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

4

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13

13 **Abstract**

14 The relationship between lipid and digestible energy content of the feed and growth  
15 performance has been exploited with great effect in Atlantic salmon (*Salmo salar*). The  
16 precise metabolic consequences of so-called “high-energy” feeds have not been fully defined,  
17 but increased and altered tissue lipid deposition patterns impacting on carcass and product  
18 quality have been reported. Recent studies on global gene expression have shown that dietary  
19 lipid and digestible energy content can have significant effects on gene expression in  
20 salmonids. In addition, we recently showed that functional feeds with reduced digestible  
21 energy significantly improved outcomes in response to inflammatory disease in salmon. The  
22 present study aimed to elucidate and clarify the effects of dietary digestible energy content  
23 (22, 20 and 18 MJ/Kg; HE, ME and LE diets, respectively) on lipid and fatty acid metabolism  
24 in salmon fed diets containing graded amounts of lipid. Specifically the effects on liver lipid  
25 and fatty acid composition, and on the hepatic expression of genes of lipid and fatty acid  
26 metabolism were determined. Final weight and weight gain were significantly higher, and  
27 FCR lower, in fish fed the HE diet. Crude lipid content was significantly lower in fish fed the  
28 LE diet compared to fish fed the two higher energy contents. Significantly lower total lipid  
29 and triacylglycerol levels were recorded in liver of fish fed the LE diet compared to fish fed  
30 the higher energy diets. Liver lipids in salmon fed the LE diet had generally significantly  
31 higher proportions of saturated fatty acids and long-chain polyunsaturated fatty acids (LC-  
32 PUFA), and lower monounsaturated fatty acids, C18 and n-6 PUFA. Consistent with this,  
33 salmon fed the LE diet showed increased liver expression of both  $\Delta 6$  and  $\Delta 5$  fatty acyl  
34 desaturases in comparison to fish fed the diets with higher energy levels. Fatty acid synthase  
35 expression showed a clear upward trend as dietary energy decreased, and sterol regulatory  
36 element binding protein 2 and liver X receptor showed reciprocal trends that were consistent  
37 with the level of dietary cholesterol that reflects digestible energy content. Although not  
38 statistically significant, these trends were biologically logical, significant and relevant.  
39 Expression of genes of fatty acid oxidation was less consistent. Overall, reduced dietary  
40 digestible energy/lipid content alone, without major changes in dietary fatty acid composition,  
41 altered the expression of key genes of lipid and fatty acid metabolism resulting in general up-  
42 regulation of biosynthetic pathways.

43

44

44 **Introduction**

45 Lipid is required in the diet of fish to supply both metabolic energy and some essential  
46 nutrients such as specific (essential) fatty acids (EFA) and, in early life stages, possibly  
47 cholesterol and intact phospholipid (Sargent et al., 2002). Consequently, it is not possible to  
48 define a single dietary lipid requirement for any species as this will vary depending upon the  
49 other dietary components supplying energy, such as protein and carbohydrate (NRC, 2011).  
50 Although an optimum level of dietary lipid cannot be defined, there are lower and upper  
51 limits within which dietary lipid should be supplied. The lower limit is the level required to  
52 supply the requirements for essential components like EFA, and will depend upon the precise  
53 lipid class and fatty acid compositions of the dietary lipid sources. Increasing dietary lipid  
54 above this minimum level will support higher growth rates due to the simple fact that lipid  
55 supplies twice as much energy per unit mass compared to other dietary energy sources, and so  
56 more energy can be supplied in the feed per unit mass, a phenomenon often referred to as  
57 “protein sparing” (Hemre et al., 1995; Bendiksen et al., 2003). However, an upper limit will  
58 be reached where the biochemical and physiological capacities of the animal to digest and/or  
59 metabolise dietary lipid is exceeded leading to reduced digestibility (undigested lipid in  
60 faeces) and/or unwanted deposition of lipid in the peritoneal cavity, liver, or other tissues  
61 (Company et al., 1999; Craig et al., 1999; Hemre and Sandnes, 1999; Gaylord and Gatlin,  
62 2000).

63 This relationship between lipid and digestible energy (DE) content of the feed and growth  
64 performance has been particularly exploited in species such as Atlantic salmon (*Salmo salar*),  
65 which deposit significant amounts of lipid in the flesh and thus are able to tolerate and utilize  
66 high lipid, such that the dietary levels have increased steadily over the years (Sargent et al.,  
67 2002). Although excess deposition and altered tissue lipid deposition patterns are known to  
68 cause various problems in terms of carcass and product quality in farmed fish like salmon  
69 (Sargent et al., 2002), the precise metabolic consequences of so-called “high-energy” feeds  
70 have not been fully defined. However, recent studies looking at global gene expression using  
71 transcriptomic (microarray) and proteomic approaches have shown that dietary lipid and DE  
72 content can have significant effects on gene expression in salmonids (Kolditz et al., 2008a,b;  
73 Panserat et al., 2008; Higgs et al., 2009).

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

78 functional foods is well known in human nutrition. These are defined as foods that contain a  
79 component (whether a nutrient or not) that could be beneficial for the state of well-being and  
80 health, or reduce the risk of a disease, beyond the basic nutritional requirement (Bellisle et al.,  
81 1980). This approach is also becoming increasingly used in aquaculture, as it could  
82 potentially lead to economic savings in terms of increased productivity and lower costs of  
83 disease treatment/management (Raynard et al., 1991; McCoy et al., 1994; Tacchi et al., 2011).  
84 In the HSMI study, we investigated the effects of functional feed formulations that contained  
85 reduced energy levels through lower lipid contents (18%) and altered levels of long-chain  
86 polyunsaturated fatty acids (LC-PUFA), including increased eicosapentaenoic acid (EPA,  
87 20:5n-3) in comparison to a standard commercial feed that contained 31% lipid. A much  
88 reduced inflammatory response to ASRV infection, and reduced severity of heart lesions were  
89 found in fish fed the functional feeds, and transcriptome (microarray) analysis of heart  
90 showed that expression of inflammation/immune related genes was greatly affected.  
91 However, in addition to effects on immune genes, it was clear that the feeds were also having  
92 significant effects on the expression of metabolic genes in the heart, including those of lipid  
93 and fatty acid metabolism (Martinez-Rubio et al., 2012, Supplementary Tables). However, it  
94 was not clear whether dietary DE and lipid content or fatty acid composition were primarily  
95 responsible for the alterations in metabolic gene expression.

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

104

## 105 **Materials and Methods**

### 106 *Fish and feeds*

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

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

#### 125 *Sampling*

126 Fish were sampled at the end of the feeding period (12 weeks) with body weight and length,  
127 and liver and viscera weights recorded for all fish culled. A total of 15 fish/tank were  
128 anaesthetised (MS222) and killed by a blow to the head with three whole fish frozen  
129 immediately for analysis of proximate composition. Livers for lipid and biomolecular analysis  
130 were collected from the remaining 12 fish. Thus, samples of liver (for fatty acid and  
131 molecular analyses) were collected and frozen immediately in liquid nitrogen and stored at -  
132 80 °C prior to analysis. Further samples of liver, specifically for lipid class analysis, were  
133 collected in 5ml glass vials containing 4 ml of chloroform/methanol (2:1, by volume).

#### 134 *Growth performance and feed utilization*

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

142 *Proximate composition of feeds and fish*

143

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 Kjeltac 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).

155

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°C. Hydrogen was used as carrier  
167 gas and temperature programming was from 50 °C to 150 °C at 40 °C min<sup>-1</sup> and then to 230  
168 °C at 2.0 °C min<sup>-1</sup>. 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

174 acid synthesis (fatty acid synthase, FAS), LC-PUFA biosynthesis (fatty acyl desaturases,  
175 *Δ6fad<sub>a</sub>* and *Δ5fad*; fatty acid elongases, *elovl2*, *elovl5a* and *elovl5b*), and β-oxidation  
176 (carnitine palmitoyl transferase-1, CPT1; acyl CoA oxidase, ACO), and their control and  
177 regulation (sterol-responsive element-binding protein 2, SREBP2; liver X receptor, LXR;  
178 peroxisome proliferator-activated receptors, PPARα, PPARβ and PPARγ). The qPCR primer  
179 sequences (obtained by literature searches), annealing temperature (T<sub>m</sub>) and size of amplicon  
180 are given in Table 3. In addition, amplification of cofilin-2 and elongation factor-1α (elf-1α)  
181 was performed and their expression was confirmed as sufficiently stable across treatments for  
182 normalization. These genes had been identified as suitable reference genes in previous qPCR  
183 studies in salmon (Morais et al., 2011).

184 For qPCR, 2 μg of column-purified total RNA per sample was reverse transcribed into cDNA  
185 using the High-Capacity cDNA RT kit (Applied Biosystems, Paisley, U.K.), following  
186 manufacturer's instructions, but using a mixture of the random primers (1.5 μl as supplied)  
187 and anchored oligo-dT (0.5 μl at 400 ng/ μl, Eurofins MWG Operon, Ebersberg, Germany).  
188 Negative controls (containing no enzyme) were performed to check for genomic DNA  
189 contamination. cDNA was then diluted 20-fold with water, after a similar amount of cDNA  
190 was pooled from all samples. qPCR analysis used relative quantification with the  
191 amplification efficiency of the primer pairs being assessed by serial dilutions of the cDNA  
192 pool. qPCR amplifications were carried out in duplicate (Quanta, Techne, Cambridge, U.K.)  
193 in a final volume of 20 μL containing either 5 μL (for most genes) or 2 μL (for the reference  
194 genes and other highly expressed genes) diluted (1/20) cDNA, 0.5 μM of each primer and 10  
195 μL Absolute<sup>TM</sup> QPCR SYBR® Green mix (ABgene). Amplifications were carried out with a  
196 systematic negative control (NTC-non template control, containing no cDNA). The qPCR  
197 profiles contained an initial activation step at 95 °C for 15 min, followed by 30 to 40 cycles:  
198 15 s at 95 °C, 15 s at the specific primer pair annealing T<sub>m</sub> and 15 s at 72 °C. After the  
199 amplification phase, a melt curve of 0.5 °C increments from 75 °C to 90 °C was performed,  
200 enabling confirmation of amplification of single products, and sizes were checked by agarose  
201 gel electrophoresis and identities confirmed by sequencing. Non-occurrence of primer-dimer  
202 formation in the NTC was also verified. Data were analyzed using the relative expression  
203 software tool (REST 2009, <http://www.gene-quantification.info/>), which employs a pair wise  
204 fixed reallocation randomization test (10,000 randomizations) with efficiency correction, to  
205 determine the statistical significance of expression ratios (or gene expression fold-changes)  
206 between two treatments (Pfaffl et al., 2002).

207 *Statistical analyses*

208 The effects of dietary treatments on fish growth performance, feed efficiency, biometry, liver  
209 lipid contents, class and fatty acid compositions were analysed by Analysis of Covariance  
210 (ANCOVA) with fish weight (or pooled fish weights) as the covariate. Briefly, the weight  
211 gain based on repeated weights on individual fish was modelled by fitting individual growth  
212 trajectories with the help of a multilevel model using the fish tag ID, tank and treatments (DE  
213 levels) as the levels of variation. Time dimension was added to the model by the day of  
214 weighing since the start of the trial and its effect was modelled with the help of cubic splines.  
215 FCR was calculated by dividing the observed feed intake by the observed weight gain and  
216 log-transformed before conducting stats. Differences in liver and visceral weights between  
217 diets were analysed including fish body weight as a covariate to account for fish size  
218 differences at the end of the trial. Afterwards the condition factor, hepatosomatic and visceral  
219 indices were calculated based on the model estimates. Differences in proximal composition  
220 analyses of the fish, fatty acid profile and lipid class composition in the liver were also  
221 analysed statistically including a covariate. Since these analyses were conducted on pool  
222 samples the average weight of each fish pool was used as a covariate in order to account for  
223 size differences. Multilevel models were fitted with the lme4 package of the R language (R  
224 Development Core Team 2008). All treatment effects were based on posterior simulation ( $n =$   
225 2,500) with 95 % credible intervals. Ninety-five percent credible intervals were interpreted as  
226 statistical significant at  $p = 0.05$  % level when the interval did not overlap the reference value  
227 in question.

228 **Results**

229 *Growth performance and body composition*

230 The salmon more than doubled their weight over the period of the trial with final weights and  
231 weight gain showing clear effects of DE content and so both were significantly higher in fish  
232 fed the HE diet compared to fish fed the diets with lower DE (Table 4). Similarly, FCR  
233 showed an increasing trend as DE decreased and so was significantly lower in fish fed the HE  
234 diet compared to fish fed the diets with lower DE. Both HSI and VSI tended to be lower in  
235 fish fed the LE diet with the lowest DE, but the differences were not statistically significant  
236 (Table 4). Crude lipid of whole fish showed a clear trend with DE content with the content  
237 being significantly lower in fish fed diet LE compared to fish fed the ME and HE diets (Table  
238 4).

### 239 *Liver lipid and fatty acid compositions*

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

### 253 *Liver gene expression*

254 Salmon fed the LE diet showed significantly increased liver expression of both  $\Delta 6$  and  $\Delta 5$   
255 fatty acyl desaturases (Fad) in comparison to fish fed the diets with higher energy levels (Fig.  
256 1). In contrast, there were no significant effects of diet on the expression of fatty acyl  
257 elongases (Elov12 or Elov15 transcripts) (Fig.2). There was a clear effect of dietary energy  
258 upon fatty acid synthase (FAS) expression, which increased with reducing DE albeit that the  
259 relatively large standard deviations rendered the effect non-significant statistically (Fig. 3A).  
260 There was no effect of dietary energy on carnitine palmitoyl transferase 1 (CPT-1), a marker  
261 of mitochondrial fatty acid  $\beta$ -oxidation, and although it appeared as though the expression of  
262 acyl-CoA oxidase (ACO), a marker of peroxisomal fatty acid oxidation, was higher in fish fed  
263 the LE diet, this was not significant (Fig.3B & C). Although statistically non-significant,  
264 reciprocal trends in the expression of sterol regulatory element binding protein 2 (SREBP2)  
265 and liver X receptor (LXR) with DE were observed (Fig. 4). Similarly, the liver expression of  
266 all three peroxisome proliferator-activated receptor subtypes ( $\alpha$ ,  $\beta$  and  $\gamma$ ) tended to show  
267 increasing expression as dietary DE decreased, but none of the differences were statistically  
268 significant (Fig.5).

### 269 **Discussion**

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

280 However, in addition to increasing growth, increased dietary DE through higher dietary  
281 lipid can also have negative impacts on other aspects of fish performance. Chief among these  
282 is based on the well-known positive correlation between dietary lipid levels and tissue/body  
283 lipid levels of fish (Sargent et al., 2002). Although the lipid data in the present study showed  
284 some variation, due to the use of an essentially ungraded stock, chosen to eliminate bias  
285 towards fast or slow growers, liver lipid contents were reduced significantly with decreasing  
286 dietary DE content. This was confirmed by the significantly lower total neutral (storage) lipid  
287 and TAG in livers of fish fed diet LE compared to fish fed HE. In this respect it is noteworthy  
288 that previous studies have reported that the level of lipid in tissues of salmon can vary  
289 considerably within a population, but the extent to which the observed biological variation is  
290 determined by environmental or genetic factors is not known (Bell et al., 1998).  
291 Notwithstanding the above, previous studies support the present data, as high dietary lipid  
292 increased lipid levels in salmonids including rainbow trout and Atlantic salmon (Bell et al.,  
293 1998; Dias et al., 1999; Hemre and Sandnes, 1999). Increased tissue lipid in response to  
294 increased dietary lipid was also shown in marine fish including sea bass (*Dicentrarchus*  
295 *labrax*) and turbot (*Psetta maximus*) (Catacutan and Coloso, 1995; Saether and Jobling,  
296 2001). Increased liver lipid deposition may have unwanted physiological effects such as the  
297 development of fatty liver pathology in marine fish (Caballero et al., 1999), possibly through  
298 mechanisms involving altered gene expression, and hence the focus on this aspect in the  
299 present study.

300 In relation to the above, a highly significant effect of dietary DE content in terms of  
301 altered gene expression was the higher expression of both  $\Delta 6$ - and  $\Delta 5$ -Fads in liver of salmon  
302 consuming the LE diet compared with fish consuming the higher energy diets. Liver  
303 transcript expression in fish fed LE was around 3-fold higher than that in fish fed HE, and

304 was highly significant (p-value = 0.002). In contrast, dietary DE did not appear to affect the  
305 expression of fatty acid elongases, consistent with the fact that these enzymes do not show the  
306 same level of nutritional regulation as desaturases (Leaver et al., 2008a; Morais et al., 2009;  
307 Tocher, 2010). In addition to being the clearest effect of DE on gene expression, this was also  
308 an important result as the effects of DE and/or lipid content on the expression of genes of LC-  
309 PUFA biosynthesis had not been reported previously in salmon. Previously, microarray  
310 analysis had shown that  $\Delta 6$ -desaturase transcript expression was down-regulated in trout fed a  
311 high energy feed compared to fish fed a lower energy feed (Kolditz et al., 2008b).  
312 Nutritional regulation of fatty acid desaturase gene expression was first reported in mammals.  
313 The levels of liver mRNA for both  $\Delta 6$ - and  $\Delta 5$ -desaturases were approximately 3-fold higher  
314 in rats fed a fat-free diet compared to animals fed either safflower oil (18:2n-6) or fish oil (n-  
315 3LC-PUFA) (Cho et al., 1999a). However, rats fed a diet containing triolein (18:1n-9) showed  
316 a similar high expression of both desaturases as observed in rats fed the fat-free diet (Cho et  
317 al., 1999a). Therefore, this suggested that it was the fatty acid composition of the diet,  
318 specifically a lack of PUFA, that was responsible for the higher expression, rather than the  
319 lipid content of the diets. Consistent with this, hepatic expression of  $\Delta 6$ -desaturase in mice  
320 fed an essential fatty acid (EFA)-deficient diet (triolein) was double that in mice fed a corn oil  
321 diet rich in 18:2n-6 (Cho et al., 1999b).

322 Previous work investigating the nutritional regulation of LC-PUFA biosynthesis in fish has  
323 focussed on dietary fatty acid composition (Leaver et al., 2008a; Tocher, 2010). The activity  
324 of the LC-PUFA biosynthesis pathway in freshwater carp cells was increased by EFA-  
325 deficiency (Tocher and Dick, 1999). *In vivo* dietary trials showed that the activity of the LC-  
326 PUFA biosynthetic pathway was increased in freshwater and salmonid fish fed vegetable oils  
327 rich in C<sub>18</sub> PUFA compared to fish fed fish oil, rich in the n-3 LC-PUFA, EPA and DHA  
328 (Tocher et al., 1997, 2002, 2003). Consistent with this, expression of  $\Delta 6$  Fad mRNA was  
329 increased in salmon fed diets lacking LC-PUFA (vegetable oil), compared to fish fed diets  
330 containing EPA and DHA (fish oil) (Zheng et al., 2004b, 2005a,b; Leaver et al., 2008b;  
331 Taggart et al., 2008). Therefore, Fad expression was increased when diets contain lower  
332 levels of the pathway end-products such as EPA and DHA. In the present study, the fatty acid  
333 compositions of the feeds were similar and, indeed the levels of the LC-PUFA, EPA and  
334 DHA were slightly higher in the LE diet, which resulted in generally higher levels of these  
335 fatty acids in fish fed LE. Therefore, the higher expression of the Fad genes in liver of fish  
336 fed the LE diet is not consistent with the previous data, supporting the view that dietary lipid  
337 content itself underpins the differences in expression observed in the present study. The only

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

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

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

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

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

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

409 In conclusion, the present study determined the effects of dietary DE on the hepatic  
410 expression of key genes of lipid and fatty acid metabolism in Atlantic salmon fed diets  
411 containing graded amounts of lipid. Dietary DE and/or lipid content had important effects on  
412 the expression of genes involved in major lipid pathways including lipogenesis (FAS), LC-  
413 PUFA biosynthesis ( $\Delta 6$  Fad,  $\Delta 5$  Fad, Elovl2 and Elovl5), and cholesterol metabolism (LXR  
414 and SREBP2) that were biologically significant and relevant, and consistent with current  
415 understanding. In contrast, the effects on fatty acid  $\beta$ -oxidation (CPT1, ACO and PPARs)  
416 were more inconclusive. Overall though, it was clear that changes in dietary DE alone,  
417 without major changes in dietary fatty acid composition, could result in altered expression of  
418 key genes of lipid and fatty acid metabolism. Combined, these changes resulted in an overall  
419 up-regulation of lipid biosynthetic pathways. Therefore, in relation to our previous application  
420 of a clinical nutrition approach to improving disease outcomes through the use of functional  
421 feeds, the present results suggest that the beneficial effects of reduced dietary DE may include  
422 positive alterations in lipid and fatty acid metabolism (Martinez-Rubio et al., 2012).

423

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429

#### 430 **References**

431 Ackman, R.G., 1980. Fish lipids. In: *Advances in Fish Science and Technology* (Connell, J.J.,  
432 ed.), pp. 87-103. Fishing News Books, Farnham.

433 AOAC, 2000. *Official methods of analysis*. Association of Official Analytical Chemists,  
434 Gaithersburg, Maryland, USA.

435 Aranda, A., Pascual, A., 2001. Nuclear hormone receptors and gene expression. *Physiol. Rev.*  
436 81, 1269–1304.

437 Arzel, J., Cardinal, M., Cornet, J., Metailler, R., Guillaume, J.C., 1993. Nutrition of brown  
438 trout (*Salmo trutta*) reared in seawater, effect of dietary lipid on growth performances,  
439 body composition and fillet quality. P. 19 in *From Discovery to Commercialization*, Special  
440 Publication no.19. Oostende, Belgium: European Aquaculture Society.

441 Bell, J.G., McEvoy, J., Webster, J.L., McGhee, F., Millar, R.M., Sargent, J.R., 1998. Flesh  
442 lipid and carotenoid composition of Scottish farmed Atlantic salmon (*Salmo salar*). *J.*  
443 *Agric. Fd. Chem.* 46, 119-127 (1998).

444 Bellisle, R., Diplock, A.T., Hornstra, G., Koletzko, B., Roberfroid, M., Salminen, S., Saris,  
445 W.H.M., 1980. Functional food science in Europe. *Br. J. Nutr.* 80 (suppl 1), 1–193.

446 Bendiksen, E.Å., Berg, O.K., Jobling, M., Arnesen, A.M., Måsøval, K., 2003. Digestibility,  
447 growth, and nutrient utilisation of Atlantic salmon (*Salmo salar* L.) in relation to  
448 temperature, feed fat content and oil source. *Aquaculture* 224, 283-299.

449 Caballero, M.J., Lopez-Calero, G., Socorro, J., Roo, F.J., Izquierdo, M.S., Fernandez, A.J.,  
450 1999. Combined effect of lipid level and fishmeal quality on liver histology of gilthead  
451 seabream (*Sparus aurata*). *Aquaculture* 179, 277–290.

452 Catacutan, M.R., Coloso, R.M., 1995. Effect of dietary protein to energy ratios on growth,  
453 survival, and body composition of juvenile Asian seabass, *Lates calcarifer*. *Aquaculture*  
454 131, 125–133.

455 Cho, H.P., Nakamura, M.T., Clarke, S.D., 1999a. Cloning, expression, and fatty acid  
456 regulation of the human  $\Delta$ -5 desaturase. *J. Biol. Chem.* 274, 37335-37339.

457 Cho, H.P., Nakamura, M.T., Clarke, S.D., 1999b. Cloning, expression, and nutritional  
458 regulation of the mammalian  $\Delta$ -6 desaturase. *J. Biol. Chem.* 274, 471-477.

459 Christie, W.W., 2003 *Lipid analysis*. 3rd Edition. The Oily Press, Bridgewater, UK. pp. 205-  
460 24.

461 Company, R., Calduch-Giner, J.A., Kaushik, S., Perez-Sanchez, J., 1999. Growth  
462 performance and adiposity in gilthead sea bream (*Sparus aurata*): Risks and benefits of  
463 high energy diets. *Aquaculture* 171, 279–292.

464 Craig, S.R., Washburn, B.S., Gatlin, D.M., 1999. Effects of dietary lipids on body  
465 composition and liver function in juvenile red drum, *Sciaenops ocellatus*. Fish Physiol.  
466 Biochem. 21, 249–255.

467 Cruz-Garcia, L., Minghetti, M., Navarro, I., Tocher, D.R., 2009. Molecular cloning, tissue  
468 expression and regulation of Liver X Receptor (LXR) transcription factors of Atlantic  
469 salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol.  
470 153B, 81-88.

471 Desvergne, B.A., Michalik, L., Wahli, W., 2006. Transcriptional regulation of metabolism.  
472 Physiol. Rev. 86, 465-514.

473 Dias, J., Corraze, G., Arzel, J., Alvarez, M.J., Bautista, J.M., Lopez-Bote, C., Kaushik, S.J.,  
474 1999. Nutritional control of lipid deposition in rainbow trout and European seabass: Effect  
475 of dietary protein/energy ratio. Cybium 23(suppl.), 127–137.

476 Diez, A., Menoyo, D., Pérez-Benavente, S., Calduch-Giner, J.A., Vega-Rubin de Celis, S.,  
477 Obach, A., Favre-Krey, L., Boukouvala, E., Leaver, M.J., Tocher, D.R., Pérez-Sanchez, J.,  
478 Krey, G., Bautista, J.M., 2007. Conjugated linoleic acid affects lipid composition,  
479 metabolism, and gene expression in gilthead sea bream (*Sparus aurata* L). J. Nutr. 137,  
480 1363-1369.

481 Einen, O., Roem, A.J., 1997. Dietary protein/energy ratios for Atlantic salmon in relation to  
482 fish size: Growth, feed utilisation and slaughter quality. Aquacult. Nutr. 3, 115–126.

483 Folch, J., Lees, M., Sloane-Stanley, G.H., 1957. A simple method for the isolation and  
484 purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.

485 Gaylord, T.G., Gatlin III, D.M., 2000. Dietary lipid level but not L-carnitine affects growth  
486 performance of hybrid striped bass (*Morone chrysops* x *M. saxatilis*). Aquaculture 190,  
487 237–246.

488 Hemre, G.-I., Sandnes, K., 1999. Effect of dietary lipid level on muscle composition in  
489 Atlantic salmon *Salmo salar*. Aquacult. Nutr. 5, 9–16.

490 Hemre, G.-I., Sandnes, K., Lie, Ø., Torrissen, O., Waagbø, R., 1995. Carbohydrate nutrition in  
491 Atlantic salmon. Growth and feed utilisation. Aquaculture Res. 26, 149-154.

492 Henderson, R.J., Tocher, D.R., 1992. Thin-layer chromatography. In *Lipid Analysis : A*  
493 *Practical Approach* (Hamilton, R.J., Hamilton, S., eds.) pp. 65-111, Oxford University  
494 Press, Oxford.

495 Higgs, D.A., Sutton, J.N., Kim, H., Oakes, J.D., Smith, J., Biagi, C., Rowshandeli, M.,  
496 Devlin, R.H., 2009. Influence of dietary concentrations of protein, lipid and carbohydrate  
497 on growth, protein and energy utilization, body composition, and plasma titres of growth

498 hormone and insulin-like growth factor-1 in non-transgenic and growth hormone  
499 transgenic coho salmon, *Oncorhynchus kisutch* (walbaum). *Aquaculture* 286, 127-137.

500 Horton, J.D., Goldstein, J.L., Brown, M.S., 2002. SREBPs: activators of the complete  
501 program of cholesterol and fatty acid synthesis in the liver, *J. Clin. Invest.* 109, 1125–  
502 1131.

503 Kleveland, E.J., Ruyter, B., Vegusdal, A., Sundvold, H., Berge, R.K., Gjøen, T., 2006. Effects  
504 of 3-thia fatty acids on expression of some lipid related genes in Atlantic salmon (*Salmo*  
505 *salar* L.). *Comp. Biochem. Physiol.* 145B, 239-248.

506 Kolditz, C., Borthaire, M., Richard, N., Corraze, G., Panserat, S., Vachot, C., Lefevre, F.,  
507 Medale, F., 2008. Liver and muscle metabolic changes induced by dietary energy content  
508 and genetic selection in rainbow trout (*Oncorhynchus mykiss*). *Am. J. Physiol.* 294,  
509 R1154-R1164.

510 Kolditz, C., Paboeuf, G., Borthaire, M., Esquerre, D., San Cristobal, M., Lefevre, F., Medale,  
511 F., 2008. Changes induced by dietary energy intake and divergent selection for muscle fat  
512 content in rainbow trout (*Oncorhynchus mykiss*), assessed by transcriptome and proteome  
513 analysis of the liver. *BMC Genomics* 9, 506.

514 Leaver, M.J., Bautista, J.M., Björnsson, T., Jönsson, E. Krey, G., Tocher, D.R., Torstensen,  
515 B.E., 2008a. Towards fish lipid nutrigenomics: current state and prospects for fin-fish  
516 aquaculture. *Rev. Fisheries Sci.* 16(S1), 71-92.

517 Leaver, M.J., Boukouvala, E., Antonopoulou, E., Diez, A., Favre-Krey, L., Ezaz, M.T.,  
518 Bautista, J.M., Tocher, D.R., Krey, G., 2005. Three peroxisomal proliferator-activated  
519 receptor (PPAR) isotypes from each of two species of marine fish. *Endocrinology* 146,  
520 3150-3162.

521 Leaver, M.J., Tocher, D.R., Obach, A., Jensen, L., Henderson, R.J., Porter, A.R., Krey, G.,  
522 2006. Effect of dietary conjugated linoleic acid (CLA) on lipid composition, metabolism  
523 and gene expression in Atlantic salmon (*Salmo salar*) tissues. *Comp. Biochem. Physiol.*  
524 145A, 258-267.

525 Leaver, M.J., Villeneuve, L.A.N., Obach, A., Jensen, L., Bron, J.E., Tocher, D.R. Taggart,  
526 J.B., 2008b. Functional genomics reveals increases in cholesterol biosynthetic genes and  
527 highly unsaturated fatty acid biosynthesis after dietary substitution of fish oil with  
528 vegetable oils in Atlantic salmon (*Salmo salar*). *BMC Genomics* 9, 299.

529 Luzzana, U., Serrini, G., Moretti, V.M., Giancesini, C., Valfre, F., 1994. Effect of expanded  
530 feed with high fish oil content on growth and fatty acid composition of rainbow trout.  
531 *Aquacult. Int.* 2, 239–248.

532 McCoy, M., McLoughlin, M., Rice, D., Kennedy, D., 1994. Pancreas disease in Atlantic  
533 salmon *Salmo salar* and vitamin E supplementation. *Comp. Biochem. Physiol.* 109, 905-  
534 912.

535 Martinez-Rubio, L., Morais, S., Evensen, Ø., Wadsworth, S., Vecino, J.L.G., Bell, J.G.,  
536 Tocher, D.R., 2012. Clinical nutrition reduces heart pathology of Atlantic salmon (*Salmo*  
537 *salar* L.) following challenge with heart and skeletal muscle inflammation (HSMI). *Public*  
538 *Library of Science (PLoS) ONE*, in press.

539 Michalik, L., Auwerx, J., Berger, J.P., Chatterjee, V.K., Glass, C.K., Gonzalez, F.J., Grimaldi,  
540 P.A., Kadowaki, T., Lazar, M.A., O'Rahilly, S., Palmer, C.N.A., Plutzky, J., Reddy, J.K.,  
541 Spiegelman, B.M., Staels, B., Wahli, W., 2006. International union of pharmacology. LXI.  
542 Peroxisome proliferator-activated receptors. *Pharmacolog. Rev.* 58, 726-741.

543 Minghetti, M., Leaver, M.J., Tocher, D.R., 2011. Transcriptional control mechanisms of  
544 genes of lipid and fatty acid metabolism in the Atlantic salmon (*Salmo salar* L.)  
545 established cell line, SHK-1. *Biochim. Biophys. Acta.* 1811, 194-202.

546 Morais, S., Monroig, O., Zheng, X., Leaver, M.J., Tocher, D.R., 2009. Highly unsaturated  
547 fatty acid synthesis in Atlantic salmon: characterisation of ELOVL5- and ELOVL2-like  
548 elongases. *Marine Biotechnol.* 11, 627-639.

549 Morais, S., Pratoomyot, J., Taggart, J.B., Bron, J.E., Guy, D.R., Bell, J.G., Tocher, D.R.,  
550 2011. Genotype-specific responses in Atlantic salmon (*Salmo salar*) subject to dietary fish  
551 oil replacement by vegetable oil: A liver transcriptomic analysis. *BMC Genomics* 12, 255.

552 National Research Council (NRC). 2011. Nutrient requirements of fish and shrimp. National  
553 Academy Press, Washington D.C.

554 Panserat, S., Ducasse-Cabanot, S., Plagnes-Juan, E., Srivastava, P. P., Kolditz, C., Piumi, F.,  
555 Esquerré, D., Kaushik, S., 2008. Dietary fat level modifies the expression of hepatic genes  
556 in juvenile rainbow trout (*Oncorhynchus mykiss*) as revealed by microarray  
557 analysis. *Aquaculture* 275, 235-241.

558 Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST)  
559 for group-wise comparison and statistical analysis of relative expression results in real-  
560 time PCR. *Nucleic Acids Res* 30, e36.

561 R Development Core Team, 2008. R: A Language and Environment for Statistical  
562 Computing. R Foundation for Statistical Computing. Vienna, Austria, 2008. Available at:  
563 <http://www.Rproject.org>

564 Raynard, R.S., McVicar, A.H., Bell, J.G., Youngson, A., Knox, D., Fraser, C.O., 1991.  
565 Nutritional aspects of pancreas disease of Atlantic salmon: The effects of dietary vitamin E  
566 and polyunsaturated fatty acids. *Comp. Biochem. Physiol.* 98, 125-131.

567 Saether, B.S., Jobling, M., 2001. Fat content in turbot feed: Influence on feed intake, growth  
568 and body composition. *Aquac. Res.* 32, 451-458.

569 Salhi, M., Izquierdo, M.S., Hernandez-Cruz, C.M., Gonzalez, M., Fernandez-Palacios, H.,  
570 1994. Effect of lipid and n-3 HUFA levels in microdiets on growth, survival and fatty acid  
571 composition of larval gilthead sea bream (*Sparus aurata*). *Aquaculture* 124, 275-282.

572 Sargent, J.R., Tocher, D.R., Bell, J.G., 2002. The lipids. Pp. 181-257 in *Fish Nutrition*, 3rd  
573 Edition, Halver, J.E., Hardy, R.W., eds. San Diego, CA: Academic Press.

574 Shimeno, S., Hosokawa, H., Takeda, M., 1996. Metabolic response of juvenile yellowtail to  
575 dietary carbohydrate to lipid ratios. *Fish. Sci.* 62, 945-949.

576 Shimeno, S., Kheyyali, D., Shikata, T., 1995. Metabolic response to dietary lipid to protein  
577 ratios in common carp. *Fish. Sci.* 61, 977-980.

578 Tacchi, L., Bickerdike, R., Douglas, A., Secombes, C.J., Martin, S.A.M., 2011.  
579 Transcriptomic responses to functional feeds in Atlantic salmon (*Salmo salar*), *Fish*  
580 *Shellfish Immunol.* 31, 704-715.

581 Taggart, J.B., Bron, J.E., Martin, S.A.M., Seear, P.J., Hoyheim, B., Talbot, R., Villeneuve, L.,  
582 Sweeney, G.E., Houlihan, D.F., Secombes, C.J., Tocher, D.R., Teale, A.J., 2008. A  
583 description of the origins, design and performance of the TRAITs/SGP Atlantic salmon  
584 (*Salmo salar* L.) cDNA microarray. *J. Fish Biol.* 72, 2071-2094.

585 Takeuchi, T., Shiina, Y., Watanabe, T., 1992a. Suitable levels of n-3 highly unsaturated fatty  
586 acids in diet for fingerlings of red sea bream. *Nippon Suisan Gakk.* 58, 509-514.

587 Takeuchi, T., Shiina, Y., Watanabe, T., Sekiya, S., Imaizumi, K., 1992b. Suitable levels of n-  
588 3 highly unsaturated fatty acids in diet for fingerlings of yellowtail. *Nippon Suisan Gakk.*  
589 58, 1341-1346.

590 Tocher, D.R., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish.  
591 *Aquac. Res.* 41, 717-732.

592 Tocher, D.R., Dick, J.R., 1999. Polyunsaturated fatty acid metabolism in a cell culture model  
593 of essential fatty acid deficiency in a freshwater fish, carp (*Cyprinus carpio*). *Fish. Physiol.*  
594 *Biochem.* 21, 257-267.

595 Tocher, D.R., Harvie, D.G., 1988. Fatty acid composition of the major phosphoglycerides  
596 from fish neutral tissues: (n-3) and (n-6) polyunsaturated fatty acids in rainbow trout

597 (*Salmo gairdneri* L.) and cod (*Gadus morhua* L.) brains and retinas. Fish Physiol.  
598 Biochem. 5, 229-239.

599 Tocher, D.R., Agaba, M., Hastings, N., Bell, J.G., Dick, J.R., Teale, A.J., 2002. Nutritional  
600 regulation of hepatocyte fatty acid desaturation and polyunsaturated fatty acid composition  
601 in zebrafish (*Danio rerio*) and tilapia (*Oreochromis nilotica*). Fish Physiol. Biochem. 24,  
602 309–320.

603 Tocher, D.R., Bell, J.G., Dick, J.R., Crampton, V.O., 2003a. Effects of vegetable oil diets on  
604 Atlantic salmon hepatocyte desaturase activities and liver fatty acid compositions. Lipids  
605 38:723–732.

606 Tocher, D.R., Bell, J.G., Dick, J.R., Sargent, J.R., 1997. Fatty acyl desaturation in isolated  
607 hepatocytes from Atlantic salmon (*Salmo salar*): Stimulation by dietary borage oil  
608 containing  $\gamma$ -linolenic acid. Lipids 32, 1237–1247.

609 Tocher, D.R., Bell, J.G., McGhee, F., Dick, J.R., Fonseca-Madriral, J., 2003b. Effects of  
610 dietary lipid level and vegetable oil on fatty acid metabolism in Atlantic salmon (*Salmo*  
611 *salar* L.) over the whole production cycle. Fish Physiol. Biochem. 29, 193-209.

612 Tocher, D.R., Bendiksen, E.Å., Campbell, P.J., Bell, J.G., 2008. The role of phospholipids in  
613 nutrition and metabolism of teleost fish. Aquaculture 280, 21-34.

614 Zheng, X., Tocher, D.R., Dickson, C.A., Bell, J.G., Teale, A.J., 2004. Effects of diets  
615 containing vegetable oil on expression of genes involved in highly unsaturated fatty acid  
616 biosynthesis in liver of Atlantic salmon (*Salmo salar*). Aquaculture 236, 467–483.

617 Zheng, X., Tocher, D.R., Dickson, C., Dick, J.R., Bell, J.G., Teale, A.J., 2005a. Highly  
618 unsaturated fatty acid synthesis in vertebrates: new insights with the cloning and  
619 characterization of a  $\Delta 6$  desaturase of Atlantic salmon. Lipids 40, 13-24.

620 Zheng, X., Torstensen, B.E., Tocher, D.R., Dick, J.R., Henderson, R.J., Bell, J.G., 2005b.  
621 Environmental and dietary influences on highly unsaturated fatty acid biosynthesis and expression  
622 of fatty acyl desaturase and elongase genes in liver of Atlantic salmon (*Salmo salar*). Biochim.  
623 Biophys. Acta 1734:13–24.

624

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  $\Delta 6$  Fad (A) and  $\Delta 5$  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-1 $\alpha$ ). 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-1 $\alpha$ ). 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 transferase-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-1 $\alpha$ ). 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-1 $\alpha$ ). 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), PPAR $\alpha$  (A), PPAR $\beta$  (B) and PPAR $\gamma$

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-1 $\alpha$ ). Different letters  
659 represent significant differences between diets (ANOVA,  $p < 0.05$ ).

660

661

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
Pea protein conc.	70.0	70.0	70.0
EWOS premix <sup>1</sup>	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
<u>Proximate composition</u>			
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

<sup>1</sup>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 <sup>1</sup>	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 <sup>2</sup>	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 <sup>3</sup>	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 <sup>4</sup>	18.0	19.0	19.8
Total PUFA	32.0	32.7	34.1

Data are means of duplicate analyses. <sup>1</sup>Totals include 15:0, 20:0 and 22:0 at up to 0.3 %; <sup>2</sup>Totals include 20:1n-7, 22:1n-9 and 24:1n-9 at up to 0.8%; <sup>3</sup>Totals include 18:3n-6, 20:2n-6, 20:3n-6, 22:4n-6 and 22:5n-6 at up to 0.2%; <sup>4</sup>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 (T<sub>m</sub>) and fragment sizes produced by the primer pairs used for real-time quantitative PCR (qPCR)

Transcript	Primer name	Primer sequence	Fragment (bp)	T <sub>m</sub> (°C)	Accession No.	Source
<i>Δ5fad</i>	D5DES-F	5'-GTGAATGGGGATCCATAGCA-3'	192	56	AF478472 <sup>1</sup>	Hastings et al. (2005)
	D5DES-R	5'-AAACGAACGGACAACCAGA-3'				
<i>Δ6fad<sub>a</sub></i>	D6DES-F	5'-CCCAGACGTTTGTGTGTCAG-3'	181	56	AY458652 <sup>1</sup>	Zheng et al. (2005)
	D6DES-R	5'-CCTGGATTGTTGCTTTGGAT-3'				
<i>elov15a</i>	Elo1UTR-SM-1F	5'-ACAAGACAGGAATCTCTTCAGATTA-3'	137	60	AY170327 <sup>1</sup>	Morais et al. (2009)
	Elo1UTR-SM-1R	5'-TCTGGGGTTACTGTGCTATAGTGAC-3'				
<i>elov15b</i>	Elo2UTR-5F	5'-ACAAAAAGCCATGTTTATCTGAAAGA-3'	141	60	DW546112 <sup>1</sup>	Morais et al. (2009)
	Elo2UTR-5R	5'-AAGTGGGTCCTCTGGGGCTGTG-3'				
<i>elov12</i>	Elo2-SM-1F	5'-CGGGTACAAAATGTGCTGGT-3'	145	60	TC91192 <sup>2</sup>	Morais et al. (2009)
	Elo2-SM-1R	5'-TCTGTTTGCCGATAGCCAT-3'				
<i>SREBP2</i>	SREBP2-1F	5'-GACAGGCACAACACAAGGTG-3'	215	60	DY733476 <sup>1</sup>	Leaver et al. (2008)
	SREBP2-1R	5'-CAGCAGGGGTAAGGTAGGT-3'				
<i>cpt1</i>	CPT1-1F	5'-CCTGTACCGTGGAGACCTGT-3'	212	60	AM230810 <sup>1</sup>	Leaver et al. (2008)
	CPT1-1R	5'-CAGCACCTCTTTGAGGAAGG-3'				
<i>aco</i>	ACO-2F	5'-AAAGCCTTACCACATGGAC-3'	230	60	TC49531 <sup>2</sup>	Leaver et al. (2008)
	ACO-2R	5'-TAGGACACGATGCCACTCAG-3'				
<i>PPAR<math>\alpha</math></i>	SsPPAR-A-F1	5'-TCCTGGTGGCCTACGGATC-3'	111	60	DQ294237 <sup>1</sup>	Kleveland et al. (2006)
	SsPPAR-A-R1	5'-CGTTGAATTTTCATGGCGAACT-3'				
<i>PPAR<math>\beta</math></i>	SsPPAR-B-F1	5'-GAGACGGTCAGGGAGCTCAC-3'	151	60	AJ416953 <sup>1</sup>	Kleveland et al. (2006)
	SsPPAR-B-R1	5'-CCAGCAACCCGTCCTTGT-3'				
<i>PPAR<math>\gamma</math></i>	SsPPAR-G-F1	5'-CATTGTCAGCCTGTCCAGAC-3'	144	60	AJ416951 <sup>1</sup>	Kleveland et al. (2006)
	SsPPAR-G-R1	5'-TTGCAGCCCTCACAGACATG-3'				
<i>FAS</i>	SsFAS-F4	5'-GTGCCCACTGAATACCATCC-3'	212	60	CK876943 <sup>1</sup>	Morais et al. (2011)
	SsFAS-R4	5'-ATGAACCATTAGGGGACAG-3'				
<i>LXR</i>	SsLXR-F	5'-GCCCGCGCTATCTGAAATCTG-3'	210	58	FJ470290	Cruz-Garcia et al. (2009)
	SsLXR-R	5'-CAATCCGGCAACCAATCTGTAGG-3'				
Reference genes:						
<i>elf-1<math>\alpha</math></i>	ELF-1A jbt2	5'-CTGCCCTCCAGGACGTTTACAA-3'	175	60	AF321836 <sup>1</sup>	Morais et al. (2009)
	ELF-1A jbt2	5'-CACCGGGCATAGCCGATTCC-3'				
<i><math>\beta</math>-actin</i>	BACT-F	5'-ACATCAAGGAGAAGCTGTGC-3'	141	56	AF012125 <sup>1</sup>	Morais et al. (2009)
	BACT-R	5'-GACAACGGAACTCTCGTTA-3'				
<i>Cofilin-2</i>	B2F	5'-AGCCTATGACCAACCCACTG-3'	224	60	TC63899 <sup>2</sup>	Morais et al. (2009)
	B2R	5'-TGTTACAGCTCGTTTACCG-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)
<u>Proximate composition (percentage of wet weight)<sup>1</sup></u>			
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.

<sup>1</sup>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)
<u>Lipid class</u>			
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 statistically 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 <sup>1</sup>		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 <sup>2</sup>		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 <sup>3</sup>		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 <sup>4</sup>		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:  $\Delta 6$  FAD B:  $\Delta 5$  FAD)

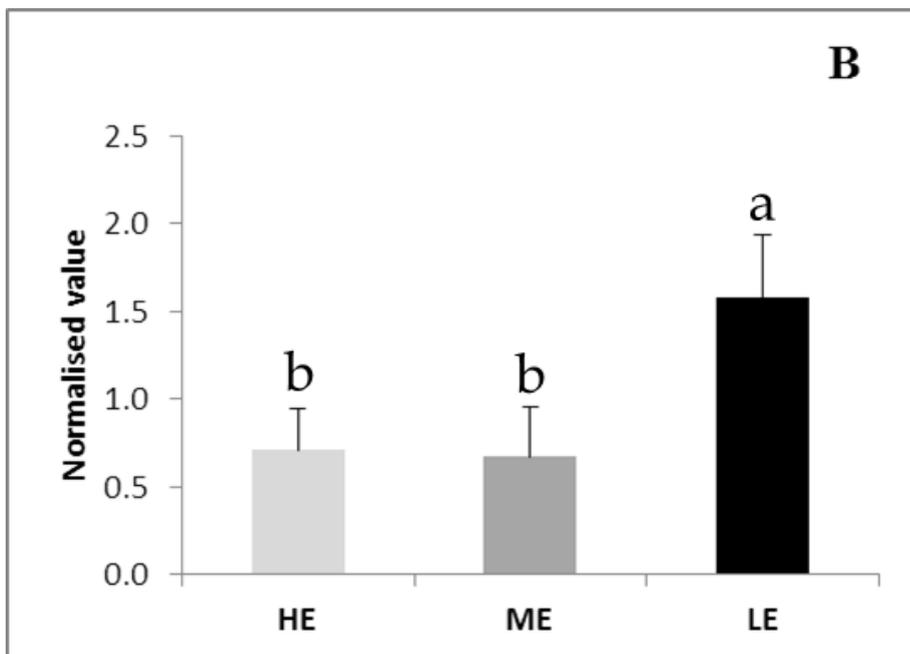
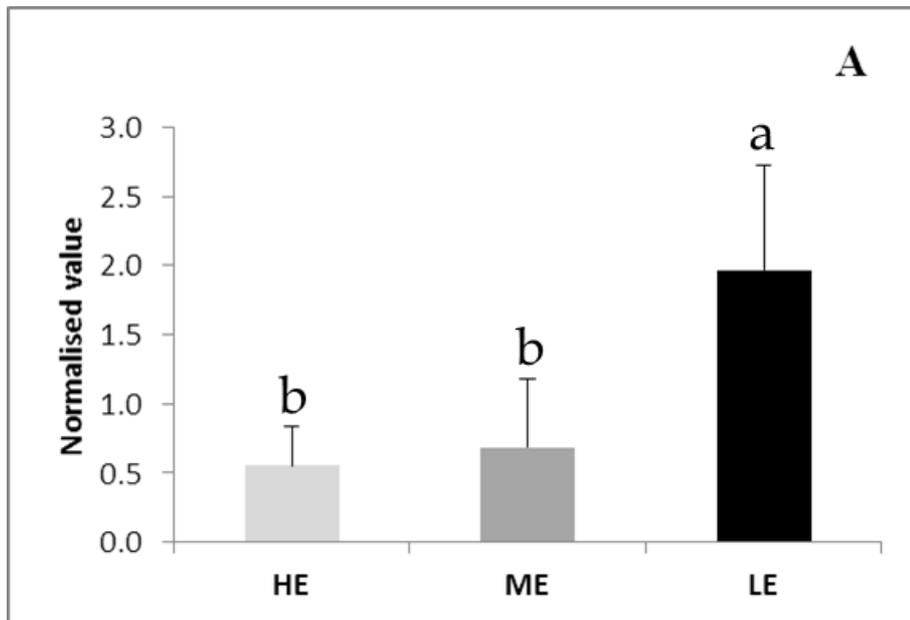


Fig 2. Elongases (A: ELOVL2 B: ELOVL5a C: ELOVL5b)

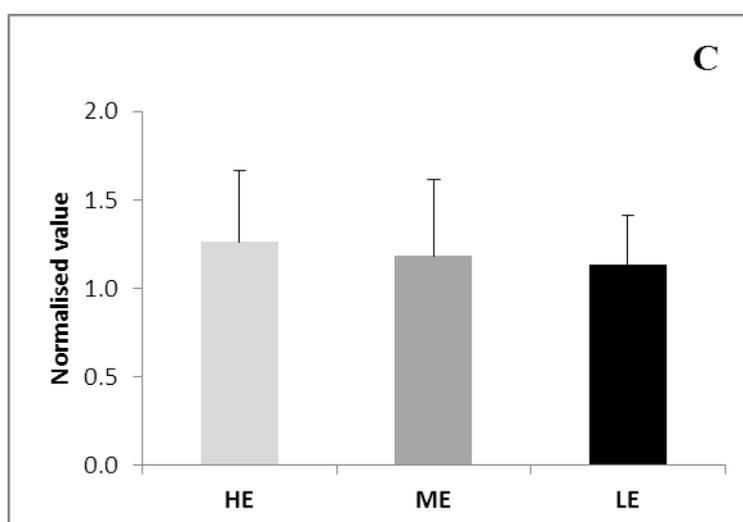
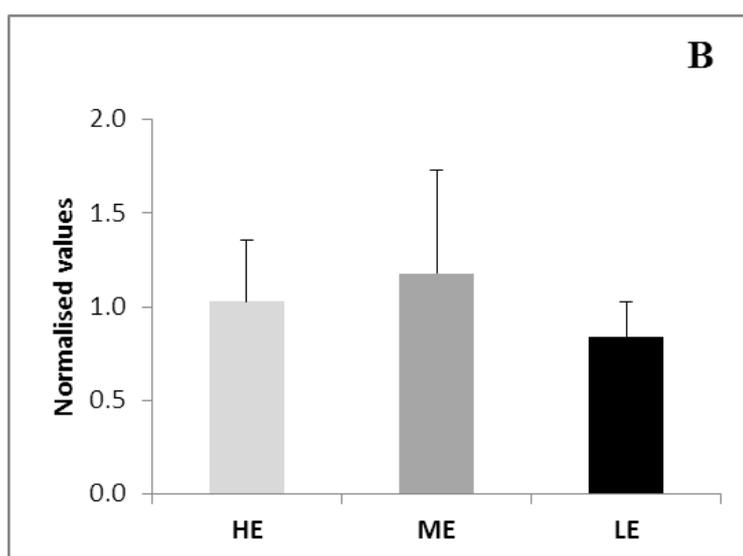
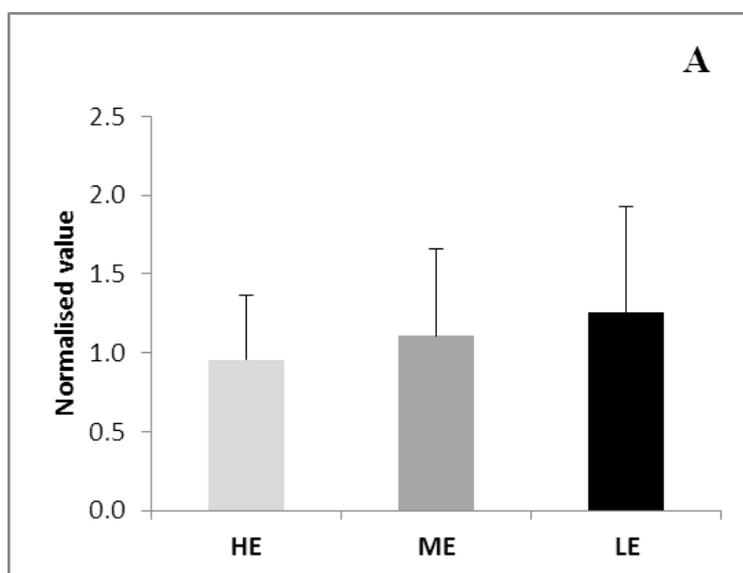


Fig. 3 (A: FAS B: CTP1 C: ACO)

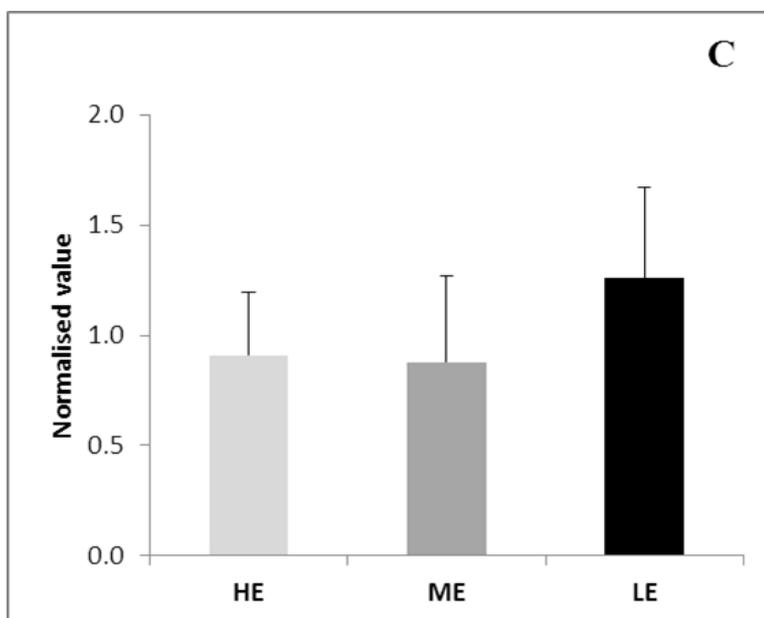
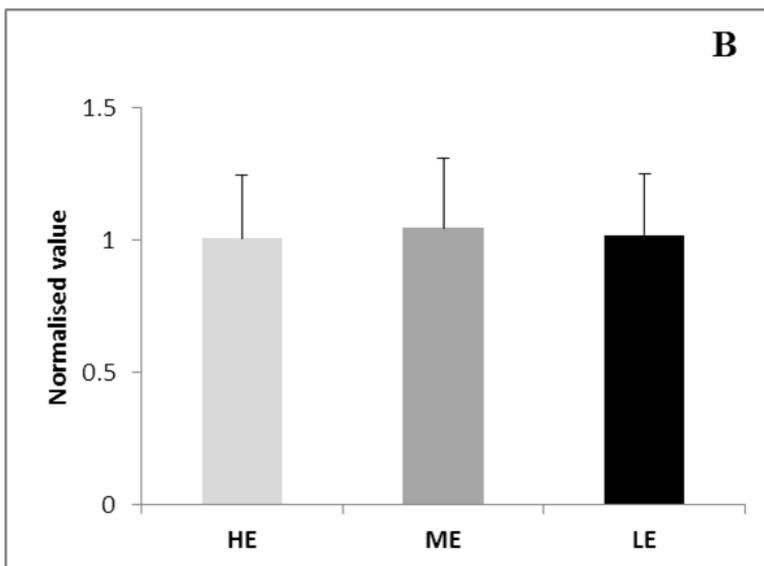
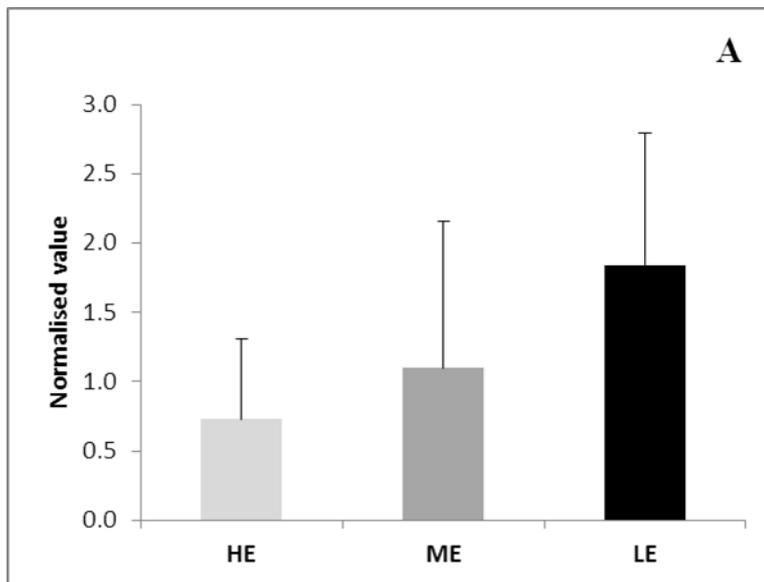


Fig. 4 (A: SREBP2 B: LXR)

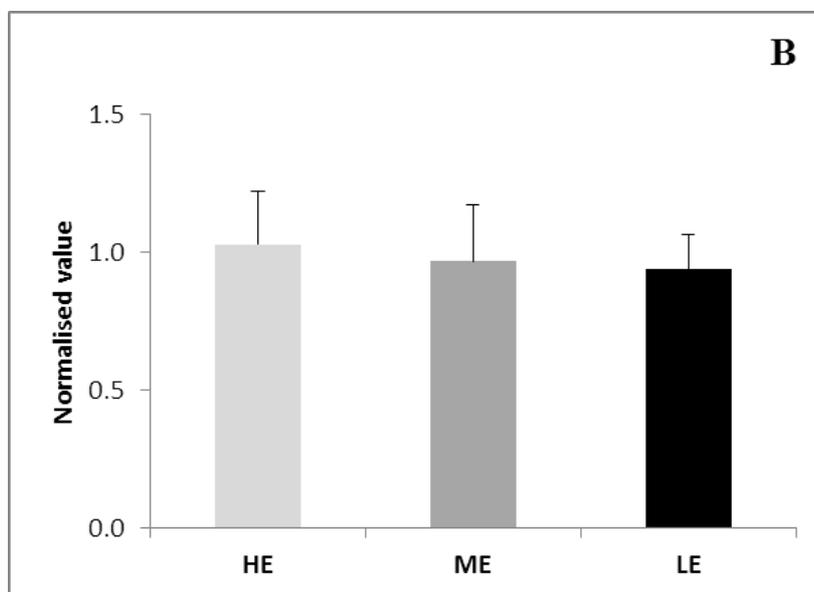
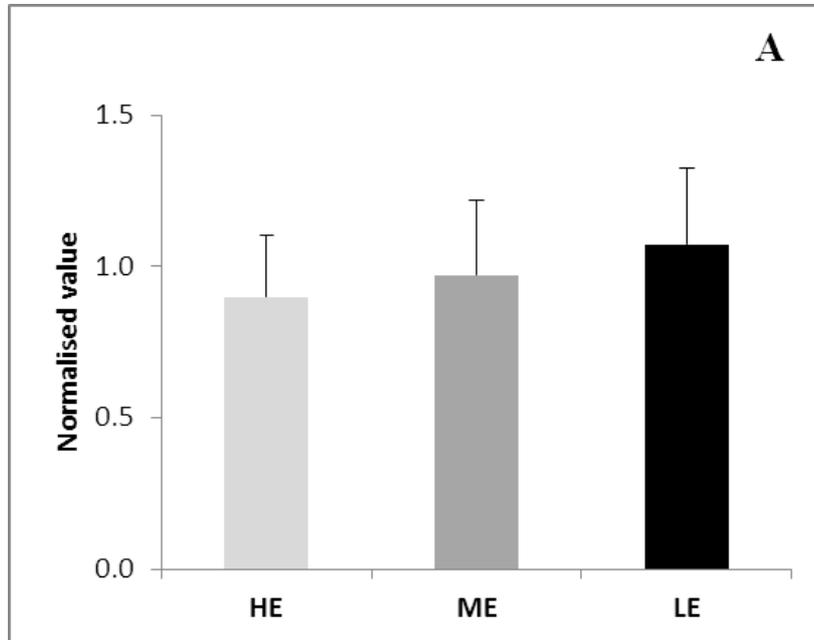


Fig. 5 PPARS

