Effect of dietary digestible energy content on expression of genes of lipid metabolism and LC-PUFA biosynthesis in liver of Atlantic salmon (*Salmo salar* L.)

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Abstract

The relationship between lipid and digestible energy content of the feed and growth performance has been exploited with great effect in Atlantic salmon (*Salmo salar*). The precise metabolic consequences of so-called "high-energy" feeds have not been fully defined, but increased and altered tissue lipid deposition patterns impacting on carcass and product quality have been reported. Recent studies on global gene expression have shown that dietary lipid and digestible energy content can have significant effects on gene expression in salmonids. In addition, we recently showed that functional feeds with reduced digestible energy significantly improved outcomes in response to inflammatory disease in salmon. The present study aimed to elucidate and clarify the effects of dietary digestible energy content (22, 20 and 18 MJ/Kg; HE, ME and LE diets, respectively) on lipid and fatty acid metabolism in salmon fed diets containing graded amounts of lipid. Specifically the effects on liver lipid and fatty acid composition, and on the hepatic expression of genes of lipid and fatty acid metabolism were determined. Final weight and weight gain were significantly higher, and FCR lower, in fish fed the HE diet. Crude lipid content was significantly lower in fish fed the LE diet compared to fish fed the two higher energy contents. Significantly lower total lipid and triacylglycerol levels were recorded in liver of fish fed the LE diet compared to fish fed the higher energy diets. Liver lipids in salmon fed the LE diet had generally significantly higher proportions of saturated fatty acids and long-chain polyunsaturated fatty acids (LC-PUFA), and lower monounsaturated fatty acids, C18 and n-6 PUFA. Consistent with this, salmon fed the LE diet showed increased liver expression of both Δ6 and Δ5 fatty acyl desaturases in comparison to fish fed the diets with higher energy levels. Fatty acid synthase expression showed a clear upward trend as dietary energy decreased, and sterol regulatory element binding protein 2 and liver X receptor showed reciprocal trends that were consistent with the level of dietary cholesterol that reflects digestible energy content. Although not statistically significant, these trends were biologically logical, significant and relevant. Expression of genes of fatty acid oxidation was less consistent. Overall, reduced dietary digestible energy/lipid content alone, without major changes in dietary fatty acid composition, altered the expression of key genes of lipid and fatty acid metabolism resulting in general up-regulation of biosynthetic pathways.

Introduction

Lipid is required in the diet of fish to supply both metabolic energy and some essential nutrients such as specific (essential) fatty acids (EFA) and, in early life stages, possibly cholesterol and intact phospholipid (Sargent et al., 2002). Consequently, it is not possible to define a single dietary lipid requirement for any species as this will vary depending upon the other dietary components supplying energy, such as protein and carbohydrate (NRC, 2011). Although an optimum level of dietary lipid cannot be defined, there are lower and upper limits within which dietary lipid should be supplied. The lower limit is the level required to supply the requirements for essential components like EFA, and will depend upon the precise lipid class and fatty acid compositions of the dietary lipid sources. Increasing dietary lipid above this minimum level will support higher growth rates due to the simple fact that lipid supplies twice as much energy per unit mass compared to other dietary energy sources, and so more energy can be supplied in the feed per unit mass, a phenomenon often referred to as "protein sparing" (Hemre et al., 1995; Bendiksen et al., 2003). However, an upper limit will be reached where the biochemical and physiological capacities of the animal to digest and/or metabolise dietary lipid is exceeded leading to reduced digestibility (undigested lipid in faeces) and/or unwanted deposition of lipid in the peritoneal cavity, liver, or other tissues (Company et al., 1999; Craig et al., 1999; Hemre and Sandnes, 1999; Gaylord and Gatlin, 2000).

This relationship between lipid and digestible energy (DE) content of the feed and growth performance has been particularly exploited in species such as Atlantic salmon (*Salmo salar*), which deposit significant amounts of lipid in the flesh and thus are able to tolerate and utilize high lipid, such that the dietary levels have increased steadily over the years (Sargent et al., 2002). Although excess deposition and altered tissue lipid deposition patterns are known to cause various problems in terms of carcass and product quality in farmed fish like salmon (Sargent et al., 2002), the precise metabolic consequences of so-called "high-energy" feeds have not been fully defined. However, recent studies looking at global gene expression using transcriptomic (microarray) and proteomic approaches have shown that dietary lipid and DE content can have significant effects on gene expression in salmonids (Kolditz et al., 2008a,b; Panserat et al., 2008; Higgs et al., 2009).

Recently, we investigated the effects of functional feeds, which included alteration of dietary lipid and DE content, in the control of Heart and Skeletal Muscle Inflammatory (HSMI) disease in Atlantic salmon experimentally infected with the causative agent, Atlantic salmon reovirus (ASRV) (Martinez-Rubio et al., 2012). The concept of clinical nutrition and

functional foods is well known in human nutrition. These are defined as foods that contain a component (whether a nutrient or not) that could be beneficial for the state of well-being and health, or reduce the risk of a disease, beyond the basic nutritional requirement (Bellisle et al., 1980). This approach is also becoming increasingly used in aquaculture, as it could potentially lead to economic savings in terms of increased productivity and lower costs of disease treatment/management (Raynard et al., 1991; McCoy et al., 1994; Tacchi et al., 2011). In the HSMI study, we investigated the effects of functional feed formulations that contained reduced energy levels through lower lipid contents (18%) and altered levels of long-chain polyunsaturated fatty acids (LC-PUFA), including increased eicosapentaenoic acid (EPA, 20:5n-3) in comparison to a standard commercial feed that contained 31% lipid. A much reduced inflammatory response to ASRV infection, and reduced severity of heart lesions were found in fish fed the functional feeds, and transcriptome (microarray) analysis of heart showed that expression of inflammation/immune related genes was greatly affected. However, in addition to effects on immune genes, it was clear that the feeds were also having significant effects on the expression of metabolic genes in the heart, including those of lipid and fatty acid metabolism (Martinez-Rubio et al., 2012, Supplementary Tables). However, it was not clear whether dietary DE and lipid content or fatty acid composition were primarily responsible for the alterations in metabolic gene expression.

The primary objective of the present study was to elucidate and clarify the effects of dietary DE on lipid and fatty acid metabolism in Atlantic salmon fed diets containing graded amounts of lipid. Specifically the effects on liver lipid and fatty acid composition, and on the hepatic expression of genes of lipid and fatty acid metabolism were determined. The expression of key genes involved in the major lipid metabolic pathways including lipogenesis, fatty acid β-oxidation, and LC-PUFA biosynthesis, and the major transcription factors and nuclear receptors controlling and regulating their expression, were investigated by quantitative real-time PCR (qPCR).

Materials and Methods

Fish and feeds

Three hundred and sixty pit-tagged Atlantic salmon (*Salmo salar* L.) post-smolts were distributed equally into 9 tanks of 1.5m diameter, 1.5m^3 volume (40 fish /tank) at the University of Stirling, Marine Environmental Research Laboratory, (Machrihanish, Argyll,

Scotland, U.K.). The tanks were supplied with flow-through seawater at ambient temperature, average 12 ^{o}C (± 1 ^{o}C). After a 3-week acclimatisation period, the fish in triplicate tanks were fed in excess (i.e. feed was not limiting) for 12 weeks with one of three feeds supplied by automatic feeders every 30 min 23 h per day. The three fishmeal-based diets were manufactured by EWOS Innovation (Dirdal, Norway), and were formulated to be isoproteic (40 % crude protein), but deliver three levels of DE being 22 (high, HE), 20 (medium, ME) and 18 (low, LE) MJ/Kg by replacing dietary oil (a 50:50 mix of fish and rapeseed oils) with starch (Table 1). The fatty acid compositions of the feeds reflected the formulations with trends of increased saturated fatty acids, 20:1n-9, 22:1n-11 and n-3 LC-PUFA, and decreased 18:1n-9, as DE decreased, reflecting the lower level of rapeseed oil and increased proportion of lipid derived from marine sources (Table 2). All the fish in each tank were individually weighed at the initiation of the experiment (415g average weight) and the mid-point and the feed ration adjusted to 0.9%. Lights and feeders were on 24h/day and waste feed was collected using an airlift system. Feed fed, waste feed, water temperature and quality were monitored daily. No mortalities or health issues were associated with the study.

Sampling

Fish were sampled at the end of the feeding period (12 weeks) with body weight and length, and liver and viscera weights recorded for all fish culled. A total of 15 fish/tank were anaesthetised (MS222) and killed by a blow to the head with three whole fish frozen immediately for analysis of proximate composition. Livers for lipid and biomolecular analysis were collected from the remaining 12 fish. Thus, samples of liver (for fatty acid and molecular analyses) were collected and frozen immediately in liquid nitrogen and stored at -80 ^{o}C prior to analysis. Further samples of liver, specifically for lipid class analysis, were collected in 5ml glass vials containing 4 ml of chloroform/methanol (2:1, by volume).

Growth performance and feed utilization

The effects of feeds on growth performance, biometry and feed utilization efficiency were calculated according to the following formulae. Weight gain (g/fish) = final weight – initial weight. Specific growth rate (SGR, % day) = 100 × [ln (final mean weight) − ln (initial mean weight)] × days^{-1}. Feed consumption (g/day) = feed intake (g) × [number of fish × days]$^{-1}$, and Feed conversion ratio (FCR) = feed intake (g) × [final biomass − initial biomass + dead fish]$^{-1}$. Hepato-somatic index (HSI, %) = 100 × [weight of liver (g)] × [weight of fish (g)]$^{-1}$. Viscero-somatic index (VSI, %) = 100 × [weight of viscera (g)] × [weight of fish (g)]$^{-1}$

142 *Proximate composition of feeds and fish*

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144 The proximate compositions of feeds and whole fish at the end of the trial were determined
145 by standard procedures (AOAC, 2000). For fish, the three fish per tank were pooled and
146 minced prior to analysis (n = 3 per dietary treatment). Moisture content was determined after
147 drying to constant weight in an oven at 105 °C for 24 h. The samples were then rigorously
148 blended into a homogeneous crumb and used for determination of feed or whole body lipid,
149 protein and ash contents. Lipid content of dried crumb was determined using the Soxhlet
150 method with extraction using petroleum ether at 120 °C (Avanti Soxtec 2050 Auto Extraction
151 apparatus; Foss, Warrington, UK). Crude protein content (N × 6.25) was determined using the
152 automated Kjeldahl method (Tecator Kjeltec Auto 1030 Analyser; Foss, Warrington, UK).
153 Ash contents were determined after heating at 600 °C for 24 h. The gross energy content of
154 the feeds was determined by Bomb Calorimetry (Gallenkamp Autobomb System).

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156 *Lipid content, lipid class and fatty acid compositions*

157 Lipid content, lipid class and fatty acid compositions of total lipid were determined in liver.
158 Liver samples were pooled according to tank (n = 3), and total lipid from approximately 1g of
159 pooled liver was extracted by homogenization in chloroform/methanol (2:1, by volume)
160 according to Folch et al. (1957), and determined gravimetrically. Liver lipid class
161 compositions were determined by single-dimension double-development high-performance
162 thin-layer chromatography (HPTLC) and densitometry (Henderson and Tocher, 1992). Fatty
163 acid methyl esters (FAME) of total lipid were prepared by acid-catalyzed transmethylation
164 (Christie, 2003), and separated and quantified by gas-liquid chromatography (Carlo Erba
165 Vega 8160, Milan, Italy) using a 30m x 0.32 mm i.d. capillary column (CP Wax 52CB,
166 Chrompak, London, U.K.) and on-column injection at 50^{o}C. Hydrogen was used as carrier
167 gas and temperature programming was from 50 ^{o}C to 150 ^{o}C at 40 ^{o}C min^{-1} and then to 230
168 ^{o}C at 2.0 ^{o}C min^{-1}. Individual methyl esters were identified by comparison with known
169 standards and by reference to published data (Ackman, 1980; Tocher and Harvie, 1988). Data
170 were collected and processed using Chromcard for Windows (version 1.19).

171 *Determination of gene expression by quantitative real-time PCR*

172 Reverse transcription quantitative real-time PCR (qPCR) analysis was performed to evaluate
173 the relative expression of genes involved in major lipid metabolism pathways including fatty

acid synthesis (fatty acid synthase, FAS), LC-PUFA biosynthesis (fatty acyl desaturases, *Δ6fad_a* and *Δ5fad*; fatty acid elongases, *elovl2*, e*lovl5a* and *elovl5b*), and β-oxidation (carnitine palmitoyl transferase-1, CPT1; acyl CoA oxidase, ACO), and their control and regulation (sterol-responsive element-binding protein 2, SREBP2; liver X receptor, LXR; peroxisome proliferator-activated receptors, PPARα, PPARβ and PPARγ). The qPCR primer sequences (obtained by literature searches), annealing temperature (Tm) and size of amplicon are given in Table 3. In addition, amplification of cofilin-2 and elongation factor-1α (elf-1α) was performed and their expression was confirmed as sufficiently stable across treatments for normalization. These genes had been identified as suitable reference genes in previous qPCR studies in salmon (Morais et al., 2011).

For qPCR, 2 µg of column-purified total RNA per sample was reverse transcribed into cDNA using the High-Capacity cDNA RT kit (Applied Biosystems, Paisley, U.K.), following manufacturer's instructions, but using a mixture of the random primers (1.5µl as supplied) and anchored oligo-dT (0.5 µl at 400 ng/ µl, Eurofins MWG Operon, Ebersberg, Germany). Negative controls (containing no enzyme) were performed to check for genomic DNA contamination. cDNA was then diluted 20-fold with water, after a similar amount of cDNA was pooled from all samples. qPCR analysis used relative quantification with the amplification efficiency of the primer pairs being assessed by serial dilutions of the cDNA pool. qPCR amplifications were carried out in duplicate (Quantica, Techne, Cambridge, U.K.) in a final volume of 20 µL containing either 5 µL (for most genes) or 2 µL (for the reference genes and other highly expressed genes) diluted (1/20) cDNA, 0.5 µM of each primer and 10 µL AbsoluteTM QPCR SYBR® Green mix (ABgene). Amplifications were carried out with a systematic negative control (NTC-non template control, containing no cDNA). The qPCR profiles contained an initial activation step at 95 °C for 15 min, followed by 30 to 40 cycles: 15 s at 95 °C, 15 s at the specific primer pair annealing Tm and 15 s at 72 °C. After the amplification phase, a melt curve of 0.5 °C increments from 75 ºC to 90 °C was performed, enabling confirmation of amplification of single products, and sizes were checked by agarose gel electrophoresis and identities confirmed by sequencing. Non-occurrence of primer-dimer formation in the NTC was also verified. Data were analyzed using the relative expression software tool (REST 2009, http://www.gene-quantification.info/), which employs a pair wise fixed reallocation randomization test (10,000 randomizations) with efficiency correction, to determine the statistical significance of expression ratios (or gene expression fold-changes) between two treatments (Pfaffl et al., 2002).

Statistical analyses

The effects of dietary treatments on fish growth performance, feed efficiency, biometry, liver lipid contents, class and fatty acid compositions were analysed by Analysis of Covariance (ANCOVA) with fish weight (or pooled fish weights) as the covariate. Briefly, the weight gain based on repeated weights on individual fish was modelled by fitting individual growth trajectories with the help of a multilevel model using the fish tag ID, tank and treatments (DE levels) as the levels of variation. Time dimension was added to the model by the day of weighing since the start of the trial and its effect was modelled with the help of cubic splines. FCR was calculated by dividing the observed feed intake by the observed weight gain and log-transformed before conducting stats. Differences in liver and visceral weights between diets were analysed including fish body weight as a covariate to account for fish size differences at the end of the trial. Afterwards the condition factor, hepatosomatic and visceral indices were calculated based on the model estimates. Differences in proximal composition analyses of the fish, fatty acid profile and lipid class composition in the liver were also analysed statistically including a covariate. Since these analyses were conducted on pool samples the average weight of each fish pool was used as a covariate in order to account for size differences. Multilevel models were fitted with the lme4 package of the R language (R Development Core Team 2008). All treatment effects were based on posterior simulation (n = 2,500) with 95 % credible intervals. Ninety-five percent credible intervals were interpreted as statistical significant at p = 0.05 % level when the interval did not overlap the reference value in question.

Results

Growth performance and body composition

The salmon more than doubled their weight over the period of the trial with final weights and weight gain showing clear effects of DE content and so both were significantly higher in fish fed the HE diet compared to fish fed the diets with lower DE (Table 4). Similarly, FCR showed an increasing trend as DE decreased and so was significantly lower in fish fed the HE diet compared to fish fed the diets with lower DE. Both HSI and VSI tended to be lower in fish fed the LE diet with the lowest DE, but the differences were not statistically significant (Table 4). Crude lipid of whole fish showed a clear trend with DE content with the content being significantly lower in fish fed diet LE compared to fish fed the ME and HE diets (Table 4).

Liver lipid and fatty acid compositions

The liver lipid contents showed a clear relationship with dietary DE content, increasing as DE increased, with fish fed HE having significantly higher lipid contents (Table 5). The lower lipid contents in liver of fish fed the diets with lower DE were reflected in lower levels of TAG, with significantly lower levels in fish fed diet LE. Consistent with this, liver polar lipids (phospholipids) and cholesterol, reflecting membrane lipids, were generally significantly higher in salmon fed the LE diet (Tables 5). The effects of the different feeds on fatty acid compositions of liver is shown in Table 6. Livers of salmon fed diet LE showed generally increased saturated fatty acids and LC-PUFA, and decreased monounsaturated fatty acids, C18 PUFA and n-6 PUFA. Thus, 16:0, 18:0, and DHA were all generally increased, whereas, 18:1n-9, 18:2n-6 and 18:3n-3 were generally decreased in liver of salmon fed diet LE compared to livers of fish fed the HE diet. Due to the variation observed in the data not all of these effects were statistically significant, but the clear overall pattern observed supported the general conclusion.

Liver gene expression

Salmon fed the LE diet showed significantly increased liver expression of both Δ6 and Δ5 fatty acyl desaturases (Fad) in comparison to fish fed the diets with higher energy levels (Fig. 1). In contrast, there were no significant effects of diet on the expression of fatty acyl elongases (Elovl2 or Elovl5 transcripts) (Fig.2). There was a clear effect of dietary energy upon fatty acid synthase (FAS) expression, which increased with reducing DE albeit that the relatively large standard deviations rendered the effect non-significant statistically (Fig. 3A). There was no effect of dietary energy on carnitine palmitoyl transferase 1 (CPT-1), a marker of mitochondrial fatty acid β-oxidation, and although it appeared as though the expression of acyl-CoA oxidase (ACO), a marker of peroxisomal fatty acid oxidation, was higher in fish fed the LE diet, this was not significant (Fig.3B & C). Although statistically non-significant, reciprocal trends in the expression of sterol regulatory element binding protein 2 (SREBP2) and liver X receptor (LXR) with DE were observed (Fig. 4). Similarly, the liver expression of all three peroxisome proliferator-activated receptor subtypes (α, β and γ) tended to show increasing expression as dietary DE decreased, but none of the differences were statistically significant (Fig.5).

Discussion

The use of high energy feeds in aquaculture is based on their ability to promote growth and feed efficiency and this was observed in the present study with increased final weights and weight gain, and lower FCR, in fish fed diet HE. It is important to note that this was observed despite the fish not being limited by ration. That is, the fish were fed in excess so that fish had the opportunity to vary feed intake to compensate for the different dietary DE. Increased weight gain in response to increased dietary lipid content has been consistently shown in salmonids including brown (*Salmo trutta*) and rainbow (*Oncorhynchus mykiss*) trout, and Atlantic salmon (Arzel et al., 1993; Luzzana et al., 1994; Hemre and Sandnes, 1999). As a result, dietary lipid in commercial feeds for salmon doubled in a twenty-year period reaching around 35% of total diet by the mid 1990s (Einen and Roem, 1997).

However, in addition to increasing growth, increased dietary DE through higher dietary lipid can also have negative impacts on other aspects of fish performance. Chief among these is based on the well-known positive correlation between dietary lipid levels and tissue/body lipid levels of fish (Sargent et al., 2002). Although the lipid data in the present study showed some variation, due to the use of an essentially ungraded stock, chosen to eliminate bias towards fast or slow growers, liver lipid contents were reduced significantly with decreasing dietary DE content. This was confirmed by the significantly lower total neutral (storage) lipid and TAG in livers of fish fed diet LE compared to fish fed HE. In this respect it is noteworthy that previous studies have reported that the level of lipid in tissues of salmon can vary considerably within a population, but the extent to which the observed biological variation is determined by environmental or genetic factors is not known (Bell et al., 1998). Notwithstanding the above, previous studies support the present data, as high dietary lipid increased lipid levels in salmonids including rainbow trout and Atlantic salmon (Bell et al., 1998; Dias et al., 1999; Hemre and Sandnes, 1999). Increased tissue lipid in response to increased dietary lipid was also shown in marine fish including sea bass (*Dicentrarchus labrax*) and turbot (*Psetta maximus*) (Catacutan and Coloso, 1995; Saether and Jobling, 2001). Increased liver lipid deposition may have unwanted physiological effects such as the development of fatty liver pathology in marine fish (Caballero et al., 1999), possibly through mechanisms involving altered gene expression, and hence the focus on this aspect in the present study.

In relation to the above, a highly significant effect of dietary DE content in terms of altered gene expression was the higher expression of both Δ6- and Δ5-Fads in liver of salmon consuming the LE diet compared with fish consuming the higher energy diets. Liver transcript expression in fish fed LE was around 3-fold higher than that in fish fed HE, and

was highly significant (p-value = 0.002). In contrast, dietary DE did not appear to affect the expression of fatty acid elongases, consistent with the fact that these enzymes do not show the same level of nutritional regulation as desaturases (Leaver et al., 2008a; Morais et al., 2009; Tocher, 2010). In addition to being the clearest effect of DE on gene expression, this was also an important result as the effects of DE and/or lipid content on the expression of genes of LC-PUFA biosynthesis had not been reported previously in salmon. Previously, microarray analysis had shown that Δ6-desaturase transcript expression was down-regulated in trout fed a high energy feed compared to fish fed a lower energy feed (Kolditz et aql., 2008b). Nutritional regulation of fatty acid desaturase gene expression was first reported in mammals. The levels of liver mRNA for both Δ6- and Δ5-desaturases were approximately 3-fold higher in rats fed a fat-free diet compared to animals fed either safflower oil (18:2n-6) or fish oil (n-3LC-PUFA) (Cho et al., 1999a). However, rats fed a diet containing triolein (18:1n-9) showed a similar high expression of both desaturases as observed in rats fed the fat-free diet (Cho et al., 1999a). Therefore, this suggested that it was the fatty acid composition of the diet, specifically a lack of PUFA, that was responsible for the higher expression, rather than the lipid content of the diets. Consistent with this, hepatic expression of Δ6-desaturase in mice fed an essential fatty acid (EFA)-deficient diet (triolein) was double that in mice fed a corn oil diet rich in 18:2n-6 (Cho et al., 1999b).

Previous work investigating the nutritional regulation of LC-PUFA biosynthesis in fish has focussed on dietary fatty acid composition (Leaver et al., 2008a; Tocher, 2010). The activity of the LC-PUFA biosynthesis pathway in freshwater carp cells was increased by EFA-deficiency (Tocher and Dick, 1999). *In vivo* dietary trials showed that the activity of the LC-PUFA biosynthetic pathway was increased in freshwater and salmonid fish fed vegetable oils rich in C_{18} PUFA compared to fish fed fish oil, rich in the n-3 LC-PUFA, EPA and DHA (Tocher et al., 1997, 2002, 2003). Consistent with this, expression of Δ6 Fad mRNA was increased in salmon fed diets lacking LC-PUFA (vegetable oil), compared to fish fed diets containing EPA and DHA (fish oil) (Zheng et al., 2004b, 2005a,b; Leaver et al., 2008b; Taggart et al., 2008). Therefore, Fad expression was increased when diets contain lower levels of the pathway end-products such as EPA and DHA. In the present study, the fatty acid compositions of the feeds were similar and, indeed the levels of the LC-PUFA, EPA and DHA were slightly higher in the LE diet, which resulted in generally higher levels of these fatty acids in fish fed LE. Therefore, the higher expression of the Fad genes in liver of fish fed the LE diet is not consistent with the previous data, supporting the view that dietary lipid content itself underpins the differences in expression observed in the present study. The only

previous study in salmon that reported LC-PUFA biosynthesis in fish fed different levels of dietary lipid gave inconclusive results. Consistent with the data in the present study, LC-PUFA synthesis in liver was higher in fish fed a low lipid diet compared to a high lipid diet when supplied as fish oil (Tocher et al., 2003). However, when the dietary lipid was supplied as vegetable oil, hepatic LC-PUFA synthesis was higher than both low and high fish oil, but there was no difference between low and high vegetable oil. This perhaps suggests a more complex interaction between lipid content and fatty acid composition, but LC-PUFA biosynthesis could be generally increased by low dietary lipid, perhaps associated with increased lipid biosynthesis in general (see below). The finding that dietary lipid content can affect the expression of the genes of LC-PUFA biosynthesis may be related to early work that suggested that the quantitative requirement for EFA may vary with dietary lipid level. Thus, the dietary requirement for n-3 LC-PUFA appeared to increase with increased dietary lipid in fingerlings of red sea bream (*Pagrus major*) and yellowtail (*Seriola quinqueradiata*) (Takeuchi et al., 1992a,b), although this was not apparent in larval gilthead sea bream (Salhi et al., 1994).

Other than the above effect on Fad genes, there were few statistically significant effects of dietary DE (lipid content) on the expression of the other genes of lipid metabolism investigated. However, several showed clear trends with the DE regression that were scientifically logical and, as argued above, in combination with each other and the growth and lipid compositional data discussed above, support the contention that some have biological significance. For instance, the effect of DE on FAS, although not statistically significant, was highly likely biologically significant as it is well established that lipogenesis and FAS, as the rate-limiting step of lipid biosynthesis pathway, is regulated by dietary lipid and is up-regulated by diets with lower lipid (DE) contents (Sargent et al., 2002). Consistent with this, early studies showed increased dietary lipid depressed lipogenesis in common carp (Shimeno et al., 1995), and high lipid diets decreased the activities of lipogenic enzymes in juvenile yellowtail (Shimeno et al., 1996). Furthermore FAS activity and gene expression was lower in trout fed a high energy diet compared to fish fed a low energy diet (Kolditz et al., 2008a).

A further example of data reinforcing each other was observed in the reciprocal responses observed in the liver expression of LXR and SREBP2, which are key regulators controlling cholesterol homeostasis. The transcription factor, LXR, regulates cholesterol catabolism, storage, absorption and transport through the transcriptional regulation of key target genes involved in these processes (Aranda and Pascual, 2001). A single LXR cDNA was recently

isolated and characterised from Atlantic salmon and shown to be similar to mammalian LXRα (Cruz-Garcia et al., 2009). The nuclear receptor, SREBP2, is activated by reduced cholesterol and is a key regulator in the biosynthesis of cholesterol (Horton et al., 2002) and, recently, SREBP2 was isolated and characterised from Atlantic salmon (Minghetti et al., 2011). In the present study, although relatively small, there were clear effects of DE on these factors with LXR showing decreased expression, and SREBP2 showing increased expression, in fish fed feeds with lower DE. This is consistent with the level of dietary cholesterol supplied by the feeds, which reflects level of dietary fish oil (Leaver et al., 2008b; Taggart et al., 2008; Tocher et al., 2008). Thus, the lower level of dietary cholesterol in the LE feed compared to the HE feed resulted in increased expression of SREBP2, promoting cholesterol biosynthesis, and lower expression of LXR, reducing cholesterol catabolism. Previously, lower expression of LXR was observed in liver of Atlantic salmon fed vegetable oil (lower cholesterol) compared to fish fed fish oil (Cruz-Garcia et al., 2009).

Peroxisome proliferator-activated receptors are ligand-activated transcription factors that have key roles in regulating lipid and fatty acid metabolism including fatty acid oxidation (esp. PPARα) and tissue lipid deposition (esp. PPARγ) in mammals (Desvergne et al., 2006). Their natural ligands include unsaturated fatty acids and their derivatives, which has led to the view that PPARs are general fatty acid sensors responding to changes in nutritional status and energy metabolism (Michalik et al., 2006). Although, compared to mammals, there is considerably less known, but available data suggests that PPARs have similar roles in the control of metabolism in fish as in mammals (Leaver et al., 2005, 2008a). It is therefore likely that PPARs would be involved in the metabolic response to dietary DE content. In the present study, although not significant, there was a clear trend for the hepatic expression of all PPAR subtypes, but especially PPARα, to increase with decreasing DE, and this was accompanied by increased expression of liver ACO, but CPT-1 expression was unaffected. Similarly, possible association of PPAR and ACO expression was observed in sea bream (*Sparus aurata*), with expression of all PPAR subtypes and ACO reduced in liver of fish fed conjugated linoleic acid (CLA) (Diez et al., 2007). In contrast, feeding Atlantic salmon with CLA increased PPARα expression in liver and this was associated with increased CPT-1 expression and β-oxidation (Leaver et al., 2006). In salmon fed the thia fatty acid, tetradecylthioacetic acid (TTA), PPARα expression in liver was decreased, but expression of ACO was unaffected (Kleveland et al., 2006). In trout fed a high energy diet, CPT-1 and ACO expression, and β-oxidation activity in liver were all increased in comparison to fish fed

a low energy diet, but PPARα expression was unaffected (Kolditz et al., 2008a). All of these data highlight the inconsistency of results obtained on expression of PPARs and genes of fatty acid oxidation. Clearly, as PPAR function is dependent upon activation by ligands, the relationship between PPAR expression, its functionality, and thus its role in controlling expression of target genes, is complicated and unclear at present (Leaver et al., 2008).

In conclusion, the present study determined the effects of dietary DE on the hepatic expression of key genes of lipid and fatty acid metabolism in Atlantic salmon fed diets containing graded amounts of lipid. Dietary DE and/or lipid content had important effects on the expression of genes involved in major lipid pathways including lipogenesis (FAS), LC-PUFA biosynthesis (Δ6 Fad, Δ5 Fad, Elovl2 and Elovl5), and cholesterol metabolism (LXR and SREBP2) that were biologically significant and relevant, and consistent with current understanding. In contrast, the effects on fatty acid β-oxidation (CPT1, ACO and PPARs) were more inconclusive. Overall though, it was clear that changes in dietary DE alone, without major changes in dietary fatty acid composition, could result in altered expression of key genes of lipid and fatty acid metabolism. Combined, these changes resulted in an overall up-regulation of lipid biosynthetic pathways. Therefore, in relation to our previous application of a clinical nutrition approach to improving disease outcomes through the use of functional feeds, the present results suggest that the beneficial effects of reduced dietary DE may include positive alterations in lipid and fatty acid metabolism (Martinez-Rubio et al., 2012).

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624 Legends to Figures

625

626 Fig. 1. Expression of fatty acyl desaturase (Fad) genes involved in long-chain polyunsaturated
627 fatty acid (LC-PUFA) biosynthesis. Expression of Δ6 Fad (A) and Δ5 Fad (B) genes in
628 liver was determined by real-time quantitative PCR. Values were normalized by dividing
629 the number of copies of the target genes by the number of copies of reference genes (an
630 average value for the expression of cofilin-2 and elf-1α). Different letters represent
631 significant differences between diets (ANOVA, $p<0.05$).

632

633 Fig.2. Expression of fatty acid elongase (Elovl) genes involved in long-chain polyunsaturated
634 fatty acid (LC-PUFA) biosynthesis. Expression of Elovl2 (A), Elovl5a (B) and Elovl5b (C)
635 genes in liver was determined by real-time quantitative PCR. Values were normalized by
636 dividing the number of copies of the target genes by the number of copies of reference
637 genes (an average value for the expression of cofilin-2 and elf-1α). Different letters
638 represent significant differences between diets (ANOVA, $p<0.05$).

639

640 Fig.3. Expression of genes involved in fatty acid biosynthesis (lipogenesis) and oxidation.
641 Expression of fatty acid synthase (A), carnitine palmitoyl tranferase-1 (B) and acyl coA
642 oxidase (C) genes in liver was determined by real-time quantitative PCR. Values were
643 normalized by dividing the number of copies of the target genes by the number of copies
644 of reference genes (an average value for the expression of cofilin-2 and elf-1α). Different
645 letters represent significant differences between diets (ANOVA, $p<0.05$).

646

647 Fig.4. Expression of genes involved in the regulation of cholesterol biosynthesis and
648 catabolism. Expression of sterol regulatory element binding protein 2 (A) and liver X
649 receptor (B) genes in liver was determined by real-time quantitative PCR. Values were
650 normalized by dividing the number of copies of the target genes by the number of copies
651 of reference genes (an average value for the expression of cofilin-2 and elf-1α). Different
652 letters represent significant differences between diets (ANOVA, $p<0.05$).

653

654 Fig.5. Expression of genes involved in the regulation of fatty acid metabolism. Expression of
655 peroxisome proliferator –activated receptors (PPAR), PPARα (A), PPARβ (B) and PPARγ

656 (C), genes in liver was determined by real-time quantitative PCR. Values were normalized
657 by dividing the number of copies of the target genes by the number of copies of reference
658 genes (an average value for the expression of cofilin-2 and elf-1α). Different letters
659 represent significant differences between diets (ANOVA, $p<0.05$).
660
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Table 1. Formulation (g/kg), proximate composition (percentage of wet weight) and digestible energy (MJ/kg) of experimental feeds with high (HE), medium (ME) and low (LE) levels of digestible energy

	HE	ME	LE
Fish meal	350.0	350.0	350.0
Wheat grain	93.1	93.1	93.1
Wheat gluten	80.0	80.0	80.0
Soy protein conc.	80.0	80.0	80.0
Pean protein conc.	70.0	70.0	70.0
EWOS premix[1]	26.9	26.9	26.9
Soy lecithin	15.0	15.0	15.0
Starch (tapioca)	10.0	80.0	150.0
Fish oil	137.5	102.5	67.5
Rapeseed oil	137.5	102.5	67.5
Proximate composition			
Moisture	4.9	5.7	6.5
Dry matter	95.1	94.3	93.5
Crude protein	40.4	40.5	40.6
Crude lipid	33.9	27.0	20.0
Digestible energy	21.9	20.1	18.2

[1]EWOS premix including minerals, vitamins, inorganic phosphorous, lysine, methione and astaxanthin to satisfy nutritional requirements (NRC, 2011).

Table 2. Fatty acid composition (percentage of total fatty acids) of total lipid of feeds containing high (HE), medium (ME) and low (LE) levels of digestible energy

	HE	ME	LE
14:0	3.3	3.5	3.7
16:0	10.0	10.2	11.4
18:0	1.9	1.8	1.9
Total saturated[1]	16.0	16.3	17.7
16:1n-7	3.2	3.5	3.8
18:1n-9	32.1	30.1	24.0
20:1n-9	7.0	7.3	7.8
22:1n-11	8.0	8.3	9.0
Total monoenes[2]	52.0	51.0	48.2
18:2n-6	12.3	12.1	12.6
20:4n-6	0.2	0.2	0.2
Total n-6 PUFA[3]	13.0	12.6	13.2
18:3n-3	5.0	4.8	4.2
18:4n-3	2.4	2.6	2.7
20:5n-3	4.4	4.8	5.2
22:6n-3	5.4	5.9	6.6
Total n-3 PUFA[4]	18.0	19.0	19.8
Total PUFA	32.0	32.7	34.1

Data are means of duplicate analyses. [1]Totals include 15:0, 20:0 and 22:0 at up to 0.3 %; [2]Totals include 20:1n-7, 22:1n-9 and 24:1n-9 at up to 0.8%; [3]Totals include 18:3n-6, 20:2n-6, 20:3n-6, 22:4n-6 and 22:5n-6 at up to 0.2%; [4]Totals include 20:3n-3, 20:4n-3 and 22:5n-3 at up to 0.6%. PUFA, polyunsaturated fatty acid.

Table 3. Sequences, annealing temperatures (Tm) and fragment sizes produced by the primer pairs used for real-time quantitative PCR (qPCR)

Transcript	Primer name	Primer sequence	Fragment (bp)	Tm (°C)	Accession No.	Source
Δ5fad	D5DES-F	5'-GTGAATGGGGATCCATAGCA-3'	192	56	AF478472 [1]	Hastings et al. (2005)
	D5DES-R	5'-AAACGAACGGACAACCAGA-3'				
Δ6fad_a	D6DES-F	5'-CCCCAGACGTTTGTGTCAG-3'	181	56	AY458652 [1]	Zheng et al. (2005)
	D6DES-R	5'-CCTGGATTGTTGCTTTGGAT-3'				
elovl5a	Elo1UTR-SM-1F	5'-ACAAGACAGGAATCTCTTTCAGATTAA-3'	137	60	AY170327 [1]	Morais et al. (2009)
	Elo1UTR-SM-1R	5'-TCTGGGGTTACTGTGCTATAGTGTAC-3'				
elovl5b	Elo2UTR-5F	5'-ACAAAAAGCCATGTTTATCTGAAAGA-3'	141	60	DW546112 [1]	Morais et al. (2009)
	Elo2UTR-5R	5'-AAGTGGGTCTCTGGGGCTGTG-3'				
elovl2	Elo2-SM-1F	5'-CGGGTACAAAATGTGCTGGT-3'	145	60	TC91192 [2]	Morais et al. (2009)
	Elo2-SM-1R	5'-TCTGTTTGCCGATAGCCATT-3'				
SREBP2	SREBP2-1F	5'-GACAGGCACAACACAAGGTG-3'	215	60	DY733476 [1]	Leaver et al. (2008)
	SREBP2-1R	5'-CAGCAGGGGTAAGGGTAGGT-3'				
cpt1	CPT1-1F	5'-CCTGTACCGTGGAGACCTGT-3'	212	60	AM230810 [1]	Leaver et al. (2008)
	CPT1-1R	5'-CAGCACCTCTTTGAGGAAGG-3'				
aco	ACO-2F	5'-AAAGCCTTCACCACATGGAC-3'	230	60	TC49531 [2]	Leaver et al. (2008)
	ACO-2R	5'-TAGGACACGATGCCACTCAG-3'				
PPARα	SsPPAR-A-F1	5- TCCTGGTGGCCTACGGATC-3'	111	60	DQ294237 [1]	Kleveland et al. (2006)
	SsPPAR-A-R1	5'-CGTTGAATTTCATGGCGAACT-3'				
PPARβ	SsPPAR-B-F1	5'-GAGACGGTCAGGGAGCTCAC-3'	151	60	AJ416953 [1]	Kleveland et al. (2006)
	SsPPAR-B-R1	5'-CCAGCAACCCGTCCTTGTT-3'				
PPARγ	SsPPAR-G-F1	5'-CATTGTCAGCCTGTCCAGAC-3'	144	60	AJ416951 [1]	Kleveland et al. (2006)
	SsPPAR-G-R1	5'-TTGCAGCCCTCACAGACATG-3'				
FAS	SsFAS-F4	5'-GTGCCCACTGAATACCATCC-3'	212	60	CK876943 [1]	Morais et al.(2011)
	SsFAS-R4	5'-ATGAACCATTAGGCGGACAG-3'				
LXR	SsLXR-F	5'-GCCGCCGCTATCTGAAATCTG-3'	210	58	FJ470290	Cruz-Garcia et al. (2009)
	SsLXR-R	5'-CAATCCGGCAACCAATCTGTAGG-3'				
Reference genes:						
elf-1α	ELF-1A jbt2	5'-CTGCCCCTCCAGGACGTTTACAA-3'	175	60	AF321836 [1]	Morais et al. (2009)
	ELF-1A jbt2	5'-CACCGGGCATAGCCGATTCC-3'				
β-actin	BACT-F	5'-ACATCAAGGAGAAGCTGTGC-3'	141	56	AF012125 [1]	Morais et al. (2009)
	BACT-R	5'-GACAACGGAACCTCTCGTTA-3'				
Cofilin-2	B2F	5'-AGCCTATGACCAACCCACTG-3'	224	60	TC63899 [2]	Morais et al. (2009)
	B2R	5'-TGTTCACAGCTCGTTTACCG-3'				

Table 4. Growth performance, feed efficiency, biometry and proximate composition of salmon fed diets containing high (HE), medium (ME) and low (LE) levels of digestible energy.

	HE	ME	LE
Initial weight (g)	420.3	420.1	407.3
95%CI	(405.9, 435.0)	(405.8, 435.3)	(392.9, 421.7)
Final weight (g)	952.5	900.4	877.4
95%CI	(910.2, 993.4)	(860.4, 945.1)	(835.5, 921.0)
Weight gain (g)	532.3	480.3	470.2
95%CI	(492.6, 569.6)	(443.9, 520.3)	(431.2, 511.4)
FCR	0.67	0.71	0.82
95%CI	(0.63, 0.70)	(0.68, 0.74)	(0.78, 0.86)
HSI	1.27	1.30	1.25
95%CI	(1.13, 1.41)	(1.19, 1.40)	(1.12, 1.39)
VSI	10.89	11.22	10.60
95%CI	(10.50, 11.31)	(10.91, 11.54)	(10.21, 10.98)
Condition factor	1.43	1.34	1.35
95%CI	(1.53, 1.33)	(1.42, 1.26)	(1.45, 1.26)
Proximate composition (percentage of wet weight) [1]			
Moisture	65.4	66.4	66.8
95%CI	(64.2, 66.6)	(65.2, 67.5)	(65.7, 67.9)
Dry matter	34.6	33.6	33.2
95%CI	(33.4, 35.8)	(32.5, 34.7)	(32.0, 34.4)
Crude protein	17.1	17.2	17.3
95%CI	(16.4, 17.7)	(16.5, 17.8)	(16.7, 18.0)
Crude lipid	14.2	12.7	10.9
95%CI	(12.3, 16.0)	(10.9, 14.5)	(9.0, 12.6)
Ash	1.9	1.7	2.0

Data are means (n = 3). Upper and lower limits for 95% credible intervals (CI) are in parentheses. Ninety-five percent CI were interpreted as statistically significant at P = 0.05% level when the interval did not overlap the reference value in question.

[1]Proximate compositions adjusted for fish weight at the end of the trial.

Table 5. Lipid content (percentage of wet weight) and lipid class composition (percentage of total lipid) of liver of salmon fed diets with high (HE), medium (ME) and low (LE) digestible energy

	Diet		
	HE	ME	LE
Lipid content	7.0	6.3	5.5
95%CI	(5.6, 8.4)	(5.7, 6.9)	(4.7, 6.3)
Lipid class			
PC	18.8	19.5	21.4
95%CI	(17.8, 19.9)	(18.6, 20.3)	(20.5, 22.4)
PE	10.2	10.9	12.0
95%CI	(9.7, 10.6)	(10.5, 11.3)	(11.6, 12.5)
PI	3.4	3.2	3.6
95%CI	(2.9, 3.9)	(2.8, 3.6)	(3.2, 4.0)
PS	2.1	1.9	2.5
95%CI	(1.9, 2.4)	(1.7, 2.1)	(2.28, 2.7)
CL/PG	2.1	2.0	2.4
95%CI	(1.8, 2.4)	(1.7, 2.3)	(2.1, 2.8)
Sphingomyelin	1.6	1.8	2.1
95%CI	(1.3, 1.9)	(1.5, 2.1)	(1.8, 2.4)
LPC	0.1	0.1	0.2
95%CI	(0.0, 0.4)	(0.0, 0.3)	(0.0, 0.5)
Total polar lipid	38.3	39.4	44.3
95%CI	(36.3, 40.3)	(37.7, 41.1)	(42.6, 46.2)
Total neutral lipid	61.7	60.7	55.7
95%CI	(59.6, 63.7)	(59.0, 62.4)	(54.0, 57.5)
Triacylglycerol	42.7	42.6	35.4
95%CI	(39.8, 45.5)	(40.1, 45.2)	(32.8, 38.1)
Cholesterol	11.8	11.6	12.5
95%CI	(11.0, 12.5)	(11.0, 12.3)	(11.8, 13.2)
Free fatty acid	0.4	0.3	0.6
95%CI	(0.0, 0.7)	(0.0, 0.6)	(0.4, 1.0)
Steryl ester	6.8	6.1	7.0
95%CI	(5.1, 8.4)	(4.6, 7.6)	(5.4, 8.6)

Data are means (n = 3). Upper and lower limits for 95% credible intervals (CI) are in parentheses. Ninety-five percent CI were interpreted as statisticanlly significant at P = 0.05% level when the interval did not overlap the reference value in question.

All effects adjusted for fish weight at the end of the trial.

CL, cardiolipin; LPC, lyso-PC; PC, phosphatidylcholine;

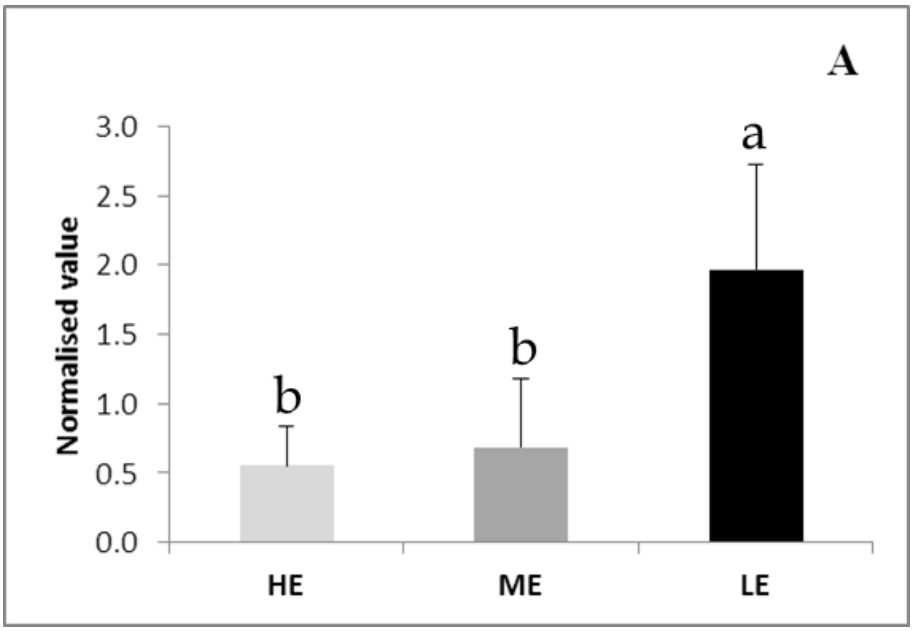
PE, phosphatidylethanolamine; PG, phosphatidylglycerol;

PI, phosphatidylinositol; PS, phosphatidylserine.

Table 6. Fatty acid composition (percentage of total fatty acids) of total lipid of liver of salmon fed diets with high (HE), medium (ME) and low (LE) levels of digestible energy

		Diet		
		HE	ME	LE
14:0		1.5	1.7	1.5
	95%CI	(1.1, 1.9)	(1.6, 1.9)	(1.2, 1.7)
16:0		9.9	11.2	13.6
	95%CI	(8.2, 11.5)	(10.5, 11.9)	(12.6, 14.6)
18:0		3.7	4.2	5.3
	95%CI	(3.2, 4.3)	(4.0, 4.5)	(5.0, 5.6)
Total saturated[1]		15.5	17.5	20.6
	95%CI	(13.6, 17.4)	(16.7, 18.4)	(19.5, 21.8)
16:1n-7		2.9	3.2	4.0
	95%CI	(2.1, 3.5)	(2.8, 3.5)	(3.5, 4.4)
18:1n-9		32.1	30.7	30.2
	95%CI	(27.5, 36.7)	(28.6, 32.7)	(27.4, 32.9)
18:1n-7		2.6	2.7	2.4
	95%CI	(1.9, 3.4)	(2.36, 3.0)	(1.9, 2.8)
20:1n-9		6.7	6.4	4.8
	95%CI	(5.9, 7.6)	(6.0, 6.8)	(4.2, 5.3)
22:1n-11		1.9	2.0	1.5
	95%CI	(1.6, 2.3)	(1.9, 2.2)	(1.3, 1.7)
Total monounsaturated[2]		47.8	46.6	44.5
	95%CI	(42.0, 53.5)	(44.1, 49.2)	(40.9, 48.0)
18:2n-6		7.7	6.6	5.0
	95%CI	(6.9, 8.6)	(6.2, 7.0)	(4.5, 5.5)
20:2n-6		1.7	1.5	1.1
	95%CI	(1.5, 1.9)	(1.4, 1.6)	(1.0, 1.2)
20:3n-6		0.4	0.5	0.7
	95%CI	(0.0, 0.7)	(0.4, 0.6)	(0.5, 0.8)
20:4n-6		0.7	0.7	0.8
	95%CI	(0.5, 0.9)	(0.7, 0.8)	(0.7, 0.9)
Total n-6 PUFA[3]		10.9	9.7	7.8
	95%CI	(9.8, 12.1)	(9.2, 10.2)	(7.0, 8.4)
18:3n-3		2.4	1.9	1.1
	95%CI	(2.1, 2.7)	(1.8, 2.0)	(0.9, 1.2)
20:4n-3		1.6	1.5	1.0
	95%CI	(1.4, 1.8)	(1.4, 1.6)	(0.9, 1.1)
20:5n-3		4.8	5.0	5.0
	95%CI	(3.7, 5.9)	(4.5, 5.5)	(4.4, 5.7)
22:5n-3		1.2	1.5	1.6
	95%CI	(1.0, 1.5)	(1.4, 1.6)	(1.4, 1.8)
22:6n-3		14.6	15.2	17.6
	95%CI	(10.5, 18.8)	(13.4, 16.8)	(15.2, 20.1)
Total n-3 PUFA[4]		25.4	25.8	26.8
	95%CI	(20.5, 30.4)	(23.6, 28.1)	(23.9, 29.8)
Total PUFA		36.9	35.9	34.9
	95%CI	(32.2, 41.6)	(33.9, 38.0)	(32.0, 37.6)
n-3/n-6		2.3	2.7	3.4
	95%CI	(1.8, 3.0)	(2.4, 3.0)	(3.0, 4.0)

Fig. 1 FAD (A: Δ6 FAD B: Δ5 FAD)



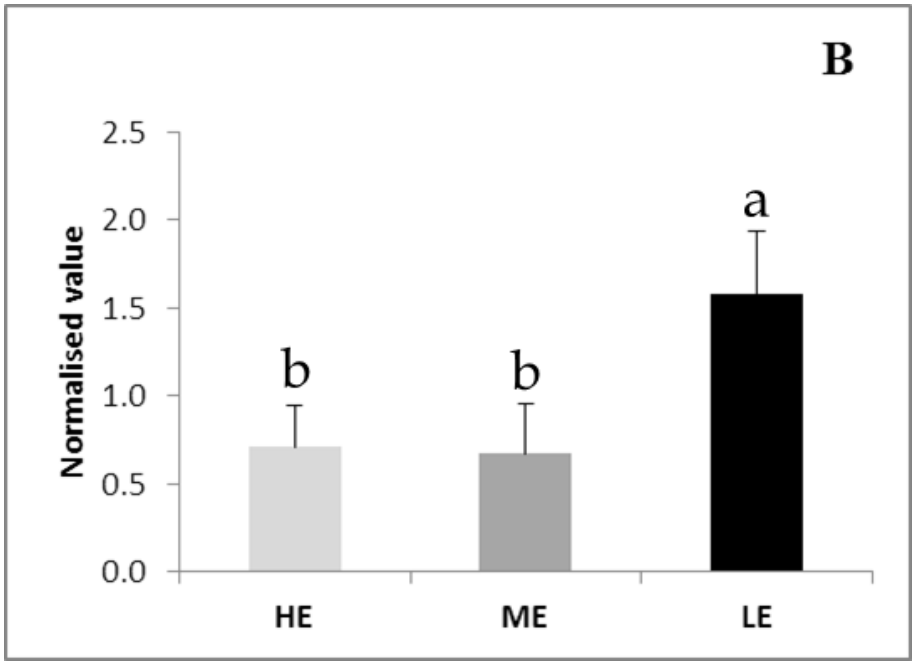


Fig 2. Elongases (A: ELOVL2 B: ELOVL5a C: ELOVL5b)

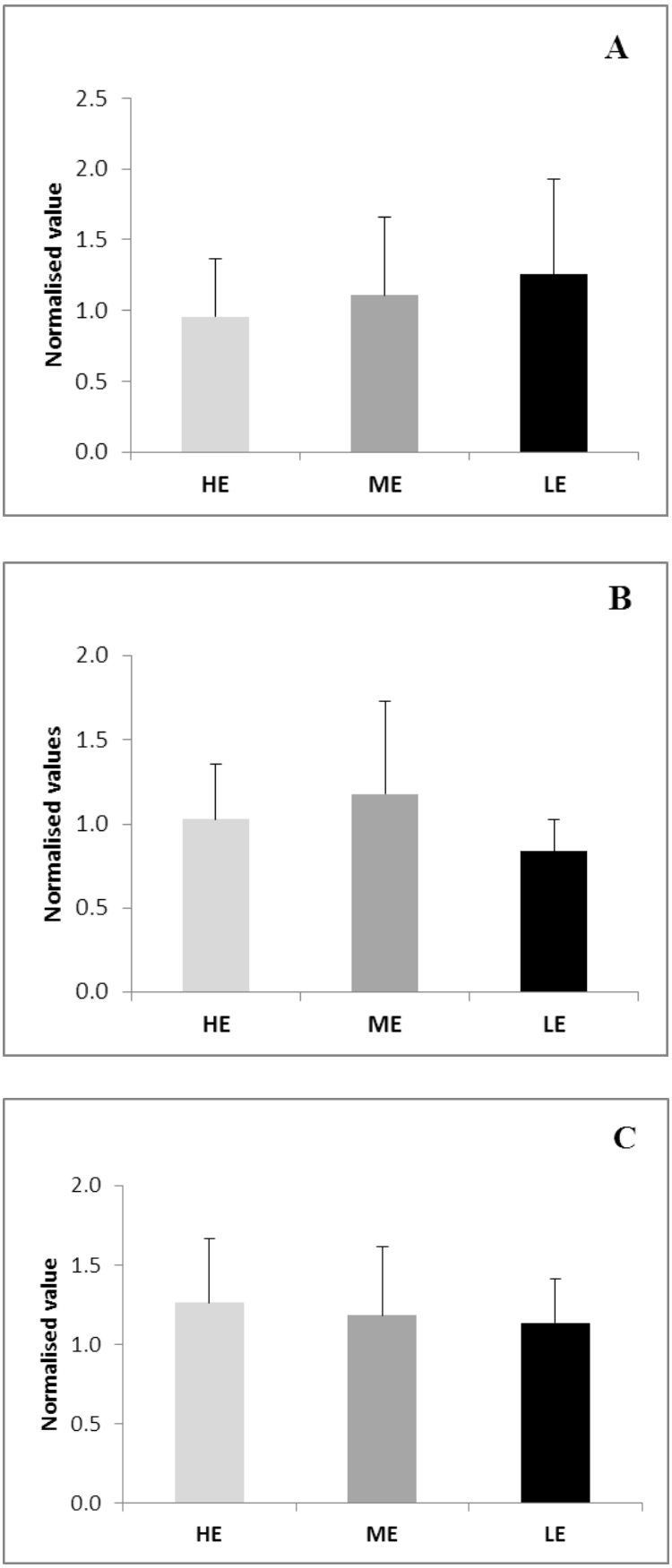
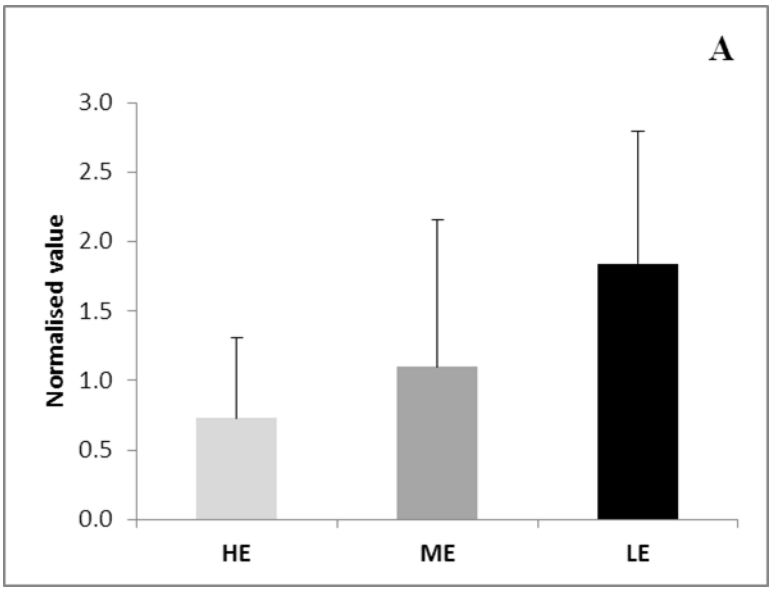
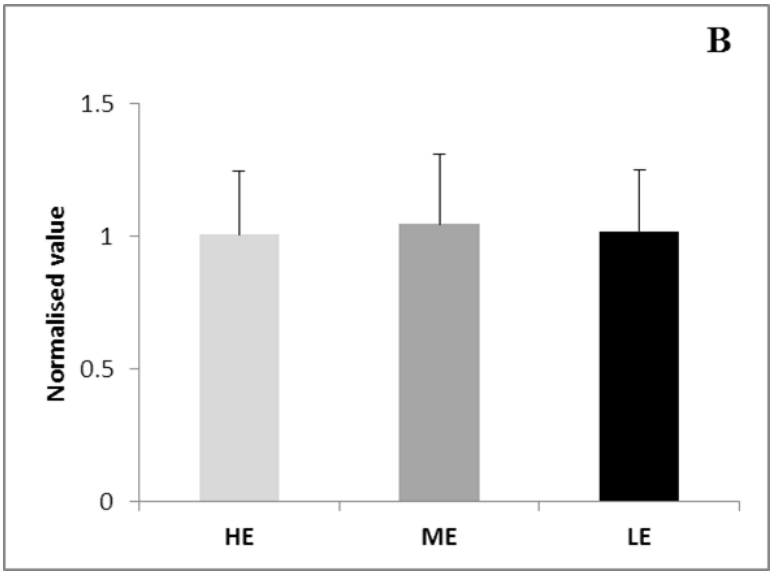


Fig. 3 (A: FAS B: CTP1 C: ACO)





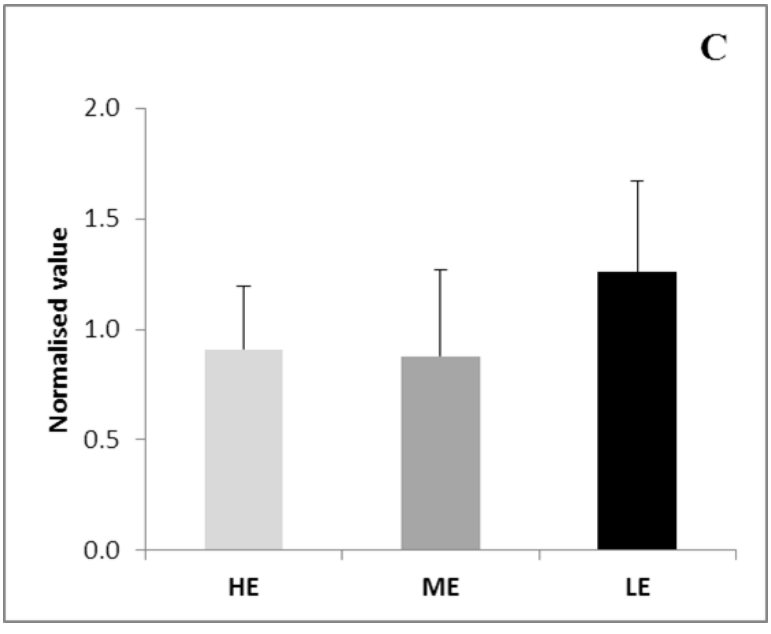
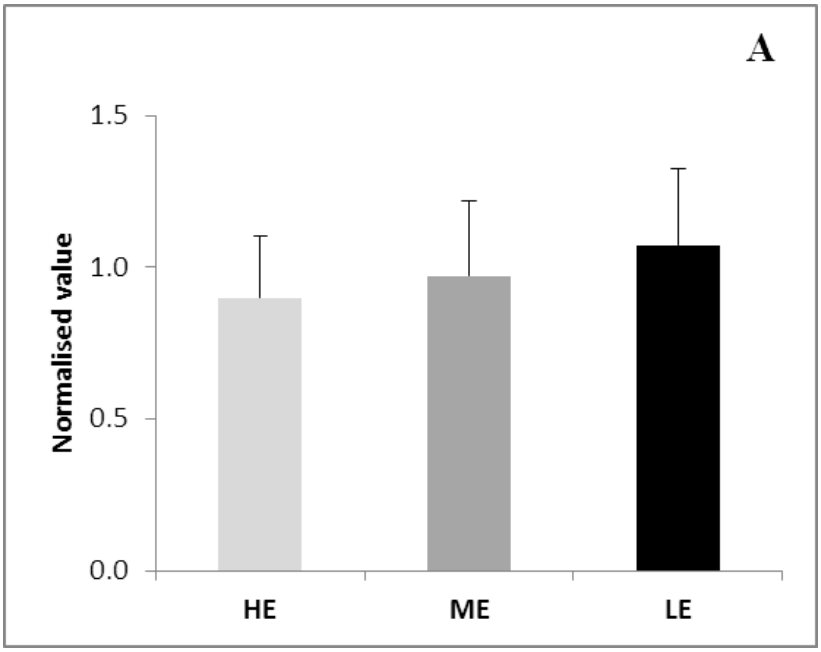


Fig. 4 (A: SREBP2 B: LXR)



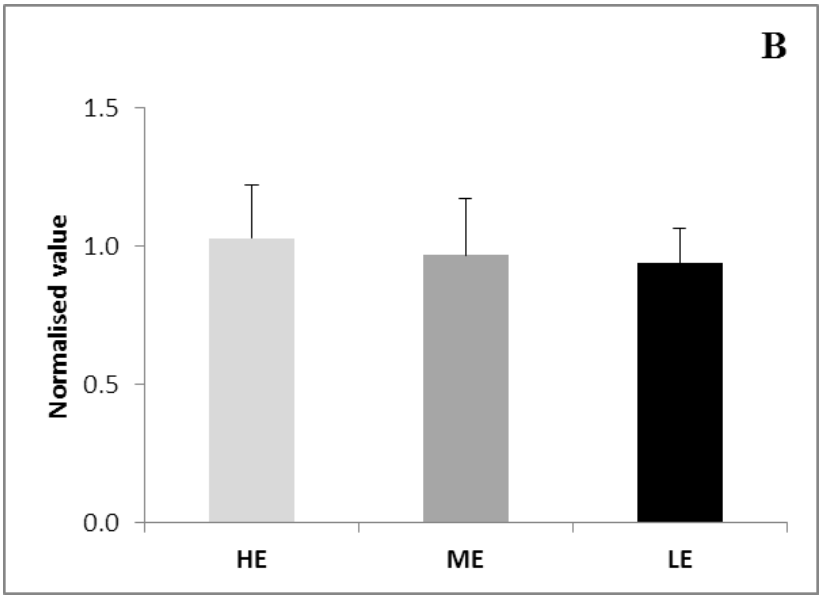
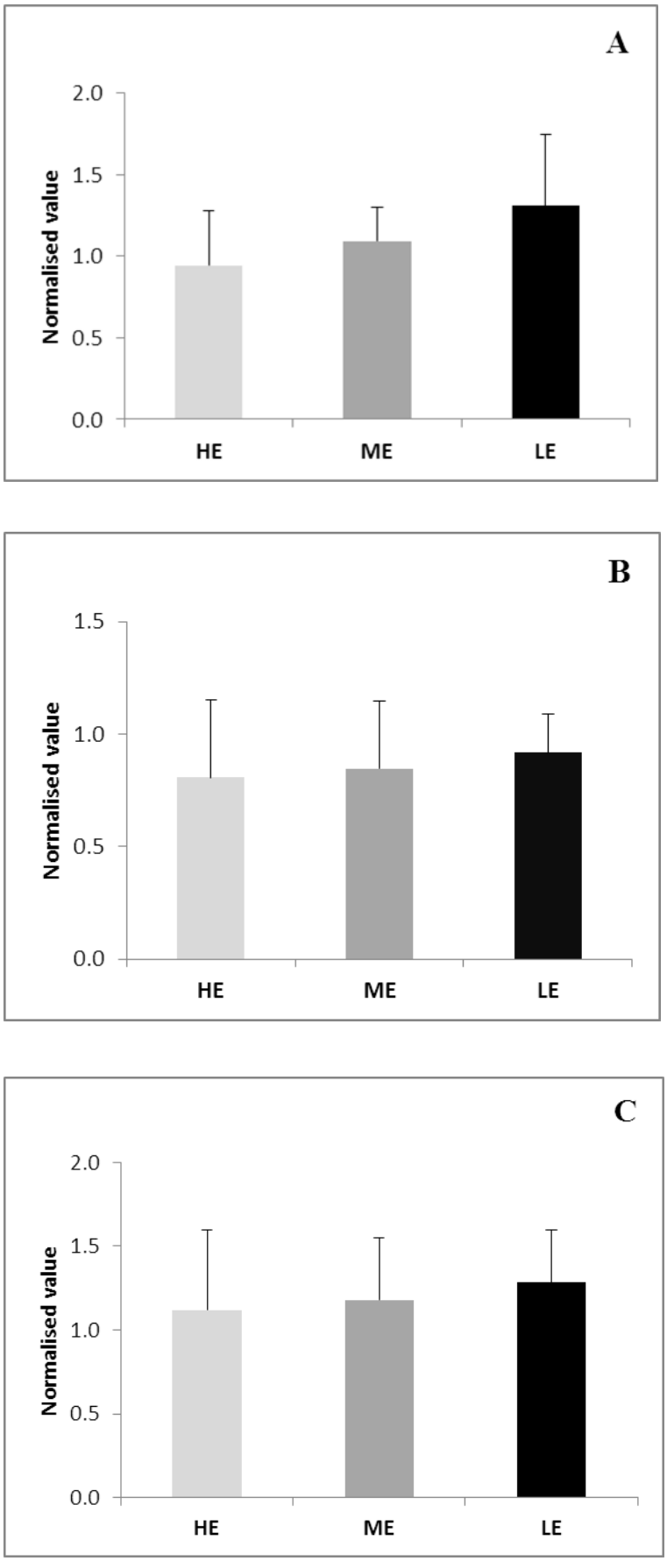


Fig. 5 PPARS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ME
LE
B
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HE
ME
LE
C
Normalised value
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1.5
1.0
0.5
0.0
HE
ME
LE