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Earlier or delayed seasonal broodstock spawning changes nutritional status and metabolic programming of growth for next-generation Atlantic salmon

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ABSTRACT

Atlantic salmon (*Salmo salar*) breeding companies depend on changing light, temperature and feeding regimes to achieve new generations outside the natural spawning season. However, there have been few conducted trials reported that have studied whether this shift affects important traits. We test whether an induced shift of two months earlier or two months later than normal spawning season affects the nutritional status (folate, methionine, vitamin B12, vitamin B6, free amino acids, N-metabolites and lipids) in broodstock liver and muscle and whether this affects the levels of the same nutrients in the offspring. The results showed significant seasonal differences in the Cahill cycle (glucose-alanine cycle), 1C metabolism and for free amino acids catabolized in the citric acid cycle all which are important for embryonic growth The broodstock nutritional status was reflected in the eggs. Nutritional status of broodstock liver and muscle and newly fertilized eggs showed two general scenarios: Advanced spawning period did not obtain optimal deposition of nutrients in the eggs. Delayed spawning broodstock displayed a metabolic profile which indicated that it had enhanced catabolization of muscle protein which led to accumulation of aminogroups from muscle breakdown to such a degree that these amino groups were increased in the eggs. The total body weight at start-feeding stage revealed best growth for both the normal and late spawning compared to early spawning. We show here that environmental alterations in broodstock husbandry influence the nutrient status of the next generation via nutritional and metabolic programming. This is an important concept which needs more careful awareness as the metabolism compensate and regulate the energy between catabolism and anabolism through the early stages of cell divisions which give rise to changes in permanent traits for the next generation.

1. Introduction

Atlantic salmon (*Salmo salar*) is an anadromous fish species which is born in freshwater and spend most of the life in seawater and thereafter return to freshwater to spawn. For salmon aquaculture, female broodstock are kept in seawater for initial sexual maturation and transferred to freshwater from four to five months prior to spawning. To keep the continuation during production cycle for aquaculture, it is important to have access to new generations of salmon throughout the year. By adjusting the time for transferring sexually mature female from sea cages to land-based freshwater cages, the breeding companies have developed protocols to expand the spawning season. Water temperature, feeding and light regimes are abiotic environmental factors that can either accelerate or prolong the time until spawning. The spawning period can thereby be shifted forward or backward in time by adjusting abiotic factors. Another possibility is a complete land-based approach, with both the initial saltwater gonad maturation and the final freshwater maturation, in recirculating aquaculture system (RAS). This system opens the possibility to copy freshwater-saltwater-timeframe (days in freshwater and days in seawater), transfer period and the abiotic factors from "*normal*" broodstock spawning in late autumn like wild salmon. Recently it has been shown that a shift from normal spawning period by five months in RAS systems affects the nutritional status and gene expression in both the salmon female broodstock and their offspring, resulting in less allocated nutrients into RAS spawned eggs followed by a deprived growth by the time for first feeding (Skjaerven et al., 2020). Here, we continue by investigating the nutritional status in broodstock and offspring when transferring mature females from sea cages to land-

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based freshwater cages but thereafter adjust the abiotic factors to obtain two months earlier and later spawning than normal.

A nutritionally imbalanced diet for fish has shown to alter growth, fecundity, chronic diseases, immune deficiencies, increase susceptibility to infectious diseases and cause changes in the metabolic homeostasis altogether altering the nutritional status in the different tissues and organs (Halver and Hardy, 2002; Kiron, 2012; Lall and Lewis-McCrea, 2007; NRC, 2011; Saito et al., 2020; Skjaerven et al., 2016; Trichet, 2010; Turchini et al., 2009; Waagbø et al., 2020). The nutritional status, in this context, refers to the tissue specific profiles of nutrients and their dynamic interaction with intermediary metabolites. The intermediary metabolic networks are conserved among eukaryotes and describe all reactions concerned with the storage and generation of metabolic energy required for the biosynthesis of molecular compounds and energy storage compounds (Braeckman et al., 2009). The intermediary metabolites change according to the need of the organism and can be short- or long-term responses, and lastly, the detection of changes in the biochemical pathways can be determined by gene expression, enzyme studies or by measuring the metabolites (Braeckman et al., 2009). Panserat et al., 2019 expresses a need to improve the understanding of the intermediary metabolism especially in order to improve aquaculture-related fish nutrition and understand how changes in the metabolites can manifest a long-term response through nutritional or metabolic programming (Panserat et al., 2019). The mechanism behind the programming includes, among others, nutrient sensitive signaling pathways and regulation of gene expression (Efeyan et al., 2015). Even if fish are fed a nutritional balanced diet, changes in abiotic factors will alter the nutritional status in tissues and organs which can direct future programming. The nutritional status is thereby an important biotic factor to investigate across generations. This is especially important during sexual and gonad maturation where a change in the nutritional status of the broodstock can lead to alterations in dietary stimuli for developing embryos altering their zygotic gene transcription (Skjaerven et al., 2016; Skjaerven et al., 2020) with intergenerational epigenetic and phenotypic consequences in mature offspring (Adam et al., 2019; Skjaerven et al., 2018). This phenomenon is called intergenerational programming whereby the nutritional status of the parents affects the next generation (Heard and Martienssen, 2014).

For vertebrate species with external fertilization (birds, reptiles and fish), the zygote is formed on top of an enormous yolk. The egg contains everything for the development of a complete embryo that eventually hatch from the egg with all necessary tissues and organs developed. The genome is important for embryo quality, but the embryo stage is very sensitive to both biotic factors and abiotic factors. For fish, environmental factors like temperature and toxicants (Olsvik et al., 2014; Skjaerven et al., 2014; Skjaerven et al., 2013) and the broodstock nutrient status (Skjaerven et al., 2020) have previously shown to alter the embryonic development and cause long-term programming of the metabolism with the potential to alter acquired phenotypes, like a fatty-liver-like phenotype (Skjaerven et al., 2018). For each cell division during embryonic development, both the inherited intrinsic and external extrinsic cell mechanisms, will promote cell type specific cytokinesis and direct the embryonic cells either to grow, divide or differentiate depending on the abiotic and biotic factors that alter the metabolic signals (Davies et al., 2018). Nutritional substances contained in the yolk are accumulated in female gonads during broodstock oogenesis (Lubzens et al., 2017; Palace and Werner, 2006), and furthermore key nutrients accumulated in the teleost oocytes have previously shown to regulate both cell number and size for *in vitro* mammalian embryo cultures (Bauer et al., 2010; Chen et al., 2018). This indicates that the yolk nutritional status, with the subsequent tissue specific nutritional profiles in the developing embryonic tissues, acts as cell signaling regulators. As such, the broodstock nutritional status in combination with the abiotic factors represents a sensitive period for long term programming (Lucas, 2005; Moody et al., 2017).

For anadromous fish species, like Atalantic salmon, it is not just the above mentioned abiotic factors like temperature, feed and RAS husbandry that can alter embryonic nutrient status. Also the duration of time spent for final freshwater maturation prior to spawning could influence broodstock liver and muscle nutrient status and influence offspring growth and nutritional status at fertilization, eye stage and yolk sac larvae. Broodstock transfer to freshwater is combined with a simultaneous drop in appetite. The reduction in appetite influences the nutritional status in broodstock at the same time when both macro- and micronutrients are accumulating in the maturing oocytes. We sampled broodstock and their embryos and investigated key nutrients and intermediary metabolites. Lipids are the major energy reserve and membrane building blocks for embryo development (Lubzens et al., 2017), although they also have specific roles, like sphingomyelin, which is vital for the development of the central nervous system (Schneider et al., 2019). Folates and other 1C nutrients (methionine, vitamin B6, vitamin B12) and metabolites (S-adenosylmethionine (SAM)/ S-adenosylhomocysteine (SAH)) are fundamental for neural tube closure and cell proliferation as well as biological methylation reactions, gene regulation, thymidine synthesis and redox regulation (Gut and Verdin, 2013; Xu and Sinclair, 2015). Amino acids are the building blocks of proteins, and embryonic development comprise a period of high biological protein synthesis for cell differentiation and organogenesis (Finn and Fyhn, 2010; Wu, 2010).

The present study revealed the nutritional basis of seasonal spawning time changes from broodstock to offspring. It emphasizes the interplay between the abiotic environmental factors and nutritional status which together control the intermediary metabolism regulating growth and robustness from broodstock to next generation. Especially, we point to the fine-tuning of the free amino acids and associated N-metabolites levels in broodstock liver and muscle as indicators of offspring yolk content. We compared three commercially availible spawning periods: early, normal and late. Our in-depth studies indicate a need for adjusting the broodstock feed to comply with the nutritional requirements when changing abiotic factors. The nutritional programming concept studied here also shows us how fragile and sensitive organisms are to a changing climate and the study illustrates possible intergenerationally consequences for aquatic vertebrates with the ongoing warming of global ocean temperatures (Masson-Delmotte et al., 2021).

2. Material and methods

2.1. Ethical considerations

The ARRIVE guidelines were applied for the design and reporting of the experiment. The broodstock and embryos sampled and analysed in this experiment were from the commercial production facility of Atlantic salmon at AquaGen's breeding station at Kyrksæterøra, Norway. The conditions and protocols for rearing and sampling used in this experiment were identical to the protocols used the in commercial production, and thereby partly commercially protected confidential information. Sampling procedures of broodstock, embryos and larvae were performed using anesthetics according to the supplier's instructions, and in accordance with the Norwegian and European legislation related to animal research. Formal approval of the experiment by the Norwegian Animal Research Authority (NARA) is not required because the experimental conditions are practices undertaken for the purpose of recognized commercial animal husbandry which are excepted from the European convention on the protection of animals used for scientific purposes (2010/63/EU), cf. article 5d. and 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".

2.2. Description of study design and analysed samples

In this study we compared the commercially produced earlier or delayed spawning with the normal spawning period, with emphasis on both broodstock and offspring. In broodstock we measured the nutritional profile in both muscle and liver. For the offspring we analysed the nutritional status of whole newly fertilized eggs and eye stage eggs as well as the larval growth at the start feeding stage (Fig. 1A and B).

All female broodstock were from the 15th generation of domesticated Atlantic salmon at AquaGen's breeding station. The broodstock originated from three separate open, sea-based net pens which held 5000 females each. These fish served as the common supply of broodstock prior to freshwater transfer. At sea, the broodstock were under ambient photoperiod and temperatures to let the broodfish mature naturally in seawater. All females were fed to satiation with broodstock feed (EWOS Breed 3500) from early June until transfer to freshwater in the middle of April for Early group and until start of August for the Normal and Late group (Fig. 1A). Artificial photoperiod and temperature were applied to advance (Early season, spawning in September) or delay (Late season, spawning in January) maturation with two months compared to natural spawning (Normal season, spawning in November). The photoperiod and temperature used for Early season broodstock were according to Naeve et al., 2018 (Naeve et al., 2018). For Normal season the broodstock were kept at 6 °C and 8 h day-light after transferring to freshwater tanks landing in August. For Late season the broodstock were landed in freshwater at the same time as Normal group, but temperature and photoperiod to post-pone maturation is commercial confidential information in the property of AquaGen AS. The fish received no feeding after landing to fresh water. The maturation process in freshwater lasted for 163 days for Early season spawners, 120 days for Normal spawners and 166 days for Late spawners before stripping and fertilization to obtain the next generation (Fig. 1B, Table A1). One female from five separate freshwater tanks (tank size: 60 m^3, fish density: 70 kg/m^3, approximately 260 broodstock per tank) from each of the three different commercially available spawning groups were sacrificed using Benzoak (200 mg/L, ACD Pharmaceuticals AS) followed by spinal transection. Each broodstock ($N = 5$ individual fish from each spawning season) was measured for weight, length, liver weight, and ovary weight. The condition factor (K) = 100 x (weight/length^3), hepatosomatic index (HSI) = 100 x (liver weight/body weight) and gonadosomatic index (GSI) = 100 x (ovary weight/body weight) were estimated for each female and compared between groups. Liver and muscle samples were flash frozen

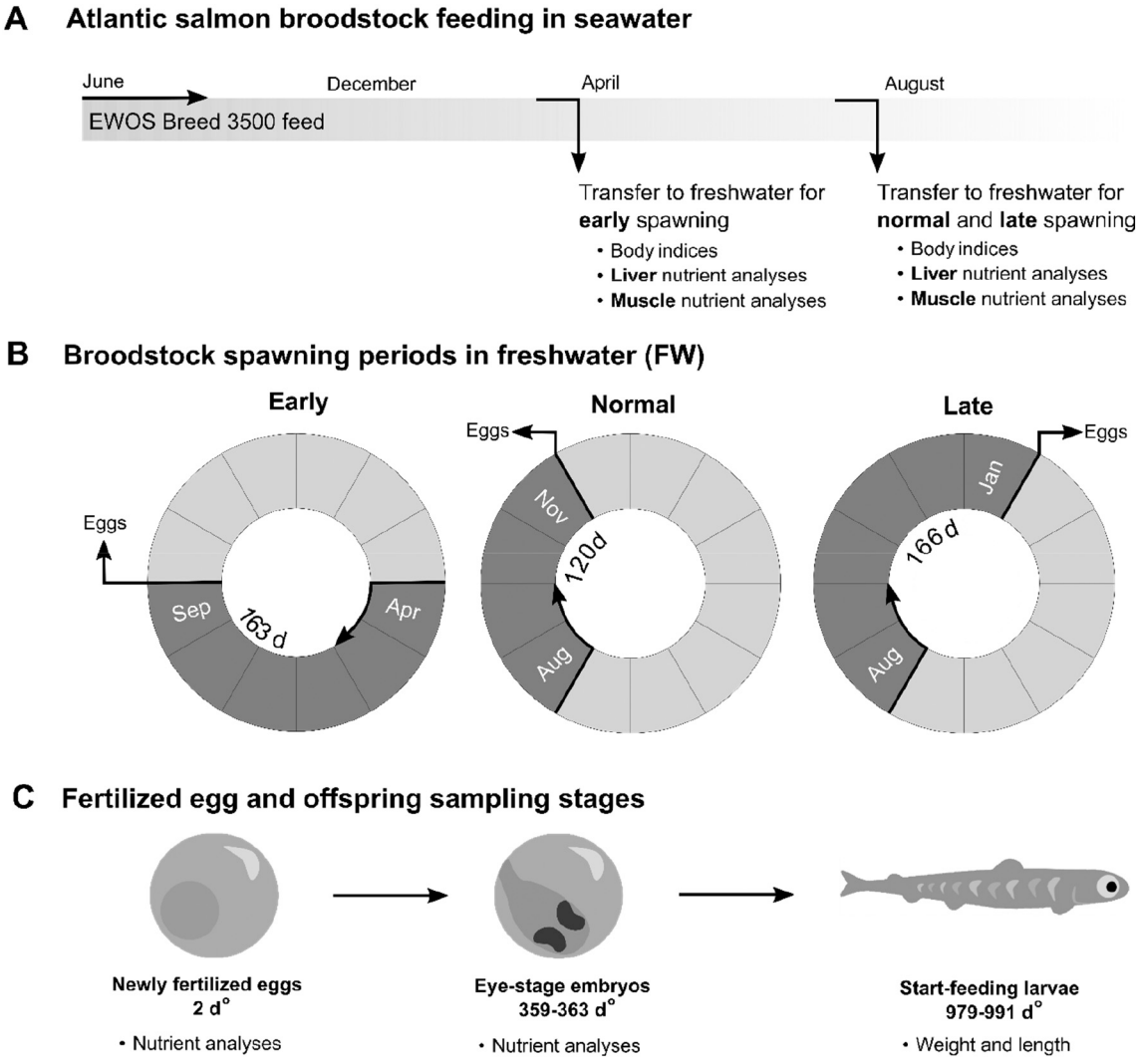

Fig. 1. Overview of the study and the samples analysed. A. Atlantic salmon feeding commercial diet in seawater prior to freshwater transfer in April for Early and August for Normal and Late spawning groups. Body indices, liver and muscle nutrient analyses were taken after spawning from five independent mature broodstock fish from each seasonal group. B. Broodstock spawning in September for Early, November for Normal, and January for Late spawning group. C. Fertilized egg and offspring sampling stages. Nutrient analyses were performed on pooled samples collected from five independent batches of newly fertilized eggs and eye-stage embryo from the five separate broodstocks from each spawning group. Weight and length were measured on start-feeding larvae.

in liquid N_2 for analysis of folate, vitamin B12, vitamin B6, free amino acids (FAA) and N-metabolites, and lipid classes.

Oocytes from all 15 females were fertilized with cryopreserved sperm originating from two males. After fertilization the embryos (N = 5) from each female were separated in three incubation tanks and 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 eyed stage embryos (359–363 d°, Table A2) were flash frozen in liquid N_2 for analysis of folate, vitamin B12, vitamin B6, FAA and N-metabolites and lipid classes (Fig. 1B, C). At the end of the endogenous feeding phase (start feeding larvae 979–991 d°), 36 larvae (12 from each of the three incubation tanks) originating from each broodstock (N = 5) were randomly selected and euthanized with an overdose of buffered tricane methanesulfonate (MS222, Pharmaq, Norway) and measured for weight and length.

2.3. Chemical analyses

All chemical analyses were measured as previously described (Skjaerven et al., 2020). In short, 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. In addition, liver and newly fertilized eggs were analysed to measure the different folate vitamers by LC-MS/MS. Samples were extracted in buffer containing folate stabilisers, and C-13 labeled folic acid, 5-CH3–THF (N^5-Methyl-tetrahydrofolate, most reduced form) and 5-CHO-THF (N^5-Formiminotetrahydrofolate, most oxidized form) were analysed as internal standards. Tri-enzymatic digestion was performed using alpha-amylase, protease and rat plasma conjugase, the latter to de-conjugate the poly-glutamate forms of folates to subsequent monoglutamate forms. The clear extract was subjected to a weak anion exchange by solid phase extraction and 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 B6 (pyridoxine, pyridoxal and pyridoxamine) were measured by ultra-performance liquid chromatography (UPLC) method as described (Albrektsen et al., 1994). Free amino acids were analysed in deproteinized tissues using the Biochrome Analyzer and post column ninhydrin reaction as described (Espe et al., 2006). Lipid class specification were analysed after separation on silica HPTLC-plates as described (Bell et al., 1993; Liaset et al., 2003).

2.4. Statistical treatment

Statistical calculations comparing the three 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 Ordinary one-way ANOVA with Tukey's multiple comparisons test using the mean of each group including Brown-Forsythe test to test for homogeneity in variance. The nonparametric Kruskall-Wallis test was used when data did not fulfil the ANOVA assumptions and indicated in descriptions of the tables given in the appendix. For all tests, differences were accepted as significant at $p < 0.05$.

3. Results

3.1. Broodstock weight, length and organ indices

To compare female broodstock we evaluated the biometry of the three different spawning seasons (Fig. 2A-C). We calculated the mean total body weight and length, and Fulton's condition factor (K) at

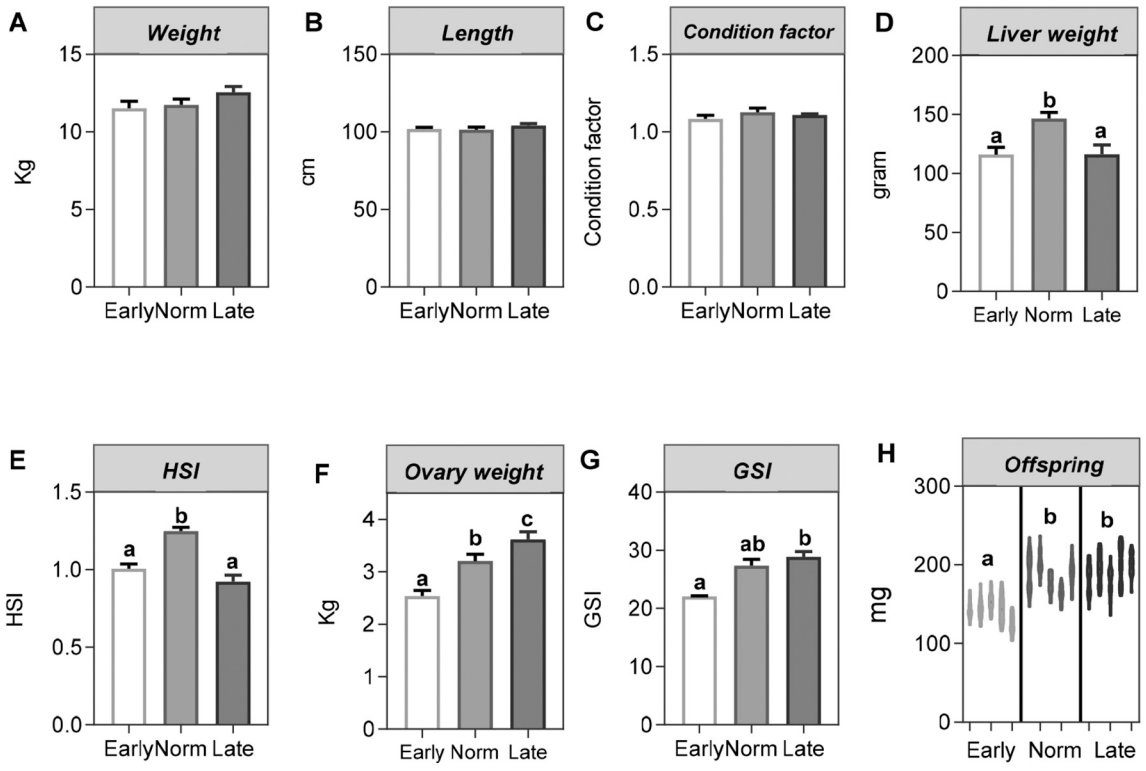

Fig. 2. Early, Normal and Late season female Atlantic salmon broodstock and offspring biometry. A. weight, B. length, C. condition factor, D. liver weight, E. HSI: hepatosomatic index, F. ovary weight, G. GSI: gonadosomatic index, and H. offspring weight. A-G: Columns represent mean values (± Standard Deviation (SD)) of five independent broodstock females. H: Mean weight (mg) of 36 first feeding stage-larvae offspring from each broodstock. Significant differences (see Materials and method section) between spawning groups are denoted by letters. For exact *p*-values see Table A2.

spawning and found no differences between the three broodstock groups. We measured organ weight of liver and gonad, calculated the hepatosomatic index (HSI) to give a predictor of the energy reserves and considered gonadosomatic index (GSI) to predict ovary maturation, fecundity in relation to total body mass, and finally we measured offspring weight at first feeding stage to evaluate if the growth of next generation was influenced by the changes in broodstock spawning time (Fig. 2D-H). Liver weight and HSI were significantly higher in Normal spawning season compared to both Early and Late spawning season. Ovary mean weight was increasing significantly from Early to Normal season while the Late season broodstock group revealed the highest ovary weight. GSI indicated significant increasing ovary maturation throughout the spawning seasons. Both the ovary weight and the GSI results suggest that a longer spawning season results in an increase in the ovary volume, maturation and fecundity. Offspring larvae weight for Normal versus Late season were on average 184 and 192 mg, respectively whereas the Early season larvae were significantly lighter ($p < 0.0001$) with an average weight of 142 mg (Fig. 2H). This result is in accordance with the higher egg count (egg/l) in the Early season compared to Normal and Late season (Table A1). Biometry results are summarized in Fig. 2 and Table A2.

3.2. Nutritional profile of broodstock skeletal muscle

To evaluate the effect on nutritional profile due to alternations in spawning season we measured selected B-vitamins, free amino acids, N-metabolites and lipids in skeletal muscle at final maturation (Table A3, A4 and A5). We observed no significant differences between the micronutrients contributing to the 1C metabolism like folate, vitamin B12, vitamin B6, serine nor methionine when comparing the three spawning seasons (Table A3). However, ten free amino acids varied significantly in the muscle tissue between the three spawning seasons, suggesting differences in skeletal muscle protein degradation due to freshwater fasting (Fig. 3, Table A4).

Increased levels of both the glucogenic and ketogenic amino acids indicate that amino acids are degraded as energy source in Late season broodstock. Muscle protein free amino acids are catabolized via transamination. The amino group is transferred to α-ketoglutarate forming α-keto acid and glutamate. Glutamate channels the amino groups either into biosynthetic reactions, or in case of hunger, into a final sequence of reactions by which nitrogenous waste products are eliminated via alanine (Fig. 4A). Alanine (not significant different in muscle tissue) and glutamine (highly significantly altered between seasons) acts as a shuttle

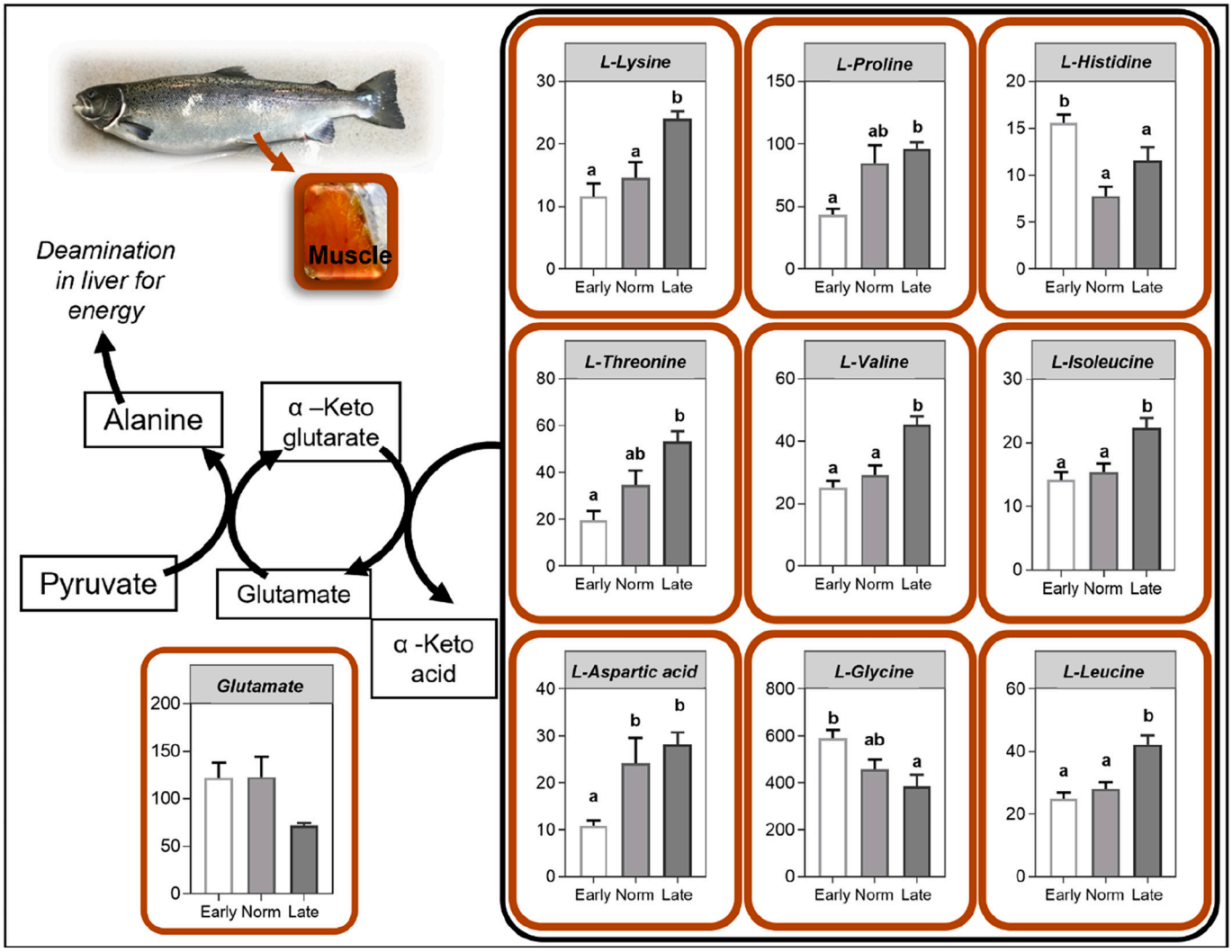

Fig. 3. Free amino acids (μmol/100 g ww) reveal significant differences in broodstock muscle due to changing Early, Normal and Late spawning season. Late season broodstock catabolize muscle protein via transamination reactions. The amino group is transferred to α-ketoglutarate forming α-keto acid and glutamate. The glutamate channels amino groups either into biosynthetic reactions, or in case of hunger, into a final sequence of reactions by which nitrogenous waste products are eliminated via alanine and to produce glucose in the liver (Fig. 3). Columns represent mean values (±SD) of five independent broodstock females. Significant differences (see Materials and method section) between spawning groups are denoted by letters. For exact *p*-values see Table A4.

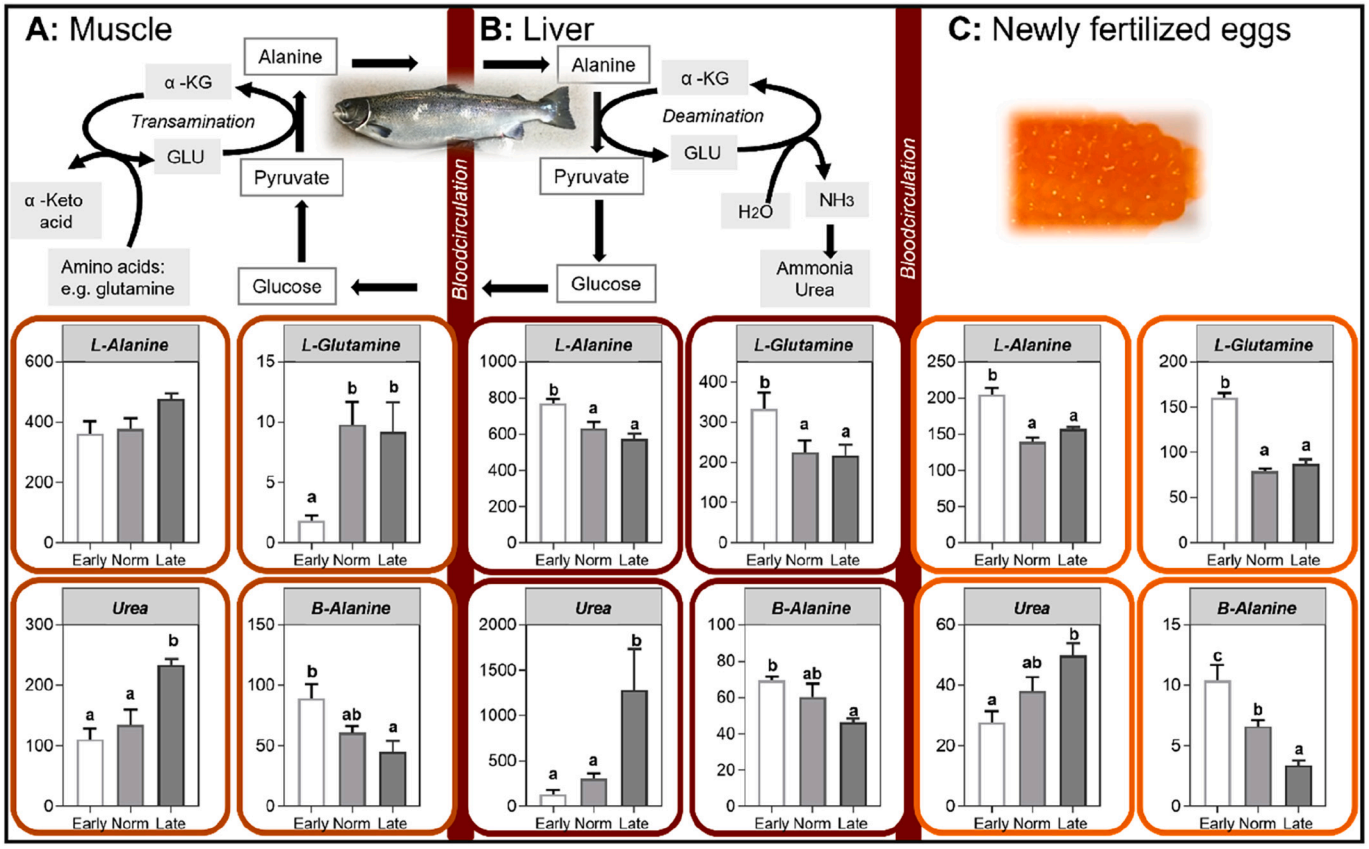

Fig. 4. Metabolites (μmol/100 g ww) in the Cahill cycle (glucose-alanine cycle) reveal significant differences due to changing spawning season. A: Muscle transamination reactions show significant differences of catabolism of protein to free amino acids (see also Fig. 2) between spawning seasons (including B-alanine). B: Significant differences in liver deamination to provide pyruvate and disposing the ammonium/urea as well as providing glucose which is then transported back to the muscle via the blood circulation to provide energy. C: Free amino acids and urea levels measured in offspring at newly fertilized egg stage. Both the Late, and to a lesser extent also Normal season, broodstock catabolize muscle protein via transamination reactions, and depletes the free amino acids from the liver to gain energy, which leads to significant differences in the deposition of nutrients between the Early, Normal and Late spawning season at the newly fertilized egg stage. Columns represent mean values (±SD) of five independent broodstock females (A and B) and their offspring (C). Significant differences (see Materials and method section) between spawning groups are denoted by letters. For exact p-values see Table A4, A7 and A9.

to provide the hepatocytes with pyruvate for gluconeogenesis (Fig. 4A and B).

The effect of fasting is more pronounced in Late season compared to Normal season, while Early season is least affected to the fasting. Aspartic acid (Fig. 3) and glutamine (Fig. 4A) had higher levels in both Normal and Late spawning season compared to Early season, whereas threonine and proline had significantly higher levels in Late season compared to Early season, whereas Normal group where intermediate to both (Fig. 3). Lysine, together with the three branched chain amino acids, valine, isoleucine, and leucine had significantly higher levels in Late season compared to Early and Normal season whereas histidine and glycine revealed the highest level in Early season compared to Normal and Late season or with Normal season intermediate to both, respectively (Fig. 3).

Among the N-metabolites, we detected significantly higher urea (Fig. 4A) and alfa-amino-n-butyric acid (AABA, Table A4) in Late season compared to Early and Normal seasons. Both DL-Beta-Aminoisobutyric acid (BAIBA, Table A4), which is a product of valine and thymine catabolism in skeletal muscle, and B-alanine (Fig. 4A), which is a part of the dipeptides carnosine and anserine, not incorporated into proteins revealed significantly highest levels in Early and lowest in Late season with Normal season intermediate to both. The concentration of other N-metabolites, like ethanolamine (ETA), NH_4 and anserine, was also significantly affected due to spawning season in broodstock muscle.

Lipid analyzes of muscle tissue are relevant indicators of the energy state and the results show that Early season had significantly ($p < 0.001$) higher levels of total lipids (mean 257 mg/g), while Normal season (mean 106,9 mg/g) and Late season (mean 49,2 mg/g) revealed lower levels (Fig. 5A). Confined by the total lipids, the analyses show that it is mostly triacylglycerol, which is the main energy depot, which decreases in Normal and Late spawning seasons compared to the Early season (Table A5). As a contrast, the signaling molecule for membrane trafficking, phosphatidylinositol, increased highly significant in both the Normal and Late season compared to Early season ($p < 0.02$).

3.3. Nutritional profile of broodstock liver

In broodstock liver, we observed significant changes in both the micronutrients in the 1C metabolism (Fig. 6, Table A6) as well as significant changes in the concentration of free amino acids and N-metabolites (Figs. 6 and 7, Table A6 and A7 (for alanine and glutamine, Fig. 4B). In detail, the results show that the Late spawning broodstock had significantly higher levels of total folates compared to Early season, with Normal season showing intermediate levels (Fig. 6A). In addition, the different folate species, 5-CHO-THF and 5-CH3-THF, varied significantly, indicating alterations in the folate cycle between broodstock spawning seasons (Table A6). More specifically, measurements reveal significantly highest level of the oxidized form 5-CHO-THF in Normal season livers compared to Early season, with Late season showing intermediate levels. The reduced 5-CH3-THF show the significantly highest levels in Late season livers compared to the two other seasons. We observed no difference in vitamin B12 between the liver samples

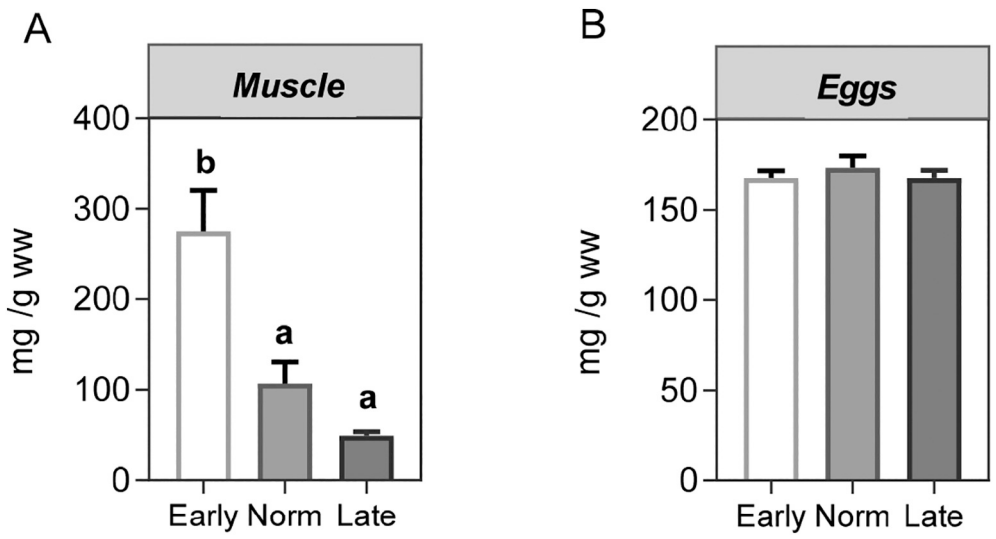

Fig. 5. Early, Normal and Late spawning seasons sum of lipids (mg/g wet weight) in broodstock muscle (A) and offspring at newly fertilized egg stage (B). Columns represent mean values (±SD) of five independent broodstock females. Significant differences (see Materials and method section) between spawning groups are denoted by letters. For exact p-values see Table A5 and A10.

from the three spawning seasons, however vitamin B6 and cystathionine had significant higher level in Late season compared to Early and Normal season which was opposite to the levels measured for free methionine in the liver (Fig. 5B and C). For vitamin B6, pyridoxal phosphate function as an intermediate carrier of amino groups, and we observed significantly higher levels of the pyridoxamine phosphate (PM) form which can donate its amino group in livers from Late season broodstock (Table A6).

We observed that the measured concentration of all free amino acids in broodstock liver were significantly affected by the spawning seasons, except from threonine (Fig. 4B: alanine, glutamine, Fig. 6A: serine, Fig. 6B: methionine, Fig. 6C: glycine and glutamate, Fig. 7: other significantly affected free amino acids). The results show that Early season had less effects of fasting as glutamate, glutamine, and alanine had significantly higher levels in Early season compared to both Normal and Late seasons. Histidine revealed the same trend, but neither of the Early nor Late seasons were significant different from the Normal season (Fig. 7). Furthermore, totally 13 free amino acids revealed a significant lower level in Late season compared to the two other spawning seasons, indicating less pronounced effect of fasting in Normal season compared to Late season. These free amino acids were serine (Fig. 6A), methionine (Fig. 6B), glycine (Fig. 6C), lysine, proline, isoleucine, valine, arginine, aspartic acid, tryptophan, leucine (Fig. 7), tyrosine, phenylalanine (Table A7).

As observed in the nutrient analyses of broodstock muscle, we again detected significantly higher urea and alfa-amino-n-butyric acid (AABA) in livers originating from Late season broodstock compared to Early and Normal seasons. AABA, which also has the name homoalanine, is biosynthesised by transamination of oxobutyrate which is a metabolite utilized in isoleucine biosynthesis. In addition, phosphoserine was significantly higher in Late season compared to the Early and Normal season, whereas the inhibitory neurotransmitter gamma-aminobutyrate and phosphoethanolamine showed decreased levels from Early, to Normal to Late season (results are summarized in Table A7).

3.4. Broodstock season changes the nutritional level in newly fertilized eggs

To evaluate if the nutritional level of folate, vitamin B12, vitamin B6, free amino acids, N-metabolites and lipid in the next generation eggs was influenced by changes in broodstock spawning season, we compared newly fertilized eggs originating from the respective broodstock spawning group (Fig. 4, Fig. 6 and Table A8, A9 and A10).

The metabolites in the 1C metabolism are especially important for embryonic cell differentiation and proliferation, and our analyses show significant differences in spawning seasons in all the micronutrients involved in the 1C metabolism (Fig. 6). More specifically, the sum of all folate species measured was significantly higher in Normal season compared to Early season, while the Late season was intermediate to both. Among the measured folate species, the oxidized 5-CHO-THF was significantly lower in the eggs originating from Early spawning season compared to both Late and Normal season, while for 5-CH3-THF and folic acid our results show the same trend however not significant (Table A8). The Late season eggs had the highest concentration of vitamin B12 (Fig. 6A). Both Late and Normal seasons revealed significant higher concentration of vitamin B6 active form pyridoxal phosphate, which can accept an amino group (Table A8). Moreover, we observed that the Early season eggs had significantly highest level of the vitamin B6 form pyridoxamine which can donate an amino group (Table A8), in addition to higher levels of methionine (Fig. 6B), and the SAM/SAH ratio (Fig. 6B).

16 out of 19 measured amino acids were significantly altered at the newly fertilized egg stage due to spawning season, except from serine (Fig. 6A), aspartic acid, and asparagine (Table A9). For 13 of these, the results reveled that the Early season eggs had higher levels compared to Normal and Late season as presented using alanine (Fig. 4C) and glutamine (Fig. 8) as one example. Two FAA deviated slightly from this pattern however Early season still had the significantly highest level for both methionine (only significant between Early and Late season) (Fig. 6B) and arginine (only significant between Early and Normal season) (Table A9). Several other N-metabolites were also altered in offspring due to spawning season, specifically cystine, phosphoserine and AABA (Table A9) which were significantly higher in Early season whereas B-alanine was significantly lower in both Early and Late compared to Normal season (Fig. 4C). The amount of urea measured increased significantly from Early to Late season and with Normal season in between the two values (Fig. 4C).

The results show that the overall sum of lipids was not affected when comparing the three spawning seasons at the newly fertilized egg stage (Fig. 5B). However, for Early season compared to the two other groups (Table A10), we detected significantly lower levels of sphingomyelin, which is important for brain development. In addition, phosphatidylinositol, which is a phospholipid in cell membranes and important for cell signaling and membrane trafficking increased highly significant in

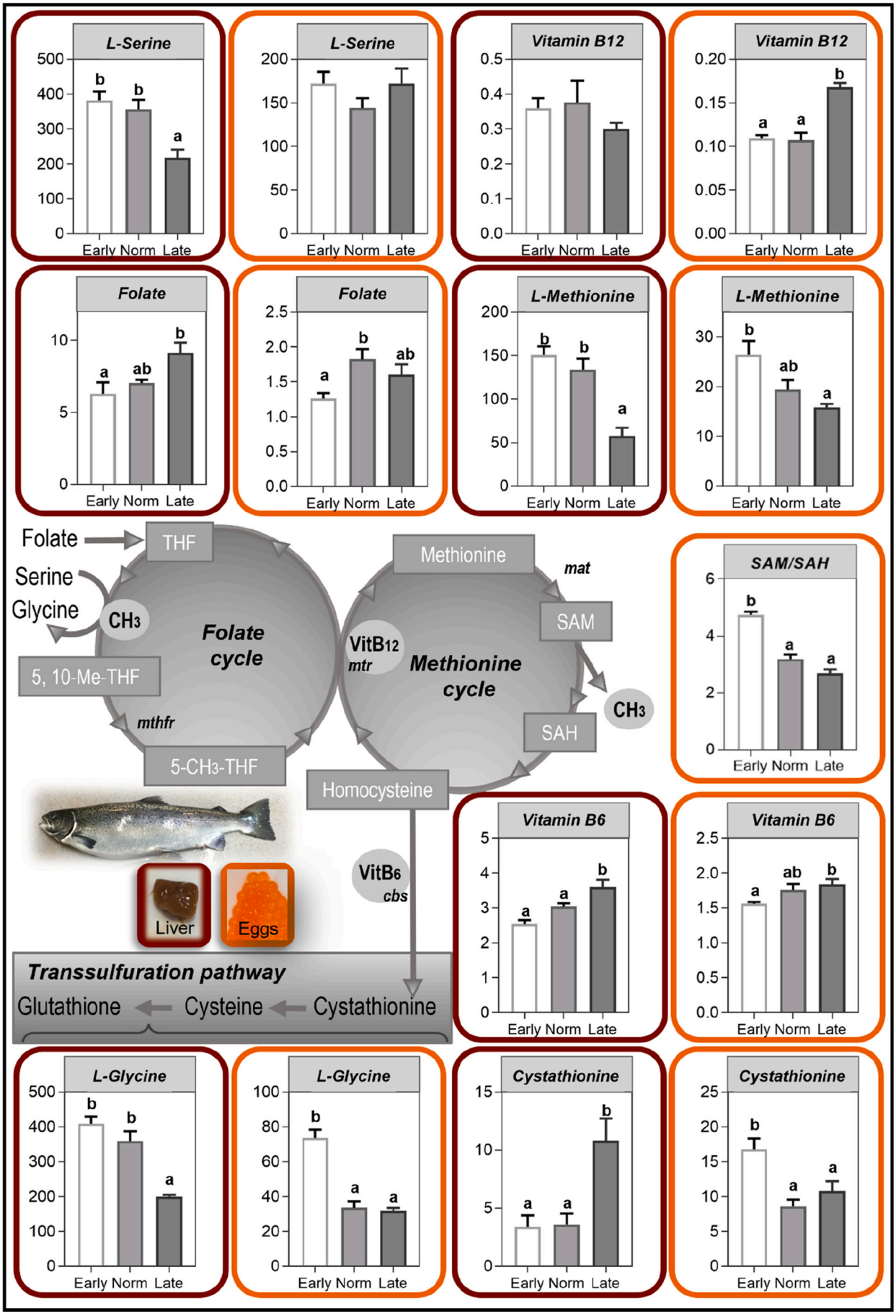

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Fig. 6. Free amino acids and N-metabolites (μmol/100 g ww), B-vitamins (mg/kg) and SAM/SAH (nmol/g) in the 1C metabolism reveal significant differences due to changing in spawning season. Both broodstock liver (dark red boxes) and newly fertilized eggs (orange boxes) reveal significant differences between the Early (white bars), Normal (light grey bars) and Late (dark grey bars) spawning season in the 1C metabolism transferring CH_3 (1C) moieties from serine or glycine through a series of reactions in the folate cycle (A) through the methionine cycle (B) to S-adenosylmethionine which transmits the methyl group to biological methylation reactions. One important route for SAH re-methylation to methionine depends on both folate and the enzymatic cofactor vitamin B12. In case of limitation in re-methylation capacity, then homocysteine can be shunted to cystathionine though the transsulfuration pathway (C) to increase the redox capacity by synthesizing glutathione through a series of reactions using both glutamic acid, glycine and the enzymatic cofactor vitamin B6. * Cystathionine results from eye-stage embryos (not detected in newly fertilized eggs). Columns represent mean values (±SD) of five independent broodstock females and their offspring. Significant differences (see Materials and method section) between spawning groups are denoted by letters. For exact p-values see Table A6, A7, A8 and A9. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

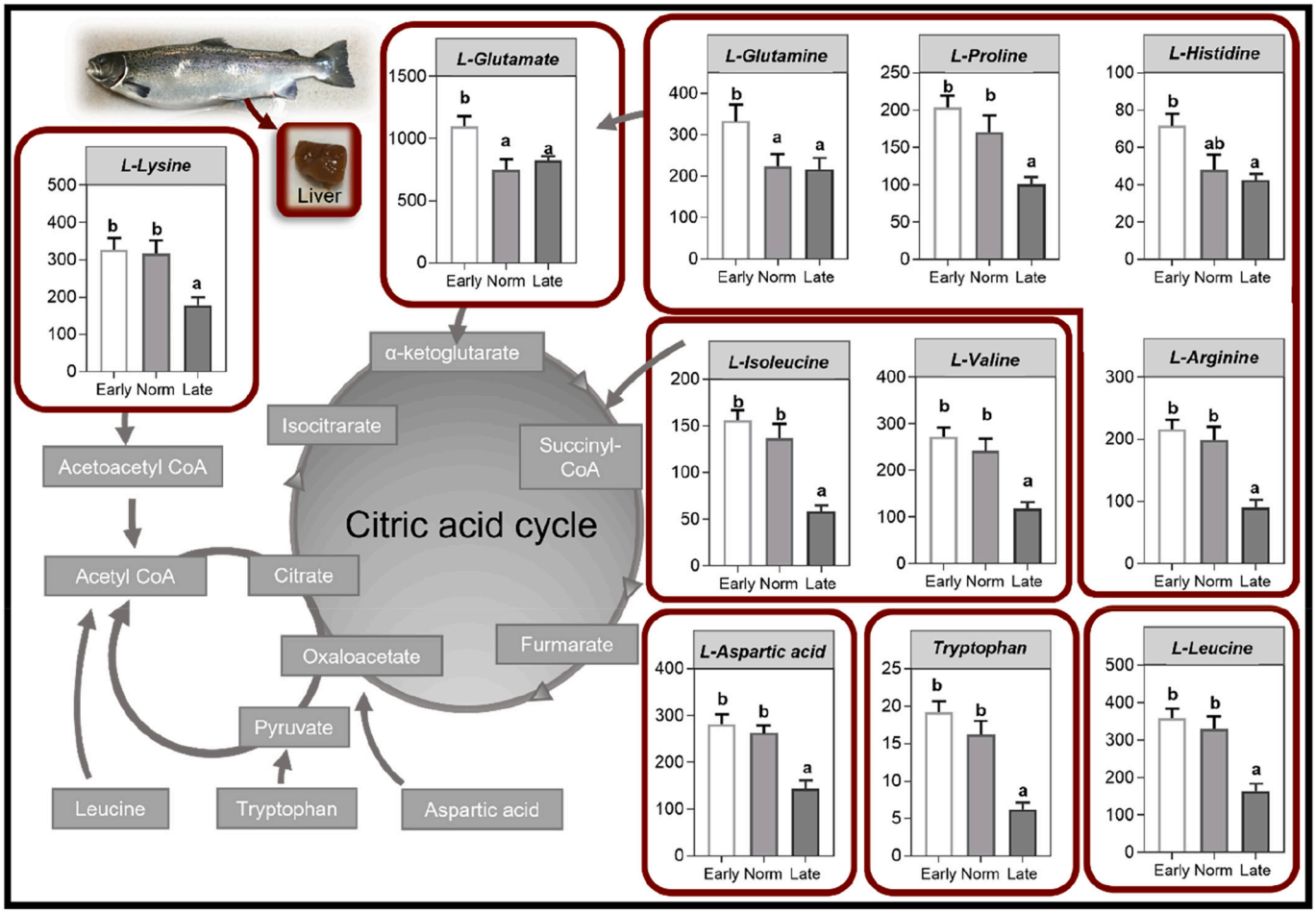

Fig. 7. Free amino acids (μmol/100 g ww) reveal significant differences in broodstock liver due to changing Early, Normal and Late spawning season. Late, and to a less degree Normal season broodstock catabolize muscle protein to free amino acids which are utilized in the citric acid cycle, acetyl CoA, pyruvate, or transaminated and converted to alanine (see Fig. 3). Note that the specific amino acids can have different routes and fates into catabolism and not all of the routes are shown in the figure above. Broodstock liver free amino acids not incorporated in this figure: alanine (Fig. 3B), serine (Fig. 5A), methionine (Fig. 5B), glycine (Fig. 5C), tyrosine, phenylalanine (Table A7). Columns represent mean values (±SD) of five independent broodstock females. Significant differences (see Materials and method section) between spawning groups are denoted by letters. For exact p-values see Table A7.

both the Normal and Late season compared to Early season (Table A10). Phosphatidylethanolamine, which is predominantly found in nervous tissue, revealed lowest level in Late season compared to the two other seasons measured (Table A10).

3.5. Spawning season influences the nutritional level in eye stage embryos

To further investigate how environmental changes during spawning influences the nutritional profile during the sensitive stage of organogenesis we analysed the embryos at 360 d° after fertilization (eye stage embryos).

As for the newly fertilized egg stage, vitamin B12 was significantly higher in Late season than in Early and Normal season. Due to lack of Early group samples obtained from AquaGen AS we only report values for Normal and Late season for folate, vitamin B6 and lipid analyses. Between these two groups, the results showed no difference in total folate, however, as for the newly fertilized egg stage, the metabolic form of vitamin B6, pyridoxamine, was significantly higher in Late compared to Normal season embryos (Table A11).

14 of the measured free amino acids and nine N-metabolites revealed significantly different levels between the three spawning seasons and for 75% of them Early season embryos had significant higher levels than both or at least one of the other two groups (Fig. 9, Table A12). Moreover, for valine, leucine, proline, phenylalanine, tyrosine, threonine, lysine, histidine, B-alanine, cystine, phosphoserine, and AABA the pattern reveled equivalent pattern as reported for newly fertilized egg

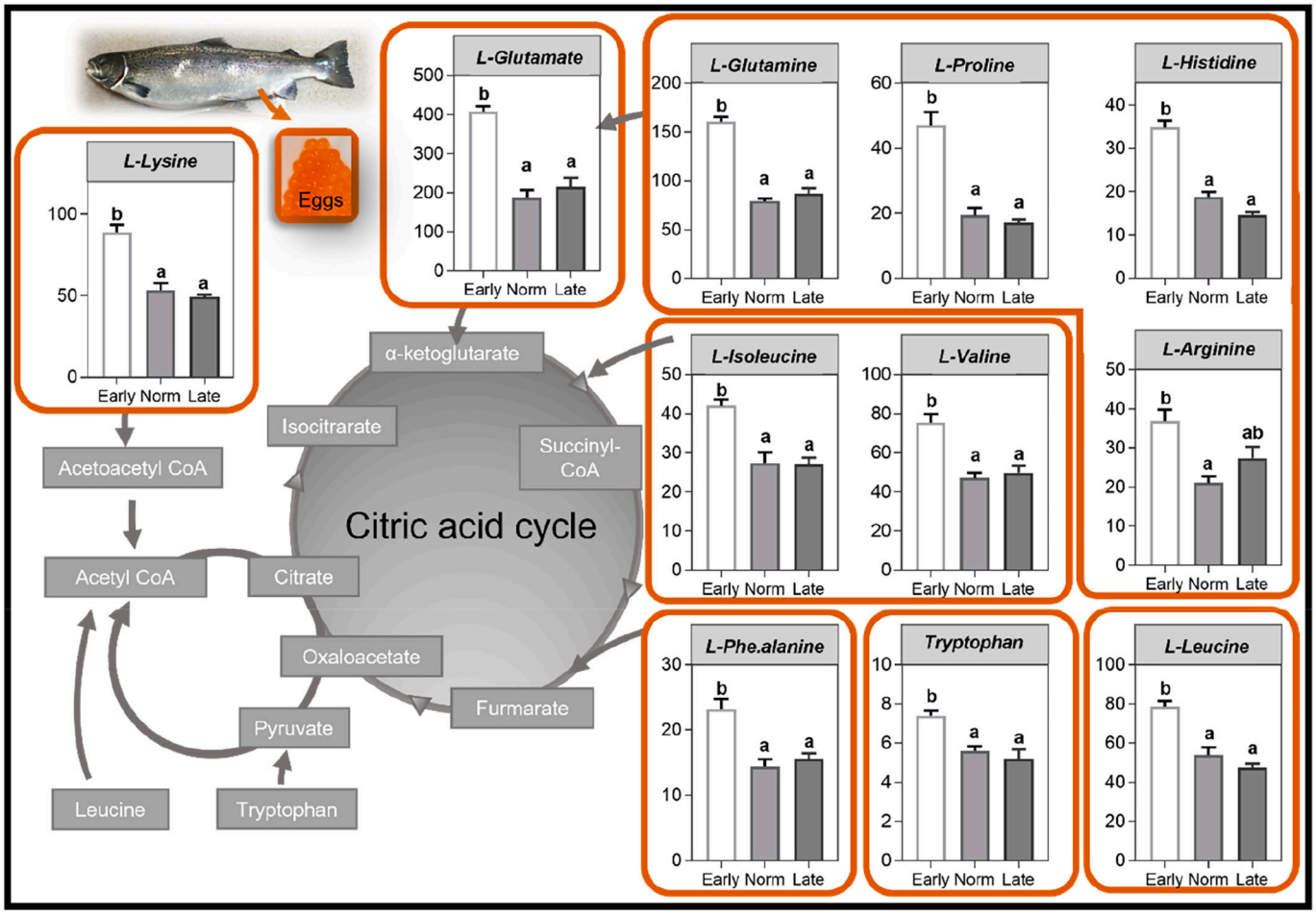

Fig. 8. 16 out of 19 free amino acids (μmol/100 g ww) reveal significant differences in newly fertilized eggs due to changing Early, Normal and Late spawning season. At Early embryo development the free amino acids are used as fuel, but also to build proteins. Depending on enzyme availability at this stage of development, free amino acids can be utilized in the citric acid cycle, acetyl CoA, pyruvate, or transaminated and converted to alanine (see Fig. 3). Note that the specific amino acids can have different routes and fates into citric acid cycle and not all of the routes are shown in the figure above. Columns represent mean values (±SD) of five independent broodstock females. Significant differences (see Materials and method section) between spawning groups are denoted by letters. For exact p-values see Table A9.

stage, and that pattern discovered that the Early season eggs had higher levels compared to Normal and Late season. Among them are the energetically important branched chain amino acids leucine, isoleucine and valine. For other amino acids, like alanine, aspartic acid, tryptophan (not sig from Early season), serine, and asparagine, reveal the significant highest level in the eye stage embryos from the Normal spawning group (Table A12). The inhibitory neurotransmitter GABA was also at the significantly lowest level in the Normal spawning group (Table A12). Interestingly both cystathionine (Fig. 6C) and methyl-histidine (Table A12) reveal significantly lower levels in both Normal and Late spawning seasons compared to eye stage embryos from the Early season. We observed no differences in lipid classes in eye stage embryos between the two analysed broodstock seasons (Table A13).

4. Discussion

Atlantic salmon spawn naturally in freshwater in the autumn. The protocols developed by breeding companies have expanded the spawning season. Our hypothesis was whether the environmental adjustments alter the heritable properties through nutritional programming, e.g. the link between the nutritional status of broodstock and embryonic nutritional profile. Vertebrates acquire developmental plasticity during sensible phases like early development whereby nutrition is a key factor for plasticity (Agosti et al., 2017; Duque-Guimaraes and Ozanne, 2013; Portha et al., 2014). We and others have previously shown that the nutritional status of parent fish influences health and/or phenotype of next generation (Adam et al., 2019; Panserat et al., 2017; Seite et al., 2019; Skjaerven et al., 2018; Skjaerven et al., 2020; Song et al., 2019; Turkmen et al., 2019; Vera et al., 2017; Wischhusen et al., 2020). We show here that environmental adjustments of rearing protocols to obtain two months advanced or delayed spawning significantly modify offspring. Importantly, we show that nutritional profile in both broodstock liver and muscle tissue sets the premises for inclusion of nutrients in newly fertilized eggs. This alters firstly the development (nutritional profile during embryonic development) and growth (total body weight) of the next generation via nutritional programming.

The nutritional profile of both broodstock and embryos point towards the normal spawning in November as favorable compared to both early and late spawning, for both the overall nutrient status which might indicate a welfare issue for broodstock and a consequence for the nutritionally acquired growth potential in newly fertilized eggs. This argument is based on several premises which we discuss below: *i)* Broodstock liver size and hepatosomatic index are significantly larger in normal spawning compared to the two other seasons. Increased hepatosomatic index indicates better nutritional condition and less hunger (Hjeltnes et al., 2018). *ii)* For broodstock nutrient status we report that muscle lipid, free amino acid and N-metabolite composition varied significantly between the three spawning seasons, but especially when

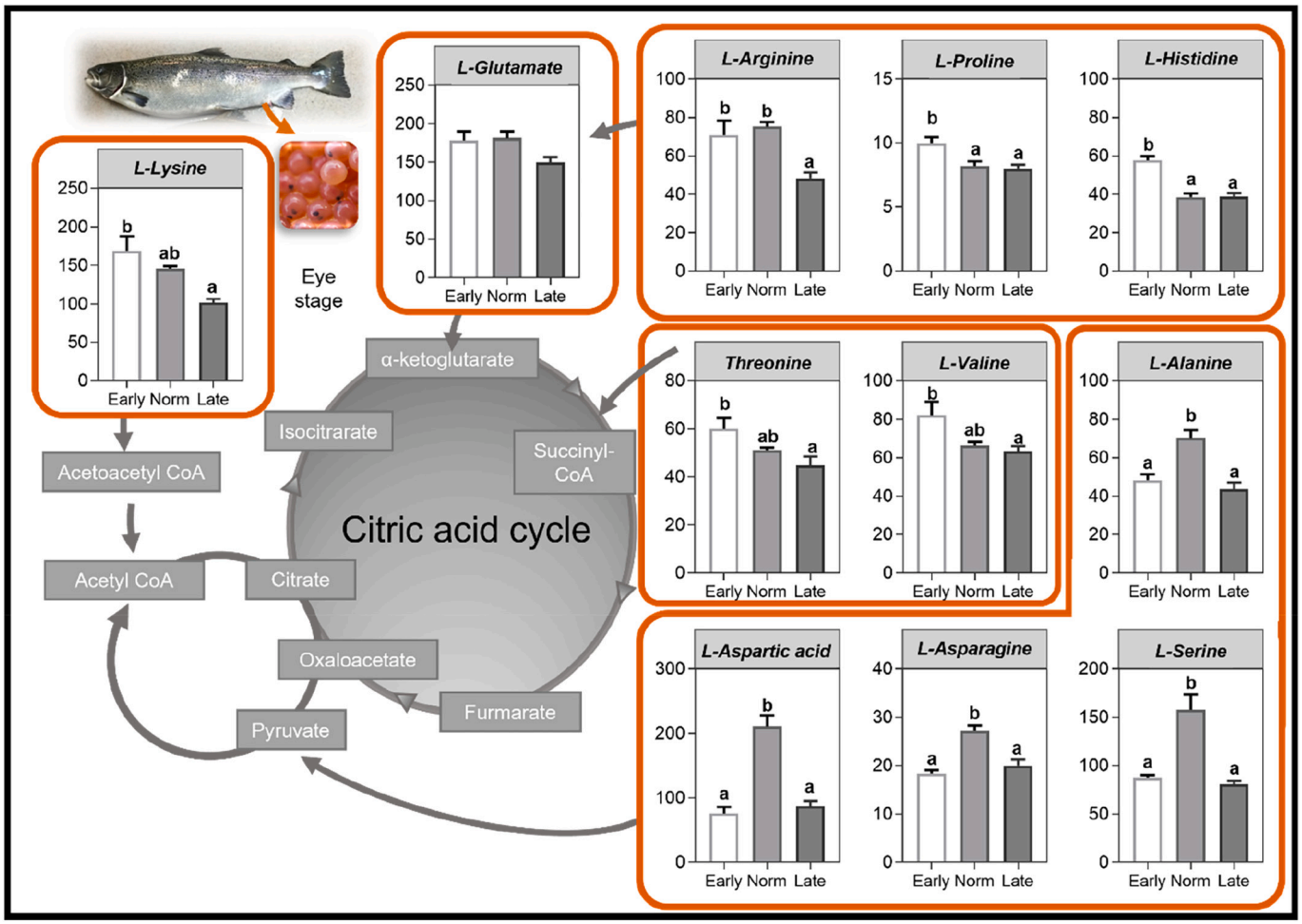

Fig. 9. 14 out of 19 free amino acids (μmol/100 g ww) reveal significant differences in eye stage eggs due to changing Early, Normal and Late spawning season. At Early embryo development the free amino acids are used as fuel, but also to build proteins. Depending on enzyme availability at this stage of development, free amino acids can be utilized in the citric acid cycle, acetyl CoA, pyruvate, or transaminated. Note that the specific amino acids can have different routes and fates into citric acid cycle and not all the routes are shown in the figure above. Columns represent mean values (±SD) of five independent broodstock females. Significant differences (see Materials and method section) between spawning groups are denoted by letters. For exact p-values see Table A12.

comparing the late season with the two other seasons, the overall results from both liver and muscle indicate hunger for the late season as we report catabolism of muscle protein via transamination reactions and free amino acids metabolism in the citric acid cycle in liver. Moreover, in muscle, lysine and the three branched-chain amino acids, leucine, isoleucine and valine were all significantly higher in late season compared to both early and normal season, whereas the lactic acid buffer B-alanine, histidine and glycine were lower in late season compared to early season. Relating these results to the free amino acid profiles measured in liver tissue show that especially the late season had significant lower levels than the two other seasons. Taken together, this indicates high liver transamination, lower levels of the lactic acid buffer B-alanine, more severe breakdown of muscle proteins (higher FAA) and higher urea levels due to the fasting period in late season broodstock. These results are indicators of muscle breakdown and high transamination (Caruso et al., 2012) in the late season compared to early and normal season. Within muscle cells, B-alanine and histidine form the dipeptide carnosine where B-alanine is considered the rate limiting N-metabolite. Both B-alanine and carnosine have multiple functions such as inhibition of protein and lipid oxidation, macrophage regulation, ATPase activation, cell membrane protection, protection against glycation and regulation of Ca^{2+} sensitivity (Caruso et al., 2012). For the late spawning broodstock, the results might be problematic in terms of fish welfare (Hjeltnes et al., 2018). *iii)* The nutritionally acquired status in newly fertilized eggs point towards the normal and late seasons as the preferred composition for increased growth potential: Measurements of overall lipid composition could not differentiate between the three groups, but the free amino acid metabolism in the citric acid cycle, the sum of folates, the SAM/SAH ratio, and the vitamin B6 level and eye stage cystathionine levels are approximately identical between normal and late seasons eggs indicating that even though the late season broodstock had severely sign of hunger the maturation of oocytes in terms of nutrient composition was appropriate to follow the growth as for normal season. Apart from these results are the results of vitamin B12, B-alanine, urea, ammonia, and AABA which reveal that the late season newly fertilized eggs have some discrepancies from the normal season which need to be further investigated in terms of impact on nutritional programming and growth potential. *iv)* The mean body weight of larvae at first feeding stage is significantly higher in normal and late season. These phenotypic changes may permanently affect the individual biological and metabolic development and may lead adaptive pathophysiological alterations at later developmental stages (Agosti et al., 2017). However, fine tuning broodstock diet and feeding protocols during the saltwater maturation, but also during freshwater maturation, might be beneficial to reduce the hunger especially for the late season.

In more detail, our results presented in this study follow the flow of nutrients from two broodstock organs to offspring at different life stages. Measuring different lipid classes in broodstock showed significant lower

levels in both normal and late spawning seasons compared to early season, however, at the newly fertilized egg stage we revealed no differences between the spawning seasons, indicating that adjusting the spawning seasons does not influence the lipid levels for the next generation. Lipids are among the most abundant nutrients in the eggs and important both as substrate for organogenesis and as energy reservoir for the embryo development (Lubzens et al., 2017; Miyares et al., 2014). However, our results show here that the sum of lipids does not set the premises for the growth differences observed at the larvae stage. Other nutrients, like the nutrients in Cahill cycle (Sarabhai and Roden, 2019) show robust data on the interaction between hunger for late spawning broodstock and the composition of the newly fertilized eggs. Especially, free amino acids, B-alanine and urea show similar trends between nutrient levels in broodstock liver and newly fertilized eggs. One might speculate and point towards the 1C metabolism and the dynamic interactions among the 1C nutrients as important factors for growth. For vertebrates, several studies have pointed to folate, vitamin B12 and the other nutrients in the 1C metabolism as important micronutrients for embryo development (Ikeda et al., 2012; Maloney et al., 2009; Maloney et al., 2011; Mikael et al., 2012; Skjaerven et al., 2018). Interestingly, we found significantly higher levels in normal and late season of several intermediate metabolites in the 1C metabolism like the oxidized 5-CHO-THF, and the active form of vitamin B6 (pyridoxal phosphate). 5-CHO-THF and pyridoxal phosphate in newly fertilized eggs showed lowest level in early season compared to the two other seasons. The free amino acids, and SAM/SAH ratio revealed the opposite pattern probably due to higher turnover and growth in normal and late season compared to early season. It is not known how these differences influence the embryonic development, but it is likely that they influence the fine-tuned differentiation processes during embryo development. Studies have shown that the 1C metabolism is highly compartmentalized within the cells (Tibbetts and Appling, 2010) and that the 1C nutrients affects cell divisions and organogenesis like neural tube defects (D'Aniello et al., 2019; Ikeda et al., 2012). Furthermore, the gene expression pattern of the mRNAs encoding several enzymes in the 1C metabolism has shown to be strictly controlled at the maternal-to-zygotic gene transition in teleost embryos (Skjaerven et al., 2014) and to be regulated differentially in embryos when broodstock mature for spawning in recirculation aquaculture system (RAS) compared to normal spawning (Skjaerven et al., 2020). More targeted gene expression studies and epigenetic studies should evaluate if the nutrient status measured here regulates specific metabolic signaling pathways depending on the nutrient status of the 1C metabolites.

The formation of the oocyte within the ovary, with the resulting mature egg which is ready to be fertilized upon spawning, is a complex process. Further on, following embryonic development from a single cell to a hatched larva rely on coordinated cell proliferation, differentiation and time to grow between each cell cycle. Signaling molecules and transcription factors are among the cell's decision architects, but the nutrients allocated to the yolk are the essential building blocks for proliferation, differentiation and growth. Here, we have analysed nutrients and metabolites regulating the 1C metabolism. From the mammalian literature we know that low folate, which is essential component of the 1C metabolism has been associated with neural tube defects related to neural tube closure (Beaudin and Stover, 2007; Dominguez-Salas et al., 2012). Besides providing 1C components for the SAM/SAH ratio, the 1C metabolism is crucial for thymidine nucleotide synthesis for cell differentiation and proliferation (D'Aniello et al., 2019; Ducker and Rabinowitz, 2017). Understanding the underlying mechanisms of nutrient incorporation into the oocytes is essential for perceiving the factors affecting the growth of the next generation through nutritional programming. Analysing gonad nutrient inclusion during the broodstock maturation process for the different spawning seasons would be a good proxy to give advice for broodstock feed optimization when adjusting abiotic factors to expand the spawning season. The number of days of starvation, which was about 40 days shorter for the normal season compared to the two other seasons, cannot explain all the seasonal changes between the spawning seasons. Further studies are needed to evaluate how changes in abiotic factors influence the nutritional status of both the broodstock and their offspring. In addition, for salmonids, further studies are needed to evaluate how the variation in nutrients between spawning seasons affect the proliferation and differentiation process of the next generation (D'Aniello et al., 2019).

Plasma cystathionine levels are known as a biomarker for folate and vitamin B12 deficiency for humans (Stabler et al., 1993). We found high levels of total folate, vitamin B12, vitamin B6 and cystathionine levels in broodstock liver from late season. For embryos we did not detect any cystathionine at newly fertilized egg stage. However, we detected cystathionine in eye stage embryos which showed the highest level in the early season which grew the least. The results report variations in cystathionine and more research is certainly needed to conclude, but we find it tempting to speculate around the measured levels of cystathionine, folate, vitamin B6, vitamin B12 and methionine, and further study the relationship between broodstock and offspring cystathionine as it is tightly linked to the 1C metabolism which is important for embryonic development (D'Aniello et al., 2019; Gos Jr. and Szpecht-Potocka, 2002; Stabler et al., 1993; Xu and Sinclair, 2015). The mature egg is a metaphase II oocyte which needs all the 1C metabolites, free amino acids and the intermediate metabolites to achieve the growth potential. In addition, to the measured nutrients, there might as well be other differences between the egg batches like other nutrients, minerals or molecular regulators like maternal mRNAs and the epigenetic regulation of DNA accessibility which needs to be studied further (D'Aniello et al., 2019; Lubzens et al., 2017).

The embryonic period is a period where the organism develops from one fertilized cell to a fully hatched larvae containing all the tissue types and organs. The period is also a period with high phenotypic plasticity. In this study, the differences in the broodstock and offspring nutritional profile eventually lead to significantly affected phenotypic plasticity in terms of growth as both the normal and late season broodstock produced significantly bigger larvae at first feeding stage. The phenotypic plasticity is probably due to some of the mechanisms underlying the nutritional programming described here, but in addition abiotic environmental variables will regulate the active signaling molecules, shift the equilibrium between metabolites which again results in cell cycle progression and turnover. All these coordinated events and the environmental driving force altering the accumulation and incorporating of yolk nutritional status, is thus greatest in terms of cellular and metabolic programming in early life stages such as the egg stage. The environment provides physiological adjustments to cellular processes that can induce diverse cellular behaviors including cell migration, tissue morphogenesis and cell fate determination, and cell division which can give rise to permanent anatomical and phenotypic consequences (Davies et al., 2018).

Alterations of abiotic factors either required for expanding the spawning season of Atlantic salmon in aquaculture settings, or as a consequence due to climate changes as reported (Masson-Delmotte et al., 2021), modify both the nutritional profile in the mother fish and affect nutrient status in developing embryos determining their future growth. The concerns raised here, by studying samples from commercial broodstock husbandry, relate to intergenerational effects when broodstock maturation is controlled by adjusting abiotic factors. The abiotic factors have a massive impact on nutritional status for both broodstock and offspring. The adjusted quantity and quality of nutritional support during early development and organogenesis may influence the robustness and welfare related to the challenging smoltification period, and as such, future research should follow the offspring seasons throughout the smoltification period.

Author contribution

Conceptualization: K.H·S., M.M; Formal analysis and data curation: M.E., K.H·S., O.E., T.S.; Funding acquisition: K.H.S.; Investigation: K.H. S, M.M., A.C.A., E.O., M.S., T.S.; Methodology: E.O., M.M., M.E., K.H.S.; Project administration: K.H.S, Writing original draft and revision; K.H. S.; Writing - review and editing; all authors.

Data availability statement

All data included.

CRediT authorship contribution statement

Kaja H. Skjærven: Conceptualization, Formal analysis, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. **Maren Mommens:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Anne-Catrin Adam:** Investigation, Writing – review & editing. **Takaya Saito:** Formal analysis, Data curation, Investigation, Writing – review & editing. **Eystein Oveland:** Formal analysis, Data curation, Investigation, Methodology, Writing – review & editing. **Marit Espe:** Formal analysis, Data curation, Investigation, Methodology, Writing – review & editing.

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.aquaculture.2022.738187.

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