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Heritability and mechanisms of n-3 long chain polyunsaturated fatty acid deposition in the flesh of Atlantic salmon

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Abstract

N-3 long chain polyunsaturated fatty acids (n-3LC-PUFA) are essential components of vertebrate membrane lipids and are crucially deficient in modern Western diets. The main human dietary source for n-3LC-PUFA is fish and seafood, and over 50% of global fish production is currently supplied by aquaculture. However, increasing pressure to include vegetable oils, which are devoid of n-3LC-PUFA, in aquaculture feeds reduces their content in farmed fish flesh. The aim of this study was to measure the heritability and infer mechanisms determining flesh n-3LC-PUFA content in Atlantic salmon. This was achieved by analysing flesh lipid parameters in 48 families of Atlantic salmon and by measuring differences, by high density microarray, in hepatic mRNA expression in families with high and low flesh n-3LC-PUFA. The results show that flesh n-3LC-PUFA composition is a highly heritable trait ($h^2 = 0.77 \pm 0.14$). Gene ontology analysis of differentially expressed genes indicate increased hepatic lipid transport, likely as very low density lipoprotein (VLDL), and implicate increased activity of a transcription factor, hepatic nuclear factor 4α (HNF4α), possibly as a result of family differences in transforming growth factor β1 (Tgfβ1) signalling. This study paves the way for identification of quantitative trait loci and gene interaction networks that are associated with flesh n-3LC-PUFA composition, which will assist the sustainable production of Atlantic salmon and provide optimal levels of critical nutrients for human consumers.

Keywords

n-3LC-PUFA, Atlantic salmon, heritability, liver, flesh, microarray, HNF4α, TGFβ1

1. Introduction

Humans, and probably all other vertebrates require preformed dietary 18:2 n-6 (linoleic acid, LA) and α–18:3n-3 (α–linolenic acid, ALA) polyunsaturated fatty acids (PUFA) (Burr and Burr, 1930;Rivers and Frankel, 1981;Cowey and Cho, 1993). These essential fatty acids cannot be biosynthesised or interconverted in vertebrates, and are primarily derived from plants. LA and ALA have vital functions in themselves, and in turn act as precursors for the long chain PUFA (LC-PUFA) 20:4n-6 (arachidonic acid, ARA), 20:5n-3 (eicosapentenoic acid, EPA) and 22:6n-3 (docosahexanoic acid, DHA). LC-PUFA are essential components of cell membranes, particularly in nervous tissue and their biosynthesis from LA and ALA can be carried out by mammals, although evidence suggests that the process of EPA and particularly DHA biosynthesis from ALA is very low in humans (Burdge and Calder, 2005). The biosynthetic pathway involves consecutive desaturation and elongation reactions that convert LA to ARA and ALA to EPA and DHA. The two main enzyme families involved in these conversions are the elongases of very long fatty acids (Elovl) and the fatty acyl desaturases (Fad) (Cook and McMaster, 2004).

Modern Western diets have an excess of n-6 PUFA, primarily LA, and because n-6 fatty acids and n-3 fatty acids cannot be interconverted in vertebrates, this has led to an increase in the tissue ratio of n-6 to n-3 LC-PUFA. Such imbalances in n-6/n-3 ratios have been linked to several chronic diseases that are particularly prevalent in Western societies including cardiovascular, inflammatory and neurological problems (Calder, 2006). One way of addressing this n-6/n-3 imbalance is to increase the levels of n-3 PUFA and especially n-3LC-PUFA in the diet of humans. Since the conversion of ALA to LC-PUFA has been found to be relatively low in humans, an increase in dietary n-3LC-PUFA would be of most benefit. In human diets seafood, particularly oily fish, is the major source of n-3LC-PUFA, and in Western countries minimum consumption levels of fish have been recommended by various health advisory bodies (Scientific Advisory Committee on Nutrition, 2004;Lichtenstein et al., 2006;Scientific Advisory Committee on Nutrition, 2004).

Currently aquaculture produces about 50% of all fish and seafood for human consumption globally. The formulation of feed is critical for finfish aquaculture, ensuring optimal growth rates and fish welfare, and until recently the bulk feed ingredients have consisted of fish meal and fish oil, extracted from industrial feed-grade capture fisheries. These capture fisheries are now fished at the maximum sustainable limit, and worldwide aquaculture currently consumes the vast majority of the

resulting fish meal and oil (Tacon and Metian, 2009). Therefore, in order to enable the aquaculture sector to grow in line with consumer demand, alternative bulk feed ingredients are being sought. In recent years, terrestrial plant seed meals and oils, such as those derived from processed soy bean or rapeseed have been used to substitute up to 70% of fish meal and oil in Atlantic salmon diets with little or no effects on fish growth rate (Torstensen et al., 2005). Like humans, fish require dietary PUFA and LC-PUFA. LC-PUFA are abundant in fish oil, having accumulated ultimately from the phytoplanktonic organisms which form the base of marine food webs (Brett and Muller-Navarra, 1997). However, the flesh of fish fed diets containing plant oils have low levels of the most beneficial n-3LC-PUFA, DHA and EPA, and this deficiency compromises the quality and health benefits of the product (Torstensen et al., 2005).

Unlike some other fish species, Atlantic salmon have the capacity to biosynthesise n-3 and n-6LC-PUFA, expressing, predominantly in liver, the Δ5fad, Δ6fad and elovl genes necessary. Furthermore, these genes and LC-PUFA biosynthesis are up-regulated after plant oil feeding (Tocher et al., 2001;Zheng et al., 2005a;Leaver et al., 2008;Morais et al., 2009). Nevertheless, the increase in n-3LC-PUFA biosynthesis during plant oil feeding is not sufficient to adjust flesh n-3LC-PUFA levels to the levels normally expected after fish oil feeding (Leaver et al., 2008). The production of strains of farmed animals with enhanced flesh n-3LC-PUFA would be of great value, and there is evidence from mammals and birds that there is a heritable genetic component governing capacity to biosynthesise and/or deposit LC-PUFA (De Smet et al., 2004;Karamichou et al., 2006;Khang et al., 2007) The aim of this study was to assess whether there is a heritable component in flesh LC-PUFA levels in families of Atlantic salmon fed plant oil, and whether flesh LC-PUFA variability can be explained by gene expression in the liver, the predominant site of LC-PUFA biosynthesis in Atlantic salmon.

2. Methods

2.1. Fish, diet and sampling

The fish chosen for analysis were from 48 families, randomly selected from a cohort of 200 families that were siblings to the broodstock fish of Landcatch Natural Selection (Alloa, Scotland). These fish were spawned in December 2004, subsequently incubated and hatched in 2005, and maintained as individual families in separate freshwater tanks at Landcatch (Ormsary, Lochgilphead, Scotland). The fish were fed a commercial salmon feed until they had reached an average weight of 71±9 in April 2006. Twenty-five smolts from each of the 48 families were transferred to a single seawater tank in April 2006 and, after acclimation, were grown for 12 weeks on an experimental diet with reduced fishmeal, and fish oil replaced with a blend of vegetable oils (50% rapeseed oil, 30% palm oil and 20% camelina oil) as described in detail previously (Petropoulos et al., 2009). The fatty acid composition of the experimental diet is given in Table 1. All fish were tagged with electronic transponders (Pit tags) to allow individual growth to be monitored, with initial fish weights and lengths recorded at tagging. Fish were maintained at ambient temperature and under ambient photoperiod. At harvest, after 12 weeks of feeding the experimental diet, fish weight and length

were recorded, and a flesh sample (Norwegian Quality Cut) collected, frozen on dry ice and stored at -20 °C until analysis. Liver samples were taken from each individual fish and immediately frozen in liquid nitrogen and stored at – 80 °C prior to RNA extraction.

2.2. Lipid and fatty acid analysis

De-boned and skinned flesh samples of equal weight from each fish were combined into 4 pools per family as described previously (Torstensen et al., 2005). Within each family the number of fish per pool was constant, ranging from one to four, with a an overall mean of 2.17 fish per pool. Total lipids were prepared according to the method of Folch et al. (1957) with lipid content determined gravimetrically. Fatty acid methyl esters (FAME) were prepared by acid-catalysed transesterification of total lipids with 19:0 as internal standard according to the method of Christie (2003). Extraction and purification of FAME was performed as described previously (Tocher and Harvie, 1988). FAME were separated and quantified by gas-liquid chromatography using a Thermo Fisher Trace GC 2000 (Thermo Fisher, Hemel Hempstead, UK) equipped with a fused silica capillary column (ZB wax, 30 m x 0.32 mm i.d.; Phenomenex, Macclesfield, UK) with hydrogen as carrier gas and using on-column injection. The temperature gradient was from 50 to 150 °C at 40 °C/min and then to 195 °C at 1.5 °C/min and finally to 220 °C at 2 °C/min. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman, 1980). Data were collected and processed using the Chromcard for Windows (version 2.00) computer package (Thermoquest Italia S.p.A., Milan, Italy). Total n-3 LC-PUFA (≥ C20 and ≥ 3 double bonds, i.e. mainly 20:4, 20:5, 22:5 and 22:6) content was expressed in relative (percentage of total fatty acids) and absolute (mg fatty acid /100 g flesh) terms.

2.3. Heritability Estimates

Heritabilities for three traits, total flesh lipid level (g/100g flesh), percentage n-3LC-PUFA and total n-3LC-PUFA (mg/100g flesh) were estimated by residual maximum likelihood techniques using the ASReml Package (Gilmour et al., 2006). Input data were the mean trait values for each pool of fish for each family. Variance component analyses of these data yields between-family and within-family variance components for each trait. Accounting for the full-sib family structure, and assuming maternal effects are negligible, the between-family variance is an estimate of $\sigma^2_g/2$ and the within-family variance is an estimate of $\sigma^2_g/2n + \sigma^2_e/n$, where σ^2_g is the additive genetic variance, σ^2_e is the residual variance and n is the harmonic mean number of fish per pool, which in this case was 1.8. Rearranging these terms allows the heritability (i.e. $\sigma^2_g/(\sigma^2_g+\sigma^2_e)$) and its standard error to be inferred.

2.4. Microarrays, Probe Preparation, Hybridization and Data Collection

Eight families were selected for transcriptomic analysis. The selection criteria ranked families in pairs, each pair with the same total flesh lipid level but with significantly different total n-3LC-PUFA levels, scored as high and low (Table 2). This produced a range of total lipid levels and a range of

total flesh n-3LC-PUFA levels. Liver was chosen as the target organ because it integrates overall body lipid uptake, dispersion and metabolism and is a major site of n-3LC-PUFA biosynthesis, whereas PUFA biosynthesis is virtually undetectable in salmon muscle tissue (Zheng et al., 2005a).

Frozen liver samples (c. 100 mg) from each individual fish (n=48) were immediately homogenised in 10 volumes of TriReagent (Sigma, Poole, UK). Total RNA was prepared according to the manufacturer's standard protocol. Following spectrophotometric quantitation and quality checking by agarose gel electrophoresis, equal quantities of RNA from each of six individuals were pooled to provide one RNA pool per family. The microarray experiment employed a dual label, pooled reference design, the reference comprising equal amounts of RNA from all samples. Labelled samples (methodology detailed below) were hybridised to the Atlantic salmon TRAITS-SGP 17K cDNA microarray v2 (ArrayExpress accession: A-MEXP-1790; originally described by (Taggart et al., 2008). A dye swap was included in the experimental design. Thus the entire experiment comprised 16 arrays; 8 families × 2 (dye flip).

RNA was reverse transcribed and labelled with either Cy3 or Cy5 using the FAIRPLAY II cDNA labelling kit (Stratagene) according to the manufacturer's instructions. Briefly, 20 µg total RNA was reverse transcribed after being primed with oligo dT. Following reverse transcription the RNA template was hydrolysed using 1M NaOH for 15 min and then neutralised with 1M HCl. The cDNA was ethanol precipitated overnight. The cDNA pellets were washed in 80% ethanol and air-dried before being resuspended in 5 µL 2× coupling buffer (Stratagene Fairplay Kit). Pre-aliquoted Cy3 and Cy5 dyes (GE HealthCare; PA23001, PA25001) were resuspended in 45µL DMSO prior to being added to coupling buffer. Once the cDNA had fully dissolved (after at least 30 min) 5 µL Cy dye suspension was added to each tube and these incubated in the dark for 30 min. To remove unincorporated dye, the labelled cDNA (total volume 10µL) was passed through a DyeEx 2.0 spin column (Qiagen). Dye incorporation was checked by spectrophotometry (NanoDrop ND-1000, NanoDrop Technologies Inc) and by electrophoresis of labelled cDNA on a mini-gel and visualisation by gel scanner (Typhoon Trio, GE Healthcare). For hybridisation, a proportion of each labeled biological replicate and corresponding pooled reference (30 pmol each dye, c. 500 ng cDNA) were combined and total volume was made up to 25 µL with nuclease-free water. After heating the labelled cDNAs at 95ºC for 3 min in a thermocycler, 225 µL of pre-heated (60ºC) hybridisation solution, comprising 185 µL 0.7X UltraHyb buffer (Ambion), 20 µL poly(A) at l0 mg mL^{-1} (Sigma-Aldrich), 10 µL herring sperm at c. 10 mg mL^{-1} (Sigma-Aldrich) and 10 µL ultra pure BSA at 10 mg mL^{-1} (Sigma-Aldrich), was added and the mixture was kept at 60ºC in the dark until being applied to the microarray. Hybridisations (8 slides per day, randomised over two days) were performed in a Lucidea Slidepro semi-automated system (GE Healthcare). Hybridisation mixture (225 µL) was applied to the microarrays previously loaded into Lucidea Slidepro chambers and maintained at 60ºC. Immediately following this, the chamber temperature was raised to 70ºC for 10 mins and then lowered to 42ºC, at which temperature hybridisation was continued for 17 hrs with pulse mixing every 15 mins. Following hybridisation, two automatic washes (800 µL per slide at 8 µL / sec) were performed with 1.0X SSC; 0.1% SDS (wash 1) and 0.3X SSC; 0.2% SDS (wash 2), after which the temperature was lowered to 40ºC. Slides were then removed from the chambers and further manual washes were performed using the EasyDip™

Slide staining system (Canemco Inc.) on an orbital shaker. Two further washes were performed with wash 2 solution for 3 mins each (125 rpm; 45 ºC), followed by 3 x 2 min-washes (125 rpm; 45 ºC) with 0.2X SSC and a final 20 sec dip (room temperature) in 0.1X SSC. Slides were then dried by centrifugation (500 xg for 5 mins) and kept in a dessicator and in the dark before being scanned.

Processed microarray slides were scanned at 10 µm resolution using a Perkin Elmer ScanArrayExpress HT scanner. Laser power was kept constant (80%) and the "auto PMT" function within the acquisition software (v.4) was enabled to adjust PMT for each channel such that less than 0.1% of features were saturated and that the mean intensity ratio of the Cy3 and Cy5 signals was close to one. BlueFuse software (BlueGnome) was then used to identify and quantify features. Following manual spot editing to remove obvious artifactual features and fusion of duplicate spot data (BlueFuse proprietary algorithm), the resulting intensity values and quality annotations, without positive and negative control features, were exported into the GeneSpring GX version 7.3.1 (Agilent Technologies) analysis platform. Dye-flipped data were subject to transformation, normalisation and quality filtering as follows: 1) all raw spot intensity values less than 1 were set to1; 2) a Per Spot and Per Chip Intensity Dependent (Lowess) normalisation was performed using software default values (20% smoothing, cutoff 10); 3) data were filtered using a BlueFuse spot confidence value ≥ 0.1 in ≥ 4 slides and BlueFuse quality >0 in ≥ 4 slides. The experimental hybridizations are archived on the EBI ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/) under accession number E-XXXXX (*number yet to be assigned*). All associated metadata comply with MIAME guidelines.

A list of differentially expressed genes was generated by examining differences between the four families with high percentage n-3LC-PUFA at each total lipid level and the four families showing low n-3LC-PUFA level at the same total lipid level (T-test, $p<0.05$). The putative identity of cDNAs on the microarray was established by comparing cDNA (EST) sequences to the Refseq database of Genbank using BLASTX homology searching. Similarity scores above e^{-10} were regarded as providing insufficient evidence for encoded protein identity. Array features were further annotated (e.g. for Gene Ontology) as described previously (Taggart et al., 2008). The probability that a particular biological process GO term was enriched in the input GO list (All GO annotations in the TRAITS-GS array) compared to the output GO list (all GO annotations in the experimentally altered gene list) was calculated using a hypergeometric distribution model as implemented by GeneSpring GX 7.3.1.

2.5. <u>Statistics</u>

Unless otherwise stated, all data are presented as means ± SD (n value as stated). The effects of family on biometry, composition and enzyme activity were analysed by one-way analysis of variance (ANOVA) followed, where appropriate, by Tukey's comparison test. Percentage data and data which were identified as non-homogeneous (Bartlett's test) were subjected to arcsine transformation before analysis. Differences were regarded as significant when $P \leq 0.05$.

3. Results

3.1. Flesh n-3LC-PUFA in families

Across all families analysed total lipid levels varied between 3.5 and 6.5 g/100g flesh and the percentage of n-3LC-PUFA in flesh varied between 9.5% and 15.6% of total fatty acids. Comparison of total lipid levels and percentage n-3LC-PUFA showed that these parameters were highly linearly inversely correlated (Table 3, Fig 1) across all of the 48 families. Final mass was also inversely correlated with percentage n-3LC-PUFA. Absolute n-3LC-PUFA levels were highly correlated with lipid levels and lipid levels were correlated to both initial and final mass (Table 3). In contrast there was no evidence of correlation between n-3LC-PUFA level and initial weights, or absolute n-3LC-PUFA during the vegetable oil feeding period.

Nevertheless, it was also apparent that some families with the same total flesh lipid level had significantly different percentage n-3LC-PUFA levels, which indicated that variation in n-3LC-PUFA composition may not be entirely due to differences in lipid deposition between families.

3.2. Heritabilities

In total 48 families and 416 fish provided data for the heritability analyses, and the inferred heritabilities are shown in Table 4. These family data indicate that total lipid levels and percentage n-3LC-PUFA are highly heritable, with absolute n-3LC-PUFA levels being moderately heritable. Therefore, in these data a high proportion of the differences seen between individual fish, and between families, are likely to be genetic in origin. Thus, although families show similar total flesh lipid levels, n-LC-PUFA composition is under genetic control.

3.3. Microarray

It was not feasible to analyse all 48 families for gene expression using microarrays. Therefore 8 families were selected, and ranked in pairs, with each pair having the same flesh total lipid levels but showing significantly different percentages of n-3LC-PUFA. This allowed a comparison between gene expression profiles associated with high and low n-3LC-PUFA, whilst minimising effects of total lipid deposition on gene expression. This comparison resulted in a list of 1345 genes that were differentially expressed between families with high percentage n-3LC-PUFA and those with low percentage n-3LC-PUFA. Application of false discovery correction (FRD, Benjamini and Hochberg, 1995) resulted in a list of only 13 genes. However, since several of the genes in the output list generated before FDR correction were present as more that one feature (eg apolipoprotein B, GAPDH, PTGS etc) it is reasonable to accept the list of 1345 genes as representative of genuine gene expression differences. To increase the robustness of this uncorrected output list, it was further filtered to exclude any gene with less than a 1.25-fold difference between high and low n-3LC-PUFA, resulting in a list of 151 genes, 96 of which had BLASTX hits above the selected cut-off criterion ($< e^{-10}$) to nr-refseq database entries (Table 5). Over-represented GO terms associated with this filtered list are presented in Table 6. Over-represented biological process categories fell into two main groups: transport, including lipid transport and cell cycle genes. This GO analysis should be treated

with some caution as it is limited by the incomplete annotation of salmon genes, and is not corrected for false discovery. Nevertheless, investigating in more detail both GO-annotated and GO-unannotated gene lists, it is clear that the largest group is involved in lipid transport and lipid metabolism, with plasma liporotein mRNAs (apoB, apoCII, apoA1), cholesterol and phospholipid transport (StarD3 and StarD2), and circulating fatty acid binding proteins (albumin and PTGDS) all having higher hepatic mRNA levels in families with high muscle n-3LC-PUFA. Two important fatty acid metabolism genes, acyl-CoA oxidase and long chain acyl-CoA dehydrogenase were expressed at lower level in families with high percentage n-3LC-PUFA, whilst two glycolytic genes (GAPDH and TPI) were higher in expression. A group of nine genes associated with cell cycle and cell growth were also expressed at lower levels in high n-3LC-PUFA families, whilst extracellular matrix proteins with cell growth functions were increased (LTBP1 and ECM1). LTPB1 was the gene with the highest expression differences between high and low n-3LC-PUFA families, and is responsible for tethering latent TGFβ1, an important growth regulating cytokine, to the extracellular matrix (Massague, 1998). A group of 14 differentially expressed genes are involved in transcription and transcriptional regulation, some higher and some lower in high n-3LC-PUFA families.

4. Discussion

Comparison of 48 randomly selected families of Atlantic salmon indicates that the level of n-3LC-PUFA, expressed as a percentage of total fatty acids in muscle, is a highly heritable trait. This and similar traits have also been measured in terrestrial livestock (Karamichou et al., 2006;De Smet et al., 2004) and significant heritabilities also demonstrated. However, the heritability for the percentage flesh n-3LC-PUFA in salmon is considerably higher than for other livestock. It is also apparent that total flesh lipid level also has a high heritable component, and this trait is also well known to be heritable in a variety of farm animal species (Kerry and Ledward, 2009).

Importantly, the absolute level of n-3LC-PUFA in salmon flesh is highly positively correlated with total lipid content because, as the lipid content increases, the content of individual fatty acids, including n-3LC-PUFAs will inevitably increase as well. Both of these parameters were also correlated with growth parameters, intial and final weights. However, the levels of flesh n-3LC-PUFA as a percentage of total fatty acids are inversely correlated with flesh total lipid and with final weight. This is because in the experiment described here the salmon are being fed diets deficient in n-3LC-PUFA and the lipid deposited in flesh is reflective of the diet content, thus the more lipid deposited, the lower the percentage n-3LC-PUFA. Nevertheless, there was still a large variation in flesh n-3LC-PUFA content between families, even amongst families showing the same total flesh lipid content. Taken together this suggests that families with high flesh n-3LC-PUFA content must either selectively incorporate it into flesh from the small amount still present in the diet, or that the n-3LC-PUFA originates from biosynthesis in either liver or intestine. Liver and intestine are the major sites of synthesis in salmon, muscle biosynthesis being negligible (Tocher et al., 2003).

Numerous previous studies have demonstrated that n-3 LC-PUFA biosynthesis in both liver and intestine is induced in salmon fed diets deficient in these nutrients (Zheng et al., 2005b;Leaver et al., 2008;Bell et al., 2002;Bell et al., 1997), and this is due to transcriptional activation of genes for the biosynthetic enzymes (Zheng et al., 2004;Leaver et al., 2008;Morais et al., 2009). The driving mechanism for this up-regulation may be the reduced cholesterol content of vegetable oil-based diets, which results in activation of SREBP transcription factors and an increase in mRNA levels of genes involved in cholesterol biosynthesis as well as in LC-PUFA biosynthesis (Leaver et al., 2008;Zheng et al., 2009). The results of the current study indicate that the mRNA levels of hepatic desaturases and elongases, and also of cholesterol biosynthetic enzymes were not different between families, presumably indicating that the vegetable oil diet had similarly increased mRNAs for these proteins in all fish. However there was a clear increase in hepatic mRNA levels for lipid transport genes in families with a high percentage of n-3LCPUFA in flesh, as well as differences in expression of cell cycle and growth related genes, and in genes for secretory pathways and various secreted proteins of the plasma and extracellular matrix.

Although there are limitations to the identification of fish-mammal homologues (or paralogues) based on BLAST, and limitations in the specificity (due to the possibility of cross-hybridisation of related genes) of cDNA arrays such as that used here, it may be possible to infer potential mechanisms for these differences between high and low LC-PUFA families. For example, in mammals several studies have shown that the nuclear receptor HNF4α (NR2A1) is a major transcriptional regulator of many of the genes listed, particularly the lipid transport lipoproteins, apoB, apoC1 and apoA1, and also of Sepp1 and various members of the Serpin protease inhibitors (Boj et al., 2006;Odom et al., 2004;Kel et al., 2008;Naiki et al., 2002), all of which are among the genes expressed more highly in high LC-PUFA families compared to low. Studies of mice defective for HNFα have shown that this transcription factor is absolutely required for constitutive expression of lipid transport genes responsible for VLDL secretion in liver (Hayhurst et al., 2001). One source of the high n-3LC-PUFA in flesh of some families is presumably liver biosynthesis, because these fatty acids are deficient in the diet. This newly synthesised LC-PUFA must be delivered to tissues as a component of lipoproteins, principally VLDL, synthesised in liver, and it is therefore possible that variations in the activity or expression of hepatic HNF4α can influence flesh n-3LC- PUFA content.

It is notable that there are significantly differentially expressed genes with homologues whose products are known to form regulatory interactions with HNFα in mammals. The gene with the greatest fold change (×3.55) of expression in high compared to low n-3LC-PUFA is LTBP1 (which encodes latent transforming growth factor beta binding protein 1). This protein binds Tgfβ1 (transforming growth factor β1) and tethers it in latent form to the extracellular matrix, this being a crucial step in regulating Tgfβ1 function (Ramirez and Rifkin, 2009). Tgfβ1 is a cytokine with a myriad of functions, including regulation of extracellular matrix synthesis, cell cycle progression and differentiation, as well as being important in cardiovascular, neoplastic and fibrotic disease processes (Massague, 1998). Tgfβ1 has also been specifically identified as a down-regulator of HNF4α activity, through activation of SMAD signalling networks (Chou et al., 2003;Cicchini et al., 2006), thus suggesting that LTBP1 might indirectly affect HNFα activity, by virtue of its regulatory

interaction with Tgfβ1. Also relevant is the appearance of Snai1 whose expression is reduced in high LC-PUFA families. Snai1 encodes a gene for a zinc finger transcription factor which also down-regulates HNF4α expression (Cicchini et al., 2006) and is involved in some of the same cellular processes as TGF1β. It is also notable, with regard to the actions of TGFβ1 and Snai1, that the mRNA expression of a group of genes involved in cell cycle progression are decreased in high n-3LC-PUFA salmon families, and a group which encode extracellular matrix components, including LTBP1 are increased.

In addition to the increased expression of lipid transport genes in the livers of salmon families with high flesh n-3LC-PUFA, there are also two genes that are instrumental in fatty acid metabolism and which are decreased in these families. These genes encode acyl-CoA oxidase (ACOX1) and a long chain acyl CoA dehydrogenase (ACADL). Since these enzymes can metabolise n-3LC-PUFA, a reduction in levels might lead to an increase in supply in the liver, and thus also increase availability for export to other tissues.

A variety of genes whose products are involved in transcription, protein processing and transport, or are involved in the regulation of these pathways, are differentially expressed when comparing high and low n-3LC-PUFA families. Since it is appears that a number of lipid transport, metabolic and cell cycle process genes differ in expression, it could be surmised that any resulting differences in these transcriptional and protein processing pathways were associated with the specific requirements of lipid transport, metabolism and growth.

In conclusion this study has established that n-3LC-PUFA level is a highly heritable trait in Atlantic salmon, and that there are specific hepatic mRNA expression patterns associated with high flesh n-3LC-PUFA, which indicate possible mechanisms for family-dependent deposition in flesh. Thus, this study opens the way to identifying quantitative trait loci for n-3LC-PUFA level in Atlantic salmon fed LC-PUFA deficient diets, an important objective given the current constraints on the supply of these valuable fatty acids, and their critical role in human nutrition.

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Figures

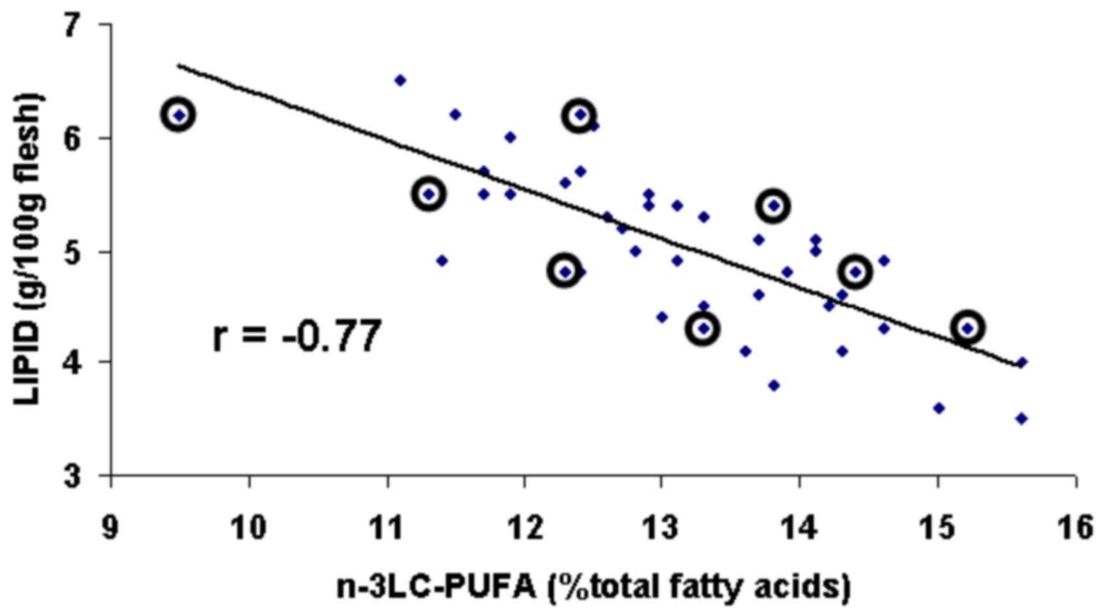

Figure 1. Correlation (Pearson) of family flesh percentage n-3LC-PUFA with family total lipid level. Ringed points indicate the data points for families used for microarray analysis.

Tables

Table 1. Fatty acid content of diet

Fatty Acid	Percentage
14:0	1.2
16:0	17.4
18:0	2.9
Total saturated	22.9
16:1n-7	1.1
18:1n-9	36.8
18:1n-7	2.0
20:1n-9	3.3
22:1	1.1
Total monoenes	44.7
18:2n-6	16.4
20:4n-6	0.2
Total n-6 PUFA	17.2
18:3n-3	9.6
20:5n-3	1.9
22:6n-3	2.3
Total n-3 PUFA	14.6
Total PUFA	32.4

Table 2. Lipid and growth parameters of families used for microarray analysis.
Percentage n-3LC-PUFA was calculated by adding percentages of 22:6n-3, 22:5n-3, 20:5n-3 and 20:4n-3 each expressed as a percentage of total fatty acids.

Family (Pairs)	Lipid (g/100g flesh)	Percentage n-3LC-PUFA	Absolute n-3LC-PUFA (mg/100g flesh)
44	4.3±0.3	13.3±0.8*(low)	481±16*
43	4.3±0.1	15.2±1.1(high)	560±38
33	4.8±0.7	12.3±1.3*(low)	504±52*
36	4.8±0.3	14.4±1.0(high)	611±64
48	5.5±1.2	11.3±0.9*(low)	557±121
10	5.4±0.6	13.8±0.9(high)	659±75
37	6.2±0.7	9.5±0.8*(low)	512±106
6	6.2±0.8	12.4±0.7(high)	639±57

* Difference for family pairs ($p<0.05$, t-test)

Table 3. Pairwise correlations coefficients (Pearson) between lipid and growth parameters in 48 salmon families.

	LIPID (g/100g)	PERCENTAGE n-3LC-PUFA	ABSOLUTE n-3LC-PUFA (mg/100g)	INITIAL MASS
PERCENTAGE n-3LC-PUFA	-0.77*			
ABSOLUTE n-3LC-PUFA	0.68*	-0.13		
INITIAL MASS	0.338*	-0.08	0.43*	
FINAL MASS	0.53*	-0.43*	0.40*	0.20

* significant correlation at p<0.05 (t-distribution)

Table 4. Heritability of flesh lipid parameters.

	Lipid (g/100g flesh)	Percentage n-3LC-PUFA	Absolute n-3LC-PUFA (mg/100g flesh)
Heritability	0.69	0.77	0.34
Standard error	0.14	0.14	0.11

Table 5. List of mRNAs from microarray analysis which are expressed at higher or lower level in salmon with high flesh n-3LC-PUFA compared to low.
Only those features which are significantly ($p<0.05$, T-test), and greater than 1.25 fold higher or lower are listed. Shaded features are significant after false discovery rate correction.

	FOLD CHANGE	p-VAL	BLASTX similarity $<e^{-10}$
LIPID TRANSPORT	1.460	0.00001	APOB apolipoprotein B
	1.504	0.00002	APOB apolipoprotein B
	1.316	0.00616	APOB apolipoprotein B
	1.272	0.00513	APOC1 Apolipoprotein C-I
	1.335	0.00153	APOA2 Apolipoprotein A-II
	1.331	0.00020	ALB Serum albumin 2
	1.311	0.01270	STARD3 StAR-related lipid transfer protein 3
	0.701	0.01310	PCTP phosphatidylcholine transfer protein (STARD2)
	0.643	0.00543	PTGDS prostaglandine D synthase
	0.525	0.00256	PTGDS prostaglandine D synthase
	0.693	0.00275	PTGDS prostaglandine D synthase
	0.772	0.02740	PTGDS prostaglandine D synthase
LIPID METABOLISM	1.329	0.00024	CYP2J2 Cytochrome P450 2K1
	1.320	0.00025	RDH4 microsomal NAD+-dependent retinol deh'ase 4
	0.701	0.02210	ACADL Acyl CoA Dehydrogenase
	0.709	0.04000	ACOX1 Acyl CoA Oxidase
GLYCOLYSIS AND CARBOHYDRATE METABOLISM	1.384	0.00001	GAPDH glyceraldehyde-3-phosphate dehydrogenase
	1.315	0.00115	GAPDH glyceraldehyde-3-phosphate dehydrogenase
	1.284	0.00593	GAPDH glyceraldehyde-3-phosphate dehydrogenase
	1.260	0.00155	TPI1 triosephosphate isomerase 1
	0.685	0.00996	AGL amylo-1, 6-glucosidase/4-alpha-glucanotransferase
	0.756	0.02500	DHDH dihydrodiol dehydrogenase (dimeric)
MITOCHONDRIAL METABOLISM	1.353	0.00019	BCAT2 branched chain aminotransferase 2
	1.343	0.01930	NDUFS1 NADH dehydrogenase (ubiquinone) Fe-S protein1
	0.788	0.01120	ATP5G1 mitochondrial ATP synthase F0 complex c1
TRANSCRIPTION	1.265	0.00249	ING5 inhibitor of growth family, member 5
	1.325	0.00187	ZNF420 zinc finger protein 420
	1.261	0.03250	ZNF782 zinc finger protein 782
	1.258	0.02610	CHRAC1 chromatin accessibility complex 1
	1.281	0.01380	POLR2I DNA-directed RNA pol II 14.5 kDa polypeptide 1
	1.332	0.01680	PBXIP1 pre-B-cell leukemia transcription factor interacting 1
	1.275	0.00037	LDB2 lim homeobox protein cofactor isoform 2
	1.307	0.01020	RNASEH2A ribonuclease H2, subunit A
	0.757	0.01110	SSBP3 single stranded DNA binding protein 3
	0.746	0.00738	CTDSP2 CTD small phosphatase 2
	0.746	0.00898	HDAC7 histone deacetylase 7
	0.797	0.00358	RBM14 RNA binding motif protein 14
	0.790	0.03370	SNAI1 snail homolog 1
	0.753	0.01330	SATB1 special AT-rich sequence binding protein 1
RNA PROCESSING	0.746	0.00509	HNRNPL heterogeneous nuclear ribonucleoprotein L1
PROTEIN SYNTHESIS, PROCESSING AND TRANSPORT	1.276	0.03580	RPL7A Ribosomal protein L7a
	1.357	0.00662	USP4 ubiquitin specific peptidase 4
	1.286	0.02410	NACA nascent polypeptide-associated complex alpha subunit
	1.329	0.03910	FKBP2 FK506 binding protein 2, 13kDa
	1.272	0.03540	GPM6B glycoprotein M6B
	1.743	0.01200	C1GALT1C1 core 1 beta3-galactosyltransferase chaperone
	1.323	0.01940	RJD9 N-acetylglucosamine-1-phosphate transferase, gamma
	0.724	0.00219	COPZ2 nonclathrin coat protein zeta2-COP
	0.783	0.04890	NSFL1C protein

	0.760	0.00986	SEC13 sec13-like 1
	0.725	0.00421	ADRM1 adhesion regulating molecule 1
CYTOSKELETON	1.534	0.03170	CPLX4 complexin 4
	1.337	0.00002	NEBL4 nebulette non-muscle isoform 4
	1.402	0.03940	NEBL nebulette
	1.421	0.01630	SEMA3F semaphorin 3fa
	0.758	0.04300	MYH1 myosin 1
	0.709	0.04740	TTN titin
CELL CYCLE AND GROWTH	3.545	0.04820	LTBP1 latent transforming growth factor beta binding protein 1
	1.351	0.00029	ENOX2 cytosolic ovarian carcinoma antigen 1b
	1.264	0.03490	ECM1 extracellular matrix protein 1
	0.782	0.04390	CCNH cyclin H
	0.697	0.01190	ZC3HC1 zinc finger, C3HC-type containing 1
	0.692	0.00633	ZC3HC1 zinc finger, C3HC-type containing 1
	0.726	0.01810	KATNA1 katanin p60 (atpase-containing) subunit a1
	0.799	0.02840	MMP19 matrix metallopeptidase 19
	0.787	0.01660	HTRA1 HtrA serine peptidase 1
	0.744	0.00314	RRM2 ribonucleotide reductase M2
	0.730	0.01440	HIST1H2AG histone macroH2A1.1
	0.777	0.03220	MAPRE1 microtubule-associated protein, RP/EB family 1
DEFENSE AND IMMUNE SYSTEM	1.329	0.00146	C3 complement C3-S
	1.334	0.01490	FGB fibrinogen, B beta polypeptide
	1.320	0.00119	SERPINC1 antithrombin
	1.286	0.00112	SERPIND1 serpin peptidase inhibitor D1
	1.296	0.02180	LYZ Lysozyme
	1.313	0.00001	SEPP1B Selenoprotein P, plasma, 1b
	1.286	0.00037	SEPP1A selenoprotein P
	0.793	0.01810	IGK immunoglobulin light chain
	0.776	0.02720	DDB1 damage-specific dna binding protein127kda
	0.770	0.01790	XPA binding protein 1 isoform 1
REPRODUCTION	0.777	0.02930	ZPC zona pellucida-like protein
	0.800	0.00974	ZPC zona pellucida protein X
	0.738	0.04970	MSMB beta-microseminoprotein
CU/FE TRANSPORT	0.479	0.01600	STEAP4 STEAP family member 4
NERVE	1.383	0.00199	ODZ2 neurestin alpha
	1.337	0.00620	GABRB2 gamma-aminobutyric acid A receptor, beta 2
TIGHT JUNCTION	1.289	0.03030	CLDN5 claudin 5b
SIGHT	0.700	0.00094	CRYGB crystallin, gamma B
UNKNOWN	1.904	0.02610	CCDC69 coiled-coil domain containing 69
	1.351	0.00078	C6orf58 Uncharacterized protein C6orf58 homolog precursor
	1.311	0.02920	CAMK2A Ca/calmodulin-dependent protein kinase II alpha
	1.326	0.00001	hypothetical protein LOC678543
	1.282	0.01190	FAM185A family with sequence similarity 185, member A
	1.281	0.04520	FAM13A1 family with sequence similarity 13, member A1
	1.340	0.01100	novel protein (zgc:55292)
	0.607	0.00137	TRIM39 tripartite motif protein 39
	0.603	0.04130	COMTD1 catechol-O-methyltransferase domain containing 1
	0.664	0.02090	LOC440330 hypothetical protein
	0.775	0.04960	FAM45A family with sequence similarity 45, member A
	0.749	0.04690	SORBS2 sorbin and SH3 domain containing 2 isoform 8
	0.748	0.00645	C8orf30A chromosome 8 open reading frame 30A
MOBILE ELEMENT	1.255	0.00060	transposase
	1.253	0.00347	ReO6 transposon
	0.573	0.01360	reverse transcriptase

Table 6. Overrepresented Gene ontology (GO) biological process categories in salmon with high flesh n-3LC-PUFA compared to low.

Category	Genes in Background	Genes in List	p-Value
GO:51234: establishment of localization	465	13	0.00119
GO:6810: transport	423	12	0.00178
GO:6869: lipid transport	13	2	0.0095
GO:6820: anion transport	26	2	0.036
GO:15698: inorganic anion transport	25	2	0.0335
GO:278: mitotic cell cycle	28	2	0.0413
GO:51325: interphase	11	2	0.0068
GO:226: microtubule cytoskeleton organization	17	2	0.0161
GO:48731: system development	52	3	0.0216
GO:45045: secretory pathway	31	2	0.0497

’In Background’, the number of genes present in the indicated GO category present on the total microarray. ‘In List’, the number of genes in the indicated GO category present in the output list (high % n-3LC-PUFA versus low % n-3LC-PUFA). The p-Value indicates the probability that the GO category is over-represented in the output list compared to the background list.