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Out-of-season spawning affects the nutritional status and gene expression in both Atlantic salmon female broodstock and their offspring

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ABSTRACT

The Atlantic salmon aquaculture industry relies on adjustments of female broodstock spawning season to meet the demand for delivery of embryos outside the natural spawning season. Earlier results from zebrafish have shown that parental micronutrient status program offspring metabolism. Therefore, the main hypothesis of this study was to investigate if out-of-season (off-season) broodstock (spawning in June, in land-based recirculation systems) and their offspring deviate in micronutrient status when compared to broodstock and offspring from normal spawning season. Both seasons of female Atlantic salmon broodstock were fed the same diet and starved for approximately the same time interval prior to spawning. We compared nutrients related to the 1C metabolism (vitamin B12, folate, vitamin B6, methionine), free amino acids (FAAs) and lipid classes in broodstock muscle and liver tissues, and during offspring ontogeny. In general, the off-season broodstock showed higher levels of folate, vitamin B6 and selected FAAs in muscle tissue, and higher levels of folate and lipids (cholesterol and sphingomyelin) in liver tissue compared to normal-season. Furthermore, embryos from off-season had reduced amounts of all the measured lipid classes, like cholesterol and sphingomyelin, and lower levels of one type of folate and changes in FAAs and N-metabolites. We discovered significant differences between the seasons in mRNA levels of genes controlling fatty acid synthesis and 1C metabolism in both broodstock liver and offspring. Moreover, for genes controlling the methylation of DNA; both maintenance and *de novo* DNA methyltransferases (DNMTs) were expressed at higher levels in off-season compared to normal-season offspring. Our results show, in general that normal spawning season broodstock allocated more nutrients to eggs than off-season. Our results indicate a potential for improved maturation for off-season group to obtain a higher offspring growth potential, and this argues for a reassessment of the nutritional influence from broodstock to offspring and the consequences through nutritional programming.

1. Introduction

Broodstock nutritional micronutrient status is a powerful environmental variable to target in order to permanently optimize offspring performance and quality. Vertebrates show a great developmental plasticity during embryogenesis, a period which is sensitive to nutritional stimuli. Nutritional stimuli can give long term metabolic consequences that persist even after the nutritional exposure terminates, a concept known as nutritional programming (Agosti et al., 2017; Duque-Guimaraes and Ozanne, 2013). In addition, embryonic development in fish accomplish a range of coordinates events consisting of intrinsic factors like nutrients accumulated during oocyte maturation, external environmental stimuli like temperature and toxicants and parental genetic material and gene regulation (Izquierdo et al., 2001; Izquierdo et al., 2015; Palace and Werner, 2006; Pelegri, 2003). We have earlier described how intrinsic factors, like zebrafish parental dietary inclusion of methionine, folate, vitamin B12 and vitamin B6, which are micronutrients known as one carbon (1C) nutrients, alter the embryonic expression of immune-, lipid and apolipoprotein genes in their offspring (Skjaerven et al., 2016). Further on, we showed that parental low 1C nutrient diet influenced zebrafish offspring at the mature stage through nutritional programming and changed both epigenetic regulation through DNA methylation, gene transcription and liver lipid accumulation to a fatty-liver-like phenotype (Skjaerven et al., 2018). For aquaculture, increased focus on broodstock feed and micronutrient status and the consequence for offspring quality might improve both

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survival and growth.

The 1C nutrients are main mediators in the 1C metabolism, which includes the folate cycle, methionine cycle and transsulfuration pathway, ameliorates cytotoxic homocysteine levels and increases simultaneously the redox capacity if the 1C nutrients depletes, as earlier reviewed (Anderson et al., 2012; Xu and Sinclair, 2015; Xu et al., 2016). In addition, the 1C metabolism includes vitamin B12 and vitamin B6 as cofactors, it includes other amino acids like serine, glycine and cysteine (Ducker and Rabinowitz, 2017) and regulates the methylation potential of the cells *via* the S-adenosylmethionine (SAM)/S-adenosylhomocysteine (SAH). SAM donates a methyl group to many biological methylation reactions, including methylation of proteins and DNA. Folate is also needed for thymidine synthesis in the DNA production during cell proliferation (Ducker and Rabinowitz, 2017). Furthermore, liver 1C metabolism capacity depends directly on the 1C nutrients, and a deficiency has been linked to accumulation of triacylglycerol and lipid content in the hepatocytes (da Silva et al., 2014; Espe et al., 2010; Espe et al., 2020). Deficiency during embryogenesis have been associated to severe developmental deformities like neural tube defects, anemia and to influence the activity of neurotransmitters in the central nervous system (Beaudin and Stover, 2007). This indicates the important role for the 1-C metabolism both for mature stages like broodstock and for early embryo stages. Early embryonic stages express and regulate almost all of the enzymes that participate in 1C metabolism (Ikeda et al., 2012), and all 1C cycle genes are highly regulated through embryonic developmental stages (Aanes et al., 2011; Skjaerven et al., 2014). Few studies have measured the 1C nutrients in fish embryos, however, of special interest one study showed variation in folates in different batches of Halibut embryos (Maeland et al., 2003).

The Atlantic salmon aquaculture industry relies on adjustments of female broodstock spawning season to meet the demand for delivery of embryos outside the natural spawning season. A variety of environmental adjustments with feeding, light and temperature regimes, or in recirculation aquaculture systems (RAS), have been developed to achieve first feeding fry all year long, as a contrast to the normal spawning season in November for wild salmon. How environmental manipulation of spawning season influences the nutritional profile in broodstock has not been studied, nor the consequence on nutritional status in offspring. The main hypothesis of this study was to investigate if broodstock spawning out-of-season, and their offspring, deviate in micronutrient status when compared to broodstock and offspring from normal spawning season. Based on our earlier studies, we focused on the micronutrients in the 1C metabolism (vitamin B12, folate, vitamin B6, methionine), other free amino acids and lipid classes in muscle and liver from female Atlantic salmon broodstock and during offspring ontogeny. In addition, we compared biometry measures and gene expression changes in both broodstock and offspring. Early embryonic stages rely on the availability of 1C stores in the yolk and tissues for appropriate cellular growth, but how five months advanced spawning in off-season group influences the micronutrient status, in both the broodstock and offspring, has, to the best of our knowledge, not been studied.

2. Material and methods

2.1. Ethical considerations

This experiment and this report are in accordance with the ARRIVE guidelines, however, the fish and sampled used in this experiment are sampled directly at a production facility for Atlantic salmon. As such, the rearing conditions and harvest protocols used in this experiment are the same as used in commercial salmon production. All procedures involving euthanization of broodstock and larvae were performed using anesthetics according to the supplier's instructions. In accordance to Norwegian and European legislation related to animal research, formal approval of the experimental protocol by the Norwegian Animal Research Authority (NARA) is not required because the experimental conditions are practices undertaken for the purpose of recognized animal husbandry. Such practices are excepted from the European convention on the protection of animals used for scientific purposes (2010/63/EU), *cf.* article 5d. Also, these practices do not require approval by the Norwegian ethics board according to the Norwegian regulation on animal experimentation, § 2, 5a, d "non-experimental husbandry (agriculture or aquaculture)" and "procedures in normal/common breeding and husbandry". Norway has implemented the European Directive as a national initiative, while waiting for a formal implementation according to the EEA agreement. This explanation may be viewed as a waiver, and as such the Norwegian Animal Research Authority does normally not give formal waivers in clear cases such as this one.

2.2. Experimental design and sampling

Sampling was performed at AquaGen's breeding station in Kyrksæterøra, Norway. Females from the 15th generation of selected Atlantic salmon representing broodstock groups with two different spawning seasons: five months advanced out-of-season (off-season) spawners and normal-season spawners were used. Off-season females were produced in a closed, land-based, recirculating aquaculture system (RAS) in brackish water (12‰ salinity) where an artificial photoperiod and temperature was applied to advance maturation with five months compared to natural spawning. Normal-season females were produced in open, sea-based net pens under ambient photoperiod and temperatures to let the broodfish mature naturally. Both groups were moved to freshwater before spawning. All females were fed to saturation with Breed 3500 (EWOS) from 1. June 2016 until 109 and 120 days of starvation before stripping for off-season and normal-season, respectively (Table S1). From each spawning group, five females from separate tanks were sacrificed using Benzoak (20%, ACD Pharmaceuticals As). After stripping, each female was measured for weight, length, liver weight, and ovary weight. Condition factor = $100 \times (\text{weight}/\text{length}^3)$, hepatosomatic index (HSI) = $100 \times$ (liver weight/body weight) and gonadosomatic index (GSI) = $100 \times$ (ovary weight/body weight) were estimated for each female and compared between groups. Liver and muscle samples were flash frozen in liquid N_2 for analysis of folate, vitamin B12, vitamin B6, free amino acids (FAAs) and N-metabolites, and lipid classes. In addition, we performed gene expression analysis of selected genes on broodstock liver tissue.

All oocytes were fertilized with cryopreserved sperm from two pooled males. Fertilized eggs were incubated (7.8 ± 0.5 °C, oxygen 102 ± 10%) until hatching and kept until start-feeding (8.2 ± 1.0 °C, oxygen 109 ± 12%). Newly fertilized eggs (2 day-degrees (2 d°)) and eye stage embryos (359–364 d°) were flash frozen in liquid N_2 for analysis of folate, vitamin B12, vitamin B6, FAAs and lipid classes. In addition, embryos at the eye stage and hatched larvae were fixated for RNA analysis in RNAlater (ThermoFisher). Overview of sampling stages and d° are given in table S2. Average egg sizes are given in table S3. At the end of the endogenous feeding phase (start feeding larvae 979–994 d°), 36 larvae from each broodstock were randomly selected and euthanized with an overdose of buffered tricane methanesulfonate (MS222, Pharmaq, Norway) and measured for total body mass and length.

2.3. Chemical analysis

Vitamin B12 and total folate were analysed microbiologically in broodstock liver and muscle, newly fertilized eggs and eye stage embryos using *Lactobacillus delrueckii* spp. *Lactis* and *Lactobacillus rhamnosus*, respectively as previously described (Maeland et al., 2000). Liver and newly fertilized eggs were analysed for folate species by LC-MS/MS analysis according to the method previously published (Bhandari et al., 2018). Briefly, the sample was extracted in buffer containing folate

stabilisers, and C-13 labeled folic acid, 5-CH3–THF and 5-CHO-THF as internal standards. Tri-enzymatic digestion was performed using alpha-amylase, protease and rat plasma conjugase, the latter to de-conjugate the polyglutamate forms of folates to corresponding monoglutamate forms. The clear extract was subjected to a weak anion exchange solid phase extraction, mono-glutamyl folates were eluted, and analysed by LC-MS/MS (1290 Infinity UHPLC and 6460 triple quadrupole, Agilent). Folate vitamers were quantified using external calibration curves and the Mashunter software (Agilent), and reported as folic acid equivalents. Vitamin B6s (pyridoxamine (PM), pyridoxal (PL), pyridoxine (PN)) were measured by ultra-performance liquid chromatography (UPLC) method as described (Albrektsen et al., 1994). FAAs and other N-metabolites were analysed in deproteinized tissues using the Biochrome Analyzer and post column ninhydrin reaction as described (Espe et al., 2006). Lipid class specification was analysed after separation on silica HPTLC-plates as described (Bell et al., 1993; Liaset et al., 2003)

2.4. RNA extraction and RT-qPCR

Liver (approximately 50 mg), embryos at the eye stage and newly hatched larvae samples (each sample containing three embryos or larvae) were added 750 µL Qiazol lysis reagent and homogenized using four ceramic beads (CK28) and a precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). The homogenization program was 3 × 10 s at 600 rcf. After homogenization, another 500 µL of Qiazol lysis reagent was added to each tube, mixed and thereafter left at −80 °C for one hour. The samples were left for 10 min at room temperature before centrifuged at 4 °C for 10 min at 12000 rcf. Another centrifugation step was added to embryo and larvae samples to ensure better separation of the transparent surface layer containing the RNA. Total RNA extraction was thereafter completed using the RNA Universal Tissue Kit (Qiagen, Hilden, Germany) combined with the BioRobot EZ1, treated with DNase according to the manufacturer's manual. RNA was eluted in RNase-Free MilliQ H_2O. The quantity and quality were assessed using the Nanodrop ND-1000UV Spectrophotometer (NanoDrop Technologies, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, USA), respectively. The mean RNA concentration obtained from liver samples was 2469 (± 483) ng/µL, from eye stage embryos it was 299 (± 87) ng/µL and from the hatched larvae stage it was 866 (± 428) ng/µL. Mean RNA Integrity Number (RIN) values were 9.8 (± 0.2) for liver samples, 9.1 (± 0.6) for eye stage embryos and 9.65 (± 0.15) for hatched larvae. Reverse transcription (RT) followed by quantitative real-time PCR (RT-qPCR) was analysed as described (Skjaerven et al., 2011). In short, RT was performed on a GeneAmp PCR 9700 (Applied Biosystems) using the TaqMan®reverse transcriptase kit with oligo(dT) primers (Applied Biosystems). The 96 well cDNA plate included triplicate wells of a six-point dilution curve from 1000 ng to 31.25 ng, duplicate wells of experimental samples at 500 ng (± 5%) and in addition, one well for non-template control and one for non-amplification control. The reverse transcription reaction included 30 µL in total (10 µL mRNA and 20 µL reaction mix) and the cDNA synthesis was performed at 25 °C for 10 min for primer annealing, at 48 °C for 60 min for reverse transcription, and at 95 °C for 5 min for transcriptase denaturation. Real-time PCR amplification and analysis were performed on a LightCycler 480 Real-time PCR system (Roche Applied Science, Basel, Switzerland) with SYBR® Green I Mastermix (Roche Applied Science). Thermal cycling was done for 45 cycles of 10 s at each of 95 °C, 60 °C and 72 °C, followed by melting curve to confirm that only one product was present. The non-template control and non-amplification control included in the cDNA reaction plate were run for each assay.

Gene expression within each sample type was normalized using *ef1α*, *β-actin* and *arp* as reference genes using GeNorm to calculate the mean normalized expression (MNE) of 15 target genes. Primer sequences used to elucidate mRNA expression profiles for reference and target genes are given in table S4.

2.5. Statistical treatment

Statistical calculations comparing the two spawning seasons were performed in GraphPad Prism 8 (GraphPad Software, USA). Differences in weight, length, liver weight, HSI, ovary weight, GSI, vitamin B12 and total folate measures were assessed by unpaired *t*-test to compare the two groups. F test and Shapiro-Wilk normality test was applied to test for homogeneity in variance between the groups. Differences in total vitamin B6 and its derivatives, free amino acids and N-metabolites, lipid classes and folate species measurements were assessed by multiple *t*-tests, using the Holm-Sidak method to correct for multiple comparisons between the two groups. For all tests, differences were accepted as significant at $p < .05$.

3. Results

3.1. Off-season broodstock had higher condition factor, lower liver weight and hepatosomatic index than normal-season broodstock at spawning

We found that the liver weight of the off-season broodstock was significantly lower than the liver weight of normal-season ($p < .045$), which reflected in a significantly lower HSI in the off-season ($p < .003$) compared to normal-season broodstock. Ovary weight and GSI did not differ between the two spawning seasons. We observed no significant differences in body weight between the two spawning seasons. However, the off-season broodstock females were significantly shorter than the normal-season females ($p < .017$), which resulted in a significantly higher condition factor for the off-season broodstock ($p < .0012$). Results are summarized in Fig. 1 and table S5.

3.2. Off-season induced spawning retained total folate, vitamin B6 and several free amino acids in broodstock muscle

Comparing the measures in muscle between the two broodstock spawning groups, we found higher folate ($p = .01$) and vitamin B6 ($p < .01$) levels in the broodstock muscle samples from off-season compared to normal-season spawners (Fig. 2A and table S6). In addition, several free amino acids (hydroxyproline, serine, glycine, L-alanine, B-alanine, histidine) displayed higher levels in off-season spawners (Fig. 2B and table S7) compared to normal-season. The level of glutamine, which is one of the energetically important amino acids, was significantly lower in off-season broodstock muscle.

The lipid classes measured in muscle were unaffected by the spawning seasons (Table S8), except for diacylglycerol that showed a significant ($p < .01$) lower level in off-season group compared to normal-season.

3.3. Off-season spawning displayed higher levels of folate, B-alanine and cholesterol in broodstock liver tissue

Comparing broodstock liver tissue from off-season to normal-season spawners we found a significantly higher level of folate ($p = .003$, microbiology method), whereas vitamin B12 and vitamin B6 were unaffected by the off-season spawning (Fig. 3A and table S9). In addition, we measured total folate and three different folate forms (Folic acid, 5-CH_3-Tetrahydrofolate (THF), 5-CHO-THF) by LC-MSMS to evaluate which forms of folate differs between the two broodstock groups. Specifically, we found a significantly higher level of 5-CHO-THF ($p < .01$) in off-season compared to normal-season (Fig. 3B).

Among the free amino acids, only B-alanine showed a statistical difference in liver tissue. Alanine displayed a significantly ($p < .05$) higher level in off-season compared to the normal-season broodstock (Fig. 3C). Among the measured N-metabolites phosphoethanolamine, citrulline and gamma-Aminobutyric acid (GABA) were also significantly different in liver when comparing two broodstock spawning seasons (Fig. 3C and table S10).

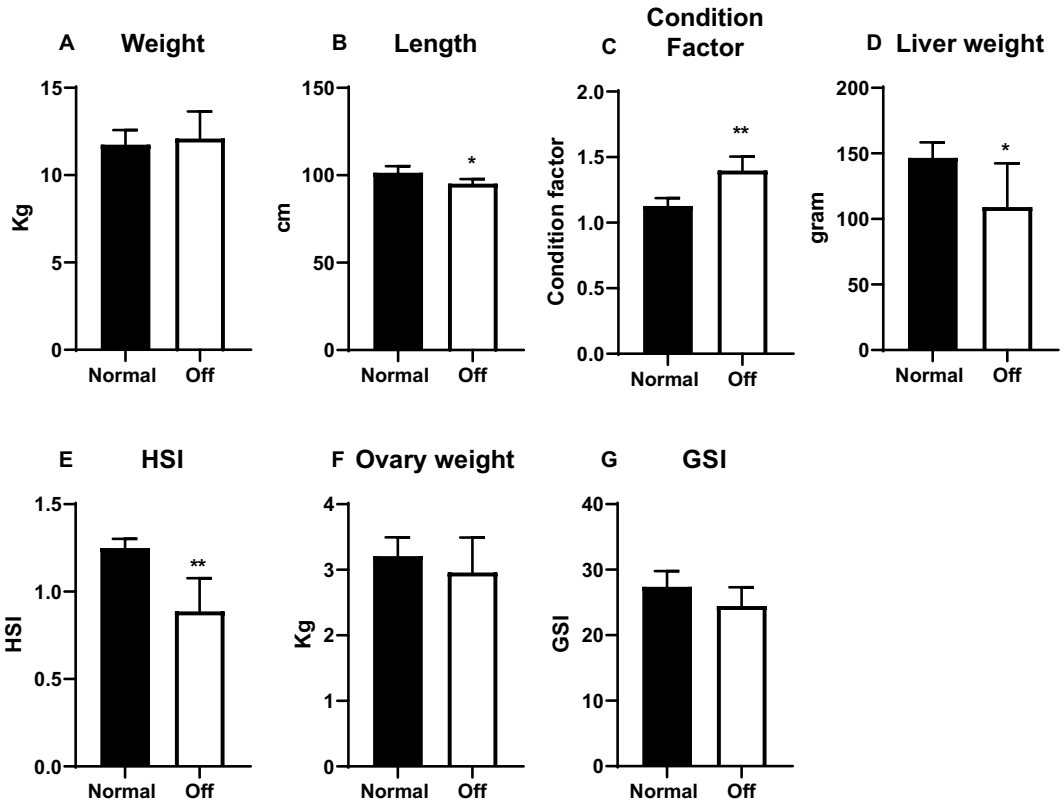

Fig. 1. Normal-season and off-season broodstock biometry. Weight (A), length (B), condition factor (C), liver weight (D), HSI: hepatosomatic index (E), ovary weight (F) and GSI: gonadosomatic index (G). Columns represent mean values (± SD) of five independent broodstock females of Atlantic salmon. Significant differences (see Materials and method section) between spawning groups are marked with asterisks (*$p < .05$, **$p < .01$).

Phosphatidylserine ($p < .05$), cholesterol ($p < .05$), and sphingomyelin ($p < .0001$) showed significantly higher levels in the off-season compared to normal-season broodstock liver, whereas free fatty acids ($p < .05$) were higher in normal-season (Fig. 3D and table S11). No other differences were present in lipid classes between the two spawning groups.

3.4. Off-season spawning exhibited lower levels of vitamin B12, certain amino acids and N-metabolites and all measured lipid classes in newly fertilized eggs

To evaluate if the artificial manipulation of spawning season alters the nutrient status in offspring, we compared newly fertilized eggs. We found that off-season spawning reduced the vitamin B12 level in the embryos compared to normal-season, whereas the active folate form, 5-CH3-THF, was significantly lower (Fig. 4A and B and table S12).

Among the amino acids, glutamic acid and glutamine were higher, whereas aspartic acid and B-alanine were lower in off-season compared to normal-season newly fertilized eggs. Several other N-metabolites were also altered, specifically taurine, phosphoethanolamine and urea were lower, whereas ammonia was higher in off-season compared to normal-season (Fig. 4C and table S13).

All measured lipid classes displayed a lower level in the off-season embryos compared to normal-season embryos, especially for

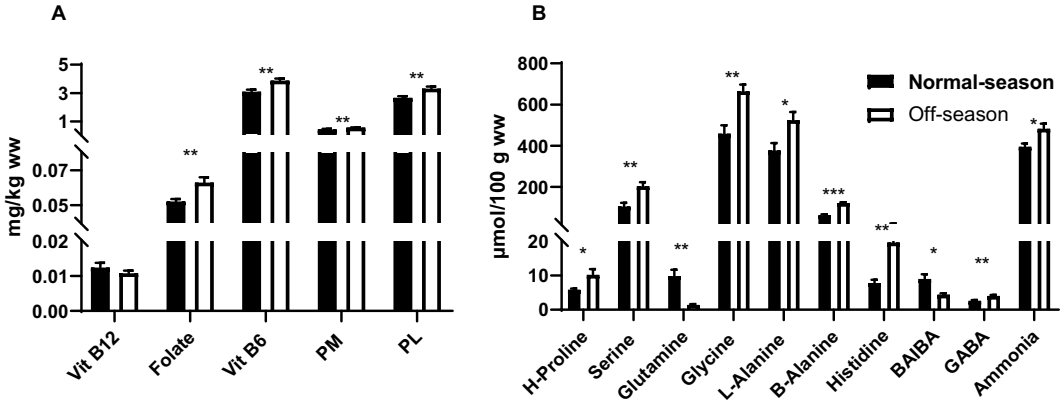

Fig. 2. Normal- and off-season broodstock muscle B-vitamins and FAA. A: Vitamin B12, total folate (microbiological method), total vitamin B6, pyridoxamine (PM) and pyridoxal (PL), and B: significant different free amino acids and N-metabolites. H-Proline: Hydroxy-L-proline, BAIBA: DL-Beta-Aminoisobutyric acid, GABA: Gamma-amino-n-butyric acid. Columns represent mean values (± SD) of five independent broodstock females of Atlantic salmon. Significant differences (see Materials and method section) between spawning groups are marked with asterisks (*p < .05, **p < .01, ***$p < .001$).

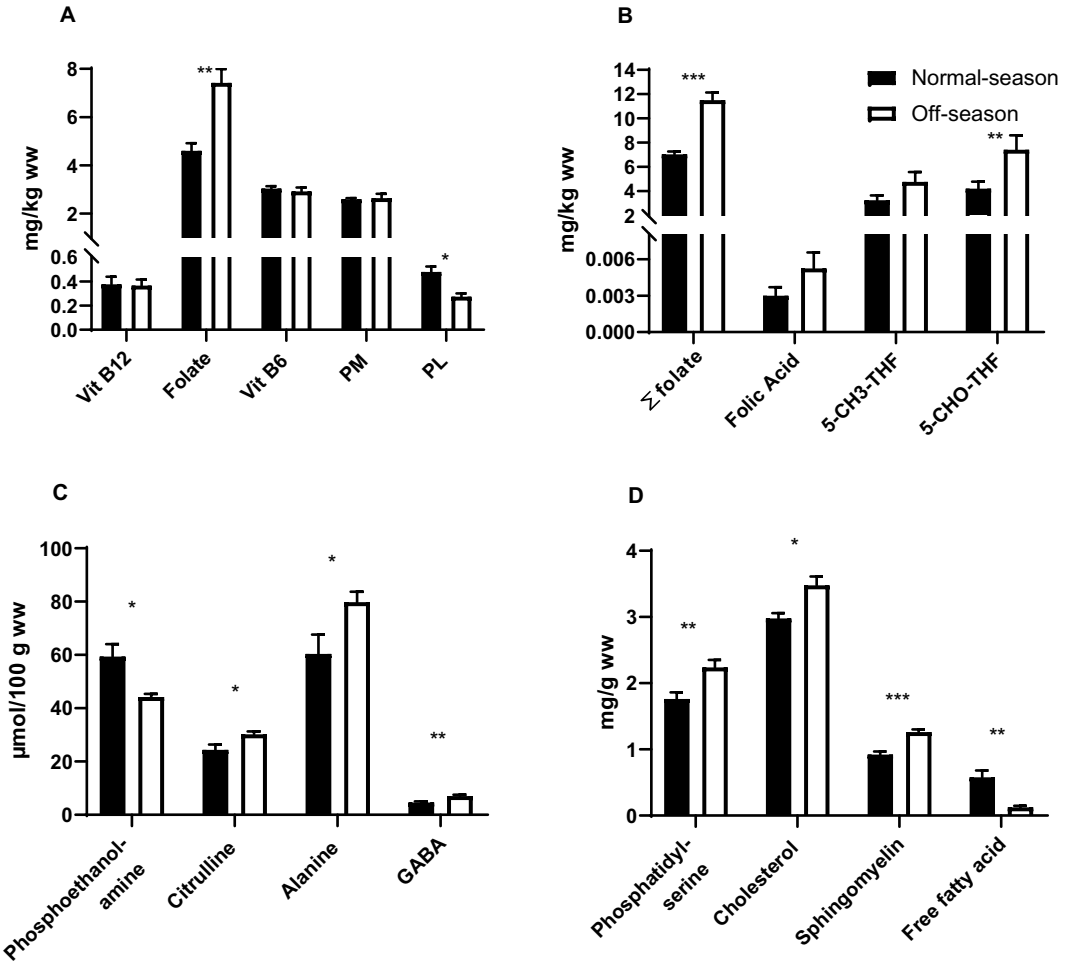

Fig. 3. Normal- and off-season broodstock livers. A: B-vitamins: vitamin B12, folate (microbiological method), total vitamin B6, pyridoxamine (PM) and pyridoxal (PL), B: LC-MSMS total folate, folic acid, 5-CH3-THF and 5-CHO-THF, C: significant different free amino acids and N-metabolites and D: significant different lipid classes. Columns represent mean values (± SD) of five independent broodstock females of Atlantic salmon. Significant differences (see Materials and method section) between spawning groups are marked with asterisks (*p < .05, **p < .01, ***p < .001).

phosphatidylinositol, sphingomyelin and phosphatidylethanolamine, which had less than half the amount in off-season compared to normal-season (Fig. 4D and table S14).

3.5. Eye stage embryos from the off-season broodstock showed changes in free amino acids and vitamin B6 (PM) compared to normal-season broodstock

To investigate if off-season spawning alters the offspring during the sensitive stage of organogenesis, we compared embryos at approximately 360 d° after fertilization (eye stage embryos). Among the B-vitamins measured, only the metabolic form of vitamin B6, pyridoxamine, was significantly higher in off-season compared to normal-season eye stage embryos (table S15).

We measured 34 FAAs and N-metabolites. The results show that 18 of them were significantly different in eye stage embryos. Approximately 78% of these were measured at a higher quantity in off-season embryos compared to normal-season. Among them were the energetically important amino acids, like glutamic acid, glutamine, glycine and B-alanine, as well as the branched chain amino acids, like isoleucine and leucine. The inhibitory neurotransmitter, GABA, was also higher in off-season embryos, whereas ammonia was significantly lower in the off-season embryos (Fig. 5 and table S16). No differences in lipid classes between the two broodstock seasons were present in eye stage embryos, except sphingomyelin, which was higher in off-season embryos (table S17).

3.6. Off-season spawning affected the weight and length in Atlantic salmon offspring at the start feeding stage

To verify if off-season spawning affects the growth of the next generation, we measured total body weights and standard lengths in larvae from both spawning groups (Fig. 6). The larvae from normal-season were 184 mg and 26 mm on average, whereas those from off-season were 154 mg and 23.5 mm on average. We observed a significant ($p < .01$) effect on both total body weight and length between the two spawning seasons. This result is in accordance with the higher egg count in the off-season group compared to normal-season group (table S3).

3.7. Off-season spawning altered the gene expression pattern in both broodstock liver and offspring

We further tested if the off-season spawning alters the mRNA gene expression of 15 selected genes in broodstock livers, in eye stage embryos and in newly hatched larvae. The selected genes are related to 1C metabolism, DNA methylation, growth and fatty acid synthesis and metabolism (table S4).

In the broodstock livers, we found that both methionine synthase (*mtr*) and S-adenosylmethionine synthase 1a (*mat1a*) in the methionine

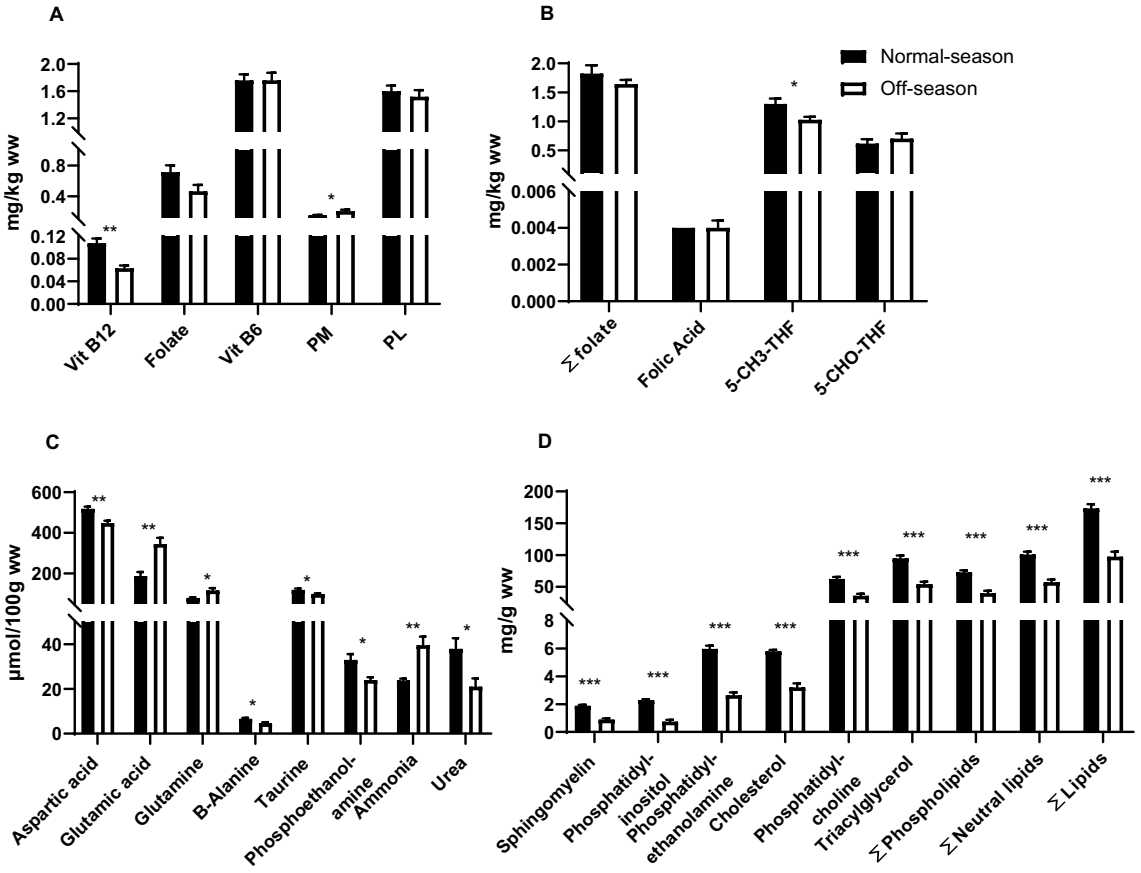

Fig. 4. Newly fertilized Atlantic salmon eggs originating from normal- and off-season broodstock. Biochemical analyses of A: B-vitamins: vitamin B12, folate (microbiological method), total vitamin B6, pyridoxamine (PM) and pyridoxal (PL), B: LC-MS/MS total folate, folic acid, 5-CH_3-THF and 5-CHO-THF, C: significant different free amino acids and N-metabolites, and D: significant different lipid classes. Columns represent mean values (± SD) of five independent broodstock females of Atlantic salmon. Significant differences (see Materials and method section) between spawning groups are marked with asterisks (*p < .05, **p < .01, ***p < .001).

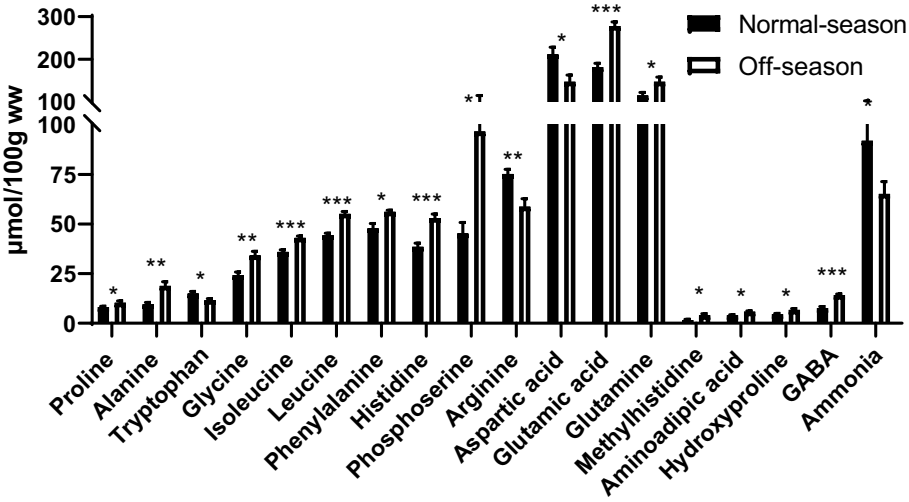

Fig. 5. Significant different free amino acids between normal-season and off-season eye stage Atlantic salmon embryos. Columns represent mean values (± SD) of five independent broodstock females of Atlantic salmon. Significant differences (see Materials and method section) between spawning groups are marked with asterisks (*p < .05, **p < .01, ***p < .001).

cycle of the 1C metabolism were significantly altered due to altered spawning season. In addition, delta-5 fatty acyl desaturase (*fads1*), delta-6 fatty acyl desaturase (*fads2*) and glucose-6-phosphate dehydrogenase (*g6pd*) showed significant lower expression levels, whereas the fatty acid binding protein 1 (*fabp1*) was significantly higher expressed in off-season compared to normal-season broodstock, respectively (Fig. 7a).

For the eye stage embryos, only the insulin-like growth factor 1 receptor 1a (*igfr1a*) showed a significant higher expression in off-season compared to normal-season ($p < .05$). None of the other selected genes were altered (data not shown).

In newly hatched larvae, seven of the selected genes measured showed a significant higher expression in the off-season compared to normal-season larvae. Among them were methylenetetrahydrofolate

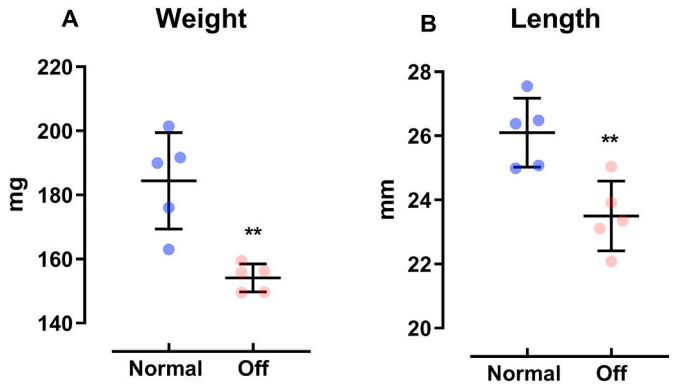

Fig. 6. Larval first feeding stage total body weight (A) and standard length (B) from normal- and off-season broodstock. Columns represent mean values of weight (± SD) of 36 individuals and length of 10 individuals originating from five female Atlantic salmon broodstock from each season. Significant differences (see Materials and method section) between spawning groups are marked with asterisks (**p < .01).

reductase (*mthfr*) and the methionine synthase (*mtr*). DNA methyltransferase 1 (*dnmt1*) and two *de novo* DNA methyltransferases (*dnmt4* and *dnmt3aa*), which methylate selected CpGs in the DNA also showed higher expression levels in the off-season. In addition, *fads2* and insulin-like growth factor 1 (*igf1*) were also significantly higher expressed in off-season compared to normal-season larvae.

4. Discussion

We show here that five months advanced spawning in the Atlantic salmon aquaculture can have long term intergenerational consequences for the offspring. The broodstock micronutrient status altered the allocation of micronutrients during oocyte maturation, which determines the nutrient status of the newly fertilized eggs and changes the metabolism, gene expression and growth during ontogeny. Adjusting light and freshwater temperature for broodstock is one possibility for altering the spawning season, but five months adjustment is only fully possible in the land-based RAS systems where all the environmental factors can be controlled. The off-season broodstock was kept in environmentally controlled rearing systems on land for both the saltwater and freshwater phases to obtain embryos in June compared to the normal-season spawners in November which were kept in open-water net pens prior to freshwater transfer and spawning. The samples analysed here, represent ongoing commercial spawning strategies used for Atlantic salmon fry production.

The main hypothesis of this study was to investigate if out-of-spawning season broodstock and their offspring deviate in micronutrient status when compared to broodstock and offspring from the normal spawning season. Based on our earlier studies, we focused on the micronutrients in the 1C metabolism (vitamin B12, folate, vitamin B6, methionine), other free amino acids and lipid classes in muscle and liver from female Atlantic salmon broodstock and during offspring ontogeny.

We studied selected nutrients in both broodstock and offspring from the two different spawning groups to compare whether seasonal adjustments alter their micronutrient status. Here, we show that both the selected tissue types in broodstock had altered nutrient status which influenced the nutrient composition of the egg. Broodstock fish analysed in this study were fed the same commercial feed and were starved for similar time interval prior to spawning. Earlier studies have pointed towards the broodstock diet and how the altered nutrients in the diet altered the dietary composition in the eggs as reviewed (Izquierdo et al., 2001). Different nutritional composition in eggs is one of the major inputs to alter the long term balance between proliferation *versus* differentiation during development, as suggested for instance for muscle growth in teleost fish (Valente et al., 2013). Here, the off-season eggs were both smaller, had altered nutritional profiles and at first feeding stage the larvae were consequently smaller than the normal-season larvae.

During vitellogenesis, vitellogenin proteins are transferred from broodstock hepatocytes to the growing oocytes as reviewed (Hipólito Fernández-Palacios et al., 2011). During this process, other molecules such as lipids, vitamins, carbohydrates, hormones, and mRNAs are also deposited in the oocytes (Lubzens et al., 2010). Our results, which specifically focus on selected B-vitamins, FAAs and lipid classes, indicate that adjustments of spawning seasons affect both broodstock and embryos. Our results show, in general, that broodstock following the normal spawning season allocated more of the measured micronutrients to newly fertilized eggs than off-season broodstock.

Overall, among the B-vitamins, FAAs and lipid classes measured in broodstock we found more differences in the liver compared to the

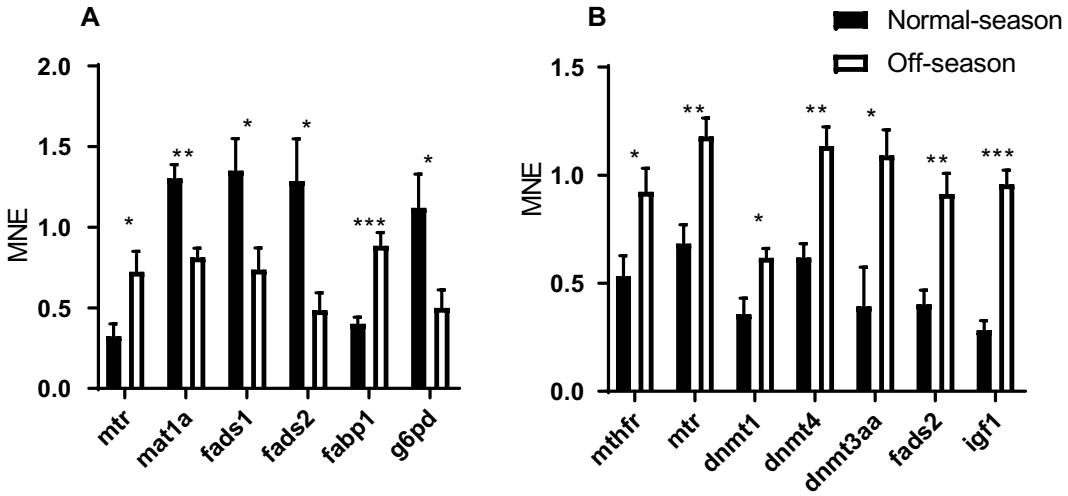

Fig. 7. Off-season spawning significantly changed gene expression in both A: broodstock liver and B: offspring (newly hatched larvae stage). Mean normalized expression (MNE) levels determined by RT-qPCR. Target genes were normalized using *ef1α*, *β-actin* and *arp* as reference genes. All data columns represent the means ± standard deviation (SD) of significantly altered mRNA expression patterns of 5 independent broodstock livers (A) and 5 independent groups of 3 pooled offspring (B). Significant differences (see Materials and method section) between spawning groups are marked with asterisks (*p < .05, **p < .01, ***p < .001). For gene abbreviations, names and functions see table S4.

muscle between the two spawning groups, as liver is the metabolic organ that allocates nutrients to the growing oocytes during vitellogenesis (Lubzens et al., 2010). However, in both tissues we found higher levels of folate, alanine and the neurotransmitter GABA in the off-season broodstock compared to the normal-season broodstock.

Folate is known to be especially important for early embryonic development. Our results show that total folate was significantly higher in off-season than normal-season broodstock liver and muscle, whereas the off-season embryos had lower levels of 5-CH_3-THF than normal-season embryos. Total folate in embryos was not different, but slightly lower levels were found in the off-season group compared to normal-season group. Folate, which is the major determinant of the capacity of the 1C metabolism, has several important functions in tissues with high proliferation rate as during early development. Besides acting as a methyl donor for the methylation of homocysteine, it is important for nucleotide synthesis (thymidine) and serine/glycine conversion (Ducker and Rabinowitz, 2017).

Vitamin B12 was unaffected in both broodstock liver and muscle tissue, but offspring at the newly fertilized egg stage had lower vitamin B12 levels in off-season compared to normal-season. Vitamin B12 acts as a cofactor for re-methylation of homocysteine to methionine and together with the results of 5-CH_3-THF this might indicate a lower capacity in the 1C metabolism during development in the off-season group. However, new studies should measure SAM/SAH ratio at early development to study if the methylation potential is altered due to adjustments of spawning season. SAM/SAH ratio is important for methylation of several biological methylation reactions including epigenetic gene regulation mechanisms like DNA methylation and histone tail methylation (Arslan, 2006; Brosnan et al., 2004; da Silva et al., 2014; Noga and Vance, 2003; Stead et al., 2004; Vance et al., 1997; Watkins et al., 2003). Deficiency in one of the 1C nutrients during embryogenesis has been linked to severe developmental deformities such as neural tube defects and anemia, and it has also been shown to affect the activity of neurotransmitters in the central nervous system. We did not detect any developmental deformities in the two spawning seasons studied here, but interestingly, the inhibitory neurotransmitter GABA was significantly higher in off-season compared to normal-season broodstock in both liver and muscle tissues as well as in eye stage embryos. In addition, several other FAAs and N-metabolites were significantly higher in off-season compared to normal-season in the eye stage embryos. Among them were the glucogenic FAAs like glutamic acid, glutamine and alanine, which all were significantly higher in off-season. Glutamine is an important α-amino acid used in biosynthesis of several other amino acids having implication for growth and health (Andersen et al., 2016; Hou and Wu, 2018; Wu, 2010). Alanine is the second most prevalent amino acid in proteins, accounting for 7.8% of the primary structure of proteins. Alanine is also a non-essential amino acid that must be readily available due to transamination reactions and thus has a close link to several metabolic pathways, such as the glucose-alanine cycle. The glucose-alanine pathway distributes alanine between the liver, blood and target tissue, and in addition incorporates glycolysis, gluconeogenesis and the citric acid cycle and is therefore important for total energy expenditure. It is difficult to conclude the overall metabolic consequence of our results, however, earlier results have suggested that if there is a reduction in growth capacity and to utilise the FAAs to build new proteins then accumulation of free amino acids might be an indicator of reduced growth potential (Espe et al., 2020). Another explanation could be that the free amino acids are used to gain energy through glucogenic FAAs and pyruvate conversion.

Newly fertilized eggs from the off-season group had lower levels than normal-season group in all measured lipid classes. Lipids are the major fuel for the growing embryo, and as such, our results indicate that off-season eye stage embryos had higher levels of free amino acids to compensate for less lipids for energy expenditure. These differences in allocation of lipids and FAAs for energy or muscle accretion should be followed up. Further on, lipids are especially important for brain development and signalling pathways such as the Wnt and Hedgehog signalling pathways (Seli et al., 2014), and here we show that the off-season group had significant lower lipid levels than the normal-season group. One of the lipid classes we measured was cholesterol. Cholesterol is an essential component of all animal membranes and serves as a precursor for the production of, for instance, steroid hormones and bile acids, and furthermore, inhibition of cholesterol biosynthesis is teratogenic and causes brain abnormalities in rats offspring (Seli et al., 2014). Cholesterol is not synthesised in the oocytes, but it originates from *de novo* synthesis in the cumulus cells in the follicle and transported to oocytes *via* gap junctions prior to spawning. Cholesterol displayed a higher level in the liver from off-season broodstock, whereas the newly fertilized eggs received about half the amount of cholesterol compared to the normal-season embryos. Another lipid class of importance for brain development is sphingomyelin (Schneider et al., 2019), which also had less than half the amount in newly fertilized eggs in off-season compared to normal-season. Sphingomyelin is important component of cell membranes but is highly enriched in the membranes of the myelin sheath that surrounds neuronal axons.

To verify if alterations in spawning seasons also affect gene expression, both in broodstock liver and in offspring embryos we measured selected genes related to the 1C metabolism, DNA methylation reactions and fatty acid synthesis. Here, we show that gene expression of these selected genes was significantly affected in both broodstock liver and in newly hatched larvae. Specifically, in the 1C metabolism, we found that the off-season broodstock liver and muscle contained higher level of folate, whereas the methionine synthase (*mtr*) gene was downregulated compared to normal season. Further on, in the offspring we found that the off-season group had lower levels of 5-CH_3-THF and vitamin B12 in the newly fertilized eggs. We could speculate that these differences in micronutrients influence the gene expression response that we observed at the hatched stage. We found an increased gene expression level for *mthfr*, *mtr*, and three of the *dnmt*s. The *mthfr* catalyses the conversion of 5,10-Me-THF to 5-CH_3-THF. The 5-CH_3-THF can be used as substrate for methionine synthase (encoded by the *mtr* gene) and is a vitamin B12 dependent enzyme that catalyzes the final step in the methionine biosynthesis (Wu et al., 2019). Interestingly, three of the DNA methyltransferases were altered in the offspring. Two of them are *de novo* methyltransferases. The function of the *de novo* methyltransferases is not fully understood, but it is suggested that one of their responsibilities is to methylate DNA at unmethylated regions. It would be interesting to investigate whether the response of the *de novo* methyltransferases could possibly be related as a nutritional programming response *via* DNA methylation as suggested (Wu et al., 2019). As both the nutrients and the genes evaluated here, are linked to the methylation potential, further studies should evaluate if these differences in both micronutrients and gene expression changes lead to permanent changes in epigenetic modifications to control the growth and metabolism as an environmentally embedded embryonic plasticity.

Broodstock diets should be formulated to ensure that all essential nutrient requirements are met for the species being cultured (Migaud et al., 2013). Here, we show that even when broodstock are fed a diet known to meet the requirement levels, the manipulation of spawning season in RAS affects the nutrient status of both the broodstock itself, and the subsequent generation. Our results indicate that these differences have long term consequences for growth, seen by the lower lipid levels in newly fertilized eggs as well as higher level of FAAs at the eye stage in off-season compared to normal-season. Earlier results have shown that exposure of suboptimal water temperatures during broodstock maturation reduces the egg quality for trout (Aegerter and Jalabert, 2004), and further that the reproductive cycle is also sensitive to photoperiod (Bromage et al., 2001). We show here that the nutrient allocation into the eggs, as well as the metabolism during embryo development are clearly influenced by the adjusted spawning season. This possibly indicates that normal-season broodstock was fed a diet that meets the requirement levels and further on, implies that the

broodstock diet or feeding regimes given off-season might need a re-evaluation to reach an equivalent growth potential in offspring as observed for the normal-season. This implies a seasonal reassessment of the nutritional influence on the maturation process from broodstock to offspring and how this affects desired phenotypes through nutritional programming.

Declaration of Competing Interest

The authors declare no competing non-financial nor financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2020.110717.

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