Altered spawning seasons of Atlantic salmon broodstock transcriptionally 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 omics data from 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: 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. Specifically, offspring from RAS-based off-season exhibited altered lipid-mediated regulation, while those from sea-pen-based early and late seasons showed changes in cellular processes, particularly in cell cycle regulation when compared to the normal season. The consequences of our findings are significant for growth and health, potentially providing information for developing valuable tools for assessing growth potential and optimizing production strategies in aquaculture.

Author Summary

This study examines the impact of manipulating Atlantic salmon spawning seasons in aquaculture on genetic and epigenetic regulations in their offspring. Manipulating water temperature and light cycles during broodstock rearing allows for year-round harvesting. In

aquaculture, altering spawning seasons is a common practice; however, recent research suggests that these changes affect the nutritional status and growth of offspring. To understand these effects at the molecular level, we analysed transcriptomic and epigenetic data from salmon offspring born to broodstock with four different spawning seasons. Our results reveal distinct transcriptomic and epigenetic patterns associated with altered seasons, influencing lipid metabolism and cell cycle regulation. These findings hold significant implications for improving aquaculture practices, potentially enhancing growth performance and nutritional quality of farmed salmon across generations as temperature and light are crucial abiotic factors influencing the next generation. Furthermore, our study potentially contributes to understanding the impact of climate change on the spawning behaviour of aquatic vertebrates, particularly in relation to increasing global ocean temperatures and the resulting changes in photoperiod as species migrate northward in the Northern Hemisphere, experiencing longer daylight in summer and shorter daylight in winter.

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

65 aquaculture, the timing of spawning is adjusted to ensure year-round harvesting availability. This
66 manipulation involves controlling abiotic factors, such as water temperature and photoperiod, to
67 influence the spawning timing (Pankhurst and King, 2010; Taranger et al., 2010). Additionally,
68 apart from the traditional open sea-based cages, a land-based approach utilising recirculating
69 aquaculture systems (RAS) is employed to more accurately replicate the salmon's natural
70 lifecycle, including both saltwater gonad maturation and final freshwater maturation periods
71 (Good and Davidson, 2016). Despite the ongoing and successful practices of spawning season
72 alterations in salmon aquaculture, recent studies have reported that these practices can impact the
73 nutritional status of broodstock, leading to negative effects on the growth performance in the
74 next generation (Skjærven et al., 2022; Skjaerven et al., 2020).

75 While various nutrients influence the health condition and growth performance of
76 Atlantic salmon (Council, 2011; Hamre et al., 2016; Hemre et al., 2016), altered spawning
77 seasons have appeared to affect the status of multiple metabolites and nutrients including
78 micronutrients, amino acids, and lipid classes (Skjærven et al., 2022; Skjaerven et al., 2020).
79 These nutrients are also closely associated with vital biological functions necessary for growth,
80 such as one carbon (1C) metabolism, the citric acid cycle, and the Cahill cycle. Multiple key
81 micronutrients, including vitamin B12, folate, vitamin B6, and methionine, are integrated with
82 the 1C metabolism (Danchin et al., 2020; Lyon et al., 2020), which regulates cellular methylation
83 potential through S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH), critical for
84 various cellular processes (Ducker and Rabinowitz, 2017). The citric acid cycle enables the
85 catabolism of carbohydrates, fats, and amino acids, releasing stored energy crucial for growth
86 and development (Akram, 2014), while the Cahill cycle facilitates the transport of amino groups
87 and carbons between muscle and liver (Felig, 1973), both playing essential roles in various

88 developmental processes. Amino acids are essential for growth and tissue development, in
89 addition to being the building blocks of proteins (Wu, 2010), while lipids serve as major energy
90 reserves and contribute to the formation of cell membranes, also supporting various
91 developmental processes (Fahy et al., 2011).

92 Beyond the importance of nutrients, understanding genetic influences provides invaluable
93 insights from a molecular biology perspective. Recent studies have revealed that alterations in
94 micronutrient levels within the feed can have transcriptomic effects on various biological
95 pathways in salmon, including lipid metabolism, protein synthesis, and post-transcriptional
96 regulation (Saito et al., 2023; Saito et al., 2021). Moreover, an additional layer of information
97 comes from the analysis of DNA methylation, which supports to reveal important epigenetic
98 regulations. DNA methylation, as an epigenetic regulatory process, influences various cellular
99 mechanisms, including cell stability, differentiation, and development, potentially in response to
100 environmental stimuli. (Amenyah et al., 2020; Moore et al., 2013). Since DNA methylation is
101 inherited during cell replication, it can result in long-term changes in epigenetic regulation (Goll
102 and Bestor, 2005; Moore et al., 2013).

103 The present study investigates the transcriptomic and epigenetic effects on offspring
104 resulting from four different spawning seasons. These seasons include the natural sea-pen-based
105 spawning in November, an accelerated RAS-based spawning occurring five months earlier, and
106 two variations of sea-pen-based spawning: one with a two-month advance and another with a
107 two-month delay relative to the natural spawning season. The main analysis of the study focuses
108 on omics analysis of liver samples collected from start-feeding larvae at the end of the
109 endogenous feeding phase. The liver serves as a key target organ for various metabolic reactions
110 crucial to growth, and using start-feeding larvae helps eliminate the influences of exogenous

feeding. To uncover both transcriptomic 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 suggest that gene expression and DNA methylation data can serve as powerful tools for assessing current and future growth performance. These tools could potentially benefit a wide range of nutritional programming studies and enhance our understanding of how aquatic species adapt to climate change.

Methods and Materials

Ethical considerations

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

As the samples were collected at the breeding station, 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, anaesthetics 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 (Skjærven et al., 2022; Skjaerven et al., 2020), we only present summarized descriptions here. The study utilized broodstock and offspring 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 brackish (12‰ salinity), while the broodstock from the early, normal, and late seasons were raised in three open sea-based net pens with ambient photoperiod and temperatures. All broodstock were fed to satiation with broodstock feed (EWOS Breed 3500) until transferred to freshwater. In freshwater, the normal season broodstock matured naturally under ambient photoperiod and temperatures, whereas artificial abiotic factors like temperature and light were applied to regulate maturation for the other spawning seasons. Specifically, while the normal season broodstock were maintained at 6 °C with an 8-hour daylight cycle upon transfer to freshwater tanks in August, the early season broodstock were maintained by following the protocol outlined by Naeve and colleagues (Naeve et al., 2018). Details about the temperature and photoperiod used to postpone maturation in the off-season and late season groups are proprietary information owned by AquaGen AS. The off-season and early season broodstock

154 were subjected to five- and two-month advance maturation, respectively, while the late season
155 broodstock were subjected to a two-month delay. The fish received no further feeding after the
156 transfer to freshwater. The maturation process in freshwater lasted for 109, 163, 120, and 166
157 days for off-season, early, normal, and late season broodstock, respectively, before fertilization to
158 obtain the next generation (Supplementary Table S1).

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

165 At the end of the endogenous feeding phase (start-feeding larvae 979–994 d°;
166 Supplementary Table S1), 36 larvae from each broodstock were randomly selected, euthanised
167 with an overdose of buffered tricane methanesulfonate (MS222, Pharmaq, Norway), and body
168 weights (n=180 per spawning season) were recorded. Additionally, 40 liver samples (two from
169 each broodstock fish) were dissected from larvae and flash frozen in liquid N2 and used for
170 either RNA or DNA analysis.

171 Growth measure and nutrient analysis

172 We collected the data on body weights for broodstock and larvae from previous studies
173 (Skjærven et al., 2022; Skjaerven et al., 2020). In addition, we obtained the data on selected
174 nutrients and metabolites from the same studies, specifically vitamin B6 & B12, folate, S-
175 adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), cholesterol, the sum of six

176 different lipids, glutamate, L-serine, L-lysine, L-alanine, L-glutamine, urea, and B-alanine
177 (Skjærven et al., 2022; Skjaerven et al., 2020). To analyse the data from all four spawning
178 seasons, we performed ANOVA followed by Tukey's HSD test.

179 DNA and RNA extraction

180 RNA extraction from 20 larvae livers (n=5 for each spawning season) was carried out using the
181 BioRobot EZ1 and EZ1 RNA Universal Tissue kit (Qiagen), followed by DNase treatment using
182 the Ambion DNA-free DNA removal kit (Invitrogen, USA) according to their respective
183 protocols. The RNA quantity was assessed using the NanoDrop ND-1000 Spectrophotometer
184 (Nanodrop Technologies) and the Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip
185 kit (Agilent Technologies). For DNA isolation from 20 larvae livers (n=5 for each spawning
186 season), the DNeasy Blood & Tissue Kit (Qiagen, Cat. No. #69506) was used as per the
187 manufacturer's protocol. Liver samples underwent RNase A treatment (provided by the Qiagen
188 kit, 50ng/µL, 10 min at room temperature) followed by proteinase K treatment (New England
189 Biolabs, #8102S 20µg/µL, 1.5 h at 55°C). DNA was eluted in Milli Q water. DNA quantification
190 was performed using the Qubit High Sensitivity Assay (Life Technologies #Q32854). Detailed
191 methods for both DNA and RNA extraction were described elsewhere (Saito et al., 2021).

192 Library preparation for RNA-seq and RRBS

193 Liver samples were sequenced at two different sequencing facilities for specific analyses. For
194 RNA-seq, the liver samples were processed at the DeepSeq sequencing facility at Nord
195 University, Bodø, Norway, and the library preparation was conducted using the NEBNext Ultra
196 II Directional RNA Library Prep Kit for Illumina (New England Biolabs). Sequencing was
197 performed on the NextSeq500 machine (Illumina). For RRBS, the liver samples were processed

198 at the CeMM Biomedical Sequencing Facility, Vienna, Austria. The genomic DNA was
199 extracted, digested by MspI, and bisulfite-converted before library preparation. The RRBS
200 libraries were sequenced on Illumina HiSeq 3000/4000 instruments. Detailed methods of the
201 library preparation for RNA-seq and RRBS were described elsewhere (Saito et al., 2021).

202 Atlantic salmon genome and genomic annotation

203 The reference genome (ICSASG version 2) and RefSeq data (version 100) for gene annotation
204 were downloaded from the NCBI web site
205 (https://www.ncbi.nlm.nih.gov/assembly/GCF_000233375.1). In cases where gene symbols were
206 either outdated or unavailable from RefSeq data (version 100), gene symbols from a newer
207 version of RefSeq data (version 102) and UniProt (Bateman et al., 2022) were used. The genome
208 regions were annotated into intron, exon, three promoter regions, and flanking regions. Promoter
209 regions were divided into three categories based on their distance from transcription start sites:
210 P250 (1-250 bp), P1K (251-1000 bp), and P5K (1001-5000 bp). Flanking regions were defined
211 as 5000 upstream from P5K and 10000 downstream from the 5' end of the gene.

212 Pre-processing of high throughput sequencing

213 For both RNA-seq and RRBS data, the same pre-processing procedures were followed as
214 described in a previous study (Saito et al., 2021). This involved trimming the reads using
215 Cutadapt (Martin, 2011) and Trim Galore! (Barbraham Institute), aligning the trimmed reads to
216 the reference genome using STAR (Dobin et al., 2013) for RNA-seq and Bismark/Bowtie 1
217 (Krueger and Andrews, 2011; Langmead et al., 2009) for RRBS with their default parameters.
218 The mapped RNA-seq reads were quantified using featureCounts (Liao et al., 2014), while the
219 mapped RRBS reads were processed by Bismark (Krueger and Andrews, 2011) for methylation

220 calling and CpG site extraction. The samples were divided into four spawning season groups for
221 further analysis: off-season, early, normal, and late seasons, and clustering analysis was
222 performed using the factoextra package (https://CRAN.R-project.org/package=factoextra). RNA-
223 seq counts were subjected to a variance stabilizing transformation (VST) using DESeq2 (Love et
224 al., 2014) before principal component analysis (PCA).

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

228 Differential gene expression analysis

229 Differentially expressed genes (DEGs) were identified using DESeq2 (Love et al., 2014) with an
230 adjusted p-value cut-off of less than 0.05. P-values were adjusted by the Benjamini-Hochberg
231 procedure (Benjamini and Hochberg, 1995). From the candidate DEGs, those with absolute log
232 fold changes (LFCs) greater than or equal to 1.2 were selected as the final set of DEGs by using
233 the lfcThreshold argument of the results function of DESeq2 (Love et al., 2014). Shrunken LFCs
234 values, calculated by the normal shrinkage method provided by DESeq2 (Love et al., 2014),
235 were used for both heatmaps showing functional annotation results and scatter plots showing
236 merged results between DNA methylation and gene expression differences.

237 Functional annotation with KEGG

238 Over-representation analysis (ORA) was performed on the Kyoto Encyclopedia of Genes and
239 Genomes (KEGG) database (Kanehisa and Goto, 2000a) using clusterProfiler (Yu et al., 2012).
240 Gene lists of DEGs identified from the three pair-wise comparisons against the normal season
241 were used as input, and enriched pathways were determined based on adjusted p-values less than

242 0.05. P-values were adjusted by the Benjamini-Hochberg procedure (Benjamini and Hochberg,
243 1995).

244 Methylation rate analysis

245 Mapped RRBS reads were filtered by methylKit (Akalin et al., 2012), discarding reads with
246 coverage less than or equal to 10 and above the 99.9th percentile. Additional RRBS data from
247 two other studies were obtained for post-smolt and harvesting stages (Saito et al., 2023; Saito et
248 al., 2021) and underwent the same pre-processing procedures to compare the distributions of
249 methylation rates. Raw data of these RRBS sequencing data can be found in SRA on the NCBI
250 site under the accession numbers of PRJNA680423 and PRJNA628740 for post-smolt and
251 harvesting stages, respectively.

252 Differential methylation analysis

253 Differentially methylated CpG sites (DMCs) were identified by methylKit (Akalin et al., 2012),
254 and Q-values were calculated using the sliding linear model (SLIM) method (Wang et al., 2011).
255 CpGs were identified as DMCs when Q-values were less than 0.01 and methylation differences
256 were greater than or equal to 15%.

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

Bioinformatics analysis

In-house R and Python scripts with Snakemake (Koster and Rahmann, 2012) were utilized for high-throughput sequence analysis, basic statistical analysis, and figure generation.

Results

Altered spawning seasons impacted the weight and nutritional status of offspring

The present study examines the effects on gene expression and DNA methylation patterns in 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). The growth performance and nutritional status of the broodstock and offspring have already been assessed and discussed elsewhere (Skjærven et al., 2022; Skjaerven et al., 2020). This section provides a summary of previous research, emphasizing the weight and the status of selected nutrients, while the primary focus of the present study is on the omics analysis.

Broodstock weights showed no significant differences at spawning (upper part of Fig. 1b), but offspring weights at the larvae stage (979 and 994 degree days) showed noticeable variation (lower part of Fig. 1b). Specifically, off-season and early season larvae had lower weights compared to normal and late seasons (Supplementary Table S2). This trend was

282 consistent with the observations in egg sizes, where eggs from off-season and early-season
283 exhibited smaller sizes compared to the other two seasons (see the number of eggs per liter in
284 Supplementary Table S1).

285 Altered spawning seasons widely impacted the nutritional status of offspring eggs,
286 particularly regarding 1C metabolism, lipid classes, citric acid cycle, and Cahill cycle (Skjærven
287 et al., 2022; Skjaerven et al., 2020), but with varying patterns across nutrients and metabolites
288 (summarised in Fig. 1c and Supplementary Tables S3-S6). Previous studies also reported that
289 altered spawning seasons affected the nutritional status of the broodstock in addition to their
290 offspring (Skjærven et al., 2022; Skjaerven et al., 2020). Thus, altered spawning seasons
291 significantly influenced the nutritional status of broodstock without impacting their weights,
292 however, subsequently affecting offspring nutritional status and growth potential of their
293 offspring.

294 Early and late spawning seasons exhibited similar gene expression patterns 295 when compared to the normal season

296 To investigate the impact of different spawning seasons, we performed gene expression analysis
297 on 20 liver samples, which were divided into four groups (n=5). We compared gene expression
298 from the normal season to that from the other three altered spawning seasons (detailed statistics
299 for each sample are provided in Supplementary Table S7).

300 Principal component analysis (PCA) differentiated the off-season from the early and
301 normal seasons when considering all mapped genes (left part of Fig. 2a). However, when
302 focusing on the top 500 genes with high variances, the off-season moderately deviated from the

303 early season but not notably from the normal season (right part of Fig. 2a). In both cases, the late
304 season overlapped partially with all other seasons.

305 In three pairwise comparisons for differential expression analysis against the normal
306 season (off-season vs. normal, early vs. normal, and late vs. normal), we identified 173, 337, and
307 265 differentially expressed genes (DEGs), respectively (Fig. 2b). All comparisons revealed a
308 higher number of down-regulated genes than up-regulated genes. Additionally, Venn diagrams
309 indicated that the DEGs from the off-season vs. normal comparison had very few overlapping
310 genes (<1%) with those from the other two comparisons (depicted in the first and second
311 diagrams in Fig. 2c). On the other hand, early vs. normal and late vs. normal comparisons shared
312 approximately 25% of the DEGs (depicted in the third diagram in Fig. 2c). These results suggest
313 that the expression pattern of the off-season differed from both the early and late seasons when
314 compared to the normal season.

315 Altered spawning seasons affected various biological pathways related to 316 metabolism, cellular processes, and organismal systems

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

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

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

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

339 In summary, the off-season appeared to down-regulate carbon metabolism, while the
340 early and late seasons primarily had strong suppressive effects on pathways associated with
341 cellular processes.

342 **Altered spawning seasons had an impact on the expression of genes in a**
343 **similar manner**

344 To further investigate the genes commonly affected by multiple spawning seasons, we used
345 overlapping DEGs (represented as intersections of Venn diagrams in Fig. 2c) by applying two
346 filtering steps: selecting DEGs found in at least two comparisons and choosing the top five
347 DEGs from each comparison based on adjusted p-values (Supplementary Table S11). This
348 filtering approach revealed a total of 13 genes (Table 1). Among them, two genes (gene IDs:
349 106612264 and 106601246) lacked corresponding gene names and symbols. All genes, except *pc*
350 and *pitpnm2*, consistently displayed up- or down-regulation across all spawning seasons,
351 suggesting that all altered spawning seasons had a similar effect on this specific set of genes
352 when compared against the normal season (Table 1).

353 These genes can be classified into three categories by relevant biological functions:
354 metabolism (*pcyt1b*, *pc*, *klf15*, and *cyp7a1*) (Cappel et al., 2019; Kanehisa and Goto, 2000b;
355 Takashima et al., 2010), tissue development (*prox3*, *crim1*, and *krt8*) (Del Giacco et al., 2010;
356 Kolle et al., 2000; Ku et al., 2007; Scott et al., 2007), and information processing, which can be
357 further classified into three sub categories as signalling (*bmp5* and *pitpnm2*) (Garner et al., 2012;
358 Kanehisa and Goto, 2000b), transcription (*tcf12*) (Hu et al., 1992), and post-translation (*rhobtb4*)
359 (Kanehisa and Goto, 2000b). As these categories are typically associated with growth, all three
360 altered spawning seasons affected a certain set of genes associated with growth regulation in a
361 similar manner, despite PCA plots (Fig. 2a) and Venn diagrams (Fig. 2c) showing a distinct gene
362 expression pattern of the off-season.

363 **Overall methylation rates increased until the harvesting stage within and**
364 **around gene bodies, except near transcription start sites**

365 We conducted DNA methylation analysis on 20 liver samples from larvae across four different
366 spawning seasons (n=5; detailed statistics in Supplementary Table S12) to investigate the
367 epigenetic effects of altered spawning seasons on DNA methylation.

368 Before analysing the effects of altered spawning seasons, we used RRBS data from two
369 independent studies on liver samples to investigate DNA methylation changes across
370 developmental stages. Specifically, we compared our RRBS data from the larvae stage with the
371 post-smolt stage (approximately 31 weeks) and the harvest stage (around 45 weeks) (Saito et al.,
372 2023; Saito et al., 2021). The post-smolt samples were divided into three groups with different
373 1C metabolism nutrient levels (Ctrl, 1C+, and 1C++) according to their experimental diets, while
374 the harvest samples were divided into three groups with varying micronutrient levels (L1, L2,
375 and L3) based on their experimental diets. We compared these six groups from post-smolt and
376 harvest developmental stages with our larvae stage samples, which were collected across four
377 spawning seasons (Fig. 4a).

378 Notably, the average methylation rates exhibited a consistent increase from larvae to
379 post-smolt to harvest stages: 83.7%, 83.9%, and 84.9% for larvae, post-smolt, and harvest stages,
380 respectively (Kruskal-Wallis, p-value < 0.001, black horizontal lines in Fig. 4a). Within the
381 larvae group, the average rate of the normal group was higher than that of the off-season group,
382 but the difference was not significant. Within the post-smolt group, the Ctrl group showed a
383 significantly lower methylation rate than the other two groups (pair-wise Wilcoxon test with the
384 Holm correction, p-values < 0.05; Supplementary Table S13). The Ctrl group was given lower

385 methionine along with B-vitamins as the 1C metabolism nutrients compared to other groups
386 (1C+ and 1C++) (Saito et al., 2023).

387 To examine regional DNA methylation patterns, CpG sites were categorised into three
388 genomic regions based on their positions relative to nearby genes: gene body (GB; including
389 exons and introns), promoter (P; including P250, P1K, and P5K), flanking regions,
390 miscellaneous regions (Mics, including various transcripts and non-coding RNA), and intergenic
391 regions (IGR) (Fig. 4b; see Methods and Materials for definitions).

392 The trend of the rate increment became more noticeable when the rates were examined
393 separately by different genomic regions (Fig. 4c). In most regions, including gene bodies,
394 promoter regions except for P250, and flanking regions, the samples from the harvest stage
395 exhibited significantly higher average rates, followed by the post-smolt samples, and then the
396 larvae samples (pair-wise Wilcoxon test with the Holm correction, p-values < 0.05;
397 Supplementary Table S14). Specifically, within the two promoter regions (P1K and P5K), both
398 the post-smolt and harvest stages showed a significant increase in methylation rates compared to
399 the larvae stage. Moreover, methylation rates were generally lower in P250 (~22%) and P1K
400 (~49%) compared to other regions (75% ~ 88%).

401 This comparative investigation suggests a potential increase in methylation rates as
402 salmon progress in growth, at least until the harvesting stage, while also noting that methylation
403 rates near transcription start sites (TSSs) tend to remain lower and more stable than in other
404 genomic regions.

405 **The off-season showed non-significant but consistently lower methylation**
406 **rates compared to the normal and early seasons**

407 To investigate the regional shift of the methylation rates among four spawning seasons, we
408 applied the same methods used for the comparison analysis between developmental stages.
409 Unlike the result of the developmental stages, spawning seasons exhibited no significant
410 differences in methylation rates when separated by genomic regions (Fig. 4d; pair-wise Wilcoxon
411 test with the Holm correction in Supplementary Table S15). Despite the non-significant
412 differences, the average rates of the off-season samples were lower than those of the normal and
413 early seasons in all regions (Supplementary Table S16). Nonetheless, the average rates of the off-
414 season were higher than those of the late season in the two promoter regions (P250 and P5K) and
415 flanking regions.
416

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

419 We conducted six pairwise comparisons to identify CpG sites with differential methylation (Fig.
420 5a). Unlike the analysis of the RNA-seq data, we considered all six possible pair-wise
421 comparisons to capture a comprehensive pattern of methylation differences with our RRBS data.
422 Remarkably, all comparisons consistently demonstrated similar characteristics, for both the
423 number of differentially methylated CpGs (DMCs) and the balanced ratio of hypo- and hyper-
424 methylation (Fig. 5a).

425 Further investigation focused on a set of 10,308 common DMCs, identified in at least one
426 comparison. Distributions of methylation rates for these common DMCs exhibited a distinct left-

427 skewed pattern across the exon, intron, P5K, and flanking regions (Fig. 5b). This observation
428 strongly suggested that methylation differences resulting from altered spawning seasons
429 primarily occurred within regions that were already highly methylated. In contrast, the P250 and
430 P1K regions displayed nearly uniform methylation rates across a range of 0-100%, indicating
431 significant methylation differences occurred irrespective of the underlying methylation rates.
432 These findings imply a potentially greater level of dynamic regulation of DNA methylation in
433 P250 and P1K compared to the other regions.

434 Filtering multiple DMCs identified key genes affected by spawning season 435 alterations

436 We examined genes with multiple DMCs in three pairwise comparisons against the normal
437 season. Since most genes had only a single DMC, especially in promoters (~75% in P250 and
438 ~70% in P1K; Supplementary Figure S1), we avoided identifying differentially methylated
439 regions (DMRs) using a sliding window approach. Instead, we filtered out genes strongly
440 supported by multiple DMCs based on specific criteria (see Methods and Materials), resulting in
441 a total of 11 genes with multiple DMCs on their promoters (Table 2; see the details in
442 Supplementary Table S17).

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

449 Literature analysis associated these genes with two biological functions: development
450 (*inf2*) and information processing, which can be further classified into four sub categories as
451 transporter (*slc31a1* and *scp-2*) (Stolowich et al., 2002; Zhou and Gitschier, 1997), signalling
452 (*stk32a* and *lrrfip2*) (Alliance of Genome Resources, 2022; Bateman et al., 2022), post-
453 translation (*klhl11* and *ttll12*) (Bateman et al., 2022; Brants et al., 2012), and transcription (*helz2*
454 and *prrx2*) (Norris et al., 2000; Tomaru et al., 2006). However, *ctl2b* and an uncharacterised gene
455 (Gene ID: 106573279) had no clear associations with specific biological functions. Although the
456 DMCs on these genes were potentially linked to the underlying gene expression, none of the 11
457 genes were identified as DEGs in our differential gene expression analysis.

458 Altered spawning seasons influenced gene expression and DNA methylation of 459 cell cycle-related genes

460 To investigate potential associations between DNA methylation and gene expression, we
461 combined DEGs and DMCs from three pairwise comparisons against the normal season (off-
462 season vs. normal, early vs. normal, and late vs. normal) in P250 and P1K. This resulted in a
463 total of seven DEGs that contain a total of 11 DMCs in their promoters (Fig. 5c, Table 3,
464 Supplementary Table S18).

465 In addition to the significant results, we expanded the list of seven DEGs with additional
466 information from all pair-wise comparisons (additional entries were marked with parentheses in
467 Table 3). Among the identified genes, three (*caprin-1*, *kifc1*, and *aurkb*) exhibited consistent
468 regulations of DMCs and DEGs compared to the normal season. Specifically, *caprin-1* and *kifc1*
469 had hypo-methylated CpG sites, while *aurkb* had hyper-methylated CpG sites; all three genes
470 showed down-regulated gene expression (Table 3). Notably, these three genes (*caprin-1*, *kifc1*,

471 and *aurkb*) were associated with cell cycle regulation (Grill et al., 2004; Walczak et al., 1997;
472 Wheatley et al., 2001), while the remaining genes were linked to metabolism (*cyp8b1*,
473 *adrenodoxin*, and *lpin1*) (Grinberg et al., 2000; Hansson and Wikvall, 1982; Kanehisa and Goto,
474 2000a) and transport (*slc43a1a*) (Babu et al., 2003). These findings indicate that altered
475 spawning seasons may have affected gene expression and epigenetic regulation of several genes
476 associated with cell cycle control.

477

478 Discussion

479 In the present study, we investigated the influence of altered spawning seasons on gene
480 expression and DNA methylation in offspring. Previous studies emphasized the significant
481 impacts of these changes on the nutritional status of both broodstock and their offspring, with no
482 observed effects on the body weights of broodstock but in offspring (Skjærven et al., 2022;
483 Skjaerven et al., 2020). Our primary aim was to reveal the transcriptomic and epigenetic effects
484 resulting from observed variations by comparing liver samples from larvae at the first feeding
485 stage.

486 What insights do our omics analyses provide?

487 Gene expression analysis revealed distinct patterns associated with altered spawning seasons.
488 Specifically, early and late seasons exhibited similar gene expression patterns when compared to
489 the normal season. The differential expression of genes associated with metabolism, cellular
490 processes, and organismal systems indicates that spawning seasons can have wide-ranging
491 effects on biological pathways related to growth and development. Furthermore, the analysis

492 with overlapped DEGs revealed that all three altered spawning seasons similarly affected genes
493 associated with metabolism, tissue development, and information processing. Hence, while early
494 and late seasons influenced a wide range of biological pathways in a similar manner, all three
495 seasons, including the off-season, also similarly affected a certain set of genes, especially those
496 strongly differentiated from the normal season.

497 DNA methylation analysis provided valuable insights into the epigenetic effects of
498 different spawning seasons. Altered spawning seasons led to increased methylation rates for the
499 early and normal seasons. Notably, these shifts primarily occurred in regions that were already
500 highly methylated, but the rates were altered independently of the underlying methylation rates
501 in promoter regions around transcription start sites. In addition, our findings across different
502 developmental stages imply that methylation rates could serve as an indicator of age. However,
503 more research is needed to understand the mechanism of methylation rate shifting and potential
504 applications in aquaculture as well as age measure for stock assessment.

505 Several genes with multiple DMCs exhibited consistent hypo- or hyper-methylation
506 patterns across multiple spawning seasons in promoter regions, suggesting a common epigenetic
507 response to altered spawning seasons. These genes were associated with key biological
508 functions, such as development and information processing, potentially indicating their role in
509 growth performance. Although both gene expression and DNA methylation analyses resulted in
510 similar biological functions impacted by altered spawning seasons, they showed little overlap
511 between DEGs and genes with DMCs. This could be attributed to regulatory differences between
512 gene expression and DNA methylation within the same biological pathway, or it might be a
513 limitation caused by the restriction enzyme used in RRBS.

514 The observed correlations between DNA methylation and gene expression in our liver
515 samples were weaker than anticipated. Note that the measurements were taken from siblings
516 rather than the same individual due to the limited size of larvae livers. Notably, our findings also
517 revealed inconsistent correspondence between hypo-methylation and active gene expression as
518 well as between hyper-methylation and gene suppression. These results suggest that the
519 regulation of DNA methylation is more intricate than commonly assumed as it is known that
520 high promoter CpG methylation reduces gene expression, while low promoter CpG methylation
521 allows for active gene expression (Lanata et al., 2018). Within promoter regions, not only DNA
522 methylation but several other factors, including underlying gene expression levels, transcription
523 factor binding sites, and chromatin remodelling, likely play a role in regulating actual gene
524 expression.

525 What are the significant biological differences observed in offspring?

526 **Off-season vs. normal:** Off-season spawning in June, facilitated through a RAS, resulted in
527 Atlantic salmon offspring with lower larval weights compared to the normal and late seasons
528 (Skjærven et al., 2022; Skjaerven et al., 2020). Offspring eggs also exhibited reduced levels of
529 vitamin B12 and lipid classes relative to normal and late seasons (Skjærven et al., 2022;
530 Skjaerven et al., 2020). Gene expression analysis indicated significant down-regulation of genes
531 associated with central carbon metabolism (CCM). CCM involves several biological pathways,
532 including the citric acid cycle, and acts as a major source of energy for growth and development.
533 Moreover, lipids are interconnected with CCM through the utilisation of acetyl-CoA as a central
534 metabolite (Westfall and Levin, 2018). Among the six genes with multiple DMCs from the off-
535 season vs. normal comparison, sterol carrier protein2-like (*scp-2*) contained four hyper-
536 methylated DMCs in its promoter. The *scp-2* gene encodes a transfer protein that plays a key role

537 in intracellular lipid transport (Stolowich et al., 2002). Overall, RAS-based off-season spawning
538 appeared to impact lipid-mediated regulations both transcriptionally and epigenetically. This
539 negative impact on lipid-related mechanisms potentially accounted for impaired growth
540 performance observed in body weight as our previous research indicated the importance of gene
541 expression and epigenetic regulation of lipid regulation, specifically involving acetyl-CoA, for
542 growth performance (Saito et al., 2021).

543 **Early season vs. normal season:** Offspring resulting from early season spawning in
544 September also exhibited lower weights at the larval stage compared to those from normal and
545 late seasons. However, the nutritional status of offspring eggs displayed higher levels of
546 SAM/SAH, lysine, glutamine, and alanine (Skjærven et al., 2022; Skjaerven et al., 2020). Gene
547 expression analysis revealed significant down-regulation of genes associated with cell cycle
548 regulation, while genes involved in metabolism showed both down- and up-regulation. Among
549 the five genes that exhibited differential expression and had at least one DMC in their promoters,
550 two were associated with cell-cycle regulation. Specifically, the carboxy-terminal kinesin 2
551 (*kifc1*) gene promotes mitotic spindle assembly (Walczak et al., 1997), and the aurora kinase B
552 (*aurkb*) gene is involved in mitotic progression (Wheatley et al., 2001). The *kifc1* gene was
553 hypomethylated, while the *aurkb* gene was hypermethylated. Consequently, the early spawning
554 season had an impact on cell-cycle regulation both transcriptionally and epigenetically. However,
555 it is challenging to hypothesise that this negative impact on cell-cycle regulation was linked to
556 impaired growth performance because similar effects were observed for the late season.

557 **Late season vs. normal season:** Late season spawning in January showed that offspring
558 weights were almost the same, but slightly heavier than those from the normal season. The
559 nutritional status of offspring eggs also exhibited similar levels of various nutrients and

560 metabolites to the normal season, except for higher vitamin B12 and lower B-alanine (Skjærven
561 et al., 2022). Gene expression patterns in the late season were comparable to those observed in
562 the early season, showing significant down-regulation of genes related to cellular processes,
563 especially cell cycle regulation. Two genes, *kifc1* and *aurkb*, showed differential expression and
564 had at least one DMC in their promoters, and both were associated with cell-cycle regulation.
565 Hence, the late season showed similar growth performance and nutritional status to the normal
566 season, while the gene expression pattern was similar to the early season.

567 **Three altered spawning seasons vs. normal season:** Although strong similarities were
568 observed in gene expression patterns between early and late spawning seasons, certain genes
569 exhibited common regulation in both gene expression and DNA methylation across all three
570 altered spawning seasons. Among the four DEGs identified in the off-season, choline-phosphate
571 cytidylyltransferase B (*pcyt1b*) and bone morphogenetic protein 5-like (*bmp5*) showed
572 concordant down-regulation across all three altered spawning seasons. The *pcyt1b* gene encodes
573 an enzyme involved in phosphatidylcholine biosynthesis, playing essential roles in multiple
574 metabolic pathways (Bateman et al., 2022), while the *bmp5* gene is involved in signalling
575 (Kanehisa and Goto, 2000a). Additionally, all DMCs found in the promoter of the cytotoxic T
576 lymphocyte-associated protein 2 beta (*ctl2b*) gene were hyper-methylated across all three altered
577 spawning seasons. The function of *ctl2b* remains unknown, but it shares similarities with genes
578 encoding cysteine proteinase, an enzyme involved in protein breakdown within cells (Denizot et
579 al., 1989). Furthermore, among genes that were both differentially expressed and had at least one
580 DMC in their promoters, the *caprin-1* gene exhibited consistent regulation, with all DMCs being
581 hypo-methylated and its expression being suppressed. The *caprin-1* gene is associated with cell

582 cycle regulation, playing a crucial role in cellular activation or proliferation (Grill et al., 2004).
583 This indicates that the off-season also influenced cell cycle regulation to a certain degree.

584 Why are our findings important in the context of aquaculture and nutritional 585 studies?

586 Our study unveiled the significant effects of altered spawning season on offspring gene
587 expression and DNA methylation patterns in Atlantic salmon. These findings provide valuable
588 insights into assessing current and future growth potential, which can be challenging to
589 determine using only nutritional status and growth performance measures. Understanding the
590 gene regulation and epigenetic responses to altered spawning seasons will help develop strategies
591 to optimize growth and production in aquaculture practices. Additionally, our study can be linked
592 to the potential impact of increasing global ocean temperatures on the spawning of aquatic
593 vertebrates, as well as the northward shift in habitats followed by changes in photoperiod, as
594 both temperature and light are key abiotic factors influencing spawning seasons. Even though
595 genetic and epigenetic profiles can be species- and even tissue-specific, the approaches we
596 demonstrated here could be applied to other fish species whose spawning seasons are artificially
597 altered in aquaculture practices. Nonetheless, further studies are needed to elucidate the
598 fundamental effects caused by spawning season alterations across various species.

599

600 Conclusions

601 The present study emphasises the substantial impact of altering spawning seasons on gene
602 expression and DNA methylation patterns in Atlantic salmon offspring. Our observations

603 indicate that RAS-based off-season spawning exerts an influence on lipid-mediated regulations
604 in offspring, while sea-pen based early and late seasons affect the regulation of the offspring's
605 cellular processes, especially cell cycle regulation. These effects potentially play a crucial role in
606 shaping both current and future growth performance.

607 These findings have important implications for aquaculture and nutritional studies, as
608 they provide valuable tools for assessing growth potential and optimizing production strategies.
609 Future research focusing on tissue and developmental stage-specific DNA methylation maps will
610 enhance the accuracy of growth performance estimation and provide further insights into the
611 gene regulation and epigenetic responses to altered spawning conditions.
612

613 Acknowledgements

614 We thank Amelie Nemc and Bekir Ergüner for advice on RRBS analysis, and Hui-Shan Tung
615 and Eva Mykkeltvedt for DNA and RNA extraction at the Institute of Marine Research (Bergen,
616 Norway). This work was supported by the Norwegian Research Council under project no:
617 267787 (NutrEpi) and by the Institute of Marine Research under the Nutritional programming
618 project.
619

620 Author contributions

621 K.H.S conceived and designed the research. M.E. and M.M. conducted and designed the feeding
622 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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Figures

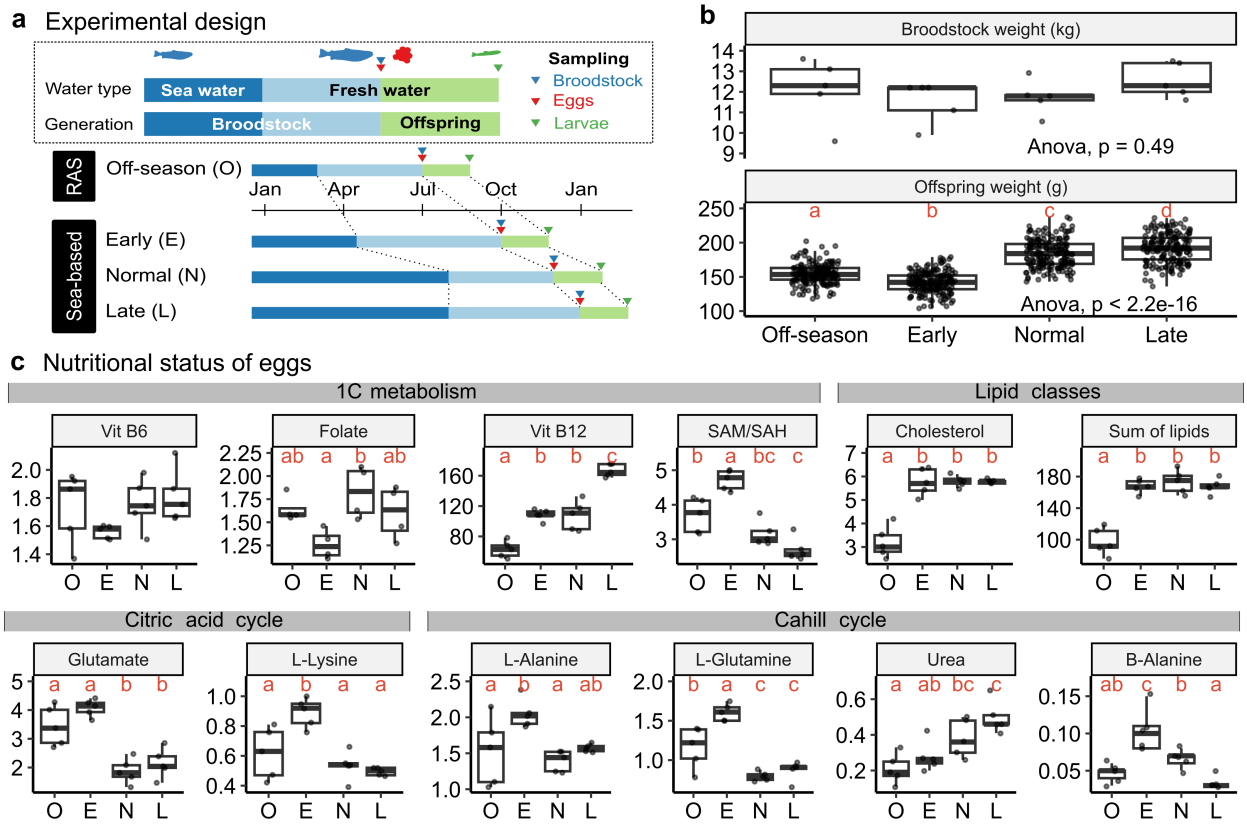

Fig. 1. Experimental design, body weights, and nutritional status across spawning seasons.

a) Schematic diagram illustrating four distinct spawning seasons: off-season (O), early (E), normal (N), and late (L). The 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) per season. 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: mg/kg 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 ($p<0.05$).

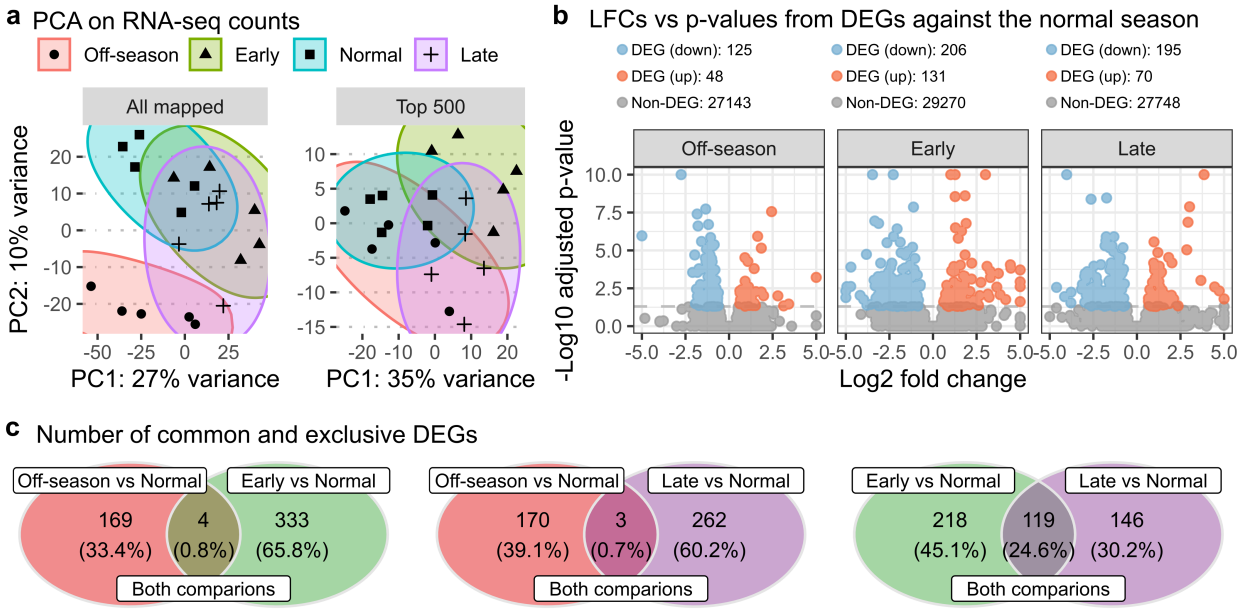

Fig. 2. Gene expression analysis comparing altered spawning seasons to the normal season.

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 45153 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). The 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.

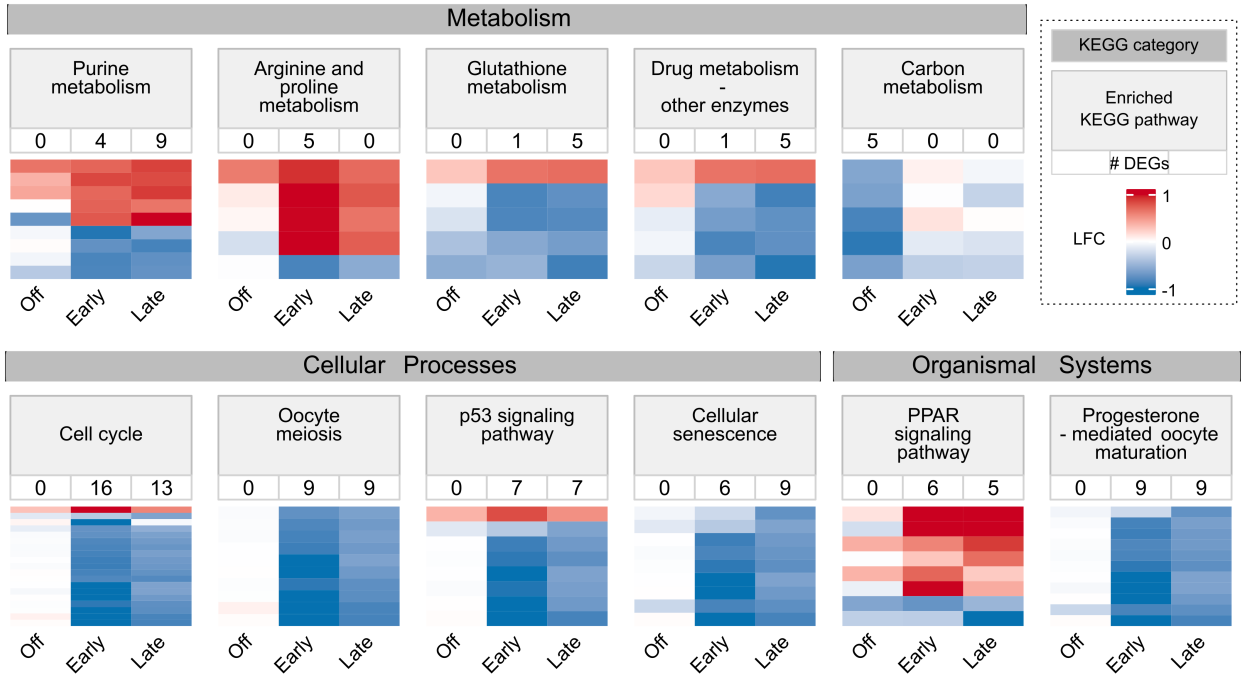

Fig. 3. Enriched KEGG pathways for DEGs identified in three pair-wise comparisons. Heatmaps showing log fold changes (LFCs) of genes associated with 11 KEGG pathways enriched through over-representation analysis. LFCs underwent normal shrinkage transformation. Genes were included if identified as DEGs in at least one of the three comparisons: off-season vs normal (Off), early vs normal (Early), and late vs normal (Late). The number below each pathway represents DEG count. KEGG pathways are categorized into metabolism, cellular processes, and organismal systems groups.

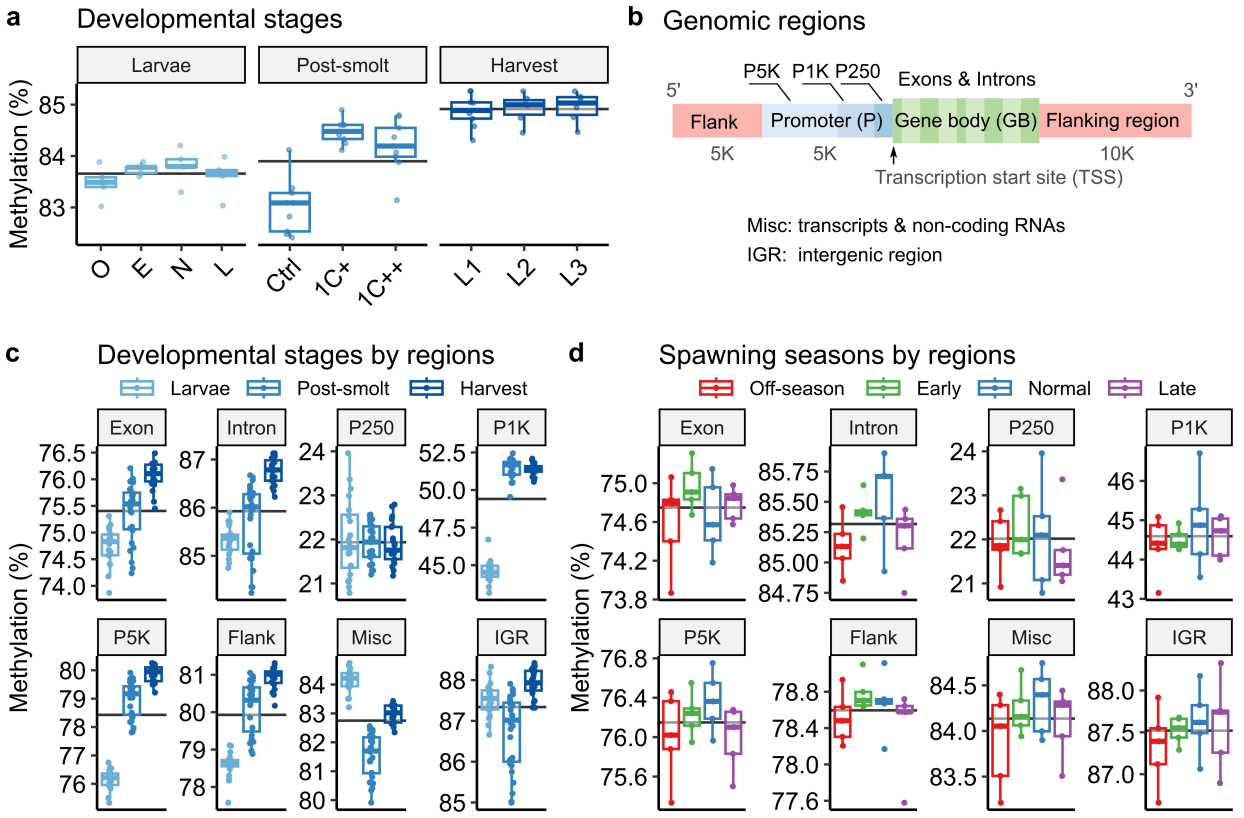

Fig. 4. Distribution of methylated CpG sites across genomic regions.

Box plots displaying liver DNA methylation status during the lifespan of Atlantic salmon; data from the present study (larvae, light blue) and two other studies: post-smolt (blue) and harvest (dark blue). In the larvae stage, O, E, N, L indicate off-season, early, normal, and late seasons, respectively. While the post-smolt stage contains three groups with different 1C-metabolism nutrient levels (Ctrl, 1C+, and 1C++), the harvest stage contains three groups with different micronutrient levels (L1, L2, and L3) in their diets. Horizontal lines indicate the average rates for each group. **a)** Box plots showing average methylation rates of the samples from three developmental stages. **b)** Genomic region diagram illustrating three regions: flanking regions (Flank, red), promoters (P, blue), and gene bodies (GB, green). The promoter region is divided into three subregions: P250, P1K, and P5K. The gene body includes exons and introns. **c)** Box plots showing average methylation rates of the samples from three developmental stages across eight genomic regions. **d)** Box plots showing average methylation rates of the samples from four spawning seasons across eight genomic regions.

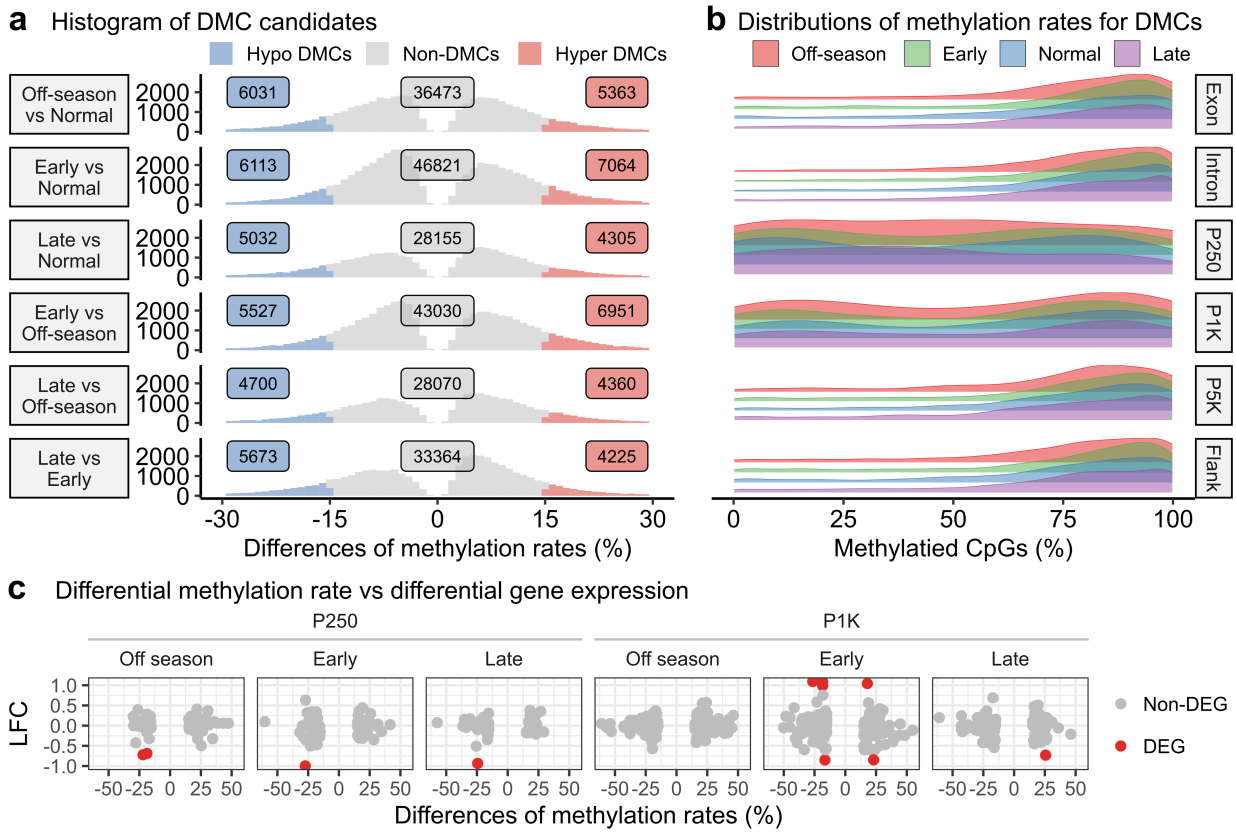

Fig. 5. DNA methylation differences and correlations with gene expression.

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 the 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 DEGs in red and non-DEGs in grey.

Tables

Table 1. List of top five DEGs identified in at least two pairwise comparisons.

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.

902 **Table 2**. List of genes with multiple DMCs in their promoter region.
903

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

904
905 [1]All hypo: All three comparisons showed hypo-methylated DMCs, Hyper & Hypo: Comparisons showed both hypo-
906 and hyper-methylated DMCs. All hyper: All three comparisons showed hyper-methylated DMCs. [2]Either hyper or
907 hypo-methylated DMC against the normal season. The number in brackets represents the number of DMCs. *: genes
908 selected by filtering of multiple DMCs.

909

910 **Table 3**. List of DEGs that contain DMCs in their promoter region.
911

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

912
913 [1]Either hypo- or hyper-methylated DMC against the normal season. Parentheses indicate that the site was identified
914 as DMC, but the corresponding genes were not identified as stringent DEG. [2]Either up- or down-regulation against
915 the normal season. Parentheses indicate that down- or up-regulation was determined by non-stringent DEGs.

916