Modulation of hepatic miRNA expression in Atlantic salmon (*Salmo salar*) by family background and dietary fatty acid composition

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Abstract
This study finds significant differences in hepatic fatty acid composition between four groups of Atlantic salmon (*Salmo salar*) consisting of offspring from families selected for high and low capacities to express the delta 6 desaturase isomer b and fed diets with 10% or 75% fish oil. The results demonstrated that hepatic lipid metabolism was affected by experimental conditions (diet/family). The fatty acid composition in the four groups mirrored the differences in dietary composition, but it was also associated with the family groups. Small RNA sequencing followed by RT-qPCR identified 12 differentially expressed microRNAs (DE miRNAs), with expression associated with family groups (miR-146 family members, miR-200b, miR-214, miR-221, miR-125, miR-135, miR-137, miR_nov_1), diets (miR-203, miR-462) or both conditions. All the conserved DE miRNAs have been reported as associated with lipid metabolism in other vertebrates. *In silico* predictions revealed 37 lipid metabolism pathway genes, including desaturases, transcription factors and key enzymes in the synthesis pathways as putative targets (e.g., srebp-1 and 2, Δ6fad_b and c, hmdh, elovl4 and 5b, cdc42). RT-qPCR analysis of selected target genes showed expression changes that were associated with diet and with family groups (d5fad, d6fad_a, srebp-1). There was a reciprocal difference in the abundance of ssa-miR-203a-3p and srebp-1 in one group comparison, whereas other predicted targets did not reveal any evidence of being negatively regulated by degradation. More experimental studies are needed to validate and fully understand the predicted interactions and how the DE miRNAs may participate in the regulation of hepatic lipid metabolism.

KEYWORDS
Atlantic salmon, diet, lipid metabolism, miRNA, small-RNA sequencing

1 | INTRODUCTION

MicroRNAs (miRNAs) are short RNA molecules, typically 21–24 nucleotides in length, that regulate gene expression as part of the miRNA-induced silencing complex (miRISC). The function of the miRNA is to guide the miRISC to the target transcripts by partial base pairing between the miRNA and the target mRNA [usually the 3'UTR (untranslated region)]. The binding of the miRISC to an mRNA results

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in the degradation of the target gene transcript or inhibition of its translation. This leads to a post-transcriptional downregulation of the target gene protein (Chekulaeva & Filipowicz, 2009; Hauser & Zavolan, 2014; Krol et al., 2010). Research over the past decade has revealed several hundreds of miRNA genes, each of which has the potential to target several target transcripts. Thus, miRNAs control the expression of a large number of genes and seem to be the major post-transcriptional regulators of cellular gene networks in vertebrates (Friedman et al., 2009). Studies in teleost fish have shown that miRNAs participate in the regulation of early development, apoptosis, the maintenance of tissue-specific functions, reproduction and immune response (Andresen & Hoyheim, 2017; Bizuayehu & Babiak, 2014; Chen et al., 2019). Studies on miRNAs in commercially important fish species have indicated that they also participate in the regulation of economically interesting traits like growth or food conversion (Andresen et al., 2016; Menningen, 2016).

The long-chain polyunsaturated omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are essential nutrients for the health of both Atlantic salmon (Salmo salar) and humans. These fatty acids exert a range of health benefits through their molecular, cellular and physiological actions (Bou et al., 2017a; Bou et al., 2017b; Calder, 2014). EPA and DHA are synthesized from the essential fatty acid 18:3n-3 in a cascade of reactions consisting of elongation (catalysed by elongase2 and elongase5), desaturation (by delta 5 desaturase and delta 6 desaturase) and a final peroxisomal beta-oxidation step (by acetyl co-A oxidase) (Sprecher, 2000).

Factors such as diets, life stage, genotype and growth are known to influence the capacity for EPA and DHA syntheses in Atlantic salmon (Tocher et al., 2000; Rosenlund et al., 2001; Bell et al., 2002; Torstensen et al., 2005; Leaver et al., 2011; Thomassen et al., 2012). The dietary content of marine oil and vegetable oil will affect both the deposition of fatty acids in the fish liver and muscle and the synthesis of the fatty acids EPA and DHA. Marine fish oils typically contain high levels of EPA and DHA, which are devoid of vegetable oils. Vegetable oils, on the contrary, are usually high in linoleic acid (18:2n-6) and monounsaturated fatty acids such as 18:1n-9. Specific fatty acids of the dietary oil fraction can act inhibitory or stimulatory on the synthesis of EPA and DHA in Atlantic salmon. Feeding salmon high-dietary levels of EPA and DHA results, e.g., in downregulation of gene expression of desaturases and elongases involved in omega-3 synthesis, whereas high dietary levels of vegetable oil and low levels of EPA and DHA result in the upregulation of the expression (Kjaer et al., 2008; Morais et al., 2009; Moya-Falon et al., 2005; Ruyter et al., 2003). The genetic background is also important for the EPA and DHA composition in salmon, and the DHA content of salmon fillet was recently identified as a highly heritable trait (h = 0.46) (Horn et al., 2018). Nonetheless, the correlation between the liver and muscle content of EPA and DHA seems to be low (Horn et al., 2019), indicating that the capacity for EPA and DHA syntheses in liver is less important for the levels of these fatty acids in the muscle. Various genes are involved in hepatic lipid metabolism (Morais et al., 2011; Torstensen et al., 2009). Many of these lipogenic genes are regulated by the transcription factors, sterol regulatory element binding proteins (SREBP) srebp1 and srebp2 (Minghetti et al., 2011). Srebp1 plays a crucial role in the regulation of fatty acid biosynthesis, whereas cholesterol biosynthesis is regulated by Srebp2. Several other genes and gene networks involved in lipid metabolism have been identified in transcriptome studies of salmon, and a connection between fatty acid accumulation, dietary lipid content and immune response has been revealed (Martinez-Rubio et al., 2012; Martinez-Rubio et al., 2013; Skugor et al., 2010).

Characterizations of miRNAs associated with lipid metabolism in teleost fish have been carried out by manipulation of dietary lipids in tilapia, rabbit fish and rainbow trout (Mennigen et al., 2014; Tao et al., 2017; Zhang et al., 2014). These studies have revealed smaller groups of differentially expressed miRNAs (DE miRNAs) likely to be involved in the regulation of lipid metabolism. Genes that might be the target transcripts were identified in a few cases and shown to be among the key genes in lipid metabolism gene networks. The miRNAome is well characterized in Atlantic salmon (Andresen et al., 2013; Woldemariam et al., 2019), and several miRNAs that respond to viral infection and that are likely to regulate inflammatory response have been identified (Andresen et al., 2017; Woldemariam et al., 2020). Nonetheless, to authors’ knowledge, there are no studies of miRNAs and their putative regulatory roles in the salmon lipid metabolism. The molecular mechanism leading to the reported dietary effect from EPA and DHA on expression of genes in the omega-3 synthesis pathway (Kjaer et al., 2008; Morais et al., 2009; Moya-Falon et al., 2005; Ruyter et al., 2003) could, e.g., involve post-transcriptional regulation by miRNAs.

This study investigates how a diet that was either rich in fish oil and low in rapeseed oil (75FO) or low in fish oil and rich in rapeseed oil (10FO) affects the hepatic fatty acid composition in two family groups of Atlantic salmon with different capacities to produce EPA and DHA (HIGH and LOW family groups). The individual miRNA expression in the HIGH and LOW family groups fed either of the two diets (75FO/HIGH, 75FO/LOW, 10FO/HIGH or 10FO/LOW) was subsequently revealed. Comparisons between the four diet/family groups could then uncover whether a difference in dietary fatty acids (diet) and/or a difference in family background was associated with the different expression of individual miRNAs. The identification of DE miRNAs was carried out applying small-RNA sequencing followed by RT-qPCR. The small-RNA Illumina sequencing and differential expression analysis of a smaller number of samples selected from all four groups was carried out to allow for identification of any Atlantic salmon miRNAs with putative regulatory functions in the lipid metabolism pathways. The miRNAs identified by this approach were subsequently validated as differentially expressed by additional RT-qPCR analysis in the larger complete materials. Even if not pointed out as putative DE miRNAs by the small-RNA sequencing, a small number of miRNAs known to have important roles in lipid metabolism in other vertebrates were also analysed by RT-qPCR in the larger complete materials to confirm the negative results in the small-RNA-sequenced materials. The putative target genes of the DE miRNAs were predicted, and some of these predicted targets were further analysed using RT-qPCR. Finally, the expression changes in the DE miRNAs and their predicted targets were compared to further elucidate the role(s) of individual miRNAs as regulators of lipid metabolism in Atlantic salmon.
2 | MATERIALS AND METHODS

2.1 | Family fish groups and feeding trial

The feeding trial was carried out at Nofima Research Station for Sustainable Aquaculture (Sunndalsøra, Norway). The experimental fish were from a Norwegian Research Council project (Towards a sustainable salmonid aquaculture – Salmon as a net producer of n-3 fatty acids). In this project, 100 families of Atlantic salmon (S. salar L.) from SalmoBreed AS Elite stock were tested for their expression of the Δ6 desaturase isomer Δ6fad_b, and families with average high expression (HIGH) and low expression (LOW) were used as parental individuals to produce new families as described in Berge et al. (2015). The materials used in this study are progeny (first-generation fish) from crosses within the HIGH families (HIGH family group) and within the LOW families (LOW family group). HIGH and LOW groups with 150–204 fish per group were included in the Norwegian Research Council project, and 18 individuals from the HIGH family group and 18 individuals from the LOW family group were included in this study. The fish were fed a diet with either 10% (10FO) or 75% (75FO) of the oil fraction for 17 weeks and increased weight from 76 ± 14 to 525 ± 75 g. All fish were individually tagged (PIT tags, passive integrated transponder, Biosonic, Seattle, USA), and fish from both family groups were equally distributed in triplicate tanks for each of the two diets, in total 12 tanks. At the end of the feeding trial, the fish were anaesthetized, and livers from nine fish per diet and family group (three fish per tank) were snap frozen in liquid nitrogen and stored at −80°C until further analysis. The fish materials, thus, consisted of four groups divided by their family background and diet: (a) 10FO/LOW, (b) 10FO/HIGH, (c) 75FO/LOW and (d) 75FO/HIGH.

The trial was performed in accordance with the national regulations for the use of animals in experiments (Ministry of Agriculture and Food, 2015). The experiment was classified as not requiring a specific licence (Commission, 2010) because the experiment was not expected to cause any distress or discomfort to the fish.

2.2 | Fatty acid composition of the diets 10FO and 75FO

The diets were formulated to contain two different levels of fish oil, 10% fish oil (10FO) or 75% (75FO) of the oil fraction. Table 1 provides the chemical composition, whereas Table 2 provides the fatty acid composition of the two diets. The resulting fatty acid composition of the 75FO diets thereby contained a higher percentage of EPA (C20:5n-3), docosapentaenoic acid (DPA, C22:5n-3) and DHA (C22:6n-3) than the 10FO.

The sum of EPA and DHA in the 75FO was 22.1% (of total fatty acids) compared to 6.9% in the 10FO. The 18:3n-3 was, nonetheless, higher in 10FO (7.5%) than in 75FO (2.8%). The 75FO had a higher percentage of saturated fatty acids (SFA) than 10FO, constituted mainly by 14:0, 16:0 and 18:0. The percentage of monounsaturated fatty acids (MUFA) was also higher in 10FO than in 75FO, resulting mostly from a 2.3 times higher level of 18:1n-9 in the 10FO. The sum of n-6 fatty acids was higher in 10FO compared to 75FO and was dominated by 18:2n-6 (linoleic acid) that was 2.4 times higher in 10FO than in 75FO.

2.3 | Measurements of fatty acid composition of the liver

The fatty acid composition of the 36 liver samples (9 fish per group) was analysed by trans-methylating the lipids re-dissolved in

| TABLE 1  | Chemical composition and EPA + DHA (g per 100) of the diets |
|-----------|-----------------------------------------------------------|
|           | 10FO            | 75FO            |
| Dry matter| 92.9            | 93              |
| Fat       | 27.6            | 27.1            |
| Protein   | 43.3            | 43.9            |
| Ash       | 8               | 8               |
| EPA + DHA (g per 100 g) | 1.7            | 5.4            |

Note. DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid.

| TABLE 2  | Fatty acid composition (% of total fatty acids) of the diets |
|-----------|-----------------------------------------------------------|
| Fatty acid| 10FO            | 75FO            |
| 14:0      | 1.5             | 5.7             |
| 16:0      | 7.3             | 14.1            |
| 18:0      | 2.6             | 3.1             |
| Sum SFAa  | 12.7            | 24.5            |
| 16:1 n-7  | 1.8             | 6.9             |
| 17:1 n-7  | 0.3             | 1.1             |
| 18:1 n-11 | Not detected   | 1.6             |
| 18:1 n-9  | 45.9            | 19.7            |
| 18:1 n-7  | 2.3             | 2.9             |
| 20:1 n-11 | 0.5             | 1.8             |
| 20:1 n-9  | 1.9             | 1.4             |
| 22:1 n-11 | 0.1             | 0.7             |
| Sum MUFAb | 53.4            | 37.5            |
| 18:2 n-6  | 17.3            | 7.1             |
| 20:4 n-6  | 0.2             | 0.8             |
| Sum n-6 PUFAc | 17.7  | 8.4             |
| 18:3 n-3  | 7.5             | 2.8             |
| 20:5 n-3  | 4.2             | 12.9            |
| 22:5 n-3  | 0.4             | 1.5             |
| 22:6 n-3  | 2.7             | 9.2             |
| Sum n-3 PUFAd | 14.9 | 26.8            |
| EPA + DHA | 6.9             | 22.1            |
| Ratio n6/n3 | 2.6            | 0.4             |

Note. DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid.

aIncludes 15:0, 17:0, 20:0, 22:0, 24:0.
bIncludes 14:1n-5, 15:1, 16:1n-9, 16:1n-5, 20:1n-7, 22:1n-7, 22:1n-9, 24:1n-9.
cIncludes 16:2 n-6, 18:3n-6, 20:3n-6, 20:2n-6, 22:4n-6.
dIncludes 16:2n-3, 20:4n-3, 18:4n-3, 20:3n-3.
chloroform using 2,2-dimethoxypropane, methanolic HCl and benzene at room temperature, as described by Mason (Mason & Waller, 1964). The methyl esters of fatty acids were then separated in a gas chromatograph (Hewlett Packard 6890) with a split injector, SGE BPX70 capillary column (length 60 m, internal diameter 0.25 mm and thickness of the film 0.25 μm), flame ionization detector and HP Chem Station software. The carrier gas was helium. The injector and detector temperatures were 300°C. The oven temperature was raised from 50 to 170°C at a rate of 4°C min⁻¹ and thereafter raised to 200°C at a rate of 0.5°C min⁻¹ and finally to 300°C at a rate of 10°C min⁻¹. The relative quantity of each fatty acid was determined by measuring the area under the peak in the gas chromatograph spectrum corresponding to the specific fatty acids.

2.4 | Isolation of RNA

RNA was isolated from 36 salmon liver samples (9 fish per group) using mirVana miRNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Concentration and purity were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the integrity of the RNA using an Agilent 2100 bioanalyzer/Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA). The 36 samples showed concentrations of total RNA ranging from 324 to 1200 ng μl⁻¹. All samples showed 260/280 ratios of 2.0 or more. Thirty-one samples showed 260/230 ratios above 2.0, whereas the remaining five samples ranged from 0.95 to 1.76. Downstream analysis (RT-qPCR) did not reveal any of these five samples as outliers within their groups. The RNA concentrations as well as quality measurements for all samples are provided in Supporting File S1.

2.5 | cDNA synthesis and miRNA expression measurements using RT-qPCR

The miScript assays were used for cDNA synthesis and qPCR as described by the manufacturer (Qiagen, Hilden, Germany). In the cDNA synthesis, 200 ng of total RNA was reverse transcribed utilizing the miScript II RT kit. The procedure was performed according to the manufacturer's protocol. The reaction mixture consisted of 4 μl of 5x HiSpec Buffer, 2 μl of 10x Nucleics Mix, 2 μl of Reverse Transcriptase Mix, a variable amount of RNase free water and template RNA to a total volume of 20 μl for each reaction. Incubation of the RT reactions was carried out in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) at 37°C for 60 min followed by an inactivation step at 95°C for 5 min and finally holding it to cool down at 4°C. All samples were placed on ice, then instantly diluted with 200 μl of RNase free water and stored at -20°C. A universal primer (reverse primer), provided with the miScript qPCR kit, was used in combination with the authors' custom-designed forward primer in the qPCR assays amplifying each of the mature miRNAs. The sequence of the mature miRNAs investigated in this study was utilized to produce miRNA-specific forward primers. All primers were purchased from Sigma-Aldrich (Darmstadt, Germany), purified by desalt only and provided as a liquid solution of 100 μM from the manufacturer. They were diluted to 10 μM for use in each of the qPCR assays. The qPCR analysis was run on an Mx3000p (Stratagene, San Diego, CA, USA). The qPCR reaction mixture consisted of 12.5 μl 2x Quantitect Syber Green Master Mix, 2.5 μl 10x miScript Universal Primer, 2.5 μl of 10 μM forward miRNA-specific primer, 5 μl of RNase free water and 2.5 μl of cDNA (template). The following programme was used in qPCR: one thermal cycle at 95°C for 15 min followed by 40 cycles at 94°C for 15 s, 55°C for 30 s and 70°C for 30 s. The Mx3000p software package was used for qPCR analysis. The SYBR Green assay module includes a final melting point analysis that follows the 40 cycles of qPCR. Plots from melting point analysis were manually inspected for all the miRNA assays tested to verify that forward primers were specific. The efficiency was calculated using the LinRegPCR software (Ramakers et al., 2003). The primer sequences of all the miRNA-specific primers (forward primers) used for measurements of miRNA expression using RT-qPCR, the efficiencies of the assays and the melting curves are provided in Supporting File S2. The relative change in miRNA expression between groups was calculated using efficiency-adjusted Ct values and the comparative Ct method (ΔΔCt method) (Schmittgen & Livak, 2008). The normalized read counts from the small-RNA-sequenced samples were utilized to compare the stability of ssa-miR-25-3p, ssa-miR-92a-3p, ssa-miR-181a-3p, ssa-miR-455-5p, ssa-miR-107-3p and ssa-miR-17-5p across all groups, an approach similar to the one applied in Johansen and Andreassen (2014) to select candidate reference miRNAs. They all showed high stability across all groups, and three of these miRNAs (ssa-miR-25-3p, ssa-miR-92a-3p and ssa-miR-181a-3p) were analysed in all samples using RT-qPCR. The following normfinder analysis carried out as described in Johansen and Andreassen (2014) showed that ssa-miR-25-3p and ssa-miR-92a-3p were the best reference gene combination with a combined stability value of 0.002. These two miRNAs were used as reference genes in the miRNA expression analysis.

Nine individuals from each group, i.e., 36 individuals, were analysed using RT-qPCR. Based on the findings from the expression analysis of the small-RNA-sequenced samples (DESeq2 analysis, see Section 2.6), the miRNAs ssa-miR-146a-5p, ssa-miR-146a-3p, ssa-miR-146a-3p, ssa-miR-462b-5p, ssa-miR-462b-5p, ssa-miR-203a-3p, ssa-miR-203a-3p, ssa-miR-214-5p, ssa-miR-214-5p, ssa-miR-122-5p, ssa-miR-122-5p, ssa-miR-145-3p, ssa-miR-145-3p, ssa-miR-10b-5p, ssa-miR-10b-5p, ssa-miR-192a-5p, ssa-miR-192a-5p were analysed using RT-qPCR in the complete materials to validate that they were DE miRNAs. Some miRNAs have been pointed out by studies in other vertebrates as associated with lipid metabolism. Although these were not significantly different in the DESeq2 analysis, they were included in the RT-qPCR analysis and investigated in a larger sample to rule out the possibility of type II errors (false positives) in the DESeq2 analysis. These miRNAs were ssa-miR-10b-5p, ssa-miR-10b-5p, ssa-miR-15a-5p, ssa-miR-15a-5p, ssa-miR-17-5p, ssa-miR-17-5p, ssa-miR-21a-5p, ssa-miR-21a-5p, ssa-miR-214-5p, ssa-miR-214-5p, ssa-miR-27a-3p, ssa-miR-27b-3p, ssa-miR-30c-5p, ssa-miR-30e-5p, ssa-miR-30e-5p, ssa-miR-33a-3p, ssa-miR-33a-3p, ssa-miR-122-5p, ssa-miR-122-5p, ssa-miR-143-3p, ssa-miR-143-3p, ssa-miR-145-3p and ssa-miR-192a-5p (Ahn et al., 2013; Casas-Agustench...
et al., 2015; Chen et al., 2014; Fernandez-Hernando, 2013; Karbiener et al., 2014; Mennigen et al., 2014; Sala et al., 2014; Shin et al., 2014; Smolle & Haybaek, 2014; Soh et al., 2013; Sun et al., 2015; Yang et al., 2015; Zhang et al., 2014). One miRNA, ssa-miR-nov-1-3p, showed a significant differential expression in the DESeq2 analysis, but the RT-qPCR assays developed for this miRNA did not pass the performance criteria (specificity, efficiency). Thus, this miRNA was not further analysed.

2.6 | Small-RNA sequencing and expression analysis (DESeq2)

The library construction was performed at the Norwegian Genomics Consortium’s genomics core facility. The Illumina NEBnext Small RNA Library Preparation Kit (New England Biolabs, Inc., Ipswich, MA, USA) was used for the library preparations as described by the manufacturer with 1 μg of total RNA input. After adapter ligation and cDNA synthesis the products were purified on a gel, and the fractions between 145 and 160 bp were used for sequencing. Twelve small-RNA libraries were constructed from 12 samples (3 samples from each of the 4 groups: 10FO/LOW, 10FO/HIGH, 75FO/LOW and 75FO/HIGH). The libraries were successfully subjected to high-throughput sequencing using Illumina Genome Analyser IIx sequencing platform as described in Andreassen et al. (2017). FastQC toolkit was used to assess reads quality (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Cutadapt (Martin, 2011) was used for trimming of adapter sequences from raw sequence reads and for removing adapter-only sequences (5'TGGAATTCTCGGGTGCCAAGGAACTCCAGTCAC 3'). Finally, an additional filtering of reads outside of the 18–25 nucleotide range was applied on all samples.

Next, the reads from each of the 12 samples were aligned to a reference miRNAome that consisted of all known S. salar mature miRNAs (Andreassen et al., 2013; Woldemariam et al., 2019). The reads mapping with edit distance 1 or less to the mature reference sequences were counted. DE miRNAs were identified using the DESeq2 package (Anders & Huber, 2010). Rows with fewer than two reads for each condition were discarded from the analysis. The four groups were compared, and a threshold of P-adjusted value <0.1 (adjusted according to Benjamini–Hochberg procedure) was applied to report the putative DE miRNAs.

2.7 | In silico predictions of target transcripts

Target gene predictions were carried out using RNAhybrid. The analysis was performed with conditions of helix constraint 2–8 and no G:U in seed, allowing only target genes that had perfect “seed” matches to be detected (Rehmsmeier et al., 2004). The minimum free energy threshold for RNA hybrids was set to −18 kcal mol⁻¹ to retrieve results (target site matches) from RNA hybrids that had a high stability (Peterson et al., 2014). The 3'UTR sequences from all S. salar transcripts in Genbank (Non-redundant mRNA, NM entries in RefSeq, NCBI) were used as an input in the in silico analysis.

Gene ontology (GO) annotations for the predicted target genes (biological process and molecular function) were received for each of the target genes using UniProt database (http://www.uniprot.org/uploadlists/). These GO annotations were used to identify the sub-set of predicted targets that were associated with lipid metabolism.

2.8 | Gene expression analysis of predicted target genes

cDNA was synthesized from 1000 ng of total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems) in a 20 μl reaction. The reaction mixture consisted of 1x TaqMan RT Buffer, 1.75 mM MgCl₂, 0.5 mM deoxynucleotide triphosphate mixture, 2.5 μM Oligo d(T)₁₅, 1 U μl⁻¹ RNase inhibitor and 2.5 U μl⁻¹ Reverse Transcriptase. The reaction was run in a Veriti 96-well thermal cycler (Thermo Fisher Scientific) with the following conditions: 25°C for 10 min, 48°C for 60 min and 95°C for 5 min. The PCR mixture consisted of 4 μl of cDNA (1:10 dilution), 5 μl of LightCycler480 SYBR Green I Master (Roche Diagnostics Gmbh, Mannheim, Germany) and 0.5 μM forward and reverse primers (Thermo Fisher Scientific). The reaction was run in a LightCycler480 (Roche Diagnostics GmbH) with the following conditions: 95°C for 5 min, 45 cycles of 95°C for 15 s and 60°C for 1 min. A melting curve analysis (95°C for 5 s, 65°C for 1 min, increasing temperature up to 97°C and finally cooling down) was performed to confirm the presence of only one amplified product (see Supporting File S3 for pictures of melting curve analysis and references). The stability of the reference genes (ef1α, rpo2 and elf3) was evaluated using RefFinder (https://github.com/fuxie/RefFinder), which integrates the computational programmes geNorm, NormFinder and BestKeeper and the comparative ΔΔCt method. According to the RefFinder results, eukaryotic transcription initiation factor 3 (elf3) was ranked as the most stable reference gene (Supporting File S3). Primer efficiency was evaluated by making a standard curve (consisting of six dilutions of cDNA), and calculation of the amplification efficiency was performed using the software included in LightCycler480 (see Supporting File S3 for primer efficiency and references for primer efficiencies evaluated in previous studies, primer sequences and GenBank accession numbers).

The expression of eight genes from lipid metabolism gene networks was measured in all individuals using RT-qPCR. These were delta 5 fatty acid desaturase (Δ5fad), delta 6 fatty acid desaturase-a (Δ6fad-a), delta 6 fatty acid desaturase-b (Δ6fad-b) and delta 6 fatty acid desaturase-c (Δ6fad-c) that are enzymes involved in the omega-3 fatty acid synthesis pathway; sterol regulatory element binding proteins 1 and 2 (srebp1, srebp2) that are transcription factors; carnitine palmitoyltransferase I (cpt1) and acyl-CoA oxidase 1 (acox) that are involved in fatty acid oxidation. The relative gene expression level was calculated according to the ΔΔΔCt method adjusting for differences in primer efficiency (Pfaffl, 2004).
2.9 Statistical methods applied to test group differences in fatty acid composition, miRNA expression and mRNA gene expression

The effects of diet and genetic background and the interaction between the main factors for fatty acid composition, miRNA expression and gene expression data were assessed using a two-way factorial design. Data were statistically analysed using the general linear model (GLM) procedure in SAS® software (SAS Institute, Cary, NC, USA). The differences in miRNA expression and mRNA gene expression were ranked using Duncan's multiple range test. Statistical analyses were conducted using the software package UNISTAT (Unistat Ltd, London, England).

3 RESULTS

3.1 Hepatic fatty acid composition is affected by diet, genetic background and their interaction

The analysis of the fatty acid composition in liver showed a significant effect of both diets and family background on multiple fatty acids, demonstrating that the experimental conditions had affected the hepatic lipid metabolism in the four groups compared. The hepatic fatty acid composition largely mirrored the diet composition when comparing 10FO and 75FO groups, but comparisons within the same diet conditions showed that there were also significant differences due to family background (LOW/HIGH). The complete results from

| TABLE 3 Hepatic fatty acid composition |
|---------------------------------------|
|                                       |
| 10FO | 75FO | Two-way ANOVA |
| LOW  | HIGH | LOW  | HIGH | Diet | Genetics | Interaction |
| 14:0 | 1.0 ± 0.0 | 1.8 ± 0.3 | 1.0 ± 0.0 | 1.8 ± 0.1 | <0.001 | NS | NS |
| 16:0 | 5.7 ± 0.4 | 11.0 ± 0.4 | 6.6 ± 0.4 | 14.9 ± 0.7 | <0.001 | <0.001 | <0.05 |
| 18:0 | 5.0 ± 0.2 | 6.5 ± 0.1 | 4.8 ± 0.1 | 6.0 ± 0.3 | <0.001 | <0.05 | NS |
| ∑SFA | 12.3 ± 0.5 | 20.1 ± 0.6 | 13.0 ± 0.5 | 23.5 ± 0.5 | <0.001 | <0.05 | <0.05 |
| 16:1-9 | 1.8 ± 0.2 | 3.4 ± 0.2 | 1.5 ± 0.1 | 2.2 ± 0.3 | <0.001 | <0.05 | NS |
| 18:1-9 | 48.3 ± 0.3 | 243 ± 0.5 | 43.9 ± 1.4 | 145.5 ± 1.7 | <0.001 | <0.001 | NS |
| 18:1-7 | 3.5 ± 0.1 | 4.2 ± 0.2 | 3.3 ± 0.1 | 3.1 ± 0.2 | NS | <0.001 | <0.05 |
| 20:1-11 | 0.2 ± 0.0 | 0.6 ± 0.0 | 0.2 ± 0.0 | 0.4 ± 0.1 | <0.001 | <0.05 | <0.05 |
| 20:1-9 | 6.0 ± 0.0 | 28.8 ± 0.1 | 59.2 ± 0.2 | 19.2 ± 0.2 | <0.001 | <0.05 | 0.05 |
| 22:1-7 | 0.6 ± 0.0 | 1.1 ± 0.1 | 0.6 ± 0.0 | 0.8 ± 0.0 | <0.001 | <0.05 | <0.05 |
| 24:1-9 | 0.3 ± 0.0 | 0.9 ± 0.0 | 0.5 ± 0.0 | 1.4 ± 0.1 | <0.001 | <0.0001 | <0.05 |
| ∑MUFA | 61.8 ± 0.2 | 39.0 ± 0.8 | 56.8 ± 1.8 | 25.4 ± 2.4 | <0.001 | <0.0001 | <0.05 |
| 18:2n-6 | 10.2 ± 0.4 | 4.2 ± 0.3 | 10.3 ± 0.2 | 3.1 ± 0.2 | <0.001 | NS | NS |
| 20:2n-6 | 25.0 ± 1.0 | 1.1 ± 0.0 | 2.6 ± 0.0 | 0.9 ± 0.0 | <0.001 | NS | <0.05 |
| 20:3n-6 | 12.0 ± 1.0 | 0.4 ± 0.0 | 12.0 ± 1.0 | 0.4 ± 0.0 | <0.001 | NS | NS |
| 20:4n-6 | 0.6 ± 0.1 | 2.2 ± 0.1 | 0.9 ± 0.1 | 3.2 ± 0.3 | <0.001 | <0.05 | NS |
| ∑n-6 PUFA | 14.9 ± 0.6 | 8.4 ± 0.4 | 15.2 ± 0.2 | 8.0 ± 0.2 | <0.001 | NS | NS |
| 18:3n-3 | 2.5 ± 0.1 | 1.1 ± 0.1 | 2.5 ± 0.1 | 0.8 ± 0.1 | <0.001 | NS | NS |
| 20:3n-3 | 0.6 ± 0.0 | 0.3 ± 0.0 | 0.6 ± 0.0 | 0.3 ± 0.0 | <0.001 | NS | NS |
| 20:5n-3 | 1.3 ± 0.1 | 7.0 ± 0.4 | 1.7 ± 0.1 | 8.6 ± 0.3 | <0.001 | <0.05 | NS |
| 22:5n-3 | 0.3 ± 0.0 | 2.6 ± 0.1 | 0.4 ± 0.0 | 2.5 ± 0.1 | <0.001 | NS | NS |
| 22:6n-3 | 4.4 ± 0.2 | 18.2 ± 0.6 | 7.9 ± 1.1 | 27.8 ± 1.6 | <0.001 | <0.001 | <0.05 |
| ∑n-3 PUFA | 9.1 ± 0.2 | 29.3 ± 0.9 | 13.1 ± 1.2 | 39.9 ± 1.8 | <0.001 | <0.001 | <0.05 |
| EPA + DHA | 5.7 ± 0.2 | 25.2 ± 0.9 | 9.6 ± 1.3 | 36.4 ± 1.9 | <0.001 | <0.001 | <0.05 |
| Ratio n-6/n-3 | 5.7 ± 0.2 | 25.2 ± 0.9 | 9.6 ± 1.3 | 36.4 ± 1.9 | <0.001 | <0.001 | <0.05 |

Note. Fatty acid composition in liver (% of total fatty acids) of salmon fed 10FO or 75FO diets in LOW and HIGH family groups. The two-way ANOVA statistics are shown with P-values for the effect of diets (10FO and 75FO), family groups (LOW and HIGH family groups) and the interaction between diet and family groups. NS: not significant (P > 0.05). Data are shown as mean ± S.E. (n = 3).

DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid.

aIncludes 15:0, 17:0, 20:0, 22:0, 24:0.
bIncludes 14:1n-5, 16:1n-7, 16:1n-5, 17:1n-7, 19:1, 20:1n-7, 22:1n-11, 22:1n-9.
cIncludes 16:2n-6, 18:3n-6.
dIncludes 20:4n-3.
measurements of fatty acid composition along with results from two-way ANOVA tests for significant differences are provided in Table 3. The relative liver composition of DHA, the sum of the polyunsaturated n-3 fatty acids (PUFA) and the sum of EPA and DHA, was significantly affected by diets, family groups and their interaction. The relative EPA levels (20:5n-3) were 1.2–1.3 times higher in the HIGH groups than in the LOW groups showing EPA levels of 1.7% (10FO/HIGH) and 8.6% (75FO/HIGH). The relative DHA levels were 1.5–1.8 times higher in the HIGH groups than in the LOW groups with DHA levels of 7.9% (10FO/HIGH) and 27.8% (75FO/HIGH). Nevertheless, the increased content of fish oil in the diet of the 75FO groups resulted in a much higher percentage of PUFA (including EPA and DHA) in the 75FO group than in the 10FO group (Table 3).

The diet, family background and their interaction also affected the relative level of the sum of SFAs ($\Sigma$SFA, Table 3), with a higher percentage in the 75FO group (20.1%–23.5%) than in the 10FO group (12.3%–13.0%) and in the HIGH group compared to the LOW group within the 75FO group.

The percentage of MUFAs ($\Sigma$MUFA, Table 3) was affected by both diets, family groups and their interaction, with the 10FO groups (56.8% and 61.8%) showing 1.6–2.2 times higher level than the 75FO groups (39.0% and 25.4%). The HIGH groups had a significantly lower relative level of MUFAs compared to the LOW groups (0.7–0.9 times). Within the 75FO group, the HIGH group had a lower liver level of several fatty acids such as 16:1n-9, 18:1n-9, 18:1n-7 and 20:1n-9 than the LOW group.

3.2 | Small-RNA sequencing followed by RT-qPCR analysis identified 12 DE miRNAs

Small-RNA sequencing followed by RT-qPCR analysis was applied to identify DE miRNAs when comparing the salmon groups fed 10FO or 75FO and the two family groups HIGH and LOW. Three samples from each group were small-RNA sequenced. The descriptive data from the 12 samples used in the small-RNA sequencing along with the results from FASTQC analysis are provided in Supporting File S4. All samples were successfully sequenced with per base Phred quality scores of 32 or more. The percentage of reads mapped as miRNAs in the size-filtered and adapter-processed samples ranged from 83.1% to 87.5%. The expression of all the known salmon miRNAs was investigated by differential expression analysis (DESeq2) (Supporting File S5). The results from DESeq2 analysis pointed out 14 putative DE miRNAs. Thirteen of these were further analysed by RT-qPCR, whereas one, ssa-miR-nov1-3p, was not further validated as the RT-qPCR assay failed.

Two-way ANOVA analysis of the results from RT-qPCR revealed that 12 of the 13 miRNAs investigated (ssa-miR146a-3p, ssa-miR-146a-5p, ssa-miR-146b-5p, ssa-miR-200b-5p, ssa-miR-203a-3p, ssa-miR-203a-5p, ssa-miR-214-5p, ssa-miR-221-5p, ssa-miR-125a-3p, ssa-miR-137a-3p, ssa-miR-135c-5p and ssa-miR-nov1-5p) showed significant differences associated with diet (10FO/75FO), family group (HIGH/LOW) or both. The results from the two-way ANOVA are provided in Table 4.

Despite being associated with lipid metabolism in other vertebrates, 16 miRNAs (ssa-miR-10b-5p, ssa-miR-15a-5p, ssa-miR-17-5p, ssa-miR-21a-5p, ssa-miR-26a-5p, ssa-miR-27a-5p, ssa-miR-27b-3p, ssa-miR-30c-5p, ssa-miR-30e-5p, ssa-miR-33a-3p, ssa-miR-33b-3p, ssa-miR-92b-3p, ssa-miR-122-5p, ssa-miR-143-3p, ssa-miR-145-3p and ssa-miR-192a-5p) did not reveal any differences when analysed by small-RNA sequencing. Neither was their expression associated with the conditions investigated when analysed by RT-qPCR in the complete materials (Ahn et al., 2013; Casas-Agustench et al., 2015; Chen et al., 2014; Fernandez-Hernando, 2013; Karbiener et al., 2014; Mennigen et al., 2014; Sala et al., 2014; Shin et al., 2014; Smolle & Haybaeck, 2014; Soh et al., 2013; Sun et al., 2015; Yang et al., 2015; Zhang et al., 2014).

The two-way ANOVA analysis (Table 4) showed that the miR-146 family (ssa-miR-146a-3-3p, ssa-miR-146a-3p and ssa-miR-146a-5p) was associated with expression differences between family groups, whereas miR-146a-3-3p and ssa-miR-146a-5p were differentially expressed when comparing the diet groups. The family group comparisons showed a relative decreased expression in the HIGH groups compared to the LOW groups, whereas in the diet comparison there was a relative decrease in expression in the 75FO groups when compared to the 10FO groups. The expression of the two miRNAs ssa-miR-462b-5p and ssa-miR-203a-3p was significantly associated only with diet. ssa-miR-462b-5p showed a relative decrease in the 75FO groups, whereas ssa-miR-203a-3p revealed a relative increase in the same groups.

Six miRNAs showed relative differences associated with only family groups (ssa-miR-200b-5p, ssa-miR-214-5p, ssa-miR-221-5p, ssa-miR-125a-3p, ssa-miR-135c-5p and ssa-miR-137a-3p). Whereas ssa-miR-135c-5p and ssa-miR-137a-3p showed a relative decrease in expression in the HIGH groups when compared to the 10FO groups. The expression of the two miRNAs ssa-miR-462b-5p and ssa-miR-203a-3p was significantly associated only with diet. ssa-miR-462b-5p showed a relative increase in the 75FO groups whereas ssa-miR-203a-3p revealed a relative increase in the same groups.

3.3 | In silico analysis predicted 37 lipid metabolism genes as putative target genes

The putative target genes of the DE miRNAs were predicted using the 3’UTR sequences of all S. salar transcript sequences in GenBank as input (see Section 2). This analysis showed 1069 predicted target genes for the 12 DE miRNAs, ranging from 20 matches by ssa-miR-125a-3p to 167 matches by ssa-miR-203a-3p and ssa-miR-nov1-5p (data not shown).

The UniProt database (GO annotations) was used to identify a sub-set of target genes with functions in the lipid metabolism gene pathways. One of the DE miRNAs, ssa-miR-200b-5p, did not reveal any matches to such lipid metabolism genes. The remaining 11 DE miRNAs showed one or more matches to 37 lipid metabolism genes.
The target genes along with the DE miRNAs predicted to target their 3′UTRs are provided in Table 5. Supporting File S6 also shows GO annotations and GenBank accession numbers.

Acyl-CoA oxidase 1 (acox1) was predicted as target for four DE miRNAs, whereas acyl-CoA synthetase long-chain family member 1 (acsl1), lysosomal acid lipase/cholesteryl ester hydrolase (lich) and acyl-protein thioesterase (miRNAs. Each of nine other genes (including elongation of very long-chain fatty acids-like 4 (elovl4), sterol regulatory element binding transcription factor 1 (srebp-1), and srebp-2, 6desaturase gene (Δ6fad_c), regulation of lipid synthesis as transcription factors (srebp1, srebp2) or lipid degradation (oxidation) (cpt1, acox). The two genes acox and cpt1 did not reveal any significant changes when groups were compared, whereas the effect of diet on Δ6fad_c was on the borderline of significance (P = 0.07). In the remaining five genes, there was at least one group comparison that showed a significant difference. The results from the two-way ANOVA analysis of these genes are summarized in Table 6.

The ANOVA analysis showed a highly significant effect of the diet on the expression of the three genes Δ5fad, Δ6fad_a and Δ6fad_b, with decreased expression in 75FO compared to 10FO. A similar trend towards a dietary effect on gene expression was observed in Δ6fad_c (towards a decrease in 75FO groups). A significant effect of family background (HIGH vs. LOW groups) was also detected on the gene expression of Δ5fad and Δ6fad_a. When the two family groups were fed 75FO, the gene expression of Δ5fad and Δ6fad_a was down-regulated in HIGH compared to LOW.

There was a significant effect of family background on the gene expression of sterol regulatory element binding transcription factor 1 (srebp1) with a relative increase in HIGH compared to LOW and a strong trend towards a dietary effect. The expression of srebp2 was affected only by the diet with a downregulation in fish fed 75FO vs. 10FO in the HIGH group.

The revealed differences in the expression of some of the target genes allowed for a comparison of the abundance of the miRNAs and their predicted targets. Sterol regulatory element binding transcription factor 1 (srebp-1) was predicted as target for ssa-miR-203a-3p. The results showed that there was a relative decrease in srebp-1 in the 75FO diet group (significant in 10FO/HIGH vs. 75FO/LOW, Table 6). In accordance with srebp-1 being negatively regulated (degraded) by an ssa-miR-203a-3p-guided RISC, this miRNA showed a relative

### Table 4: Comparison of miRNA expression in diet and family groups

|          | 10FO LOW | 10FO HIGH | 75FO LOW | 75FO HIGH | Two-way ANOVA |
|----------|----------|-----------|----------|-----------|---------------|
| miR-146a-3-3p | 0.00a ± 0.09 | 0.35b ± 0.20 | 0.42b ± 0.17 | 1.13a ± 0.26 | 0.004 | 0.009 | 0.37 |
| miR-146-a-3p | 0.00a ± 0.16 | 0.58b ± 0.17 | 0.08a ± 0.27 | 0.85a ± 0.14 | 0.37 | 0.001 | 0.60 |
| miR-146a-5p | 0.00a ± 0.16 | 0.49b ± 0.21 | 0.31a ± 0.21 | 1.19a ± 0.14 | 0.01 | 0.0007 | 0.30 |
| miR-462b-5p | 0.00ab ± 0.07 | 0.10b ± 0.14 | 0.30b ± 0.07 | 0.12ab ± 0.10 | 0.02 | 0.17 | 0.71 |
| miR-203a-3p | 0.00a ± 0.17 | 0.18b ± 0.16 | 0.36ab ± 0.07 | 0.34b ± 0.14 | 0.003 | 0.48 | 0.54 |
| miR-221-5p | 0.00a ± 0.17 | 0.36b ± 0.22 | 0.49ab ± 0.15 | 0.17b ± 0.26 | 0.11 | 0.02 | 0.46 |
| miR-214-5p | 0.00a ± 0.22 | 1.25b ± 0.22 | 0.52b ± 0.27 | 0.93b ± 0.24 | 0.09 | <0.0001 | 0.69 |
| miR-222-5p | 0.00a ± 0.13 | 1.08b ± 0.14 | 0.10a ± 0.25 | 0.54ab ± 0.18 | 0.25 | 0.003 | 0.10 |
| miR-125a-3p | 0.00a ± 0.21 | 1.07b ± 0.20 | 0.08a ± 0.36 | 0.94b ± 0.28 | 0.69 | 0.0005 | 0.92 |
| miR-135c-5p | 0.00a ± 0.12 | 0.39b ± 0.40 | 0.13 ± 0.15 | 0.45 ± 0.15 | 0.88 | 0.05 | 0.67 |
| miR-137a-3p | 0.00ab ± 0.58 | −1.58b ± 0.31 | 0.62a ± 0.91 | −1.14a ± 0.25 | 0.36 | 0.007 | 0.88 |
| miR-nov-1-5p | 0.00a ± 0.31 | 1.45b ± 0.31 | −1.66a ± 0.25 | 0.99b ± 0.15 | 0.0003 | <0.0001 | 0.03 |

Note: All changes are relative to the group 10FO/LOW which is set as 0.00. Significant difference in expression between groups is indicated by different letters in superscript.

Diet: P-values for comparisons of diet (10FO vs. 75FO).
Family: P-values for comparisons of family groups selected for LOW or HIGH desaturase activity.
Interaction shows the P-values for combined effect of diet and family selection for desaturase.

### 3.4 Gene expression changes in predicted target genes and miRNA/target comparisons

The seven predicted target genes acox1, cpt1, srebp-1, srebp-2, Δ5fad_b, Δ6fad_c and Δ5fad and the Δ6 desaturase gene Δ6fad_a were further analysed by RT-qPCR in the complete materials. These are key genes involved in omega-3 fatty acid synthesis (Δ5fad, Δ6fad_a, Δ6fad_b, Δ6fad_c), regulation of lipid synthesis as transcription factors (srebp1, srebp2) or lipid degradation (oxidation) (cpt1, acox). The two genes acox and cpt1 did not reveal any significant changes when groups were compared, whereas the effect of diet on Δ6fad_c was on the borderline of significance (P = 0.07). In the remaining five genes, there was at least one group comparison that showed a significant difference. The results from the two-way ANOVA analysis of these genes are summarized in Table 6.

The ANOVA analysis showed a highly significant effect of the diet on the expression of the three genes Δ5fad, Δ6fad_a and Δ6fad_b, with decreased expression in 75FO compared to 10FO. A similar trend towards a dietary effect on gene expression was observed in Δ6fad_c (towards a decrease in 75FO groups). A significant effect of family background (HIGH vs. LOW groups) was also detected on the gene expression of Δ5fad and Δ6fad_a. When the two family groups were fed 75FO, the gene expression of Δ5fad and Δ6fad_a was down-regulated in HIGH compared to LOW.

There was a significant effect of family background on the gene expression of sterol regulatory element binding transcription factor 1 (srebp1) with a relative increase in HIGH compared to LOW and a strong trend towards a dietary effect. The expression of srebp2 was affected only by the diet with a downregulation in fish fed 75FO vs. 10FO in the HIGH group.

The revealed differences in the expression of some of the target genes allowed for a comparison of the abundance of the miRNAs and their predicted targets. Sterol regulatory element binding transcription factor 1 (srebp-1) was predicted as target for ssa-miR-203a-3p. The results showed that there was a relative decrease in srebp-1 in the 75FO diet group (significant in 10FO/HIGH vs. 75FO/LOW, Table 6). In accordance with srebp-1 being negatively regulated (degraded) by an ssa-miR-203a-3p-guided RISC, this miRNA showed a relative
increase in expression when same groups were compared (Table 4). Sterol regulatory element binding transcription factor 2 (srebp2) was predicted as target for ssa-miR-nov-1-5p. Nevertheless, both the miRNA and the predicted target showed expression changes in the same direction (10FO/HIGH vs 75FO/HIGH). Both Δ6fad_b and Δ6fad_c were also predicted as target for ssa-miR-nov-1-5p. These genes showed a lower expression in the 10FO/LOW vs. 75FO/LOW comparisons, which was significant in Δ6fad_b (Table 6). Nevertheless, also in this case the DE miRNA showed a change in the same direction (decrease, Table 4). The gene Δ5fad was predicted as target for ssa-miR-462b-5p, but also in this case, both the gene and the miRNA expression changed in the same direction in group comparisons (Tables 4 and 6). Peroxisomal acyl-coenzyme A oxidase 1 (acox1) and carnitine-o-palmitoyltransferase 1 (cpt1) were also among the predicted targets. Nonetheless, they did not reveal any significant expression changes in their mRNAs (Table 6).

### TABLE 5 Predicted target genes that are part of lipid metabolism gene networks

| Gene                                                                 | miRNA                                                                 |
|----------------------------------------------------------------------|-----------------------------------------------------------------------|
| Peroxisomal acyl-coenzyme A oxidase 1 (acox1)                        | miR-146a-3p or 3-3p, miR-137a-3p, miR-203a-3p                          |
| Acyl-CoA synthetase long-chain family member 1 (acs1)                | miR-146a-3p, miR-146a-3-3p, miR-135c-5p                               |
| Lysosomal acid lipase/cholesteryl ester hydrolase (lich)             | miR-nov-1-5p, miR-135c-5p, miR-137a-3p                               |
| Acyl-protein thioesterase 1 (lypa1)                                   | miR-146a-3p, miR-146a-3-3p, miR-214-5p                               |
| S-Acyl fatty acid synthase thioesterase, medium chain (sost)         | miR-137a-3p, miR-203a-3p                                             |
| Fumarylacetoacetate hydrolase domain containing 1 (fahd1)           | miR-nov-1-5p, miR-221-5p                                             |
| Acyl-coenzyme A thioesterase 5 (acot5)                                | miR-146a-3p, miR-146a-3-3p                                           |
| Very long-chain acyl-CoA synthetase (s27a2)                          | miR-146a-3p, miR-146a-3-3p                                           |
| Retinol-binding protein II, cellular (ret2)                           | miR-146a-5p, miR-135c-5p                                             |
| Apolipoprotein D (apol)                                              | miR-221-5p                                                           |
| 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (hmhd)               | miR-221-5p                                                           |
| Cell division control protein 42 (cdc42)                             | miR-221-5p                                                           |
| Solute carrier family 25 member 28 (slc25a28)                       | miR-221-5p                                                           |
| Sterol regulatory element binding transcription factor 2 (srebp2)    | miR-221-5p                                                           |
| Delta-6 fatty acyl desaturase (d6fad-c)                              | miR-221-5p                                                           |
| Delta-6 fatty acyl desaturase (d6fad-b)                              | miR-221-5p                                                           |
| Delta-5 fatty acyl desaturase (fadsd5)                               | miR-221-5p                                                           |
| Fatty acyl-CoA reductase 1 (facr1)                                    | miR-221-5p                                                           |
| Elongation of very long-chain fatty acids-like 4 (elovl4)            | miR-221-5p                                                           |
| Apolipoprotein F (apolf)                                             | miR-221-5p                                                           |
| Serine incorporator 1 (serc1)                                        | miR-221-5p                                                           |
| ORM1-like protein 1 (orml1)                                          | miR-221-5p                                                           |
| Lipase member H (liph)                                               | miR-221-5p                                                           |
| Acyl-CoA dehydrogenase family member 11 (acad11)                     | miR-214-5p                                                           |
| Sterol regulatory element binding transcription factor 1 (srebp1)    | miR-203a-3p                                                           |
| Lathosterol oxidase (sc5d)                                           | miR-203a-3p                                                           |
| AP-2 complex subunit mu-1 (ap2m1)                                    | miR-203a-3p                                                           |
| Carnitine O-palmitoyltransferase 1 (cpt1)                             | miR-203a-3p                                                           |
| Phospholipid scramblase 2 (pls2)                                     | miR-146a-5p                                                           |
| Phospholipase C delta 4 (plcd4)                                      | miR-146a-3p                                                           |
| StAR-related lipid transfer protein 5 (star5)                        | miR-135c-5p                                                           |
| Polyunsaturated fatty acid elongase elovl5b (LOC100192340)           | miR-135c-5p                                                           |
| Proteinase-activated receptor-2a (par-2)                             | miR-135c-5p                                                           |
| Probable palmitoyltransferase ZDHHC11 (zdh11)                        | miR-135c-5p                                                           |
| Phosphatidylcholine transfer protein (ppct)                           | miR-135c-5p                                                           |
| Lipolysis-stimulated lipoprotein receptor (lsr)                      | miR-125a-3p                                                           |
| Cardiolipin synthase 1 (crls1)                                       | miR-125a-3p                                                           |
TABLE 6 Comparison of hepatic gene expression of predicted target genes

|                | 10FO LOW | 10FO HIGH | 75FO LOW | 75FO HIGH | Two-way ANOVA |
|----------------|----------|-----------|----------|-----------|---------------|
| d5fad          | 0.0 ± 0.1 a | -0.4 ± 0.1 ab | -0.7 ± 0.2 b | -1.3 ± 0.2 c | <0.0001 |
| d6fad a        | 0.0 ± 0.2 a | -0.2 ± 0.2 ab | -0.6 ± 0.2 b | -1.3 ± 0.1 c | <0.0001 |
| d6fad b        | 0.0 ± 0.3 a | 0.1 ± 0.2 a  | -0.8 ± 0.2 b | -1.0 ± 0.3 c | <0.0007 |
| d6fad c        | 0.0 ± 0.1  | 0.0 ± 0.3   | -0.5 ± 0.3  | -0.6 ± 0.4  | 0.07   |
| acox           | 0.0 ± 0.2  | -0.2 ± 0.1  | -0.5 ± 0.2  | -0.2 ± 0.2  | 0.31   |
| cpt1           | 0.0 ± 0.1  | 0.1 ± 0.1   | 0.0 ± 0.1   | 0.0 ± 0.1   | 0.25   |
| srebp-1        | 0.0 ± 0.2 ab| 0.6 ± 0.4 a  | -0.6 ± 0.2 b | 0.0 ± 0.4 ab | 0.08   |
| srebp-2        | 0.0 ± 0.2 ab| 0.1 ± 0.1 a  | -0.3 ± 0.2 ab| -0.5 ± 0.1 b | 0.01   |

Note. All changes are relative to the group 10FO/LOW which is set as 0.0. Significant difference in expression between groups is indicated by different letters in superscript.

Diet: P-values for comparisons of diet (10FO vs. 75FO).
Family: P-values for comparisons of family groups selected for LOW or HIGH desaturase activity.
Interaction shows the P-values for combined effect of diet and family selection for desaturase.

4 | DISCUSSION

4.1 | Diet, family selection, target gene expression and fatty acid composition

The reported feeding trial found that both diets and family background affected the fatty acid composition of the liver. The 75FO diet group showed much higher percentages of EPA and DHA than the 10FO diet group. Nonetheless, comparisons within both dietary groups demonstrated that the family background also contributed to the differences. The two-way ANOVA tests also revealed a significant interaction between family groups and diets, e.g., the level of DHA and EPA + DHA in the liver (Table 3).

A higher inclusion level of fish oil, and therefore EPA and DHA, in the 75FO diet induced downregulation of all the desaturases, except for d6fad_c, compared to the 10FO diet high in rapeseed oil (Table 6). This is in agreement with findings in other studies reporting that there is a reduced synthesis capacity of EPA and DHA and a decreased gene expression of desaturases, elongases and sterol regulatory element binding protein 1 (srebp1) when Atlantic salmon is fed high-dietary levels of fish oils, whereas high-dietary vegetable oil levels resulted in the opposite effect (Kjaer et al., 2008; Morais et al., 2009; Moya-Falon et al., 2005; Ruyter et al., 2003). Even though the 10FO groups had a higher gene expression level of the desaturases than the 75FO groups, the expression differences in srebp1, which regulate desaturase expression, were less affected by diet (P = 0.08) in the present study. Also, despite a selection of parental individuals into HIGH and LOW groups based on d6fad_b expression, the differences in the gene expression that were observed between the two family groups were not significant for this desaturase in the materials. The first-generation offspring did not show any obvious added effect on d6fad_b expression from crossing the parental individuals from families with high-average d6fad_b expression. This could indicate that there is genetic variation in several genes within the parental generation that affects the d6fad_b expression and not necessarily variation in the same genes that led to the average high expression in the different parental HIGH families. On the contrary, both the EPA/DHA percentage and other key genes like d5fad, d6fad_a and srebp-1 did reveal significant differences associated with family background.

Studies have shown that other fatty acids also influence omega-3 synthesis (and desaturase expression). The synthesis capacity for EPA and DHA is partly regulated by the availability of the precursors 18:3n-3. The increased expression of genes of the omega-3 synthesis pathway was, e.g., observed in Atlantic salmon fed a vegetable oil high in 18:3n-3 compared to salmon fed a fish oil low in 18:3n-3 (Gillard et al., 2018). Another fatty acid, 18:1n-9, which is the most abundant fatty acid in rapeseed oil and is found at high levels in the 10FO diet, has also been shown to stimulate the gene expression of the Δ5- and Δ6fads in salmon hepatocytes (Kjaer et al., 2016). Compared to the 75FO diet, the rapeseed oil–based 10FO diet had higher levels of both 18:3n-3 and 18:1n-9. In accordance with other studies (Gillard et al., 2018), the gene expression of the Δ5- and Δ6fads was higher in the 10FO compared to the 75FO (Table 6). Nonetheless, as revealed in the measurements of fatty acid composition (Table 3), the increased availability of 18:3n-3 and 18:1n-9 in the 10FO diet did not stimulate omega-3 synthesis to produce EPA and DHA to levels gained from the 75FO diet. Atlantic salmon has all the enzymes for EPA and DHA synthesis, but, as demonstrated here, the capacity is limited. Zheng et al. (2009) showed that the molecular mechanism that leads to EPA suppressing the omega-3 synthesis in salmon cells could be by suppressing the activity of the Δ6fad promoter. Nonetheless, five of the miRNAs identified as differentially expressed in this study could, as discussed in Section 4.2, also be involved in such diet-triggered regulation by targeting key lipid metabolism genes.

The transcription factor Srebp-2 predominantly regulates cholesterol biosynthesis, and its expression is increased by the depletion of cholesterol (Horton et al., 2002). A suppression of the cholesterol synthesis pathway was also observed in Atlantic salmon fed a...
differences in the expression of several lipid key genes. These findings
background resulted in different hepatic fatty acid compositions and
β-oxidation (Table 6) that could have explained the differences in hepatic DHA content of the four groups. There were also no effects of either diets or family background on Δ6fad_b expression level involved in mitochondrial β-oxidation (Table 6). In conclusion, the differences in diet and family background resulted in different hepatic fatty acid compositions and differences in the expression of several lipid key genes. These findings are largely in agreement with previous studies.

4.2 Putative regulatory roles of DE miRNAs in S. salar lipid metabolism

The analysis of relative expression differences revealed 12 mature miRNAs with changed expressions. Some of these miRNAs were differentially expressed when comparing family groups, others were differentially expressed when comparing differences in diets and the expression of four miRNAs was associated with both family groups and diet. As the expression of several miRNAs was affected by family background independent of diet, the initial selection of the parental individuals in two family groups using Δ6fad_b expression levels led to a selection into two family groups that also differed in their expression of certain miRNAs. Nonetheless, as the diet affected the expression of six miRNAs, their expression seems to be modulated by dietary levels of fish oil and vegetable oil.

One species-specific mature miRNA (ssa-miR-nov1-5p) and the teleost-specific miR-462b-5p were among the DE miRNAs, indicating they may have regulatory roles in the salmon lipid metabolism. All the remaining 10 evolutionary-conserved DE miRNAs have been identified as miRNAs that are important in lipid metabolism and/or adipogenesis in other vertebrates (Ahn et al., 2013; Arner & Kulyte, 2015; Chen et al., 2014; Cheng et al., 2018; Hu et al., 2012; Shin et al., 2014; Ye et al., 2014). In most cases their particular functions have not been experimentally established but rather assumed from target gene predictions. Nonetheless, the finding that same evolutionary-conserved orthologous miRNAs are discovered as differentially expressed in other vertebrate studies of lipid metabolism indicates that they may have similar regulatory functions in Atlantic salmon. The miRNA ssa-miR-135c-5p (identical to 135a-5p in Chen et al.) controlled, e.g., adipogenesis and lipid droplet accumulation in a mammalian cell line study (Chen et al., 2014). Also, ssa-miR-146a (identical to miR-146b in Ahn et al., 2013) was reported as a regulator of adipogenesis by suppressing the SIRT1-FOXO cascade.

In silico predictions of target genes are a common approach to further elucidate the role of a certain DE miRNA. One limitation of such predictions when studying non-model species is that the 3’UTRs of most genes, including those genes suggested as targets in other vertebrate studies, are poorly characterized. Also, a large proportion of the predicted targets will be false positives (Andreassen & Hoyheim, 2017). Nevertheless, such predictions may narrow down the genes relevant to study further by experimental approaches. One finding in the present in silico analysis was that ssa-miR-137a-3p was predicted to target cdc42. If there is a conserved miRNA/target interaction in vertebrates, one would expect that the target site sequence in the 3’UTR of the target transcript is also conserved across species. Interestingly, the same miRNA ortholog (miR-137) has been suggested by Shin et al. to control adipogenesis in human adipose cell lines, also by targeting the same orthologous target transcript (cdc42) (Shin et al., 2014).

When there is a lack of evidence from comparative studies of particular miRNA–target interactions, direct measurements of the expression changes in the predicted targets could add evidence that they are true targets. One predicted DE miRNA–target gene pair did reveal reciprocal change in abundance in one group comparison (ssa-miR-203a-3p and srebp-1), whereas the others did not. Nonetheless, the negative regulation mechanism executed by the miRISC when directed to a target gene by the guide miRNA depends on the kind of Argonaute homologue that are part of RISC. There are several Argonaute proteins in fish, and whereas one slices the target transcript (leading to degradation of the target mRNA), the others lead to repression of target gene protein expression by, e.g., translational inhibition. The Argonaute proteins are not well characterized in Atlantic salmon, but investigations in other teleosts indicate that cleavage of the target mRNA is not the dominant regulatory mechanism in fish (Chen et al., 2017). Thus, although one predicted DE miRNA–target gene interaction showed signs of reciprocal change in abundance in the groups compared as expected for RISC-mediated degradation of target mRNA, the absence of such relationship in the other predicted DE miRNA–target gene pairs does not rule out that they are true targets. This is also the case for the two genes acox1 and cp1 as the mRNA expression, not protein expression, was investigated here.

Some orthologues of the DE miRNAs identified are known to be important, not only in lipid metabolism and adipogenesis but also in low chronic inflammatory processes associated with pathological accumulation of lipids in vertebrates (Arner & Kulyte, 2015). The 10FO diet group, and particularly the 10FO/Low group, showed a significantly lower percentage of the anti-inflammatory EPA (Table 3). Reduced omega-3 levels and increased proinflammatory omega-6 fatty acid levels may be associated with metabolic imbalance in the liver (Scorletti & Byrne, 2013). Interestingly, the miRNAs ssa-miR-125a-3p and ssa-miR-221 and the three miR-146 family members are all associated with inflammation in the adipose tissue (Arner & Kulyte, 2015). For example, Mir-146a is shown to inhibit oxidized low-density lipoprotein-induced lipid accumulation and inflammatory response by targeting toll-like receptor 4c (Chen et al., 2016; Yang et al., 2011). All these miRNAs, as well as the teleost-specific ssa-miR-462b-5p, showed changed expressions in the inflammatory phase of viral disease in Atlantic salmon (Woldemariam et al., 2020). Whether the observed differential expression in these particular miRNAs and in
the relative level of EPA could influence the immune response capacity of the groups would be an interesting topic for future work.

In summary, applying the 12 DE miRNAs as an input in target gene predictions, a limited number of key genes in the Atlantic salmon hepatic lipid metabolism were predicted as putative targets. One of these miRNA–target gene interactions was supported by a similar study in mammals. More experimental studies in Atlantic salmon are, nonetheless, needed to validate and fully understand the predicted miRNA–target gene interactions. Such knowledge may further help understand whether the identified DE miRNAs participate in the regulatory networks that control lipid metabolism in Atlantic salmon.

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AUTHOR CONTRIBUTION
Conceived and coordinated the study: R.A. and T.-K.K.Ø.; methodology: R.A. and T.-K.K.Ø.; software: R.A. and N.T.W.; validation: R.A. and N.T.W.; formal analysis: R.A., T.-K.K.Ø., C.E.L., G.M.B. and N.T.W.; investigation: R.A. and N.T.W.; writing original draft: R.A. and T.-K.K.Ø.; review and editing: R.A., T.-K.K.Ø., C.E.L., G.M.B. and N.T.W.; supervision: R.A. and T.-K.K.Ø.; project administration: R.A.; funding acquisition: R.A. All authors revised and approved the final draft.

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REFERENCES
Ahn, J., Lee, H., Jung, C. H., Jeon, T. I., & Ha, T. Y. (2013). MicroRNA-146b promotes adipogenesis by suppressing the SIRT1-FOXO1 cascade. EMBO Molecular Medicine, 5, 1602–1612.
Anders, S., & Huber, W. (2010). Differential expression analysis for sequence count data. Genome Biology, 11, R106.
Andreasen, R., & Hoyheim, B. (2017). miRNAs associated with immune response in teleost fish. Developmental and Comparative Immunology, 75, 77–85.
Andreasen, R., Rangnes, F., Sivertsen, M., Chiang, M., Tran, M., & Worren, M. M. (2016). Discovery of miRNAs and their corresponding miRNA genes in Atlantic cod (Gadus morhua): Use of stable miRNAs as reference genes reveals subgroups of miRNAs that are highly expressed in particular organs. PLoS One, 11, e0153324.
Andreasen, R., Woldemariam, N. T., Egeland, I. O., Agafonov, O., Sindre, H., & Hoyheim, B. (2017). Identification of differentially expressed Atlantic salmon miRNAs responding to salmonid ahpavirus (SAV) infection. BMC Genomics, 18, 349.
Andreasen, R., Worren, M. M., & Hoyheim, B. (2013). Discovery and characterization of miRNA genes in Atlantic salmon (Salmo salar) by use of a deep sequencing approach. BMC Genomics, 14, 482.
Arner, P., & Kulyte, A. (2015). MicroRNA regulatory networks in human adipose tissue and obesity. Nature Reviews. Endocrinology, 11, 276–288.
Bell, J. G., Henderson, R. J., Tocher, D. R., McGhee, F., Dick, J. R., Porter, A., ... Sargent, J. R. (2002). Substituting fish oil with crude palm oil in the diet of Atlantic salmon (Salmo salar) affects muscle fatty acid composition and hepatic fatty acid metabolism. The Journal of Nutrition, 132, 222–230.
Berge, G. M., Østbye, T. K., Kjær, M. A., Sonesson, A., Mørkøre, T., & Ruyter, B. (2015). Betydning av genetisk bakgrunn og ulike nivåer av omega-3-fettsyrer i før i tidligere livsfaser for fiskehelse. Tidsskrift for Kremlin, 105, 12–28.
Bizuaye, T. T., & Babiak, I. (2014). MicroRNA in teleost fish. Genome Biology and Evolution, 6, 1119–1937.
Bou, M., Berge, G. M., Baeverfjord, G., Sigholt, T., Østbye, T. K., Romarheim, O. H., ... Ruyter, B. (2017a). Requirements of n-3 very long-chain PUFAs in Atlantic salmon (Salmo salar L): Effects of different dietary levels of EPA and DHA on fish performance and tissue composition and integrity. The British Journal of Nutrition, 117, 30–47.
Bou, M., Berge, G. M., Baeverfjord, G., Sigholt, T., Østbye, T. K., & Ruyter, B. (2017b). Low levels of very-long-chain n-3 PUFAs in Atlantic salmon (Salmo salar) diet reduce fish robustness under challenging conditions in sea cages. Journal of Nutritional Science, 6, e32.
Calder, P. C. (2014). Very long chain omega-3 (n-3) fatty acids and human health. European Journal of Lipid Science and Technology, 116, 1280–1300.
Casas-Agustench, P., Fernandes, F. S., Tavares do Carmo, M. G., Visioli, F., Herrera, E., & Davalos, A. (2015). Consumption of distinct dietary lipids during early pregnancy differentially modulates the expression of microRNAs in mothers and offspring. PLoS One, 10, e0117858.
Chekulaeva, M., & Filipowicz, W. (2009). Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. Current Opinion in Cell Biology, 21, 452–460.
Chen, C., Peng, Y., Peng, Y., Peng, J., & Jiang, S. (2014). miR-135a-5p inhibits 3T3-L1 adipogenesis through activation of canonical Wnt/-beta-catenin signaling. Journal of Molecular Endocrinology, 52, 311–320.
Chen, G. R., Sive, H., & Bartel, D. P. (2017). A seed mismatch enhances Argonaute2-catalyzed cleavage and partially rescues severely impaired cleavage found in fish. Molecular Cell, 68(1095–1107), e1095.
Chen, L., Huang, R., Zhu, D., Yang, C., He, L. L., Li, Y., ... Wang, Y. (2019). Deep sequencing of small RNAs from 11 tissues of grass carp Ctenopharyngodon idella and discovery of sex-related microRNAs. Journal of Fish Biology, 94, 132–141.
Chen, Y., Zeng, Z., Shen, X., Wu, Z., Dong, Y., & Cheng, J. C. (2016). MicroRNA-146a-5p negatively regulates pro-inflammatory cytokine secretion and cell activation in lipopolysaccharide stimulated human hepatic stellate cells through inhibition of toll-like receptor 4 signaling pathways. International Journal of Molecular Sciences, 17(7), 1–13. https://doi.org/10.3390/ijms17071076.
Cheng, X. Y., Liu, J. D., Lu, X. Y., Yan, X., Huang, C., Meng, X. M., & Li, J. (2018). miR-203 inhibits alcohol-induced hepatic steatosis by targeting Lipin1. Frontiers in Pharmacology, 9, 275.
Commission, T. E. P. a. (2010). Directive on the Protection of Animals Used for Scientific Purposes. Official Journal of the European Union, EU.
Fernandez-Hernando, C. (2013). Emerging role of microRNAs in the regulation of lipid metabolism. Hepatology, 57, 432–434.
Friedman, R. C., Farh, K. K., Burge, C. B., & Bartel, D. P. (2009). Most mammalian miRNAs are conserved targets of microRNAs. Genome Research, 9, 19–105.
Gillard, G., Harvey, T. N., Gjøslands, A., Jin, Y., Thomassen, M., Lien, S., ... Sandve, S. R. (2018). Life-stage-associated remodelling of lipid metabolism regulation in Atlantic salmon. Molecular Ecology, 27, 1200–1213.
Hauser, J., & Zavolaj, M. (2014). Identification and consequences of miRNA-target interactions: Beyond repression of gene expression. Nature Reviews. Genetics, 15, 599–612.

Horn, S. S., Ruyter, B., Meuwissen, T. H. E., Hillestad, B., & Sonesson, A. K. (2018). Genetic effects of fatty acid composition in muscle of Atlantic salmon. Genetics Selection Evolution, 50, 23.

Horn, S. S., Sonesson, A. K., Krasnov, A., Moghadam, H., Hillestad, B., Meuwissen, T. H. E., & Ruyter, B. (2019). Individual differences in EPA and DHA content of Atlantic salmon are associated with gene expression of key metabolic processes. Scientific Reports, 9, 3889.

Horton, J. D., Goldstein, J. L., & Brown, M. S. (2002). SREBPs: Activators of the complete program of cholesterol and fatty acid synthesis in the liver. Journal of Clinical Investigation, 109, 1125–1131.

Hu, Z., Shen, W. J., Kraemer, F. B., & Azhar, S. (2012). MicroRNAs 125a and 455 repress lipoprotein-supported steriodogenesis by targeting scavenger receptor class B type I in steroidogenic cells. Molecular and Cellular Biology, 32, 5035–5045.

Johansen, I., & Andreassen, R. (2014). Validation of miRNA genes suitable as reference genes in qPCR analyses of miRNA gene expression in Atlantic salmon (Salmo salar). BMC Research Notes, 8, 945.

Karbieri, M., Pisani, D. F., Frontini, A., Oberreiter, L. M., Lang, E., Vegiopoulos, A., ... Scheideler, M. (2014). MicroRNA-26 family is required for human adipogenesis and drives characteristics of brown adipocytes. Stem Cells, 32, 1578–1590.

Kjaer, M. A., Ruyter, B., Berge, G. M., Sun, Y., & Østbye, T. K. (2016). Regulation of the Omega-3 fatty acid biosynthetic pathway in Atlantic salmon hepatocytes. PLoS One, 11, e0168230.

Kjaer, M. A., Vegusdal, A., Gjøen, T., Rustan, A. C., Todorevic, M., & Ruyter, B. (2008). Effect of rapeseed oil and dietary n-3 fatty acids on triacylglycerol synthesis and secretion in Atlantic salmon hepatocytes. Biochimica et Biophysica Acta-Molecular and Cell Biology of Lipids, 1781, 112–122.

Kortner, T. M., Björkhem, I., Krasnov, A., Timmerhaus, G., & Krogdahl, A. (2014). Dietary cholesterol supplementation to a plant-based diet suppresses the complete pathway of cholesterol synthesis and induces bile acid production in Atlantic salmon (Salmo salar L.). The British Journal of Nutrition, 111, 2089–2103.

Krol, J., Loedige, I., & Filipowicz, W. (2010). The widespread regulation of microRNA biogenesis, function and decay. Nature Reviews. Genetics, 11, 597–610.

Leaver, M. J., Taggart, J. B., Villeneuve, L., Bron, J. E., Guy, D. R., Bishop, S. C., ... Tocher, D. R. (2011). Heritability and mechanisms of n-3 long chain polyunsaturated fatty acid deposition in the flesh of Atlantic salmon. Comparative Biochemistry and Physiology Part B: Biochemistry & Molecular Biology, 154, 323–340.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBO Journal, 17, 10–12.

Martinez-Rubio, L., Morais, S., Evensen, O., Wadsworth, S., Ruohonenn, K., Vecino, J. I., ... Tocher, D. R. (2012). Functional feeds reduce heart inflammation and pathology in Atlantic Salmon (Salmo salar L.) following experimental challenge with Atlantic salmon reovirus (ASRV). PLoS One, 7, e40266.

Martinez-Rubio, L., Morais, S., Evensen, O., Wadsworth, S., Vecino, J. G., Ruohonenn, K., ... Tocher, D. R. (2013). Effect of functional feeds on fatty acid and eicosanoid metabolism in liver and head kidney of Atlantic salmon (Salmo salar L.) with experimentally induced heart and skeletal muscle inflammation. Fish & Shellfish Immunology, 34, 1533–1545.

Mason, M. E., & Waller, G. R. (1964). Dimethoxypyruvone induced trans-esterification of fats oils in preparation of methyl esters for gas chromatographic analysis. Analytical Chemistry, 36, 583–586.

Mennigen, J. A. (2016). Micromanaging metabolism-a role for miRNAs in teleost energy metabolism. Comparative Biochemistry and Physiology, Part B: Biochemistry & Molecular Biology, 199, 115–125.

Mennigen, J. A., Plagnes-Juan, E., Figueredo-Silva, C. A., Selliez, I., Panserat, S., & Skiba-Cassy, S. (2014). Acute endocrine and nutritional co-regulation of the hepatic omi-miRNA-122b and the lipogenic gene fac in rainbow trout, Oncorhynchus mykiss. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 169, 16–24.

Minghetti, M., Leever, M. J., & Tocher, D. R. (2011). Transcriptional control mechanisms of genes of lipid and fatty acid metabolism in the Atlantic salmon (Salmo salar L) established cell line, SHK-1. Biochimica et Biophysica Acta-Molecular and Cell Biology of Lipids, 1811, 194–202.

Ministry of Agriculture and Food, N. (2015). Regulations on the Use of Animals in experiments. P. 761.

Moraes, S., Monroig, O., Zheng, X. Z., Leaver, M. J., & Tocher, D. R. (2009). Highly unsaturated fatty acid synthesis in Atlantic Salmon: Characterization of ELOVL5 and ELOVL2-like Elongases. Marine Biotechnology, 11, 627–639.

Moraes, S., Pratoomjot, Y., Taggart, J. B., Bron, J. E., Guy, D. R., Bell, J. G., & Tocher, D. R. (2011). Genotype-specific responses in Atlantic salmon (Salmo salar) subject to dietary fish oil replacement by vegetable oil: A liver transcriptomic analysis. BMC Genomics, 12(255), 1–17. https://doi.org/10.1186/1471-2164-12-255.

Moya-Falon, C., Thomassen, M. S., Jakobsen, J. V., & Ruyter, B. (2005). Effects of dietary supplementation of rapeseed oil on metabolism of (1-14C)-18: 1 n-9, (1-14C)-20: 3n-6, and (1-14C)-20: 4n-3 in Atlantic salmon hepatocytes. Lipids, 40, 709–717.

Pelorson, S. M., Thompson, J. A., Ufkin, M. L., Sathyarayanan, P., Liaw, L., & Congdon, C. B. (2014). Common features of microRNA target prediction tools. Frontiers in Genetics, 5, 23.

Pfaffl, M. W. (2004). Quantification strategies in real-time PCR. In S. A. Bustin & C. La Jolla (Eds.), A-Z of Quantitative PCR (pp. 87–120). La Jolla, CA: International University Line.

Ramakers, C., Ruijter, J. M., Deprez, R. H., & Moorman, A. F. (2003). Assumption-free analysis of real-time polymerase chain reaction (PCR) data. Neuroscience Letters, 339, 62–66.

Rehmsmeier, M., Steffen, P., Hochsmann, M., & Giegerich, R. (2004). Fast and effective prediction of microRNA/target duplexes. RNA, 10, 1507–1517.

Rosenlund, G. O., Sandberg, A., Strandal, M. G., & Tveit, H. (2001). Effect of alternative lipid sources on long-term growth performance and quality of Atlantic salmon (Salmo salar L.) Aquaculture Research, 32, 323–328.

Ruyter, B., Rosjo, C., Grisdale-Helland, B., Rosenlund, G., Obach, A., & Thomassen, M. S. (2003). Influence of temperature and high dietary linoleic acid content on esterification, elongation, and desaturation of PUFA in Atlantic salmon hepatocytes. Lipids, 38, 833–840.

Sala, F., Aranda, J. F., Rottlan, N., Ramirez, C. M., Aryan, B. Ela, L., ... Norata, G. D. (2014). miR-143/145 deficiency attenuates the progression of atherosclerosis in Ldlr−/− mice. Thrombosis and Haemostasis, 112, 796–802.

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. Nature Protocols, 3, 1101–1108.

Scorletti, E., & Byrne, C. D. (2013). Omega-3 fatty acids, hepatic lipid metabolism, and nonalcoholic fatty liver disease. Annual Review of Nutrition, 33, 231–248.

Shin, K. K., Kim, Y. S., Kim, J. Y., Bae, Y. C., & Jung, J. S. (2014). miR-137 controls proliferation and differentiation of human adipose tissue stromal cells. Cellular Physiology and Biochemistry, 33, 758–768.

Skugor, S., Skugor, A., Todorcevic, M., Torgersen, J., Ruyter, B., & Krasnov, A. (2010). Exposure to lipopolysaccharide induces immune genes in cultured preadipocytes of Atlantic salmon. Fish & Shellfish Immunology, 29, 817–824.

Smolle, E., & Haybaeck, J. (2014). Non-coding RNAs and lipid metabolism. International Journal of Molecular Sciences, 15, 13494–13513.

Soh, J., Iqlabal, J., Queiroz, J., Fernandez-Hernando, C., & Hussain, M. M. (2013). MicroRNA-30c reduces hyperlipidemia and atherosclerosis in mice by decreasing lipid synthesis and lipoprotein secretion. Nature Medicine, 19, 892–900.

Sprecher, H. (2000). Metabolism of highly unsaturated n-3 and n-6 fatty acids. Biochimica et Biophysica Acta, 1486, 219–231.
Sun, C., Huang, F., Liu, X., Xiao, X., Yang, M., Hu, G., ... Liao, L. (2015). miR-21 regulates triglyceride and cholesterol metabolism in non-alcoholic fatty liver disease by targeting HMGCR. *International Journal of Molecular Medicine*, 35, 847–853.

Tao, Y. F., Qiang, J., Yin, G. J., Xu, P., Shi, Q., & Bao, J. W. (2017). Identification and characterization of lipid metabolism-related microRNAs in the liver of genetically improved farmed tilapia (GIFT, *Oreochromis niloticus*) by deep sequencing. *Fish & Shellfish Immunology*, 69, 227–235.

Thomassen, M. S., Rein, D., Berge, G. M., Østbye, T. K., & Ruyter, B. (2012). High dietary EPA does not inhibit Delta 5 and Delta 6 desaturases in Atlantic salmon (*Salmo salar*) fed rapeseed oil diets. *Aquaculture*, 360, 78–85.

Tocher, D. R., Bell, J. G., Dick, J. R., Henderson, R. J., McGhee, F., Michell, D., & Morris, P. C. (2000). Polyunsaturated fatty acid metabolism in Atlantic salmon (*Salmo salar*) undergoing parr-smolt transformation and the effects of dietary linseed and rapeseed oils. *Fish Physiology and Biochemistry*, 23, 59–73.

Torstensen, B. E., Bell, J. G., Rosenlund, G., Henderson, R. J., Graff, I. E., Tocher, D. R., ... Andreassen, R. (2009). Gene expression of fatty acid-binding proteins, fatty acid transport proteins (cd36 and FATP) and beta-oxidation-related genes in Atlantic salmon (*Salmo salar*) flesh lipids and sensory quality by replacing fish oil with a vegetable oil blend. *Journal of Agricultural and Food Chemistry*, 53, 10166–10178.

Torstensen, B. E., Nanton, D. A., Olsvik, P. A., Sundvold, H., & Stubhaug, I. (2009). Gene expression of fatty acid-binding proteins, fatty acid transport proteins (cd36 and FATP) and beta-oxidation-related genes in Atlantic salmon (*Salmo salar*) flesh fish oil or vegetable oil. *Aquaculture Nutrition*, 15, 440–451.

Woldemariam, N. T., Agafonov, O., Hoyheim, B., Houston, R. D., Taggart, J. B., & Andreassen, R. (2019). Expanding the miRNA repertoire in Atlantic Salmon: discovery of IsomiRs and miRNAs highly expressed in different tissues and developmental stages. *Cell*, 8, 1–23. https://doi.org/10.3390/cells8010042.

Woldemariam, N. T., Agafonov, O., Sindre, H., Hoyheim, B., Houston, R. D., Robledo, D., ... Andreassen, R. (2020). miRNAs predicted to regulate host anti-viral gene pathways in IPNV-challenged Atlantic Salmon fry are affected by viral load, and associated with the major IPN resistance QTL genotypes in late infection. *Frontiers in Immunology*, 11, 2113.

Yang, K., He, Y. S., Wang, X. Q., Lu, L., Chen, Q. J., Liu, J., ... Shen, W. F. (2011). miR-146a inhibits oxidized low-density lipoprotein-induced lipid accumulation and inflammatory response via targeting toll-like receptor 4. *FEBS Letters*, 585, 854–860.

Yang, Z., Cappello, T., & Wang, L. (2015). Emerging role of microRNAs in lipid metabolism. *Acta Pharmacologica Sinica B*, 5, 145–150.

Ye, Y., Deng, L., Liang, M., Xu, L., Zhang, L., Ma, Y., & Li, Y. (2014). Micro-RNAs expression profiles in adipose tissues and liver from sex-linked dwarf and normal chickens. *Acta Biochimica et Biophysica Sinica (Shanghai)*, 46, 723–726.

Zhang, Q., Xie, D., Wang, S., You, C., Monroig, O., Tocher, D. R., & Li, Y. (2014). miR-17 is involved in the regulation of LC-PUFA biosynthesis in vertebrates: Effects on liver expression of a fatty acyl desaturase in the marine teleost *Siganus canaliculatus*. *Biochimica et Biophysica Acta*, 1841, 934–943.

Zheng, X. Z., Leaver, M. J., & Tocher, D. R. (2009). Long-chain polyunsaturated fatty acid synthesis in fish: Comparative analysis of Atlantic salmon (*Salmo salar*) and Atlantic cod (*Gadus morhua*) Delta 6 fatty acyl desaturase gene promoters. *Comparative Biochemistry and Physiology B-Biochemistry and Molecular Biology*, 154, 255–263.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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