Metabolic and molecular signatures of improved growth in Atlantic salmon (*Salmo salar*) fed surplus levels of methionine, folic acid, vitamin B6 and B12 throughout smoltification

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Abstract

A moderate surplus of the 1C nutrients methionine, folic acid, vitamin B6 and B12 above dietary recommendations for Atlantic salmon has shown to improve growth and reduce hepatosomatic index in the on-growing salt water period when fed throughout smoltification. Metabolic properties and molecular mechanisms determining the improved growth are unexplored. Here, we investigate metabolic and transcriptional signatures in skeletal muscle taken before and after smoltification to acquire deeper insight into pathways and possible nutrient-gene-interactions. A control feed (Ctrl) or 1C nutrient surplus feed (1C+) were fed to Atlantic salmon six weeks prior to smoltification until three months after salt water transfer. Both metabolic and gene expression signatures revealed significant 1C nutrient-dependent changes already at pre-smolt, but differences intensified when analysing post-smolt muscle. Transcriptional differences revealed a lower expression of genes related to translation, growth, and amino acid metabolization in post-smolt muscle when fed additional 1C nutrients. The 1C+ group showed less free amino acid and putrescine levels, and higher methionine and glutathione (GSH) amounts in muscle. For Ctrl muscle, the overall metabolic profile suggests a lower amino acid utilization for protein synthesis, and increased methionine metabolization in polyamine and redox homeostasis, whereas transcription changes are indicative of compensatory growth regulation at local tissue level. These findings point to fine-tuned nutrient-gene-interactions fundamental for improved growth capacity through better amino acid utilization for protein accretion when salmon was fed additional 1C nutrients throughout smoltification. It also highlights potential nutritional programming strategies on improved post-smolt growth through 1C+ supplementation before and throughout smoltification.

Keywords: muscle; micronutrients; one-carbon metabolism, RNA-sequencing; metabolomics

Abbreviations: 1C, one-carbon; SAM, S-adenosylmethionine

Introduction

In Atlantic salmon (*Salmo salar*) farming, the feed currently consists of 60% plant-based material, which is a substantial increase from 22% in 2000 [1]. The change in feed raw materials from marine to plant-based alters the requirement levels of certain micronutrients. Recommendations have thereby been re-evaluated through the EU-funded ARRAINA project [2; 3; 4; 5; 6], and recent research also points to methionine as a key micronutrient along with folic acid, vitamin B6 (pyridoxine) and vitamin B12 (cobalamin) for improved and healthy growth through smoltification [7; 8]. B vitamin supplementation above the NRC recommendations improved overall growth performance, protein retention and reduced relative liver size of Atlantic salmon [3; 4; 6; 9; 10]. Dietary micronutrients including the B vitamins have been proposed as the most probable candidates affecting fillet texture and increasing muscle cell size observed in post-smolts [5].

Methionine, folic acid, vitamin B6 and vitamin B12, are important substrates and cofactors in the one-carbon (1C) metabolism, hereinafter collectively referred to as 1C nutrients. The 1C metabolism links several pathways that allocate 1C groups by S-adenosylmethionine (SAM) that is synthesised from methionine by the enzyme methionine adenosyltransferase. In mammals, SAM affects energy metabolism, synthesis of proteins, phosphatidylcholines, creatine and polyamines, but also redox defence, cell signalling through post-translational protein modification, and epigenetic control of gene expression through methylation of DNA and histones [11; 12; 13; 14; 15]. S-adenosylhomocysteine (SAH) is formed after donation of the methyl group from SAM to a methyl acceptor and hydrolysed to homocysteine. Homocysteine can be re-methylated back to methionine by methionine synthase that requires vitamin B12 and 5-methyltetrahydrofolate, re-methylated through the betaine homocysteine methyltransferase pathway, or irreversibly transsulfurated through the vitamin B6-dependent cystathionine beta-synthase to glutathione. [16; 17]

Skeletal muscle mass represents a dynamic balance between protein synthesis and degradation controlled by numerous factors, including growth hormone (GH), insulin-like growth factors (IGF-1, IGF-2) and their cell surface receptors, and IGF binding proteins (IGFBPs) in the GH/IGF system in fish [18; 19; 20; 21]. A shift towards increased protein synthesis leads to positive protein accretion, and the availability of nutrients is one of the most important factors influencing muscle growth performance in fish [22]. GH activates IGF-1 production through GH receptors

(GHR) mainly in liver, whereas IGF function greatly depends on IGFBP activity in target tissues such as muscle [19; 23]. During protein synthesis in muscle, myofibrillar content increases along with the enlargement of muscle fibres. The formation of muscle tissue, called myogenesis, is regulated by several myogenic regulatory factors and myocyte-specific enhancer factor 2 (MEF2) transcription factors that stimulate differentiating myocytes to fuse to multinucleated myofibers during myofibrillogenesis [20; 24; 25]. Endocrine regulation of growth, particularly circulating plasma GH levels are nutritionally regulated, however, downstream signalling and nutritional regulation of IGFBP receptors and GHR is tissue-specific that remains to be understood [26]. The availability of certain nutrients such as lysine, methionine or B vitamins through the diet has been shown to modulate GH/IGF responses by modulating gene expression and protein deposition, turnover thus affecting growth performance in fish [7; 27; 28]. The crosstalk between the GH/IGF system and sirtuins, which de-acylate histones or non-histone proteins, is coupled to the energy status of a cell via NAD+ and is involved in tuning growth energy-demanding processes [26].

Factors such as photoperiod [29], temperature [30; 31; 32], circadian clock [33], dietary macronutrients such as protein, but also dietary micronutrients [3; 5; 7; 10; 16; 34] affect muscle growth and quality in teleost species. The role of dietary 1C nutrients when feeding plant-based feed has broad consequences for amino acid availability utilized for growth [16], for regulating mRNA levels of genes encoding enzymes in the 1C metabolism both intra- [10], and intergenerationally [17], and moreover it has shown to enhance growth by altering the metabolism in both liver and muscle of Atlantic salmon [7; 35]. In a recently published study, two 1C nutrient surplus levels were given in a Med-1C and a High-1C diet on top of the current recommendations as for the Low-1C diet [7]. When salmon was fed the highest surplus level, no additional benefit for growth was reported in the respective trial. Feeding a moderate surplus of 1C nutrients (Med-1C) during the fresh water period, through smoltification until the on-growing salt water period, increased body weight and decreased liver weight of salmon, which was reflected in a higher condition factor and specific growth rate compared to the Low-1C group [7]. The present study compares muscle from Low-1C and Med-1C fed salmon, hereinafter named as Ctrl and 1C+, respectively. Describing the metabolic signatures and gene expression profiles in muscle from better growing 1C+ fed salmon will improve our knowledge on active pathways and interactions to explain how surplus 1C nutrients fed to pre-smolts throughout smoltification improves growth already at the start of the on-growing salt water period.

Material and methods

Feeding trial

The Atlantic salmon feeding trial was performed at Lerang Research Station (Skretting Aquaculture Research Centre, Norway). In accordance to Norwegian Regulation on Animal Experimentation (FOR-2015-06-18-761) and European legislation (Directive 2010/63/EU), formal ethical approval of the experiment by the Norwegian Animal Research Authority was not required as experimental conditions were practices undertaken for the purpose of recognized animal husbandry, and experimental feed was not deficient in nutrient composition with regard to health and welfare of the animals. Two earlier described experimental feeds [7], Ctrl (Low-1C) and 1C+ (Med-1C), contained varying levels of methionine, folate, vitamin B6 and B12, collectively named as 1C nutrients. Analysed proximate composition and 1C nutrient levels in the feed are given in Table 1. Feed composition is provided in Table S1 and Figure S1 (Supplementary information). For the Ctrl feed, B vitamins were included at recommended levels [6] and methionine at known NRC requirement levels [9]. The 1C+ feed contained a moderate surplus of four 1C nutrients to support maximal performance as suggested in previous work [6; 36].

Primary outcomes of the feeding experiment, i.e. growth performance, body indices, nutrient retention, feed conversion ratio and protein utilization of the same experimental fish were described by Espe et al. [7].

The feeds were fed in triplicate tanks six weeks prior to smoltification until 3 months after salt water transfer (Figure 1). Pre-smolt salmon (SalmoBreed strain) were randomly assigned to experimental tanks for a two weeks long acclimatization period prior to the start of the experiment as earlier described [7]. Tanks from all treatment groups were placed in random order. To avoid poor tank environment, abnormal behaviour and poor welfare, 90 fish per tank were initially kept during the freshwater period (mean body weight of 32.1 g (bulk weight), tank diameter 0.6 m, tank volume 70 L, water flow 300–450 L/hr; rearing temperature 11.9 ± 0.4°C), and then moved to larger tanks for the salt water period (mean body weight of 95.0 ± 3.9 g, tank diameter 0.6 m, tank volume 450 L, water flow 450–900 L/hr; rearing temperature 12.0 ± 0.1°C) [7]. Fish were fed continuously until day 94 of the experiment, and thereafter fed three meals daily for two hours per meal (08:00, 12:00 and 20:00) [7]. The fish received 12 hours light and 12 hours darkness during the first 6 weeks, and continuous light for the rest of the experiment [7]. Fish were vaccinated at day 38 and starved for five days during vaccination. Early signs of disease, pain or

distress were regularly monitored to avoid suffering. 40 mg/L tricaine (Pharmaq, Norway) was used to anesthetize fish before all handling, and samples were collected from salmon 24 hours after feeding to address effects on metabolism rather than reflecting the feed itself [7]. Tricaine solved in salt water requires no additional buffering, but tricaine solved in fresh water was buffered with 40 mg/L Na_2CO_3.

Pre-smolt and post-smolt muscle sampling

Fast muscle from Ctrl and 1C+ fed salmon were taken in the end of the fresh water period (pre-smolt, day 95) and in the on-growing salt water period (post-smolt, day 220) as illustrated in Figure 1. Muscle tissue was obtained from the same epaxial area in the filet between dorsal and anal fin from three fish of each of the three replicate tanks per group. mRNA was extracted from muscle from individual fish for RNA-sequencing (Table S2). Global metabolic profiling and SAM/SAH analysis were performed on the same pooled samples containing muscle tissue from five fish derived from the same tank (Table S3).

SAM and SAH analysis

SAM and SAH were determined in twelve pooled muscle samples (each contained five muscles from fish of the same tank) on a reverse phase HPLC after deproteinization in 0.4 M $HClO_4$ and quantified using standards of the respective metabolites (Sigma) as earlier described [35; 37].

Global metabolic profiling

Twelve muscle samples containing pooled muscle tissue of five individual fish from the same tank were analysed in a MxP® Global Profiling by Metanomics Health GmbH (Berlin, Germany). Samples were weighed, freeze dried, homogenized, and dry weight was determined before extraction. After extraction and protein precipitation, samples were separated into lipid and polar fractions before further analysis using gas chromatography-mass spectrometry (GC-MS; Agilent 6890 GC coupled to an Agilent 5973 MS System, Agilent, Waldbronn, Germany) and liquid chromatography-MS/MS (LC-MS/MS; Agilent 1100 HPLC-System, Agilent, Waldbronn, Germany, coupled to an Applied Biosystems API4000 MS/MS-System, Applied Biosystems, Darmstadt, Germany). Integration and validation of chromatographic data were performed by Metanomics Health GmbH (Berlin, Germany). Metabolite levels were normalized

against the median of the pool reference samples (derived from aliquots of all samples) to give pool-normalized ratios performed for each sample per metabolite. GraphPad Prism (version 8.3.0) was used for illustration of pool-normalized metabolite data. Evaluation of all metabolites was semi-quantitative and metabolite levels were reported as pool-normalized ratios (Table S4). Results were given for free (non-covalently bound) metabolites as sample preparation did not involve hydrolysation.

mRNA extraction

For mRNA extraction, muscle tissue of 36 individual fish was homogenized each in Qiazol (Qiagen, Germany) and 3 ceramic beads (CK28) using a Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 6000 rpm. Total RNA was extracted following the EZ1 RNA Tissue Mini Kit (Qiagen, Germany) including a DNAase treatment using the RNAse-Free DNAse set (Qiagen, Germany). RNA quantity and quality were verified using NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, USA) and Agilent 2100 Bioanalyser (RNA 6000 Nano LabChip kit, Agilent Technologies, USA), respectively.

RNA library preparation and high-throughput RNA-sequencing

Total RNA quality was assessed before creating mRNA fragments for high-throughput sequencing (RNA-seq). RIN values of in total 36 samples were on average 9.3 (RINmin=8.2; RINmax=9.7, Table S2). The libraries were divided in two sets of 18 samples each, which were prepared simultaneously and were balanced for gender, dietary group, and stage. See Supplementary methods (File S1) for library preparation and sequencing details. The generated libraries of an approximately 200 nt insert size were PCR enriched and quality of the libraries was assessed, they were barcoded, pooled and denatured according to the NextSeq System Denature and Dilute Libraries Guide (Illumina, USA). The two library pools were sequenced on a NextSeq500 (Illumina, USA) at Nord University (Bodø, Norway) to generate 76 bp single-end reads. Fastqc v0.11.8 (Babraham Bioinformatics) was used for quality assessment. Quality Control assessment indicated that sequences were of high quality with minimal adapters present and trimming was not required. Quality-trimmed reads were mapped to the latest Atlantic salmon RefSeq reference genome and annotation (ICSASG_v2. 6/10/2016). This genome was indexed using the latest version of HISAT2 (v2.1.0).

Statistical analyses

Differences in metabolites between treatment groups (pre-smolt 1C+ vs. pre-smolt Ctrl, and post-smolt 1C+ vs. post-smolt Ctrl) were determined by calculating the ratio of the group means from log10-transformed data for each of the metabolites (Table S4). Significantly different metabolites in each group comparison were determined using ANOVA with Tukey adjustment and false discovery rate (FDR) by Benjamini and Hochberg multiple comparison adjustment [38]. The enrichment for significant altered metabolites (FDR<0.1) in a metabolite class shows the largest differences in the metabolic profiles using enrichment scores (Table S5). One score corresponds to the ratio between the odds of the number of altered metabolites among the number of detected metabolites in one class, and the odds of all altered metabolites among all detected in the whole screening. The significance of enrichment (adjusted $p<0.05$) was assessed by Fisher's exact test with Benjamini and Hochberg multiple comparison correction [38] using the functions fisher.test and p.adjust in R, respectively.

Statistically significant changes in SAM and SAH levels comparing the 1C+ with the Ctrl group were assessed by an unpaired t-test (two-tailed) with $p<0.05$. F test and Shapiro-Wilk test were applied to test variance and normality of the data.

For RNA-seq analysis, differential gene expression analysis was determined using the R package DESeq2 with default parameters (version 1.24.0) that estimates variance-mean dependence in count data from high-throughput sequencing assays and tests for differential expression based on a model using the negative binomial distribution [39]. Two differential expression comparisons were made: (1) pre-smolt 1C+ vs. pre-smolt Ctrl, and (2) post-smolt 1C+ vs. post-smolt Ctrl. Statistical significance of over-representation of differentially expressed genes (DEGs, $|\log2FC| >1$ and adjusted $p<0.05$) in KEGG pathways and Gene Ontology (GO) terms was determined using a hypergeometric distribution test (over-representation analysis, ORA) using the package clusterProfiler [40]. Since ORA is ratio-based, many genes can be annotated to multiple pathways and these tests can also lead to false-positives when species and pathways are poorly annotated. Gene Set Enrichment Analysis (GSEA) relies on the whole set of genes detected, allows the detection of smaller differences and being able to indicate either up- or downregulation for pathways instead of selecting DEGs based on fold change thresholds for defining differences as in case of ORA [10; 41]. Both ORA and GSEA were performed to examine enrichment in KEGG pathways.

No data points or samples were excluded in the analysis of metabolites, SAM and SAH, or RNA-seq.

Analysis and bioinformatics environment

For metabolomics and SAM/SAH analyses, the statistical software R (version 3.4.4) and GraphPad Prism 8.3.0 (GraphPad Software, USA) were used. RNA-seq analysis was completed using Linux tools (see supplementary methods in File S1) and Bioconductor packages for downstream analysis using the R programming language version 3.6.1 (2019-07-05, https://www.r-project.org/).

Data availability

Unaligned RNA-seq raw data is accessible through accession number PRJNA680206 (https://www.ncbi.nlm.nih.gov/sra/). Supplementary tables have been made available on *figshare* (DOI: 10.6084/m9.figshare.14484489).

Results

Metabolic and transcriptomic changes in pre-smolt and post-smolt muscles

Metabolic profiles from both Ctrl and 1C+ muscle were analysed using a mass spectrometric-based metabolomic approach, where in total 536 free, non-covalently bound metabolites were detected (Table S4). The most significantly different metabolites were found in post-smolt (37 metabolites) than in pre-smolt (13 metabolites) muscle when comparing 1C+ and Ctrl groups (Figure 2A). However, for both pre- and post-smolts, the differences between the dietary groups were found in the free amino acid profile and their conjugates, and again the results show that this effect was less pronounced in pre-smolt than in post-smolt muscle (Figure 2B and Table S5).

For RNA-seq analysis, on average 94.2% of 24.6 million reads were mapped to the reference genome (Table S6). Samples were controlled for outliers and mRNA libraries showed no batch effects by sex, flow cell or tanks (Figure S2). Gene expression profiles clustered by dietary treatment and particularly by life-stage of Atlantic salmon (Figure S3). Hierarchical clustering of the samples shows a clear separation between the 1C+ and Ctrl group at post-smolt (Figure S3). More DEGs between dietary groups were found in post-smolt (902 DEGs) than in pre-smolt muscle (95 DEGs, Figure 2C). The majority of the DEGs show lower mRNA levels in

1C+ than in Ctrl for both pre- and post-smolt muscle (Figure 2C). The annotated lists of DEGs are given in Table S7 and volcano plots in Figure S4 to visualize differential expression in pre- and post-smolt muscle.

Metabolic characteristics of muscle from pre-smolt and post-smolt salmon

The amino acids lysine, histidine, and proline were the metabolites showing the biggest differences (highest pool-normalized ratio), whereas alanine, proline and arginine were the most significant metabolites (lowest adjusted p-value) between the dietary groups (Figures 2A and 3, Table S4). 14 out of in total 19 detected amino acids, including the N-metabolite taurine showed lower levels in 1C+ compared to Ctrl post-smolt muscle (Figure 3). Lysine, histidine, alanine, proline, leucine, isoleucine, phenylalanine, tryptophan, serine, glycine, glutamine, arginine, valine, and glutamate had significantly lower levels, while methionine had significantly higher levels in post-smolt muscle (Figure 3 and Table S4).

Lysine, generally present in high proportions in fish muscle, is involved in growth and cross-linking of proteins such as collagen. Another major component of collagen is trans-4-hydroxyproline, which was here elevated in 1C+ (Figure 2A). Post-translational acetylation of lysine, or lysine residues of histones and non-histone proteins forms derivates like Ɛ-acetyllysine, which was decreased in 1C+. 1C+ comprised lower nicotinamide levels, which is an important epigenetic mediator of deacetylases and part of nicotinamide adenine dinucleotide (NAD^+) functioning in oxidation-reduction reactions of intermediary metabolism. 1C+ showed higher levels of beta-alanine and the histidine conjugate 1-methylhistidine, which are together constituents of anserine important for muscle homeostasis, pH buffering and antioxidant capacity. The amine oxide trimethylamine N-oxide, in short TMAO, was elevated in 1C+. TMAO is biosynthesized from choline and phosphatidylcholine and known to protect against the adverse effects of temperature, salinity, high urea, and hydrostatic pressure. Choline was not different, but 1C+ exhibited higher levels of choline phosphate (other name for phosphocholine), which is an intermediate in the synthesis of phosphatidylcholine. Phosphatidylcholine C32:3 was significantly lower in 1C+. SAM acts as a methyl donor in the synthesis of phosphatidylcholine from phosphatidylethanolamine. Levels of phosphatidyl-myo-inositols and maltotriose were lower in 1C+ than in Ctrl muscle.

1C+ muscle revealed lower levels of arginine, ornithine, and putrescine, which is the direct precursor for polyamines (Figure 2A). Polyamine synthesis is dependent on the availability

of SAM, which was not detected in the metabolic profiling, but additional analysis of SAM revealed increased levels in 1C+ in post-smolt muscle (Figure 4). SAH, synthesised upon donation of a methyl group from SAM, was not different between the dietary groups (Table S4 and Figure 4). The ratio of SAM to SAH was higher in 1C+ than in Ctrl. SAM synthesis depends on methionine, which levels follow the dietary treatment with higher amounts in 1C+ than Ctrl (Figure 2A). Methionine can get irreversible metabolized through transsulfuration into cysteine, taurine, and the antioxidant glutathione (GSH), which was significantly increased in 1C+ (Figure 2A).

Pre-smolt muscle shares most of their changed metabolites with those that were different in post-smolt muscle (Figure 2A). Common metabolites show the same direction of change between 1C+ and Ctrl muscle. Methionine, TMAO and trans-4-hydroxyproline levels were increased, whereas levels of alanine, serine, putrescine, proline, histidine, lysine and Ɛ-acetyllysine were decreased in 1C+ compared to Ctrl (Figure 2A). Betaine, an osmolyte and methyl group donor in the re-methylation of homocysteine to methionine, was decreased in pre-smolt 1C+, but not in post-smolt (Figure 2A and Table S4).

Common differentially expressed genes between pre-smolt and post-smolt muscle

Twenty-five DEGs were shared between pre- and post-smolt muscle differences (Table S7). Among them (Table 2), decreased mRNA levels in 1C+ muscle were found for two S-adenosylmethionine synthase paralogous genes (*mat2a, mat2a-like*), two genes encoding ferritin subunit paralogs (*frim*, *frim-like*), and four sodium-coupled amino acid transporter paralogs (*slc6a9-like, slc38a3-like, slc6a15-like, slc6a15-like*). The gene encoding galactosidase alpha (*gla*), which is active in lysosomes and catalyzes the removal of terminal α-galactose groups from glycoproteins and glycolipids, was expressed at significantly lower levels in 1C+ muscle from both pre- and post-smolts. Three paralogs of the interferon-induced guanylate-binding protein 1 (*gbp-1-like*) involved in endosomal trafficking, cytoskeleton regulation, and autophagy were expressed at lower levels in 1C+. Two overlapping DEGs encode Rho family GTPase-activating proteins (*arhgap12-like, arhgap30-like*) that function in a variety of cytoskeleton-dependent cell functions.

Lower expressed genes linked to mRNA translation and amino acid metabolization in 1C+ muscles from post-smolts

DEGs between 1C+ and Ctrl (Table S7) have been associated with biological processes and molecular functions by overrepresentation analysis (ORA). ORA results from pre- and post-smolt muscles are listed separately in Table S8 and S9, respectively. For post-smolt enrichment, solely downregulated genes encoding amino acid specific tRNA synthetases and ligases central in translation for protein synthesis account for the most enriched KEGG pathway, aminoacyl-tRNA biosynthesis (Figure 5), and a few enriched GO term categories listed in Table S9. Figure 5 shows also DEG enrichment in the KEGG pathway biosynthesis of amino acids entirely by lower expressed genes encoding a diverse group of enzymes driving amino acid metabolization (Figure 5, Table S9). Among them were genes like *glna*, *cgl*, *shmt2*, *cbs-like*, *mat1a-like*, *mat2a-like*, *pycr1-like*, *p5cs-like*, *cps1*, *otc* and *arg2*, which were listed in Table 3 and categorized after their functional roles or pathway affiliation. Concordant results were found with GSEA (Figure S5) revealing aminoacyl-tRNA biosynthesis and biosynthesis of amino acids as the most downregulated KEGG pathways in the post-smolt gene expression profiles.

Folate cycle key genes are expressed at lower levels in 1C+ muscle from post-smolts

Genes encoding mitochondrial homologs of folate cycle enzymes enriched the KEGG pathway one carbon pool by folate (Figure 5, Table S9). Compared to the Ctrl, 1C+ muscle showed lower expression of *shmt2*, *mthfd1l*, *mthfd2-like*, *aldh1l2-like*, and *mtdc-like* (Table 3).

1C+ muscle from post-smolts reveal lower gene expression in transmethylation and transsulfuration reactions

Transmethylation reactions depend on the availability of SAM. *peam3* (Table 3) encoding a N-methyltransferase that methylates phosphoethanolamine to phosphocholine using SAM as substrate, was among the top DEGs with the largest fold change showing lower mRNA levels in 1C+ muscle. Table 3 also lists the transsulfuration pathway and vitamin B6 dependent genes *cgl* and *cbs-like* that were lower expressed in 1C+. The transsulfuration pathway removes undesirable amounts of homocysteine through cystathionine and cysteine.

1C+ muscle from post-smolts show lower expression of genes linked to polyamine homeostasis regulation

Polyamine homeostasis is maintained by the spermidine/spermine N1-acetyltransferase family member 1 and 2, also known as diamine acetyltransferases 1 and 2. *sat2, sat2-like* and its paralog *sat1-like* (Table 3) were lower expressed in 1C+ than in the Ctrl muscle.

Ferroptosis and glutathione metabolizing genes are expressed at lower levels in post-smolt 1C+ muscle

The ferroptosis KEGG pathway was enriched by solely lower expressed genes in both pre-smolt and post-smolt muscle comparisons (Figure 5, Table S8). Decreased expression in 1C+ was found for ferritin middle subunit paralogs (*frim, frim-like*), ferritin heavy polypeptide 1-1 (*frih*), *sat2, sat2-like, sat1-like,* and *slc40a1* (Table S9). One regulatory pathway of ferroptosis is the metabolism of glutathione. 1C+ showed decreased expression of the glutathione regulating and conjugating enzymes encoded by *gstp1*, *chac1-like*, *mgst3-like* and *osgin1-like* (Table 3).

Differential expression of myofibrillar proteins and genes regulating cytoskeletal organization in post-smolt muscle

Actin cytoskeleton, striated muscle thin filament and the troponin complex were among the DEG enriched cellular component ontology terms (Figure 5). Several genes encoding various proteins building the actin cytoskeleton such as myosin and troponins I and T were differentially expressed between 1C+ and Ctrl muscle (Table S9). Transgelin-3 is an actin binding protein of the cytoskeleton and differences in *tagln*-3-*like* expression showed the highest fold change in the post-smolt data, where it was expressed in Ctrl, but not expressed in 1C+ muscle (Figure 6A). Contrarily, 1C+ muscle revealed an increased expression of *myoz2* encoding an α-actinin- and γ-filamin-binding Z line protein involved in myofibrillogenesis. Fibrillar proteins and extracellular matrix regulating proteins involved in homeostasis and organization of the sarcomeric cytoskeleton were more highly expressed in 1C+ than in Ctrl muscle. Among them, there were DEGs encoding various collagen alpha paralogs, collagenase 3 and cathepsin S (Figure 6A and Table S7). A few other DEGs encoding muscular cytoskeleton assembly proteins such as integrin alpha and beta subunits (*itgae*, *itb2*, LOC106586548, LOC106602819) and tubulin alpha chain-like (LOC106565439) showed increased expression in 1C+ (Table S7).

Differential expression of factors mediating growth and myogenesis in post-smolt muscle

Expression of *igfbp-1a1* encoding a protein that inhibits or potentiates IGF-1 growth promoting functions was decreased in 1C+ muscle (Figure 6B). The early growth response 1 encoding gene (*egr1*) encodes a transcription factor that can be induced by growth factors in the IGF-1 cascade and that acts in a wide range of growth and cell differentiation related processes. *egr1* showed decreased expression in 1C+ muscle. Maintenance of muscle fibre differentiation is regulated by myogenic regulatory factor 4 encoded by *mrf4* that was lower expressed in 1C+ (Figure 6B). Lower expression was also found for *mef2b-like* that encodes a paralog of myocyte-specific enhancer factor 2B, a transcriptional activator of growth-related genes (Figure 6B).

Discussion

A moderate surplus of dietary methionine, folic acid, vitamin B6 and B12 above the current requirements and recommended levels for Atlantic salmon given with the Med-1C feed improved growth, i.e. increased body weight in the on-growing salt water period as recently described by Espe et. al [7]. We aimed to understand how salmon fed the Med-1C feed, which is identical to the 1C+ feed in the present study improved growth during the on-growing salt water period [7]. We investigated metabolic and molecular signatures of muscle from the same fish as in the previous study [7] from the Ctrl (Low-1C) and 1C+ (Med-1C) group before and after smoltification to acquire deeper insight in metabolic properties, pathways and possible nutrient-gene-interactions involved in the observed difference in growth.

We found a higher number of different metabolites and DEGs in muscle of salmon from the on-growing salt water period (post-smolt) than in the fresh water period (pre-smolt). That is in line with the phenotype earlier described for the same experimental fish between the two dietary groups after smoltification [7]. Espe et al. reported higher protein accretion (deposition of muscle protein) and higher retention of indispensable amino acids in post-smolts compared to pre-smolts [7]. Moreover, the retention of methionine was higher when dietary inclusion was lowest while none of the other indispensable amino acid levels differed between the two feeds [7]. Results from the present study were in line with Espe et al. showing lower methionine levels in Ctrl muscle when dietary inclusion was lower. Given that most of the free amino acids in Ctrl muscle from the present study were increased, confirms the results from Espe et al. showing that utilization or deposition of amino acids was lower when the Ctrl diet was fed to salmon. This

points to free amino acids accumulation as an indicator of a reduced growth capacity in both salmon and zebrafish [7; 16; 42]. Conformably, increased mRNA expression of genes involved in translation and amino acid metabolization suggests a readiness for translation with a following mobilization of amino acids for protein synthesis in Ctrl muscle. Amino acids have the ability to influence gene expression through modulating the initiation phase of mRNA translation, but the likelihood that an aminoacyl-tRNA synthetase acts in translation for protein synthesis may also depend on other transcription factors that can explain the reduced growth capacity in the Ctrl group [43; 44].

Since the demand of methionine is shared between protein synthesis and transmethylation reactions, efficient metabolization of methionine requires both dietary methionine and B vitamin supply in the 1C metabolism, and the endogenous supply of betaine from choline. For 1C+ muscle, the demand of methionine for protein growth was most likely met through dietary and endogenous supply of methionine and the B vitamins. Contrarily, Ctrl muscle showed increased expression of key genes in the mitochondrial folate cycle to meet the demand for methionine in protein synthesis and other reactions. Espe et al. reported lower cystathionine levels in Ctrl muscle, which indicates that the dietary levels of B vitamins were not sufficient [7]. When methionine gets less incorporated into protein, it will be metabolized through SAM to a variety of other metabolites. In the present study, 1C+ muscle showed increased levels of both SAM, the mediator of transmethylation reactions, and glutathione (GSH) synthetized through the transsulfuration pathway. Ctrl muscle revealed increased expression of S-adenosylmethionine synthase isoforms and vitamin B6 dependent enzymes suggesting stronger regulation of methionine metabolization, transsulfuration and folate cycle in the 1C metabolism. Further, *peam3* expression was increased suggesting increased synthesis of phosphatidylcholine from phosphoethanolamine, however, we found only one phosphatidylcholine (C32:3) significantly increased in the Ctrl group. Except for increased phosphatidylcholine and decreased choline-phosphate levels in Ctrl muscle, we hardly found an effect on lipid or fatty acid composition in muscle that is in line with the previous results from the same experimental fish [7]. Overall, metabolic and RNA-seq results from muscle in this study show that the demand for dietary folic acid and methionine seems larger in Ctrl fed salmon to counterbalance the need for protein synthesis and growth-related investments, which was more pronounced in the salt water period. However, we cannot be certain whether the amounts of all 1C nutrients together or solely one of

them were the main driving factors that improved growth when fed the 1C+ feed consisting of a combined surplus package of methionine, folic acid, vitamin B6 and B12.

Skeletal muscle growth in fish is plastic and involves the enlargement of already existing muscle fibres (hypertrophy) and the recruitment of new fibres (hyperplasia). The balance between both is dependent on genetic differences and extrinsic factors such as early nutritional history [31; 45]. It has been suggested that increased muscle hypertrophic growth in salmon is also associated with the stimulation of protein synthesis by diet balanced in micronutrients, particularly in B vitamins [5]. In general, fish growth and muscle maturation are centrally regulated through GH acting on receptors on the sarcolemma that indirectly initiates IGFBP production by IGF from the liver, and by myogenic regulatory factors, respectively [20; 21; 26]. We found significantly higher mRNA expression of genes encoding factors involved in growth and transcription, which suggests stronger stimulation of myogenesis and growth in the Ctrl than in the 1C+ group. Other genes that encode GHR1 and GHR2 precursors, IGF-1 and a few more IGFBPs had higher mRNA levels in Ctrl muscle but were either below the chosen threshold $|log2FC|<1$ or were not significant differentially expressed. Espe et al. reported from targeted RT-qPCR analysis in muscle that *ghr* was significantly increased, whereas *igf*-1 and its receptor were not different but showed the same trend with higher expression in Ctrl muscle [7]. Mechanisms explaining the increased expression of growth stimulating factors in Ctrl muscle and the lower growth reported for this group are indicative of compensatory growth regulation at local tissue level. A compensatory regulation of systemic and local components of the GH/IGF axis has been shown for gilthead sea bream fed diets highly replaced marine ingredients by alternative raw materials [46]. The ability of increased free amino acids in muscle to stimulate translation and protein accretion on the one hand, and a likely higher methionine, SAM, glutathione and B vitamin demand on the other hand, may account as factors for the reduced growth capacity in the Ctrl group [44].

Muscle tissue consists of muscle fibres and myofibrils with a complex and highly organized network, the sarcomeric cytoskeleton that interlinks filaments with contractile, structural and regulatory proteins [20]. RNA-seq analysis revealed differences in expression of genes encoding a wide range of actin-binding and structural proteins such as troponin and myosin, but also proteins regulating cytoskeletal re-organization. Interestingly, in case of transgelin-3, which is involved in membrane trafficking and actin-binding, we could not detect

any mRNAs in 1C+, but its transcripts were present in Ctrl muscle. Tissue remodelling involves both protein synthesis and degradation through proteolytic activity that are central to nutrient supply adaptation and homeostasis of a tissue [20; 47]. Here, we found increased expression of collagenase 3 and cathepsin S in 1C+ muscle that suggest increased proteolysis capacity thus tissue remodelling. The expression of proteolytic enzymes has also been associated with quality measures such as high firmness of salmon filet [48]. Overall, RNA-seq results from 1C+ post-smolts imply increased muscle tissue remodelling, but less stimulation of differentiation and growth in muscle. However, analysis of muscle cellularity is needed to provide more details on structural changes and possible quality measures of salmon muscle.

Ctrl muscle from the present study showed increased levels of the polyamine precursor putrescine, and increased expression of ferritin subunits and enzymes regulating polyamine levels. Expression of *sat1* has been linked to lipid peroxidation thus stimulation of ferroptosis due to reactive oxygen species (ROS) formation in cell culture [49]. The metabolism of amino acids is tightly linked to the regulation of ferroptosis, which is an iron-dependent form of cell death [50; 51]. Ferroptosis is characterized by the accumulation of intracellular lipid ROS [51] upon intracellular iron accumulation. Under oxidative stress conditions, methionine can be converted through the transsulfuration pathway to glutathione that further exerts antioxidant effects [52]. Ferritin contributes to decrease oxidative stress besides main functions in intracellular iron storage [50; 53; 54]. This might explain the increased mRNA levels of ferritin, and the depletion of glutathione (GSH) that suggest higher glutathione utilization with increased oxidative load in the Ctrl muscle, possibly associated with increased polyamine metabolism [13; 49; 51; 55]. Polyamines, produced from putrescine and ornithine, regulate fundamental cellular processes such as cell growth and proliferation but can also affect redox balance as polyamine acetylation (degradation) can promote ROS affecting the oxidative load [56]. However, central enzymes in polyamine synthesis were not differentially expressed, whereas *sat2* and *sat1-like* expression was increased, suggesting higher degradation to maintain polyamine homeostasis thus affecting redox balance in Ctrl muscle [13; 56]. Interestingly, Ctrl muscle showed decreased expression of glutathione S-transferase P that suggests glutathione depletion through S-glutathionylation of functional and structural proteins to maintain cellular redox homeostasis in response to ROS in the Ctrl group [52; 57].

Adequate amounts of micronutrients prior to biological transformations such as smoltification in salmonids can be a decisive factor for later growth. Feeding a 1C-nutrient surplus prior to smoltification affected growth of pre-smolts marginally but that of post-smolts significantly, which indicates metabolic, possibly epigenetic programming in muscle. Changes in methionine levels can directly impact methyl-donor availability with possible consequences for DNA and histone tail methylation [58; 59; 60]. Differences we found in Ɛ-acetyllysine amounts and *sirt5-like* mRNA expression in muscle point to effects on histone tail or non-histone protein acetylation, but also solely to non-enzymatic acetylation of free lysine [61; 62; 63]. One can speculate whether a dietary 1C-nutrient surplus as for the 1C+ feed can introduce life-long changes in epigenetic profiles that affect muscle gene expression and improve growth [64; 65]. Future studies should consider integration analysis of DNA methylation, histone methylation and acetylation marks to provide a deeper insight into nutrient-sensitive regulation of epigenetic mechanisms involved in growth, and whether post-smolt growth can be programmed prior to smoltification or during early development [12; 66; 67; 68; 69; 70; 71].

Conclusion

A dietary surplus of methionine, folate, vitamin B6 and B12 above current requirement and recommended levels throughout smoltification appears to be beneficial in protein accretion for improved growth in farmed Atlantic salmon. The results from this study support that the availability of methionine along with B vitamins is important to efficiently use amino acids for protein growth. The metabolic signatures point to improved growth and redox capacity through improved utilization and metabolization of amino acids in muscle of 1C+ fed salmon. We have also demonstrated that transcriptional changes in muscle in response to a dietary 1C-nutrient surplus relate to translation, mobilization of amino acids, cytoskeletal organization, and growth. Understanding how growth is controlled by non-genetic mechanisms becomes important for a rapidly growing aquaculture industry whose concerns are to optimize production, sustainability, and quality.

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Conflicts of Interest

The authors have no financial or personal conflicts of interest to declare.

Authorship statement

A.A., M.E. and K.H.S conceived and designed the research. V.V. provided the feed and performed the feeding trial. A.A. and J.M.O.F. prepared the libraries, and P.W. and T.S. performed bioinformatics analysis for the RNA-seq results. A.A., K.H.S., M.E., and T.S. analysed and interpreted data. A.A. drafted the manuscript. All authors read and approved the final manuscript.

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Figures and Tables

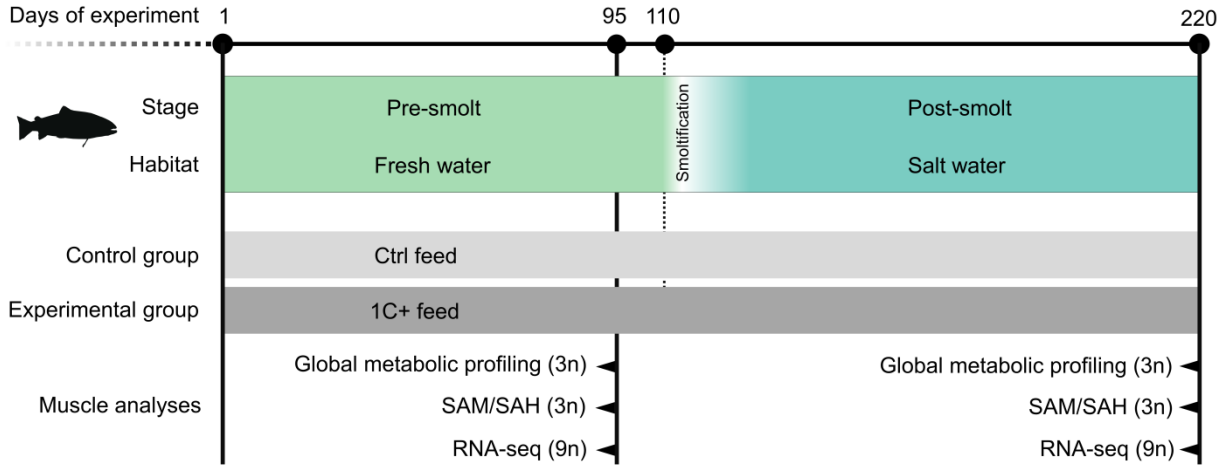

Figure 1. Experimental design and sampling of muscle from Atlantic salmon fed a control feed (Ctrl) and a feed including surplus levels of one-carbon (1C) nutrients (1C+) through the smoltification period. The Ctrl feed contained 1C nutrients (folate, methionine, vitamin B6 and B12) as recommended and on the requirement levels to support maximal performance (ARRAINA [6] and NRC [9]). The 1C+ feed included even higher levels of the 1C nutrients, which improved growth when given through smoltification [7]. Fish were fed in triplicate tanks for 6 weeks in the fresh water period, through salt water transfer and 3 months in the on-growing salt water period. Muscle tissue was sampled in the end of the fresh water and in the on-growing salt water period for further analyses: global metabolic profiling, SAM/SAH by HPLC and RNA-sequencing (RNA-seq). Figure is modified from Espe et al. [7].

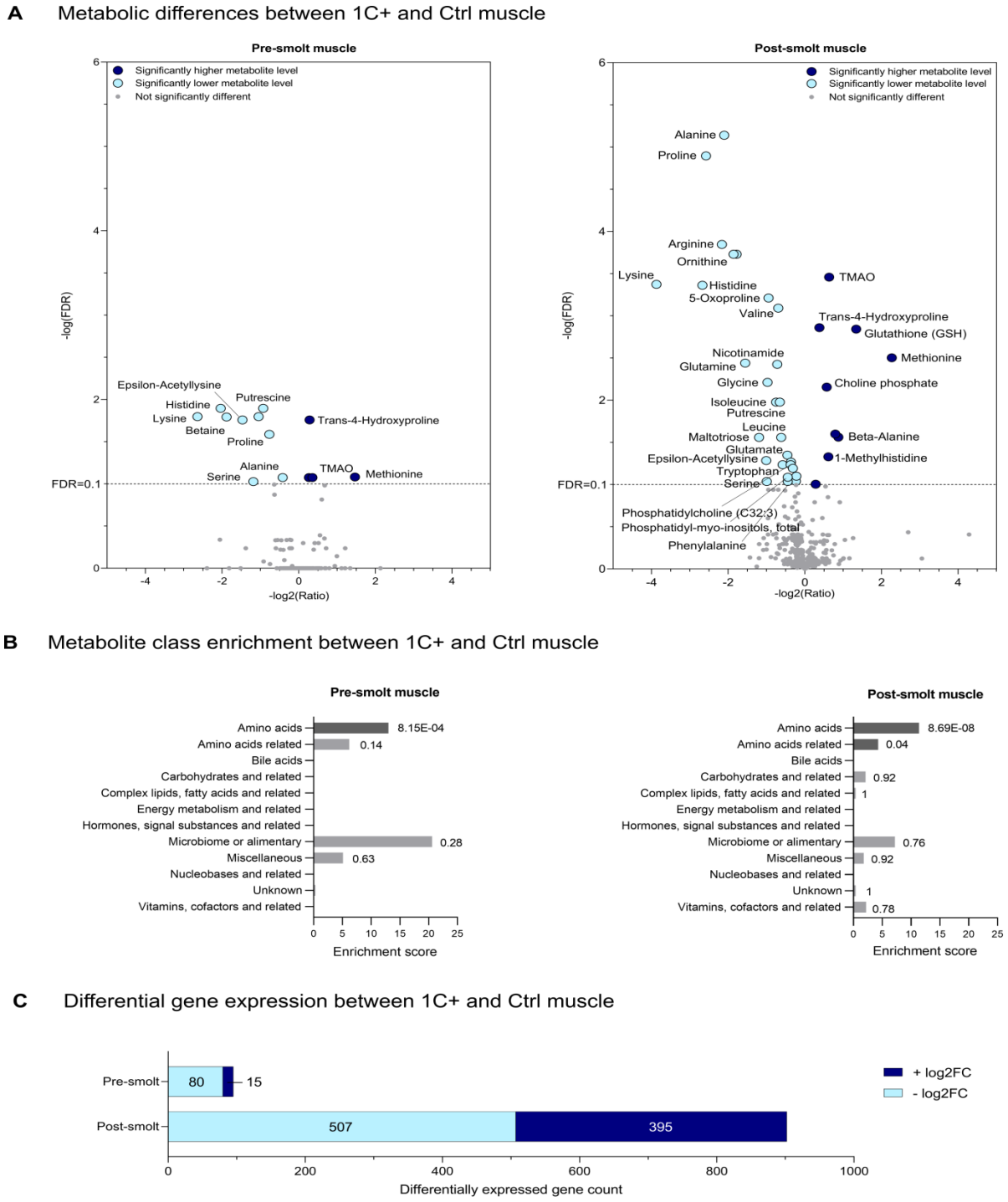

Figure 2. Feeding a 1C nutrient surplus to Atlantic salmon during the smoltification period changed metabolic and transcriptomic profiles in muscle from both pre- and post-smolts. **(A)** Volcano plots showing metabolomic data from pre-smolt and post-smolt muscle. Differences in metabolites (dots) between the 1C+ and Ctrl group were plotted as log2-transformed ratios of group means on the x-axis. Statistically different log2-ratios (FDR < 0.1, dashed line crossing the

y-axis) were highlighted in shades of blue. In total 13 and 37 out of 536 detected metabolites were different between 1C+ and Ctrl profiles at pre- and post-smolt, respectively. Metabolites with negative log2-ratios indicate lower levels (light blue) in the 1C+ compared to the Ctrl group, and vice versa for positive log2-ratios (dark blue). Metabolites with either high positive or negative log2-ratios display large magnitude differences between the groups. Metabolites-of-interest were labeled with their names (Table S4). **(B)** Metabolite class enrichment analysis shows that the largest differences between 1C+ and Ctrl muscle were in the amino acids and their conjugates, which were particularly pronounced in post-smolt muscle. Calculated enrichment scores (x-axis) underlie a ratio of significant altered metabolites (FDR < 0.1) among detected ones in a metabolite class in relation to all significant altered metabolites among all detected ones in the global metabolic profiling. Significance of enrichment was assessed using Fisher`s exact test with Benjamini and Hochberg correction [(38)] and adjusted p-values given for each bar. Bold compound classes designate significant enrichment (adjusted p-value < 0.05). **(C)** The total number of differentially expressed genes (DEGs, RNA-seq) was higher in post-smolt (902) than in pre-smolt muscle (95) when using false-discovery adjusted p-value < 0.05 and |log2-fold change| 1 as significance cut-offs. Numbers within bars represent DEGs with either higher (upregulated) or lower mRNA levels (downregulated) in 1C+ muscle compared to Ctrl muscle.

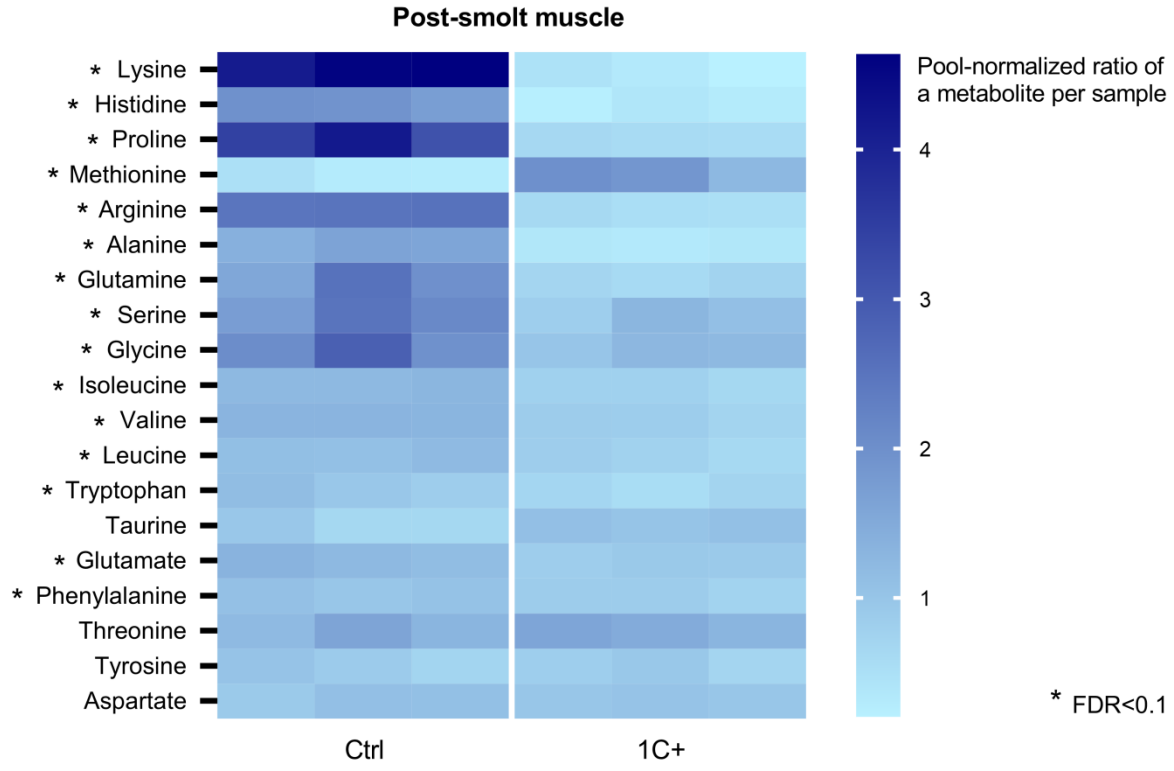

Figure 3. Relative amino acid levels in muscle from 1C+ and Ctrl fed salmon after smoltification (post-smolt). The heatmap illustrates individual sample levels of single amino acids, and the N-metabolite taurine that were detected in the global metabolic profiling. Cysteine and asparagine were not detected. Each cell illustrates the level of a single amino acid (rows) in each sample (columns) from each dietary group expressed as pool-normalized ratios in a color scale. Asterisks (*) show significantly different amino acid levels between 1C+ and Ctrl muscle (FDR<0.1). Amino acids were sorted after the effect size (magnitude of log2ratio) between group means in descending order. All metabolic data is provided in Table S4.

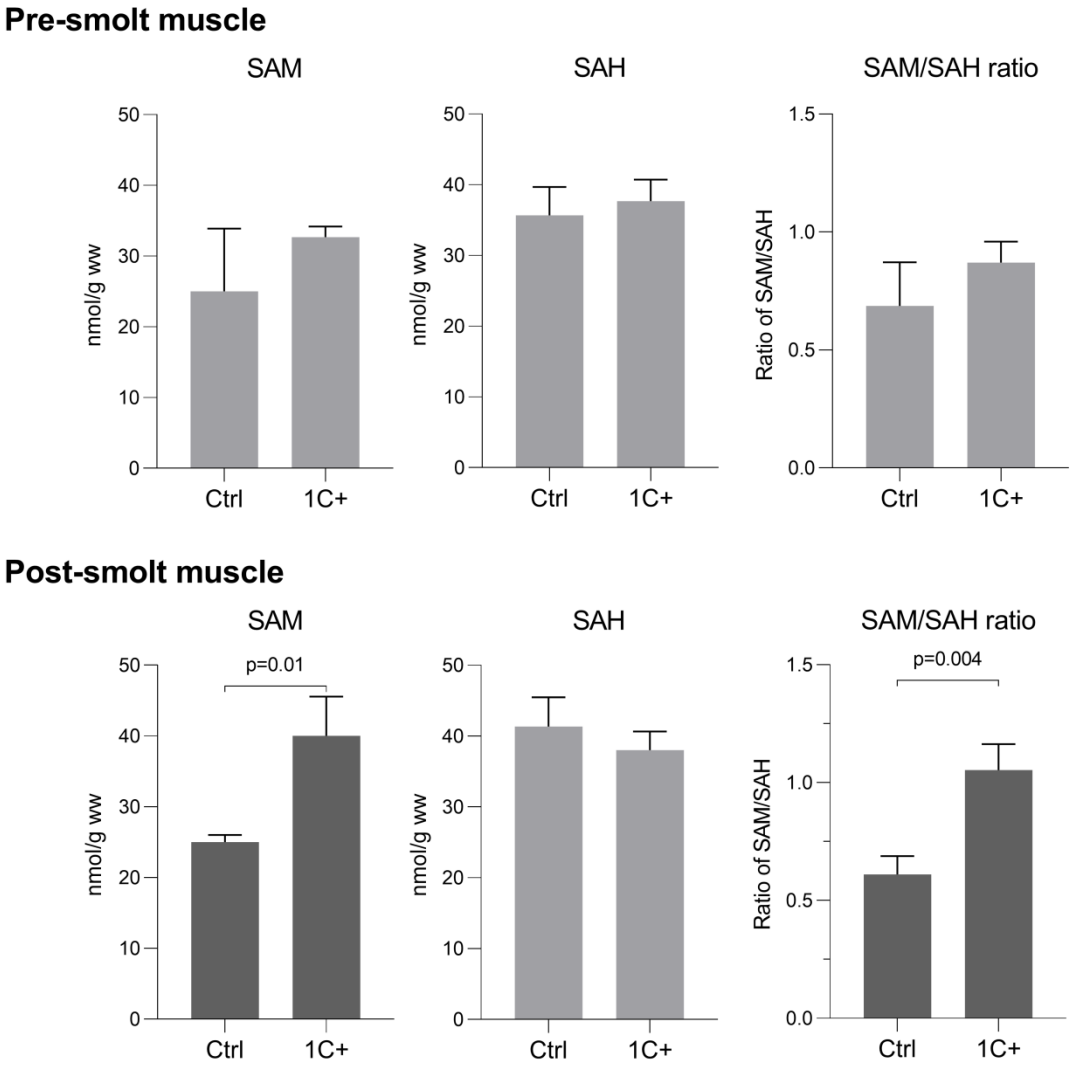

Figure 4. S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) levels comparing 1C+ and Ctrl in pre- and post-smolt salmon muscle assessed by HPLC. The ratio of SAM to SAH is given as a measure to study methylation capacity in the muscle. Statistical significance was assessed using a two-tailed t-test with $p < 0.05$. P-values of significant differences were indicated above dark grey colored bars. Non-significant differences were kept light grey.

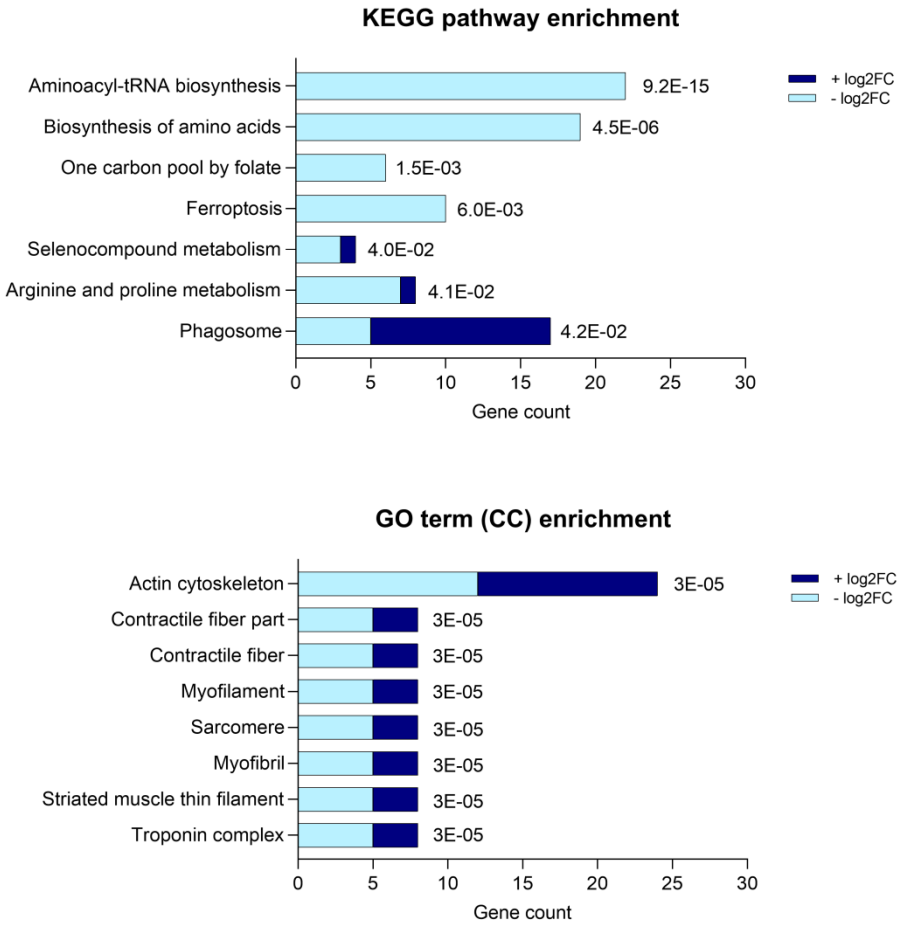

Figure 5. Overrepresentation of differentially expressed genes (DEGs) in KEGG pathways and cellular components (CC) between 1C+ and Ctrl muscle after smoltification. Overrepresentation analysis (ORA) was performed to show functional enrichment of DEGs. Bars illustrate the DEG number in a category. Shades of blue highlight DEGs either with positive (+log2FC) or negative log2-fold changes (-log2FC). DEGs with -log2FC show lower mRNA levels in 1C+ than in Ctrl muscle. Adjusted p-values for each enrichment were associated with each category (bars). ORA is based on DEGs with a |log2-fold change| >1 and adjusted p-value < 0.05 when comparing gene expression of 1C+ and Ctrl muscle.

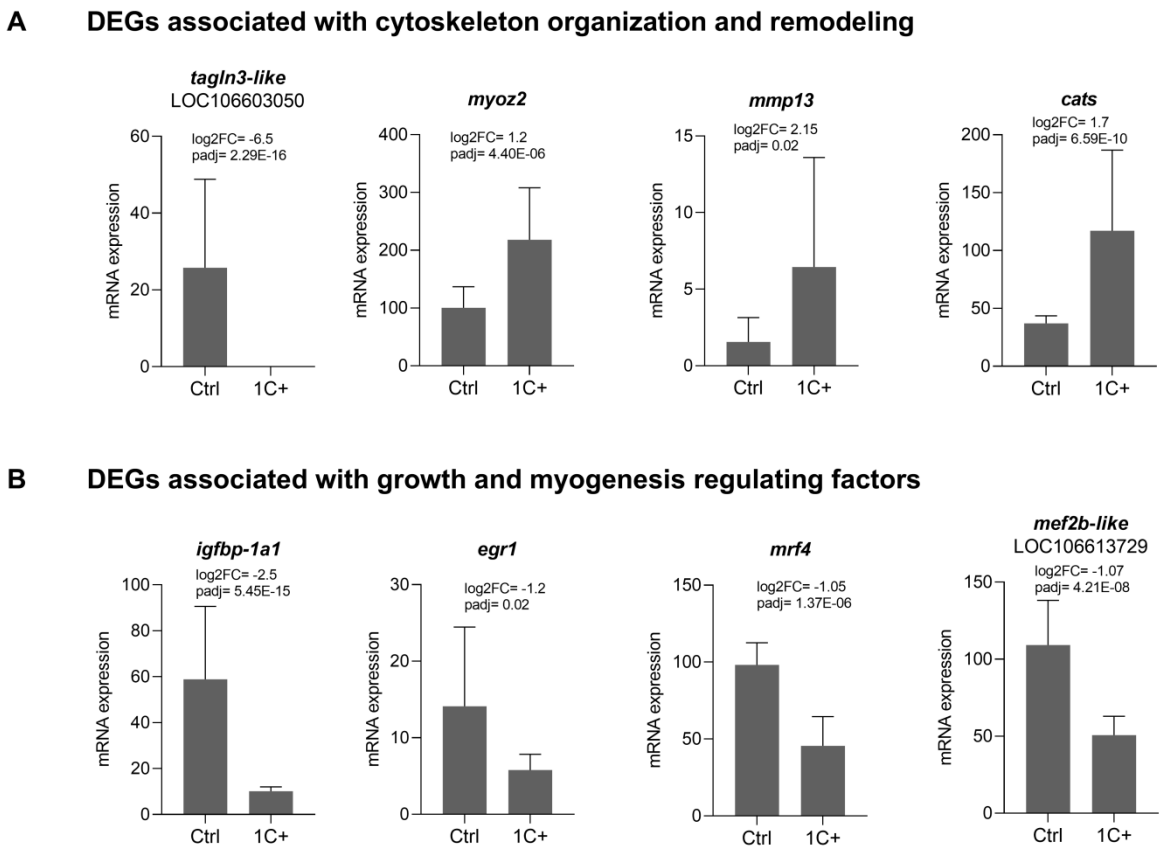

Figure 6. mRNA expression of selected differentially expressed genes (DEGs) that encode myofibrillar proteins and proteins regulating cytoskeletal organization (A), and genes associated with growth and myogenesis regulating factors (B) between 1C+ and Ctrl muscle from post-smolts. Bar graphs show the group mean (n=9) of normalized mRNA read counts detected by RNA-seq. **(A)** *tagln3-like*: transgelin-3-like, *myoz2*: myozenin 2, *mmp13*: collagenase 3, *cats*: cathepsin S; **(B)** *igfbp-1a1*: insulin-like growth factor binding protein 1 paralog A1, *egr1*: early growth response 1, *mrf4*: myogenic regulatory factor 4, *mef2b-like*: myocyte-specific enhancer factor 2B-like. Log2FC: log2-fold change; padj: adjusted p-value (DESeq2 output).

Table 1. Analyzed proximate composition and 1C nutrient levels in Ctrl and 1C+ feed. Re-used and modified table from Espe et al. [7]. Feed composition is provided in Table S1 in the supplementary information.

		3 mm		4 mm	
	Base†	**Ctrl**	**1C+**	**Ctrl**	**1C+**
Analyzed composition					
Crude protein (g/kg)	436.7[a]	-	-	-	-
Crude lipid (g/kg)	235[b]	-	-	-	-
Dry matter (g/kg)	920	-	-	-	-
Energy (MJ/kg)	22.8[c]	-	-	-	-
Vitamin B12 (mg/kg)		0.16	0.18	0.15	0.16
Folate (mg/kg)		2.90	4.80	2.60	4.60
Vitamin B6 (mg/kg)		6.75	8.45	7.01	9.31
Methionine (g/kg)		6.7	9.2	6.7	9.5

†base values indicate that all values in the same row are equivalent/similar across the feeds and the pellet sizes.

[abc] values are not equivalent but similar as determined by their relatively small SDs: ±5.2, ±5.5 and ±0.18, respectively.

Table 2. Selected genes differentially expressed with diet in both pre-smolt and post-smolt muscle. List of all overlapping genes is provided in Table S7. Log2FC, log2-fold change. Adjusted p, adjusted p-value (output of DESeq2).

Abbreviation	Entrez ID	Gene description	Pre-smolt muscle		Post-smolt muscle	
			Log2FC	Adjusted p	Log2FC	Adjusted p
frim *	106600949	ferritin, middle subunit	-1.6	3.0e-05	-2.28	3.5e-11
frim-like *	106600950	ferritin, middle subunit-like	-1.49	4.8e-03	-2.16	2.0e-06
gla	100380380	galactosidase alpha	-1.57	3.0e-03	-3.68	3.4e-61
gbp-1-like *	106582240	interferon-induced guanylate-binding protein 1-like	-2.67	1.1e-03	-1.41	1.1e-04
gbp-1-like *	106582242	interferon-induced guanylate-binding protein 1-like	-2.45	8.6e-03	-1.34	4.4e-05
gbp-1-like *	106582237	interferon-induced guanylate-binding protein 1-like	-2.21	2.3e-04	-3.54	1.5e-77
arhgap12-like *	106566136	rho GTPase-activating protein 12-like	-1.1	1.1e-03	-1.34	8.3e-07
arhgap30-like *	106605587	rho GTPase-activating protein 33-like	-1.06	4.5e-02	1.31	3.3e-05
mat2a *	106563191	S-adenosylmethionine synthase isoform type-2	-1.57	1.3e-15	-1.57	6.3e-25
mat2a-like *	106580346	S-adenosylmethionine synthase isoform type-2-like	-1.64	1.9e-06	-1.99	6.8e-20
slc6a9-like *	106599379	sodium- and chloride-dependent glycine transporter 1-like	-1.19	8.0e-03	-1.81	3.6e-14
slc38a3-like *	106572384	sodium-coupled neutral amino acid transporter 3-like	-1.45	4.7e-02	-3.42	1.1e-45
slc6a15-like *	106573109	sodium-dependent neutral amino acid transporter B(0)AT2-like	-2.33	1.5e-02	-4.54	2.3e-34
slc6a15-like *	106561596	sodium-dependent neutral amino acid transporter B(0)AT2-like	-1.8	4.8e-02	-5.39	7.3e-28

* Genes that received a human orthologue name when an official gene symbol was not available for the Atlantic salmon reference genome.

Table 3. Selected differentially expressed genes (DEGs) between 1C+ and Ctrl post-smolt muscle categorized after their functional roles or pathway affiliation. Table S7 provides the full list of DEGs between diet groups in post-smolts. Log2FC, log2-fold change. Adjusted p, adjusted p-value (output of DESeq2).

Abbreviation	Entrez ID	Gene description	log2FC	Adjusted p
Folate cycle				
mthfd1l	100306829	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1 like	-1.79	2.7e-14
shmt2	106566120	serine hydroxymethyltransferase 2	-1.55	8.8e-04
mthfd2-like *	106585270	bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase, mitochondrial-like	-1.51	1.7e-25
aldh1l2-like *	106609325	mitochondrial 10-formyltetrahydrofolate dehydrogenase-like	-1.16	9.9e-10
aldh1l2-like *	106576152	mitochondrial 10-formyltetrahydrofolate dehydrogenase-like	-1.15	2.8e-18
mtdc-like *	106580352	bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase, mitochondrial-like	-1.1	3.9e-21
Methionine metabolism				
mat1a-like *	106613069	S-adenosylmethionine synthase isoform type-1-like	-5.17	7.7e-09
mat2a *	106563191	S-adenosylmethionine synthase isoform type-2	-1.57	6.3e-25
mat2a-like *	106580346	S-adenosylmethionine synthase isoform type-2-like	-1.99	6.8e-20
mat2a-like *	106585265	S-adenosylmethionine synthase isoform type-2-like	-1.24	1.3e-10
Transsulfuration pathway				

cbs-like *	106582148	cystathionine beta-synthase-like	-1.21	4.4e-03
cgl	100194964	cystathionine gamma-lyase	-1.74	5.4e-44
Transmethylation reactions				
peam3	100380594	phosphoethanolamine N-methyltransferase 3	-5.02	4.7e-39
Polyamine metabolism and ferroptosis				
sat2	100380620	spermidine/spermine N1-acetyltransferase family member 2	-1.27	2.9e-21
sat2-like *	106608759	diamine acetyltransferase 2-like	-2.38	2.2e-07
sat1-like *	106563588	diamine acetyltransferase 1-like	-1.74	4.5e-20
Arginine and proline metabolism				
pycr1-like *	106606436	pyrroline-5-carboxylate reductase 1, mitochondrial-like	-1.34	1.0e-32
glna	100196183	glutamine synthetase	-2.93	2.3e-14
p5cs-like *	106579029	delta-1-pyrroline-5-carboxylate synthase-like	-2.13	8.5e-28
p5cs-like *	106589322	delta-1-pyrroline-5-carboxylate synthase-like	-2.35	1.7e-68
cps1	106586447	carbamoyl-phosphate synthase 1	-1.79	1.9e-27
sirt5-like *	106560408	NAD-dependent protein deacylase sirtuin-5, mitochondrial-like	-3.65	1.0e-03
otc	106586678	ornithine carbamoyltransferase	-1.94	8.6e-29

arg2	106599105	arginase 2	-1.33	1.0e-16
Glutathione metabolization and redox balance				
gstp1 *	100136484	glutathione S-transferase P	-1.69	3.1e-21
chac1-like *	106611099	glutathione-specific gamma-glutamylcyclotransferase 1-like	-1.3	7.8e-03
mgst3-like *	106600407	microsomal glutathione S-transferase 3-like	-2.6	7.5e-03
osgin1-like *	106565048	oxidative stress-induced growth inhibitor 2-like	-1.02	5.7e-07

* Genes that received a human orthologue name when an official gene symbol was not available for the Atlantic salmon reference genome.