle assembly
597 [54], and the aurora kinase B (*aurkb*) gene is involved in mitotic progression [55]. The *kifc1*
598 gene was hypomethylated, while the *aurkb* gene was hypermethylated. Consequently, the
599 early spawning season had an impact on cell-cycle regulation both genetically and
600 epigenetically. However, it is challenging to hypothesise that this negative impact on cell-
601 cycle regulation was linked to impaired growth performance because similar effects were
602 observed for the late season.

603 Late season spawning in January showed that offspring weights were almost as close
604 to, but slightly heavier than those from the normal season. The nutritional status of offspring
605 eggs also exhibited similar levels of various nutrients and metabolites to the normal season,

606 except for higher vitamin B12 and lower B-alanine. Gene expression patterns in the late
607 season were comparable to those observed in the early season, showing significant down-
608 regulation of genes related to cellular processes, especially cell cycle regulation. Two genes,
609 *kifc1* and *aurkb*, showed differential expression and had at least one DMC in their promoters,
610 and both were associated with cell-cycle regulation. Hence, the late season showed similar
611 growth performance, nutritional status, and global DNA methylation pattern to the normal
612 season, while gene expression pattern was similar to the early season.

613 Although strong similarities were observed in gene expression patterns between early
614 and late spawning seasons, certain genes exhibited common regulation in both gene
615 expression and DNA methylation across all three spawning seasons. Among the four DEGs
616 identified in the off-season, choline-phosphate cytidylyltransferase B (*pcyt1b*) and bone
617 morphogenetic protein 5-like (*bmp5*) showed concordant down-regulation across all three
618 spawning seasons. The *pcyt1b* gene encodes an enzyme involved in phosphatidylcholine
619 biosynthesis, playing essential roles in multiple metabolic pathways [25], while the *bmp5*
620 gene is involved in signalling [33]. Additionally, all DMCs found in the promoter of the
621 cytotoxic T lymphocyte-associated protein 2 beta (*ctl2b*) gene were hyper-methylated across
622 all three spawning seasons. The function of *ctl2b* remains unknown, but it shares similarities
623 with genes encoding cysteine proteinase, an enzyme involved in protein breakdown within
624 cells [61]. Furthermore, among genes that were both differentially expressed and had at least
625 one DMC in their promoters, the *caprin-1* gene exhibited consistent regulation, with all
626 DMCs being hypo-methylated and its expression being suppressed. The *caprin-1* gene is
627 associated with cell cycle regulation, playing a crucial role in cellular activation or
628 proliferation [53]. This indicates that the off-season also influenced cell cycle regulation to a
629 certain degree.

Can we link our findings with future growth performance?

Although methylation levels roughly correspond to body weights, indicating current growth levels, we can still estimate future growth performance from all kinds of observed data. Offspring from the off-season exhibited lower lipid levels and suppressed lipid related mechanism, which could hinder their growth potential, while offspring from the early season showed suppressed cellular processes, which are also important for growth. However, the early season had the highest methylation potential (SAM/SAH ratios), suggesting superior growth potential due to methionine's role in vital growth-related biology [10, 62]. Therefore, even though fish from the off-season and early seasons are expected to underperform those from the normal season in later development stages, fish from the early season potentially outperforms those from the off-season at some point in later stages.

Late and normal seasons exhibited similar overall DNA methylation rates and nutritional status measures. Nonetheless, the late season displayed suppressed expression of genes involved in cellular processes, including cell cycle regulation. Furthermore, the late season exhibited the lowest SAM/SAH ratio among the four seasons. This suggests that fish from the late season may underperform those from the normal season in later development stages.

Hence, the projected future growth levels can be represented as: off-season < early < late < normal, in comparison to the current growth levels: early ≈ off-season < normal ≈ late, without considering compensation from exogenous feeding. To enhance the accuracy of these estimations, compiling tissue and developmental stage-specific DNA methylation maps would be valuable, as specific DNA methylation levels at certain loci are likely associated with the underlying growth potential.

Why are our findings important in the context of aquaculture and nutritional studies?

Our study unveiled the significant effects of altered spawning season on offspring gene expression and DNA methylation patterns in Atlantic salmon. These findings provide valuable insights into assessing current and future growth potential, which can be challenging to determine using only nutritional status and growth performance measures. Understanding the genetic and epigenetic responses to altered spawning seasons will help develop strategies to optimize growth and production in aquaculture practices. Additionally, our study can be linked to the potential impact of increasing global ocean temperatures on the spawning of aquatic vertebrates, as temperature is a key abiotic factor influencing spawning seasons.

Conclusions

The present study emphasises the substantial impact of altering spawning seasons on gene expression and DNA methylation patterns in Atlantic salmon offspring. Our observations indicate that RAS-based off-season spawning exerts an influence on lipid-mediated regulations in offspring, while sea-pen based early and late seasons affect the regulation of the offspring's cellular processes, especially cell cycle regulation. These effects potentially play a crucial role in shaping both current and future growth performance.

These findings have important implications for aquaculture and nutritional studies, as they provide valuable tools for assessing growth potential and optimizing production strategies. Future research focusing on tissue and developmental stage-specific DNA

methylation maps will enhance the accuracy of growth performance estimation and provide further insights into the genetic and epigenetic responses to altered spawning conditions.

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Author contributions

K.H.S conceived and designed the research. M.E. and M.M. conducted and designed the feeding trial. J.M.O.F. and C.B. prepared RNA-seq and RRBS data. T.S., M.E., and K.H.S. analysed and interpreted data. T.S. drafted the manuscript, and K.H.S revised it. The authors read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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