

1 Requirement for omega-3 long-chain polyunsaturated fatty acids by Atlantic salmon is relative 2 to the dietary lipid level

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10

11 Abstract

12

13 Requirements for omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA), such as
14 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), for Atlantic salmon are typically
15 represented as an absolute level in the diet (e.g. g/kg or % of diet). Data for other species suggests that
16 requirements for n-3 LC-PUFA are actually relative to dietary lipid (e.g. % of total fatty acids). A 2 x
17 2 factorial design of dietary lipid level x n-3 LC-PUFA level was designed to examine this question.
18 Atlantic salmon post-smolts of 187 ± 4 g were fed one of four diets for 116 days that either had a low
19 or high lipid level (180 or 230 g/kg) and a low or high n-3 LC-PUFA level (7 or 14 g/kg). Fish fed the
20 diet with high-lipid + high n-3 had greater final weight and weight gain than the high-lipid + low n-3
21 diet, but no differences were noted between the two low-lipid diets. Significant effects of n-3 and a
22 lipid*n-3 interaction were observed. However, no effects on feed intake, FCR and survival were found.
23 Feeding high n-3 diets generally increased n-3 levels and retention in the whole body, especially EPA
24 and DHA. Relative expression of lipid metabolism genes in the liver showed that fish fed high lipid +
25 high n-3 had lower levels of expression of fatty acid synthesis genes (*fads2d5*, *fads2d6* and *elovl2*).
26 Upregulation of lipid transcription factor (*srebp2* and *lxr*) and fatty acid beta-oxidation (*hoad* and *aco*)
27 genes in fish fed low lipid + high n-3 further suggest that the proportion of dietary n-3 and energy level
28 in those diets were lower than the high-lipid + high n-3 treatment. In conclusion, the significant
29 interaction between lipid and n-3 levels on growth clearly shows that n-3 LC-PUFA requirements are
30 relative to the lipid level in diets for Atlantic salmon. These results support the notion that requirements
31 for this species should be defined based on a percent of total fatty acid content, implying that the
32 absolute amount of n-3 LC-PUFA needs to increase as lipid content of the diet increases.

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34 Keywords

35 Docosahexaenoic acid (DHA); Eicosapentaenoic acid (EPA); Lipid; Omega-3; Requirements

36

37 **Highlights**

- 38 • Highest growth for Atlantic salmon fed high lipid + high n-3 LC-PUFA diet.
- 39 • Significant interaction between lipid and n-3 levels for fish growth.
- 40 • Retention of n-3 LC-PUFA in the carcass was higher when fed high n-3 diets.
- 41 • Up-regulation of fatty acid synthesis genes in fish fed low n-3 diets.
- 42 • The n-3 LC-PUFA requirement is relative to the total lipid level in the diet.

45 **1. Introduction**

46
47 The omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic acid
48 (EPA) and docosahexaenoic acid (DHA), are conditionally-essential dietary nutrients for Atlantic
49 salmon (*Salmo salar*) (Glencross, 2009) (Fig. 1). Various studies have shown that n-3 LC-PUFA are
50 required by Atlantic salmon at a level between 10 and 15 g/kg of the diet for optimal growth (Bou *et*
51 *al.*, 2017, Glencross *et al.*, 2014, Ruyter *et al.*, 2000). However, this level may actually be subject to
52 varying dietary lipid levels as there is some evidence from other species that requirements may in fact
53 be relative not absolute (Glencross, 2009).

54
55 Throughout the literature, fatty acid requirement studies have been expressed both in terms of the
56 amount of these nutrients in the diet (g/kg) and/or the relative proportion they represented of the total
57 fatty acids (%TFA) (Glencross, 2009). Previous studies have indicated that fatty acid requirements are
58 better represented relative to the level of total fatty acids in other species such as rainbow trout
59 (*Oncorhynchus mykiss*) (Watanabe, 1982), red sea bream (*Pagrus major*) (Takeuchi *et al.*, 1992a),
60 yellowtail (*Seriola quinqueradiata*) (Takeuchi *et al.*, 1992b) and Giant tiger shrimp (*Penaeus monodon*)
61 (Glencross *et al.*, 2002). For example, Watanabe (1982) found that double the level of n-3 PUFA (18:3n-
62 3) was required when feeding 100 instead of 50 g/kg total lipid to rainbow trout. The important
63 implication of this observation is that given that it is typical to change the lipid level in diets as species
64 grow, then relying on a single, fixed absolute level of n-3 PUFA in the diet may in fact be pushing the
65 diets to becoming limiting in n-3 as the lipid level increases if these nutrients are not proportionally
66 increased. However, this approach to reporting fatty acid requirements has not been fully adopted by the
67 aquaculture nutrition community. This is in contrast to amino acid requirements that are typically
68 represented either or both relative to protein level and/or relative to energy level.

69
70 Therefore, the objective of this study was to determine the nature of requirement responses by Atlantic
71 salmon. To do this required a two-way factorial analysis of the effect of dietary lipid level and n-3 LC-
72 PUFA level on the respective responses by the fish, where we present an assessment of the performance,

73 nutrient utilisation and transcriptomic responses of this species. We tested the hypothesis that n-3 LC-
74 PUFA level is relative to the total lipid level, rather than absolute level, in the diet by evaluating lipid*n-
75 3 interactions on the above response parameters.

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78 **2. Materials and Methods**

79

80 *2.1 Fish management*

81

82 Atlantic salmon post-smolts were acquired from a commercial hatchery (Marine Harvest, Loch Ailort,
83 Scotland) and transferred to University of Stirling's Marine Environmental Research Laboratory
84 (Machrihanish, Scotland). Fish were sedated with MS222, weighed (187 ± 4 ; mean \pm SD) and sorted
85 into 12 circular tanks (500L) to achieve 32 fish per tank. Tanks contained 350 L of bag-filtered (100
86 μm) seawater sourced from the adjacent bay in a flow-through system. Each tank was equipped with
87 LED lighting on a 16:8 light:dark cycle, an air stone and a probe that recorded dissolved oxygen and
88 temperature every 10 min (Oxyguard A/S, Farum, Denmark). Fish were acclimatised to the tanks for
89 three weeks while they were introduced to the experimental diets. Over the 17-week (116 day; 138 days
90 including the acclimation period) experiment, temperature was 13.2 ± 0.2 °C (mean per week \pm SD)
91 and the dissolved oxygen was $92.6 \pm 2.7\%$ (8.0 ± 0.2 mg/L). The experiment was approved by the
92 University of Stirling Animal Welfare and Ethical Review Body (reference AWERB-16/17-84) in
93 accordance with the UK Home Office under the Animals (Scientific Procedures) Act 1986.

94

95 *2.2 Experimental diets and feeding*

96

97 The basic diet design was a simple 2 x 2 factorial, with high and low levels of lipid (HL and LL) and
98 high and low levels of n-3 LC-PUFA (Hn3 and Ln3). Levels of n-3 LC-PUFA were planned to be
99 slightly above and below reported critical optima (Glencross et al., 2014; Bou et al., 2017). Diets were
100 formulated (Table 1) to be isoenergetic on a digestible basis. To achieve this the level of protein was
101 increased in the low lipid diets (LL-Ln3 and LL-Hn3) to maintain the diets on this isoenergetic basis,
102 while maintaining an equal amount of fishmeal (200 g/kg) in each diet. While clearly this changed the
103 protein:energy ratio of the diets, this was considered less of an issue than not balancing the digestible
104 energy content of the diets or using starch to manipulate digestible energy density. The n-3 LC-PUFA
105 level in two of the diets (HL-Hn3 and LL-Hn3) was increased from 7 to 14 g/kg by additional inclusion
106 of linseed and fish oils. Yttrium oxide was included as a digestibility marker in the diets. The 3 mm
107 diets were produced by SPAROS I&D (Olhão, Portugal) using twin-screw extrusion, vacuum lipid
108 coating, and were air-dried and stored at 4 °C.

109

110 Each tank of fish was fed one of the four extruded diets in triplicate in a randomised block design. Diets
111 were fed twice per day for three-hour durations using automated drum feeders (Arvo-tec Oy,
112 Huutokoski, Finland) at a rate of about 1.0 to 1.5% of fish bodyweight per day. Feeding rations were
113 adjusted daily based on the uneaten feed from each tank to ensure satiation. Each morning, uneaten feed
114 was collected manually from each external tank standpipe using a sieve and was weighed. A recovery
115 and dissolution test was performed to determine a correction factor to be applied to the wet uneaten
116 feed waste in order to calculate the daily feed intake according to (Helland *et al.*, 1996), which is
117 included in the equation below.

118

119 *2.3 Sample collection*

120

121 Fish were sedated with MS222 and weighed at day 0, 21, 56 and 138 (day 116 post-acclimation period).
122 Fish were fed until the day of sampling and faeces were stripped from all fish by gently squeezing the
123 abdomen, pooled per tank and stored at -20 °C. At each weighing point, eight fish per tank were
124 euthanised by an overdose of MS222 and cervical dislocation. Four fish were measured for fork length,
125 pooled per tank (n=3/treatment) and stored at -20 °C. However, at the end of the trial one tank of fish
126 developed symptoms of amoebic gill disease and was treated with freshwater, thus this tank was
127 removed from growth performance analysis (i.e. HL-Hn3: n=2). Pooled faeces and whole carcass
128 samples were homogenised and freeze dried overnight. The other four fish were dissected, liver and
129 viscera weights were recorded to determine somatic indices and the liver was frozen in cryotubes on
130 dry ice and stored at -70 °C for gene expression analysis.

131

132 *2.4 Nutritional analyses*

133

134 Proximate, fatty acid and mineral composition of the diets, carcasses and faeces were performed at the
135 Institute of Aquaculture (Stirling, UK). Moisture and ash were analysed using ovens at 105 and 550 °C
136 for approximately 24 and 12 hours, respectively according to the Association of Official Analytical
137 Chemists (AOAC, 1995). Protein was analysed by digestion in sulphuric acid at 400 °C (FOSS A/S,
138 Hillerød, Denmark) for one hour and then addition of sodium hydroxide by a Tecator Kjeltex system
139 (FOSS A/S) according to the Kjeldahl Method (Persson, 2008). Gross energy was measured by ballistic
140 bomb calorimetry using a Parr 6200 bomb calorimeter (Parr Instrument Co., Moline, IL, USA).
141 Lipid was analysed by homogenisation in 2:1 chloroform/methanol, centrifugation, aqueous layer
142 aspiration and nitrogen evaporation (TurboVap Classic, Biotage AB, Uppsala, Sweden) according to
143 the Folch method (Folch *et al.*, 1957). Fatty acids were analysed according to methods of the American
144 Oil Chemists' Society (Christie, 2003). Fatty acid methyl esters (FAME) were made by acid-catalysed
145 esterification of 1 mg of total lipid by overnight incubation at 50 °C with an internal standard of 17:0,

146 sulphuric acid, methanol and toluene. A solution of 1:1 iso-hexane/diethyl ether was added and then
147 centrifuged. The upper layer was purified through a silica cartridge, redissolved in iso-hexane and then
148 injected onto a gas liquid chromatographer (GLC) using a Fisons GC-8160 (Thermo Scientific, Milan,
149 Italy) equipped with a 30 m × 0.32 mm i.d. × 0.25 µm ZB-wax column (Phenomenex, Cheshire, UK),
150 on-column injector and a flame ionisation detector. Individual FAMES were identified by MD800 mass
151 spectrometer (ThermoFisher Scientific, Hempstead, UK) and compared to external standards of marine
152 oil. Data were collected and processed using Chromcard software version 2.01 (Thermoquest Italia
153 S.p.A., Milan, Italy).

154

155 *2.5 Calculations of growth performance, body indices and feed efficiency*

156

157 Means for growth performance were generated based on per fish values from three replicate tanks, body
158 indices were based on four representative fish per tank and nutrient utilisation was based on a pooled
159 sample of four fish per tank. Weight gain, gain rate, feed intake (FI) and feed conversion ratio (FCR)
160 were calculated using the following equations:

161 Weight gain (g fish⁻¹) = final weight – initial weight

162 Gain rate (g fish⁻¹ day⁻¹) = weight gain / days

163 FI (g fish⁻¹) = [(Feed fed – (feed waste / correction factor)] / number of fish in each tank

164 Protein intake (g fish⁻¹) = FI x (diet protein % / 100)

165 Lipid intake (g fish⁻¹) = FI x (diet lipid % / 100)

166 FCR = FI / weight gain

167

168 Hepatosomatic index (HSI) and viscerosomatic index (VSI) were calculated according to the following
169 equations:

170 HSI (%) = (liver weight / final weight) x 100

171 VSI (%) = (viscera weight / final weight) x 100

172

173 Nutrient retention and apparent digestibility were calculated as:

174 Nutrient retention (%) = [(FW x C / 100) – (SW x C / 100)] / (FI x C / 100) x 100

175 Apparent digestibility (%) = [1 – (F / D × D_i / F_i)] x 100

176 where C is % nutrient (or MJ kg⁻¹ for energy) in whole body carcass or diet (D), F is % nutrient (or MJ
177 kg⁻¹ for energy) in faeces, D_i is % inert marker yttrium in diet and F_i is % inert marker yttrium in faeces.

178

179 *2.6 Molecular analyses using qPCR*

180

181 Liver samples were thawed on ice and approximately 50 mg of the apical tip was homogenised in 1 mL
182 of Tri Reagent (Sigma-Aldrich, Dorset, UK) using a mini-bead beater (Biospec Products, Bartlesville,

183 OK, USA) for two cycles of 45 sec with 45 sec rest period. Samples were centrifuged at 12,000 g for
184 10 min and the upper layer was transferred to new tubes containing 1-bromo-3-chloropropane (Sigma-
185 Aldrich). The RNA solution was mixed, centrifuged at 20,000 g for 15 min, precipitated with a solution
186 of sodium chloride (Merck KGaA, Darmstadt, Germany), sodium citrate sesquihydrate (Sigma-Aldrich)
187 and isopropanol. Samples were centrifuged as before and the RNA pellet was washed with two washes
188 of 70% ethanol and then air dried in a fume hood. The RNA pellet was resuspended in RNase free water
189 and the concentration and quality was checked using a spectrophotometer (ND-1000, Nanodrop
190 Technologies LLC, Wilmington, DE, USA). All samples had a 260/230 nm 260/280 ratios above 2.0
191 and 1.8, respectively, or the extraction was redone. The quality was also checked by running denatured
192 samples on a 1% agarose gel to verify RNA integrity of the two rRNA bands.

193

194 From two fish per tank (n=6/treatment), 6 µg was pooled and then diluted with RNase free water to 2
195 µg (200 ng/µL). Samples were denatured at 75 °C for 5 min and then added to 10 µL of High-capacity
196 cDNA Reverse Transcription Kit (Applied Biosystems, Paisley, UK) containing RT buffer, dNTP,
197 random primers, dT oligo primers, multiscribe reverse transcriptase (50 U/µL) and nuclease free water.
198 Non-template control (NTC) and reverse transcription negative (RT-) were included for quality control.
199 The cDNA was synthesised in a thermocycler (T Advanced, Biometra GmbH, Göttingen, Germany)
200 with the conditions: 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min.

201

202 The qPCR efficiency was determined for every set of primers by pooling 4 µL of each sample and then
203 making a dilution series from 1/5 to 1/500. In duplicate, 2.5 µL of each diluted sample (1 µL for
204 reference genes) was mixed with 5 µL of Luminaris Color HiGreen qPCR mastermix (Thermo
205 Scientific, Paisley, UK), 0.5 µL of each primer (10 pmol) and nuclease free water in 10 µL reactions,
206 along with a NTC. The qPCR was performed in a thermocycler (T Professional, Biometra GmbH) under
207 conditions: 50 °C for 2 min, 95 °C for 10 min and 35 cycles of 95 °C for 15 sec, 60 °C for 30 sec and 72
208 °C for 30 sec. All primer efficiencies (E) were between 90-105% and the Ct of each target gene was
209 calibrated against the control treatment of high lipid + high n-3 (delta Ct = calibrator Ct – sample Ct).
210 The relative gene expression was calculated based on relative quantity (RQ = E^{delta Ct}) between the
211 target and the geometric mean of two reference genes (RQ target / RQ reference) (Pfaffl et al., 2000).
212 Four reference genes (Table 2) were compared using Genorm (Vandesompele *et al.*, 2002) and *hprt* and
213 *rps5* were selected to be the most stable genes.

214

215 2.7 Statistical analysis

216

217 Normal distribution and homogeneity of each dataset were determined using Shapiro-Wilk and Levene
218 tests in Rstudio software version 1.0.143 (R-Core-Team, 2015). If needed, data were normalized by

219 log-transformation. All data are presented as means \pm SE unless otherwise specified. Akaike's An
220 Information Criterion (AIC) was used to determine the statistical model that best fitted the data.
221 Significant differences between treatments were determined using linear models (lm) for phenomic and
222 nutrient data and linear mixed effects (lme) models for gene expression data based on the nlme R
223 package (Pinheiro *et al.*, 2014). Both lm and lme models included fixed effects of lipid and n-3 LC-
224 PUFA as well as an interaction, except lme included random effect of tank since there was two pooled
225 samples per tank for the transcriptomic data. P-values of each factor and interaction were generated
226 using ANOVA tables and below 0.05 were considered significant and below 0.10 was considered to be
227 a tendency. P-values among treatments were determined using Fisher's least significant difference test
228 (LSD.test) for multiple comparisons based on the agricolae R package (de Mendiburu, 2020).

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230

231 **3. Results**

232

233 *3.1 Growth performance and feed efficiency*

234

235 The levels of dietary lipid/protein (Lipid) and n-3 LC-PUFA in this experiment influenced several
236 parameters of fish growth and feed efficiency (Table 4 and Fig. 2). Effects of n-3 and a Lipid*n-3
237 interaction were found for final weight, weight gain and gain rate. Growth of fish fed the HL-Hn3 diet
238 was significantly higher than that of fish fed the HL-Ln3 diet, while growth of fish fed the LL-Ln3 or
239 LL-Hn3 diets was similar. Protein and lipid intake, HSI and VSI were significantly affected by diet
240 lipid level. Protein intake was higher in the LL diets and lipid intake and VSI were higher in the HL
241 diets.

242

243 *3.2 Whole body composition, nutrient retention and digestibility*

244

245 Proximate composition of whole-body carcasses were only influenced by dietary lipid level, with no
246 effects of n-3 (Table 5). Ash, lipid and energy were elevated in the HL treatments, whereas protein was
247 reduced. Lipid and n-3 significantly affected almost every fatty acid level in the whole body represented
248 as % of total fatty acid, but a Lipid*n-3 interaction was found for a few monoenes and n-3 PUFA (Table
249 5). Both lipid and n-3 levels affected total saturates, monoenes, n-3 PUFA, PUFA and LC-PUFA where
250 low lipid and high n-3 typically increased levels found in the whole body. Total n-6 PUFA were only
251 influenced by lipid levels, resulting in high n-6 levels when fed low lipid diets. High n-3 diets resulted
252 in higher levels of EPA and DHA in the whole body.

253

254 Only lipid level influenced retention of protein while only n-3 level influenced the retention of fatty
255 acids in the whole body carcass (Table 6). Retention of total saturates and monoenes were not influenced
256 at all, whereas total n-6, n-3, PUFA and LC-PUFA were affected by the level of n-3 that typically
257 resulted in higher retention for the high n-3 diets. EPA retention was higher in fish fed high n-3 diets,
258 while DHA retention was unaffected.

259

260 Apparent digestibility of protein, lipid and energy were influenced by lipid and/or n-3 dietary levels,
261 while only a few fatty acids were affected (Table 7). HL diets generally increased the digestibility of
262 protein and energy, while Hn3 diets decreased the digestibility of lipid. A lipid*n-3 interaction existed
263 for protein digestibility and it was significantly higher for the HL-Hn3 diet. The digestibility of total
264 saturates were influenced by n-3 level, total monoenes were influenced by dietary lipid and no effects
265 were found on total n-6, n-3, PUFA and LC-PUFA, including EPA and DHA.

266

267 3.3 Differential gene expression in the liver

268

269 The expression of 9 out of 13 genes related to lipid metabolism in the liver were influenced by lipid, n-
270 3 and/or lipid*n-3 interaction (Fig. 2-4). For fatty acid synthesis, low lipid and low n-3 diets increased
271 expression of *fads2d5* and *fads2d6* where the HL-Hn3 diet had significantly lower expression (Fig. 3).
272 Also, LL diets tended to increase *elovl2* expression. For transcription factors, a Lipid*n-3 interaction
273 was found for *srebp1*, *srebp2* and *lxr* genes that had significantly increased expression for the LL-Hn3
274 diet (Fig. 4). For beta-oxidation of fatty acids, HL diets increased expression of *cpt1b* and a lipid*n-3
275 interaction existed for *hoad* and *aco* that showed increased expression in fish fed the LL-Hn3 diet (Fig.
276 5).

277

278 **4. Discussion**

279

280 *4.1 Dietary lipid and n-3 LC-PUFA on feed intake and growth performance*

281

282 An interaction between lipid and n-3 LC-PUFA levels on growth performance (Table 4 and Fig. 2)
283 provide further evidence that the level of n-3 LC-PUFA (i.e. EPA and DHA) required by Atlantic
284 salmon is proportional/relative to the total lipid level rather than the absolute level in the diet. These
285 results are in agreement with previous research that demonstrated that n-3 fatty acids are better
286 represented by the proportionality of total fatty acids (Glencross *et al.*, 2002, Watanabe, 1982). In this
287 study, both Hn3 diets had the same absolute level of n-3 LC-PUFA (i.e. 14 g/kg of diet), as did the two
288 Ln3 diets (i.e. 7 g/kg of diet), although each of the diets differed in their relative levels of n-3 LC-PUFA
289 (i.e. 3, 5, 7 and 9% of total fatty acids; TFA) (Table 2). Increased growth of fish fed the **HL-Hn3 relative**
290 **to the HL-Ln3 treatment** is inline with previous studies that stipulate the required level of n-3 LC-PUFA
291 in the diet is between 10 to 15 g/kg (Bou *et al.*, 2017, Glencross *et al.*, 2014). **In comparison, the equal**
292 **growth of fish fed the two LL treatments** suggests that the n-3 LC-PUFA requirement is proportional
293 and not entirely based on an absolute level between 10 to 15 g/kg. Given that the diets were formulated
294 to be equal in terms of digestible energy in order to compare the interaction between dietary lipid and
295 n-3 LC-PUFA levels, the lack of a difference between treatments in feed intake is perhaps not surprising
296 (Tables 1 and 4). Because the fish were fed to satiety, the similar feed intake across treatments indicates
297 that the fish are clearly eating to an energy demand and not an essential nutrient demand, as there was
298 no observation that the fish were adjusting appetite to compensate for any key nutrient differences
299 among the diets (see Fig. 1).

300

301 The higher dietary lipid level, even with a slightly lower proportion of dietary EPA and DHA (i.e. HL-
302 Hn3 diet) resulted in a numerically better fish growth performance than the LL-Hn3 diet, suggesting
303 that the energetic role of the dietary lipid also plays an important role beyond the n-3 LC-PUFA story.
304 This may reflect subtle differences in the net energy value of the diets, and that Atlantic salmon
305 metabolise energy from lipid more effectively than protein and therefore, despite that the digestible
306 energy levels of the diets being close, the net energy values of the diets were likely more divergent
307 (Phan *et al.*, 2019). Previous studies found that feeding higher levels of total lipid and n-3 LC-PUFA
308 increased growth of rainbow trout and shrimp, although over-supplementation of both resulted in
309 reduced growth (Glencross *et al.*, 2002, Watanabe, 1982). However, the proportion of n-3 LC-PUFA in
310 the LL-Hn3 diet in the present study was similar to previous studies (i.e. 5 to 10% TFA) that resulted
311 in optimal growth of Atlantic salmon (Glencross *et al.*, 2014, Bou *et al.*, 2017), which further supports
312 the notion of a net energy imbalance. This would also explain similar growth of fish fed both LL diets
313 with n-3 LC-PUFA levels of 5 and 9% TFA, respectively (see Fig. 6). However, seasonal effects, such
314 as water temperature, have been found to effect protein, lipid and energy retentions in post-smolt salmon

315 fed diets based on high and low protein-lipid ratio (Dessen *et al.*, 2017). Other environmental
316 conditions, such as hypoxia, may also play a role in dietary requirements (Glencross, 2009). In addition,
317 life stage is known factor as Atlantic salmon fry require a lower level of dietary lipid (e.g. 80 g/kg) and
318 hence a higher proportion of n-3 LC-PUFA (e.g. >10% TFA) (Ruyter *et al.*, 2000).

319

320 *4.2 Dietary lipid and n-3 LC-PUFA on nutrient retention and digestibility*

321

322 Altering the levels of protein, lipid and fatty acids in the diet had clear effects on the composition of the
323 whole-body carcass that reflected the diet (Table 5). These results agree with previous studies that have
324 found that feeding high levels of n-3 LC-PUFA results in higher levels in the body or muscle of salmon
325 (Betancor *et al.*, 2014, Betancor *et al.*, 2017, Hixson *et al.*, 2017, Glencross *et al.*, 2014). The retention
326 of n-3 PUFA, especially DHA, in the body indicates that deposition of these essential fatty acids are
327 preferred over others (Table 6). In contrast, previous studies have found that higher levels of n-3 PUFA,
328 such as DHA and EPA, did not result in higher retention in the whole body or flesh of salmon and can
329 even decrease with increased dietary inclusion (Glencross *et al.*, 2014, Bell *et al.*, 2004, Bell *et al.*,
330 2001).

331

332 Increased (numerical, but not significant) growth of fish fed the HL-Hn3 diet (Table 4) may be
333 explained by a higher net energy value from that diet compared to the LL-Hn3 diet (high protein), due
334 to the lower net energy values from protein. Although digestible energy values were accounted for in
335 the formulation, that protein has a higher heat increment of feeding than lipid may result in higher
336 metabolic cost and subsequently result in lower net energy values from those diets (Kaushik and
337 Médale, 1994). Higher energetic costs may also explain why fish fed the LL-Hn3 diet had numerically
338 lower growth. In addition, higher net energy values for fish fed HL diets may have resulted in slight
339 improvements in nutrient utilisation since fish fed HL diets had higher retention of protein as well as
340 higher digestibilities of protein and energy (Tables 6 and 7). However, higher protein utilisation may
341 be due to lower protein content in the HL diets and/or the quality of raw ingredients. Similar lipid, n-3
342 and lipid*n-3 interaction effects were found for protein digestibility of Atlantic salmon (Bendiksen *et al.*
343 *et al.*, 2003), although different lipid levels and oil type were fed to parr. In this study, the interaction
344 between lipid and n-3 LC-PUFA on growth performance further supports the inclusion of n-3 LC-PUFA
345 relative to lipid level, especially since lipid level can affect net energy values and feed utilisation in
346 Atlantic salmon.

347

348 The significant effect of n-3 level on lipid digestibility agrees with previous studies on salmonids
349 (Caballero *et al.*, 2002, Karalazos *et al.*, 2011), although no effects on specific n-3 PUFA or LC-PUFA
350 digestibilities were found (Table 7). Previous studies have found reduced digestibility of n-3 PUFA,
351 especially EPA, when rainbow trout were fed diets based on a mixture of vegetable oils (Caballero *et*

352 *al.*, 2002). In Atlantic salmon, replacing fish oil with rapeseed oil reduced EPA (tendency) and DHA
353 (significant) (Karalazos *et al.*, 2011). In this study, digestibility of EPA and DHA was slightly decreased
354 (not significant) for fish fed low n-3 diets, but this lack of effect may be due to the subtle difference
355 between the high and low n-3 diets as opposed to replacing large proportions of fish oil with vegetable
356 oil.

357

358 4.3 Dietary lipid and n-3 LC-PUFA influences hepatic gene expression

359

360 The results indicate that both lipid and n-3 LC-PUFA levels in the diet influence the transcriptomic
361 pathway for fatty acid synthesis, regulation and beta-oxidation in the liver of Atlantic salmon (Fig. 3-
362 5). Reduced expression of fatty acid desaturases and elongases, such as *fads2d5* and *elovl2*, in fish fed
363 high n-3 diets (Fig. 3) agrees with previous studies that have fed fish oil with higher n-3 LC-PUFA to
364 salmon in comparison to vegetable oils (Zheng *et al.*, 2005, Leaver *et al.*, 2008b, Betancor *et al.*, 2014,
365 Hixson *et al.*, 2017). Upregulation of desaturases and elongases commonly results in an increased
366 production of intermediate products (i.e. 20:4n-3 and 22:5n-3) during EPA and DHA synthesis from
367 18:3n-3, which may explain the retention greater than 100% for DHA in the present study. The reduced
368 level of expression of fatty acid elongation (*elovl2* and *elovl5a*) and desaturation (*fads2d5* and *fads2d6*)
369 genes supports that the higher level of dietary n-3 LC-PUFA was sufficient at meeting the requirement
370 for Atlantic salmon.

371

372 Upregulation of transcription factors, such as *srebp* and *lxr*, in fish fed the LL-Hn3 diet (Fig. 4) indicates
373 the activation of the cholesterol and PUFA biosynthesis pathways (Leaver *et al.*, 2008a), which may be
374 due to low levels of lipid in the diet. Previous studies have found increased expression of *srebp1* and/or
375 *srebp2* in the liver or muscle of Atlantic salmon fed diets with low n-3 PUFA (Leaver *et al.*, 2008b,
376 Hixson *et al.*, 2017, Betancor *et al.*, 2014). In contrast, expression of *srebp1* was not increased in fish
377 fed the low n-3 diets in this study although differences in n-3 levels between low and high diets were
378 considerably less than previous studies that replaced large portions of fish oil with vegetable oil (Table
379 1). In mammals, *srebp1* is involved in fatty acid metabolism and de novo lipogenesis, whereas *srebp2*
380 is involved with cholesterol metabolism (Horton *et al.*, 2003). Upregulation of *srebp2* and cholesterol
381 synthesis has been found in lean rather than fat family groups of Atlantic salmon (Morais *et al.*, 2011),
382 which agrees with fish fed the LL-Hn3 diet in this study. In addition, *lxr* is activated by a variety of
383 sterols, including intermediates in the synthesis of cholesterol (Horton *et al.*, 2003). Studies on the
384 transcriptome of Atlantic salmon in response to varying DHA levels have found that sterol synthesis
385 pathways are one of the more notable pathways affected (Glencross *et al.*, 2015). Another recent study
386 on Atlantic salmon found that high levels of n-6 and n-3 PUFA in the diet were positively correlated to
387 cholesterol synthesis and suggested PUFA and cholesterol were required together to maintain cell
388 membrane fluidity (Hixson *et al.*, 2017). Therefore, significant upregulation of *srebp2* and *lxr* in the

389 liver of fish fed the LL-Hn3 diet in this study suggests that cholesterol synthesis pathways were
390 activated, potentially to compensate for lower cholesterol supply due to low lipid in the diet while being
391 stimulated by high dietary n-3 LC-PUFA.

392

393 The upregulation of the genes for *cpt1*, *hoad* and *aco* in the liver of fish fed the LL-Hn3 diet (Fig. 5)
394 indicates that the fatty acid beta-oxidation pathway was activated to generate more energy or DHA
395 (Leaver *et al.*, 2008a). Since this diet has high n-3 and low lipid levels, it is more likely that the
396 upregulation of beta-oxidation genes is a catabolic response to provide fish with more energy. This is
397 also supported by the fact that the DHA level in the whole body carcass was similar between fish fed
398 either of the high n-3 diets (Table 5), while expression of beta-oxidation genes was only increased in
399 the LL-Hn3 diet. Previous studies have found that feeding fish oil high in n-3 PUFA, especially EPA
400 and DHA, resulted in upregulation of beta-oxidation genes, such as *cpt1* and *aco*, in the liver of Atlantic
401 salmon compared with feeding vegetable oil (Stubhaug *et al.*, 2007, Jordal *et al.*, 2005). In the beta-
402 oxidation pathway, *cpt1* activates and transports LC-PUFA into the mitochondrial matrix for
403 catabolism, *hoad* catalyses the third step of beta-oxidation in the mitochondria and *aco* catalyses the
404 rate-limiting step in the peroxisome (Jordal *et al.*, 2005, Leaver *et al.*, 2008b). Therefore, upregulation
405 of beta-oxidation genes in fish fed LL-Hn3 demonstrates an increased demand for energy rather than n-
406 3 LC-PUFA.

407

408 4.4 Conclusion

409

410 The significant interaction between dietary levels of lipid and n-3 LC-PUFA on growth in the present
411 study provides a clear indication that the requirement for n-3 LC-PUFA by Atlantic salmon is relative
412 to the total lipid level, rather than based on the absolute level in the diet. As such, we suggest that n-3
413 LC-PUFA requirements should in fact be expressed based on their proportion of the total fatty acids
414 (i.e. %TFA). Our results agreed with previous studies that found an optimal dietary level of n-3 LC-
415 PUFA was between 10 to 15 g/kg (Ruyter *et al.*, 2000, Bou *et al.*, 2017, Glencross *et al.*, 2014), or more
416 precisely a relative proportion between 5 and 8% TFA (see Fig. 6). However, this requirement is based
417 primarily on key phenomic responses under ideal conditions and further work is needed to examine
418 growth and immunological responses of Atlantic salmon under non-ideal conditions, e.g. hypoxia or
419 higher thermal regimes.

420

421 Additionally, this study also showed that a higher level of lipid in the high n-3 diet, despite being equal
422 in digestible energy, allowed better growth performance. Increased growth of fish fed HL-Hn3 diet may
423 be explained by a higher net energy value from that diet compared to the lower lipid (high protein) diets,
424 due to the lower net energy values from protein, despite that digestible energy values were accounted
425 for in the formulation. Levels and retentions of n-3 PUFA, especially EPA and DHA, were increased

426 in the whole-body carcass of fish fed the HL-Hn3 diet and indicated both energy and nutrient
427 dependencies were met. These findings were also supported by various transcriptomic responses in the
428 liver, which showed reduced expression of fatty acid desaturases and elongase in fish fed the high n-3
429 diets. In addition, elevated transcription factors and beta-oxidation in fish fed the LL-Hn3 diet further
430 shows that the n-3 and energy levels in the diet may be insufficient, consistent with an interaction story.
431

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433

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539 **Tables**

540

541 Table 1. Diet formulation and proximate composition.

	HL-Ln3	LL-Ln3	HL-Hn3	LL-Hn3
<i>Formulation (g kg⁻¹)</i>				
Fishmeal ¹	200	200	200	200
Soy protein concentrate ²	180	64	180	64
Soy protein isolate	115	300	115	300
Wheat meal	145	100	145	100
Wheat gluten	92	113	92	113
Fish oil ³	11	11	38	38
Linseed oil	2	2	8	8
Olive oil	194	144	161	111
L-Histidine	8	8	8	8
DL-Methionine	2	3	2	3
L-Lysine	2	5	2	5
L-Taurine	4	5	4	5
Dicalcium phosphate	20	20	20	20
Vitamin & Mineral Premix ⁴	10	10	10	10
Yttrium oxide	2	2	2	2
Antioxidant (Paramega TM) ⁵	1.5	1.5	1.5	1.5
Soy Lecithin	10	10	10	10
Astaxanthin (Carophyll Pink TM) ⁶	0.5	0.5	0.5	0.5
Choline chloride	1	1	1	1
<i>Proximate composition as measured (g kg⁻¹ dry matter)</i>				
Dry matter	938	947	941	950
Protein	475	590	490	591
Digestible Protein	442	542	458	548
Lipid	241	192	222	187
Ash	85	83	85	83
Carbohydrate ⁷	199	136	203	139
Gross Energy (MJ kg ⁻¹)	24.2	23.6	23.7	23.1
Digestible Energy (MJ kg ⁻¹)	21.5	20.7	21.0	20.3
Calcium (Ca)	18	17	18	18
Phosphorus (P)	13	13	13	14

542 HL; high lipid, Hn3; high n-3 LC-PUFA, LL; low lipid, Ln3; low n-3 LC-PUFA.

543 ¹Norvik LT70 (704 g kg⁻¹ protein and 63 g kg⁻¹ lipid; Sopropêche, France)544 ²Soycomil (624 g kg⁻¹ protein and 4 g kg⁻¹ lipid; ADM Animal Nutrition, Decatur, IL, USA)545 ³Savinor (10.5% EPA and 15.7% DHA; Savinor UTS, Covelas TRF, Portugal)546 ⁴Neovia (formerly Invivo); Vannes, France.547 ⁵Paramega (blend of natural mixed-tocopherols); Kemin, Herentals, Belgium.548 ⁶CarophyllPink (10% astaxanthin); DSM, Village-Neuf, France.549 ⁷Carbohydrate, calculated by difference (i.e. CHO = 1000 - protein - lipid - ash)

550

551 Table 2. Diet fatty acid composition (% of total fatty acids) .

Fatty acids ¹	HL-Ln3	LL-Ln3	HL-Hn3	LL-Hn3
14:0	0.5	0.6	1.4	1.7
16:0	12.4	13.1	13.0	14.4
18:0	2.9	2.9	3.3	3.2
20:0	0.4	0.3	0.3	0.3
Total saturates	16.5	17.2	18.3	20.0
16:1n-9	0.2	0.2	0.2	0.2
16:1n-7	1.5	1.6	2.2	2.7
18:1n-9	61.8	58.7	56.2	48.4
18:1n-7	3.8	3.6	3.0	2.5
20:1n-9	0.6	0.7	0.7	0.9
22:1n-11	0.4	0.5	0.5	0.7
24:1n-9	0.2	0.2	0.2	0.2
Total monoenes	68.9	66.0	63.6	56.3
18:2n-6	10.5	12.0	9.2	11.5
20:4n-6	0.1	0.1	0.2	0.2
Total n-6 PUFA	10.6	12.2	9.6	11.9
18:3n-3	1.5	1.7	2.8	3.5
18:4n-3	0.2	0.2	0.5	0.6
20:5n-3	1.0	1.2	2.7	3.5
22:5n-3	0.1	0.1	0.3	0.4
22:6n-3	0.9	1.1	1.8	2.5
Total n-3 PUFA	3.7	4.3	8.3	10.8
Total PUFA	14.6	16.8	18.2	23.7
Total LC-PUFA	2.1	2.6	5.3	7.0
n-6/n-3	2.9	2.8	1.2	1.1

553 HL; high lipid, Hn3; high n-3 LC-PUFA, LL; low lipid, Ln3; low n-3 LC-PUFA.

554 ¹Fatty acids <0.2% not reported.

555

Table 3. Information on the qPCR primer pairs for reference and target genes.

Function	Gene	Full name	Primers	Length	Accession Number
Reference	<i>cf12</i>	Cofilin-2	AGCCTATGACCAACCCACTG TGTTACAGCTCGTTTACCG	224	TC63899 ^b
	<i>hprt</i>	Hypoxanthine phosphoribosyl-transferase 1	GATGATGAGCAGGGATATGAC GCAGAGAGCCACGATATGG	165	XM_014212855.1 ^a
	<i>rpl2</i>	Ribosomal protein L2	TAACGCCTGCCTCTTCACGTTGA ATGAGGGACCTTGTAGCCAGCAA	112	XM_014137227.1 ^a
	<i>rps5</i>	Ribosomal protein S5	AACTCCATGATGATGCACGG GGTCTTGATGTTTCTGAAAGCA	284	XM_014142016.1 ^a
Fatty acid synthesis	<i>fads2d5</i>	Delta-5 fatty acyl desaturase	GCCACTGGTTTGTATGGGTG TTGAGGTGTCCACTGAACCA	148	NM_001123542.2 ^a
	<i>fads2d6</i>	Delta-6 fatty acyl desaturase	TCCTCTGGTGCGTACTTTGT AAATCCCGTCCAGAGTCAGG	163	NM_001123575.2 ^a
	<i>elovl2</i>	Fatty acyl elongase 2	GGTGCTGTGGTGGTACTACT ACTGTTAAGAGTCGGCCCAA	190	NM_001136553.1 ^a
	<i>elovl5a</i>	Fatty acyl elongase 5 isoform a	TGTTGCTTCATTGAATGGCCA TCCCATCTCTCCTAGCGACA	150	GU238431.1 ^a
	<i>elovl5b</i>	Fatty acyl elongase 5 isoform b	CTGTGCAGTCATTTGGCCAT GGTGTCACCCCATTTGCATG	192	NM_001136552.1 ^a
	<i>fas</i>	Fatty acid synthase	ACCGCCAAGCTCAGTGTGC CAGGCCCAAAGGAGTAGC	212	CK876943 ^a
Transcription factor	<i>lxr</i>	Liver X receptor	GCCGCCGCTATCTGAAATCTG CAATCCGGCAACCAATCTGTAGG	210	FJ470290 ^a
	<i>srebp1</i>	Sterol regulatory element binding protein 1	GCCATGCGCAGGTTGTTTCTTCA TCTGGCCAGGACGCATCTCACACT	151	TC148424 ^a
	<i>srebp2</i>	Sterol regulatory element binding protein 2	GACAGGCACAACACAAGGTG CAGCAGGGGTAAGGGTAGGT	147	DY733476 ^a
Fatty acid β -oxidation	<i>aco</i>	Acyl-CoA oxidase	AAAGCCTTCACCACATGGAC TAGGACACGATGCCACTCAG	230	TC49531 ^a
	<i>cpt1a</i>	Carnitine palmitoyl transferase 1a	TCGATTTTCAAGGGTCTTCG CACAACGATCAGCAAACCTGG	166	AF327058 ^a
	<i>cpt1b</i>	Carnitine palmitoyl transferase 1b	CCCTAAGCAAAAAGGGTCTTCA CATGATGTCACTCCCGACAG	149	AJ606076 ^a
	<i>hoad</i>	3-hydroxyacylCoA-dehydrogenase	GGACAAAGTGGCACCAGCAC GGGACGGGGTTGAAGAAGTG	145	tcad0001a.i.15 3.1.om ^c

^a GenBank database (<http://www.ncbi.nlm.nih.gov>). ^b Atlantic salmon Gene Index (<http://compbio.dfc.harvard.edu/tgi>). ^c Sigenae database (<http://www.sigenae.org>)

Table 4. Growth performance, feed efficiency and body indices of Atlantic salmon post-smolts.

	Diets				Pooled SE	Main Effect Means					P-values ¹		
	HL-Ln3	LL-Ln3	HL-Hn3	LL-Hn3		LL	HL	Ln3	Hn3	Pooled SE	Lipid	n3	Lipid x n3
Initial weight (g fish ⁻¹)*	187.7	186.4	191.7	185.0	2.3	185.7	189.3	187.0	187.7	1.7	0.160	0.676	0.293
Final weight (g fish ⁻¹)	505.3a	525.3ab	552.0b	524.8ab	7.1	525.1	524.0	515.3	535.7	8.8	0.923	0.069	0.043
Weight gain (g fish ⁻¹)	317.6a	338.9ab	360.3b	339.8ab	8.2	339.3	334.7	328.3	348.0	8.7	0.795	0.096	0.079
Gain rate (g fish ⁻¹ day ⁻¹)	2.74a	2.92ab	3.11b	2.93ab	0.07	2.93	2.89	2.83	3.00	0.07	0.809	0.099	0.086
Feed intake (g fish ⁻¹)	280.6	297.0	317.6	283.5	15.2	290.2	295.4	288.8	297.1	13.0	0.757	0.650	0.226
Protein intake (g fish ⁻¹)	125.1	166.0	146.5	159.2	7.5	162.6	133.6	145.5	154.1	7.5	0.017	0.549	0.168
Lipid intake (g fish ⁻¹)	60.6	54.3	64.0	50.0	3.0	52.2	63.0	57.5	56.7	3.2	0.026	0.937	0.221
FCR (feed:gain)	0.88	0.88	0.88	0.83	0.03	0.86	0.88	0.88	0.85	0.03	0.639	0.618	0.655
Survival (%)	98.6	95.8	97.9	98.6	1.8	97.3	98.4	97.3	98.4	1.2	0.526	0.526	0.386
HSI ²	1.13	1.06	1.13	1.02	0.04	1.04	1.13	1.10	1.06	0.03	0.079	0.649	0.612
VSI ²	8.45bc	6.83a	8.67c	6.98ab	0.36	6.90	8.54	7.64	7.65	0.38	0.010	0.715	0.944

*Initial weight is the weight at the end of the three-week acclimation period. FCR; feed conversion ratio, HSI; hepatosomatic index; HL; high lipid, Hn3; high n-3 LC-PUFA, LL; low lipid, Ln3; low n-3 LC-PUFA; VSI; viscerosomatic index.

¹P-values from linear model with lipids and n-3 LC-PUFA (n3) as fixed effects and a lipid x n3 interaction. P-values in bold are <0.10.

²n=12, 4 fish were sampled in each triplicate tank.

Table 5. Whole body proximate (g kg⁻¹ wet matter basis) and fatty acid (% of total fatty acids) composition of Atlantic salmon (n=3, pooled per tank)

	Diets					Pooled SE	Main Effect Means				Pooled SE	P-values		
	Initial	HL-Ln3	LL-Ln3	HL-Hn3	LL-Hn3		LL	HL	Ln3	Hn3		Lipid	n3	Lipid x n3
Dry matter	304	315	310	315	313	3.7	312	315	313	314	2.5	0.408	0.707	0.779
Ash	18	16ab	18b	15a	18b	0.6	18	16	17	17	0.5	0.021	0.430	0.537
Protein	167	185a	195b	182a	195b	2.2	195	184	190	189	2.3	0.002	0.634	0.481
Lipid	101	92ab	83a	102b	89ab	4.1	86	97	88	95	3.5	0.040	0.124	0.655
Energy (MJ kg ⁻¹)	8.1	8.6	8.3	8.7	8.4	1.5	8.3	8.6	8.4	8.6	0.1	0.094	0.472	0.700
<i>Fatty acids²</i>														
14:0	3.7	1.4a	1.6b	1.9c	2.1d	0.02	1.8	1.6	1.5	2.0	0.1	< 0.001	< 0.001	0.376
16:0	13.5	12.4a	13.1b	12.8ab	13.8c	0.15	13.4	12.6	12.8	13.3	0.2	0.001	0.008	0.278
18:0	3.1	3.4a	3.6ab	3.4a	3.7b	0.05	3.6	3.4	3.5	3.6	0.0	0.007	0.274	0.532
20:0	0.2	0.3a	0.2bc	0.2ab	0.2c	0.01	0.2	0.2	0.2	0.2	0.0	0.002	0.077	0.696
Total saturates	21.2	17.7a	18.7b	18.6b	20.1c	0.18	19.4	18.2	18.2	19.3	0.3	< 0.001	< 0.001	0.220
16:1n-9	0.3	0.5b	0.5b	0.4a	0.4a	0.02	0.5	0.5	0.5	0.4	0.0	0.662	< 0.001	0.773
16:1n-7	4.4	2.1a	2.3b	2.6c	2.9d	0.03	2.6	2.4	2.2	2.8	0.1	< 0.001	< 0.001	0.078
18:1n-9	29.9	53.1c	49.4b	49.1b	43.6a	0.18	46.5	51.1	51.2	46.4	1.1	< 0.001	< 0.001	0.002
18:1n-7	3.8	3.0	3.0	2.9	2.8	0.10	2.9	2.9	3.0	2.9	0.1	0.844	0.244	0.936
20:1n-9	4.2	3.5b	3.4b	3.2a	3.1a	0.05	3.2	3.3	3.4	3.2	0.1	0.103	0.001	0.855
22:1n-11	3.7	0.9a	1.0ab	1.0a	1.1b	0.04	1.1	0.9	1.0	1.0	0.0	0.022	0.173	0.449
22:1n-9	0.6	0.4a	0.4b	0.4a	0.4ab	0.01	0.4	0.4	0.4	0.4	0.0	0.014	0.253	0.803
24:1n-9	1.3	0.3a	0.4ab	0.4bc	0.4c	0.01	0.4	0.4	0.4	0.4	0.0	0.026	0.005	0.641
Total monoenes	48.9	64.5c	61.0b	60.6b	55.6a	0.24	58.3	62.5	62.8	58.1	1.0	< 0.001	< 0.001	0.022
18:2n-6	9.8	7.9a	9.4b	8.2a	9.8c	0.11	9.6	8.1	8.7	9.0	0.2	< 0.001	0.022	0.575
18:3n-6	0.2	0.3b	0.4c	0.2a	0.2a	0.01	0.3	0.2	0.3	0.2	0.0	0.008	< 0.001	0.493
20:2n-6	0.7	0.8a	1.0c	0.8b	1.1d	0.02	1.0	0.8	0.9	1.0	0.0	< 0.001	0.001	0.482
20:3n-6	0.2	0.7c	0.8d	0.4a	0.5b	0.02	0.7	0.6	0.8	0.5	0.0	0.001	< 0.001	0.629
20:4n-6	0.4	0.4b	0.5c	0.3a	0.3ab	0.01	0.4	0.3	0.4	0.3	0.0	0.006	0.001	0.406
Total n-6 PUFA	11.5	10.2a	12.2b	10.0a	12.1b	0.11	12.1	10.1	11.2	11.0	0.3	< 0.001	0.370	0.708
18:3n-3	3.0	1.2a	1.4b	2.0c	2.4d	0.03	1.9	1.6	1.3	2.2	0.1	< 0.001	< 0.001	0.004
18:4n-3	1.1	0.4a	0.4b	0.5c	0.5d	0.01	0.5	0.4	0.4	0.5	0.0	< 0.001	< 0.001	0.123
20:4n-3	0.8	0.3a	0.3b	0.4c	0.5d	0.01	0.4	0.4	0.3	0.5	0.0	< 0.001	< 0.001	0.008
20:5n-3	3.7	1.3a	1.3a	1.9b	1.9b	0.03	1.6	1.6	1.3	1.9	0.1	0.441	< 0.001	0.406
22:5n-3	1.3	0.5a	0.6b	0.8c	0.9d	0.01	0.7	0.6	0.5	0.8	0.0	0.001	< 0.001	0.157
22:6n-3	6.9	3.4a	3.7a	4.5b	5.0b	0.17	4.3	4.0	3.6	4.7	0.2	0.078	< 0.001	0.416
Total n-3 PUFA	17.4	7.3a	7.8a	10.4c	11.7d	0.20	9.7	8.8	7.5	11.0	0.5	0.003	< 0.001	0.098
Total PUFA	29.9	17.8a	20.3b	20.8b	24.4c	0.29	22.3	19.3	19.0	22.6	0.8	< 0.001	< 0.001	0.151
Total LC-PUFA	14.8	7.6a	8.4b	9.5c	10.8d	0.20	9.6	8.6	8.0	10.2	0.4	0.001	< 0.001	0.258

HL; high lipid, Hn3; high n-3 LC-PUFA, LL; low lipid, Ln3; low n-3 LC-PUFA, SE; pooled standard error of the mean.

¹P-values from linear model with lipids and n-3 LC-PUFA (n3) as fixed effects and a lipid x n3 interaction. P-values in bold are <0.10.

²Fatty acids ≤ 0.2 not detailed.

Table 6. Retention (%) of macronutrients and fatty acids in the whole-body carcass of Atlantic salmon (n=3, pooled per tank).

	Diets				Pooled SE	Main Effect Means				Pooled SE	P-values ¹		
	HL-Ln3	LL-Ln3	HL-Hn3	LL-Hn3		LL	HL	Ln3	Hn3		Lipid	n3	Lipid x n3
Protein	50.2b	43.0a	44.3ab	44.8ab	2.2	43.9	47.2	46.6	44.5	1.7	0.230	0.474	0.170
Lipid	46.4	48.2	53.4	57.4	5.4	52.8	49.9	47.3	55.4	4.0	0.650	0.228	0.865
Energy (MJ kg ⁻¹)	45.1	42.8	43.1	47.1	3.1	45.0	44.1	44.0	45.1	2.0	0.781	0.719	0.323
<i>Fatty acids</i> ²													
14:0	38.5	39.1	47.1	50.0	8.2	44.6	42.8	38.8	48.6	5.6	0.847	0.296	0.904
16:0	52.9	51.1	56.3	64.0	6.4	57.6	54.6	52.0	60.2	4.5	0.666	0.258	0.501
18:0	66.4	68.3	63.8	81.4	6.8	74.8	65.1	67.3	72.6	5.2	0.220	0.494	0.312
20:0	30.2	25.6	33.6	32.7	4.2	29.2	31.9	27.9	33.2	2.9	0.550	0.265	0.686
Total saturated	53.8	52.7	56.1	64.5	6.5	58.6	54.9	53.2	60.3	4.6	0.602	0.328	0.498
16:1n-9	184.4	178.1	131.8	140.8	23.2	159.4	158.1	181.2	136.3	16.4	0.954	0.091	0.751
16:1n-7	39.6	41.2	49.2	55.1	7.0	48.2	44.4	40.4	52.1	5.0	0.621	0.150	0.778
18:1n-9	56.9	52.2	58.7	68.2	5.7	60.2	57.8	54.6	63.4	4.3	0.697	0.174	0.265
18:1n-7	40.3a	38.1a	49.3ab	64.8b	6.7	51.4	44.8	39.2	57.1	5.6	0.372	0.034	0.244
20:1n-9	268.7	220.7	223.4	197.1	24.0	208.9	246.0	244.7	210.2	18.1	0.202	0.233	0.695
22:1n-11	-41.7a	-31.0ab	-15.6bc	-6.0c	6.5	-18.5	-28.7	-36.4	-10.8	6.0	0.186	0.006	0.939
24:1n-9	-41.0a	-32.5ab	6.0ab	12.0b	12.6	-10.3	-17.5	-36.8	9.0	11.1	0.641	0.015	0.936
Total monounsaturated	57.4	52.7	59.6	69.0	6.1	60.8	58.5	55.1	64.3	4.5	0.728	0.184	0.300
18:2n-6	37.3a	39.6a	47.4ab	56.6c	4.5	48.1	42.3	38.5	52.0	3.8	0.252	0.020	0.483
20:4n-6	258.8b	289.8b	79.8a	92.9a	27.5	191.4	169.3	274.3	86.4	32.8	0.529	0.001	0.797
Total n-6 PUFA	49.4a	53.0ab	56.8ab	68.7b	5.7	60.8	53.1	51.2	62.7	4.4	0.222	0.084	0.501
18:3n-3	14.9a	17.0a	33.4b	39.7b	2.8	28.3	24.2	16.0	36.5	3.6	0.245	<0.001	0.549
18:4n-3	7.8ab	7.0a	24.7bc	25.9c	5.1	16.4	16.2	7.4	25.3	4.3	0.970	0.862	0.590
20:4n-3	48.9a	54.8a	141.1b	155.1b	15.3	105.0	95.0	51.9	148.1	17.6	0.590	0.001	0.824
20:5n-3	10.8ab	3.2a	24.6b	20.9b	4.1	12.1	17.7	7.0	22.8	3.8	0.242	0.008	0.675
22:5n-3	72.8a	81.0a	119.2b	125.4b	14.8	103.2	96.0	76.9	122.3	12.6	0.678	0.026	0.955
22:6n-3	113.8	99.1	109.4	109.2	11.3	104.2	111.6	106.4	109.3	7.8	0.550	0.813	0.559
Total n-3 PUFA	39.4ab	35.3a	52.7ab	55.3b	5.5	45.3	46.1	37.3	54.0	4.5	0.895	0.02	0.586
Total PUFA	46.0	47.4	54.6	60.8	5.7	54.1	50.3	46.7	57.7	4.1	0.537	0.098	0.697
Total LC-PUFA	111.5	103.3	81.0	84.4	11.1	93.8	96.3	107.4	82.7	8.3	0.838	0.065	0.627
Total Fatty Acids	55.2	51.8	58.1	66.1	6.1	59.0	56.6	53.5	62.1	4.4	0.720	0.210	0.393

HL; high lipid, Hn3; high n-3 LC-PUFA, LL; low lipid, Ln3; low n-3 LC-PUFA, SE; pooled standard error of the mean.

¹P-values from linear model with lipids and n-3 LC-PUFA (n3) as fixed effects and a lipid x n3 interaction. P-values in bold are <0.10.

²Fatty acids <0.2% in the diet not detailed.

Table 7. Apparent digestibility (%) of macronutrients and fatty acids for Atlantic salmon (n=3, pooled per tank).

	Diets				Pooled SE	Main Effect Means				Pooled SE	P-value ¹		
	HL-Ln3	LL-Ln3	HL-Hn3	LL-Hn3		LL	HL	Ln3	Hn3		Lipid	n3	Lipid x n3
Protein	93.2c	92.0a	93.5d	92.8b	0.1	92.4	93.4	92.6	93.2	0.2	<0.001	<0.001	0.032
Lipid	98.3b	97.3ab	95.6a	96.2a	0.6	96.8	97.2	97.8	96.1	0.5	0.517	0.025	0.424
Energy (MJ kg ⁻¹)	89.0b	87.7a	88.9b	88.0ab	0.3	87.9	88.9	88.3	88.5	0.2	0.010	0.718	0.492
<i>Fatty acids</i> ²													
14:0	95.0	94.8	86.7	92.0	1.7	93.1	90.9	94.9	89.4	1.8	0.393	0.104	0.418
16:0	97.8	97.1	92.8	94.4	1.1	95.5	95.3	97.5	93.6	1.0	0.725	0.043	0.497
18:0	97.1	96.1	92.0	92.9	1.3	94.2	94.6	96.7	92.5	1.2	0.988	0.045	0.616
20:0	97.3	95.7	93.0	92.8	1.4	94.0	95.2	96.7	92.9	1.1	0.613	0.051	0.684
Total saturates	97.5	96.7	92.1	93.8	1.2	95.0	94.8	97.2	92.9	1.1	0.732	0.045	0.500
16:1n-9	99.7	100.0	100.0	99.8	0.1	99.9	99.8	99.8	99.9	0.1	0.889	0.689	0.264
16:1n-7	99.6	99.3	99.1	99.3	0.1	99.3	99.4	99.5	99.2	0.1	0.823	0.153	0.202
18:1n-9	99.7	99.3	99.6	99.5	0.1	99.4	99.7	99.5	99.6	0.1	0.043	0.749	0.365
18:1n-7	99.6b	99.3ab	99.2ab	98.9a	0.1	99.1	99.4	99.5	99.0	0.1	0.137	0.042	0.898
20:1n-9	98.8	97.9	98.3	98.3	0.3	98.1	98.6	98.5	98.3	0.2	0.212	0.648	0.219
22:1n-11	98.9	98.0	98.3	98.4	0.3	98.3	98.6	98.5	98.4	0.2	0.481	0.718	0.209
24:1n-9	96.0	92.7	90.0	92.7	1.5	96.7	96.1	96.5	96.3	1.0	0.986	0.165	0.205
Total monoenes	99.6	99.3	99.5	99.4	0.1	99.3	99.6	99.5	99.4	0.1	0.063	0.797	0.386
18:2n-6	99.4	99.2	99.2	99.1	0.2	99.1	99.3	99.3	99.1	0.1	0.522	0.556	0.774
20:4n-6	97.1a	96.5a	99.6b	98.9ab	0.7	97.9	98.3	96.9	99.2	0.6	0.457	0.017	0.928
Total n-6 PUFA	99.3	99.1	99.1	99.0	0.2	99.1	99.2	99.2	99.1	0.1	0.565	0.569	0.740
18:3n-3	99.6	99.4	99.7	99.7	0.1	99.6	99.6	99.5	99.7	0.1	0.416	0.195	0.435
18:4n-3	99.2	99.1	99.4	99.6	0.2	99.4	99.3	99.1	99.5	0.1	0.726	0.072	0.485
20:5n-3	99.7	99.6	99.8	99.8	0.1	99.7	99.7	99.6	99.8	0.1	0.707	0.155	0.774
22:5n-3	99.3	98.6	99.8	99.3	0.3	99.0	99.6	99.0	99.6	0.3	0.209	0.180	0.845
22:6n-3	98.5	98.1	99.2	99.0	0.4	98.6	98.8	98.3	99.1	0.3	0.529	0.129	0.823
Total n-3 PUFA	99.3	99.1	99.6	99.5	0.2	99.3	99.4	99.2	99.5	0.1	0.527	0.134	0.701
Total PUFA	99.3	99.1	99.3	99.3	0.2	99.2	99.3	99.2	99.3	0.1	0.552	0.646	0.676
Total LC-PUFA	98.8	98.5	99.5	99.3	0.3	99.0	99.2	98.7	99.4	0.2	0.607	0.100	0.807
Total	99.2	98.8	98.1	98.2	0.3	98.5	98.7	99.1	98.2	0.3	0.767	0.070	0.532

HL; high lipid, Hn3; high n-3 LC-PUFA, LL; low lipid, Ln3; low n-3 LC-PUFA, SE; pooled standard error of the mean.

¹P-values from linear model with lipids and n-3 LC-PUFA (n3) as fixed effects and a lipid x n3 interaction. P-values in bold are <0.10.

²Fatty acids <0.2% in the diet not detailed.

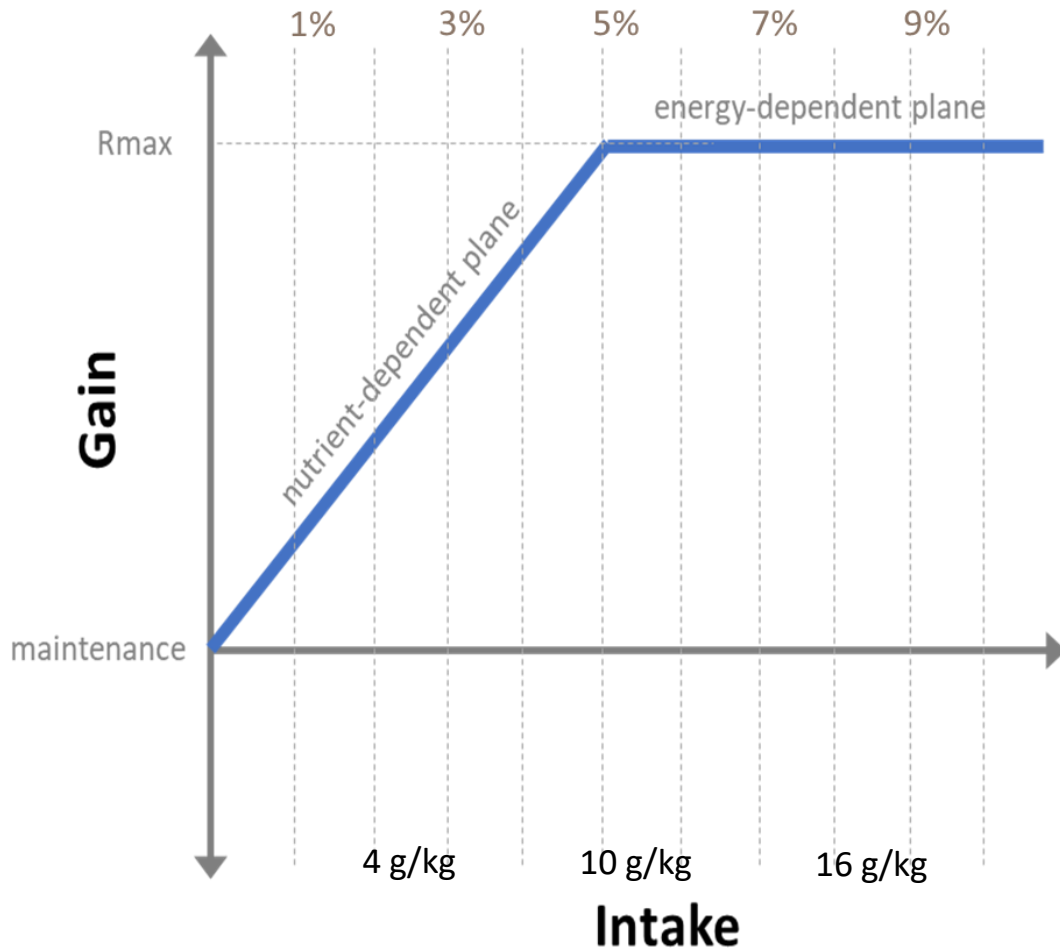


Figure1. General nutrient response schematic showing the approximate relative levels at which responses by Atlantic salmon to variable n-3 LC-PUFA supply were observed based on the results from Glencross et al. (2014). In this example performance is projected to decline to a maintenance point consistent with the needs for conditionally-essential nutrients. Across the top of the figure are the relative levels of n-3 LC-PUFA (% of total fatty acids; TFA) in a diet with 200 g/kg of lipid, whereas along the bottom of the figure are the commensurate absolute n-3 LC-PUFA levels (g/kg).

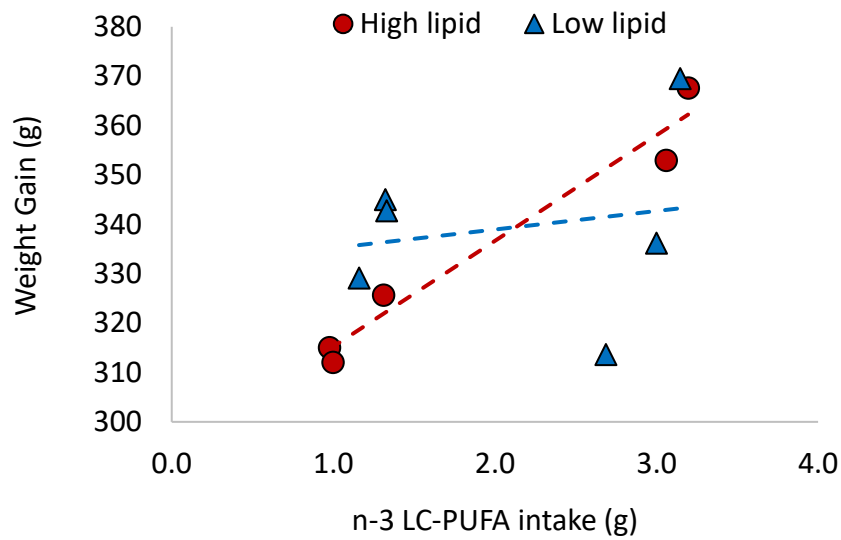


Figure 2. Interaction plot of mean weight gain based on total n-3 LC-PUFA intake of each tank of fish fed either high-lipid (red) or low-lipid (blue) diets. The cross-over of slopes indicate an interaction between dietary lipid and n-3 levels, where weight gain was more increased for fish fed high than low lipid diets at a similar n-3 intake. The higher slope of the high-lipid data shows that these diets are on a nutrient-dependent plane, whereas the low-lipid data are more closely representing responses on an energy-dependent plane (see Fig. 5).

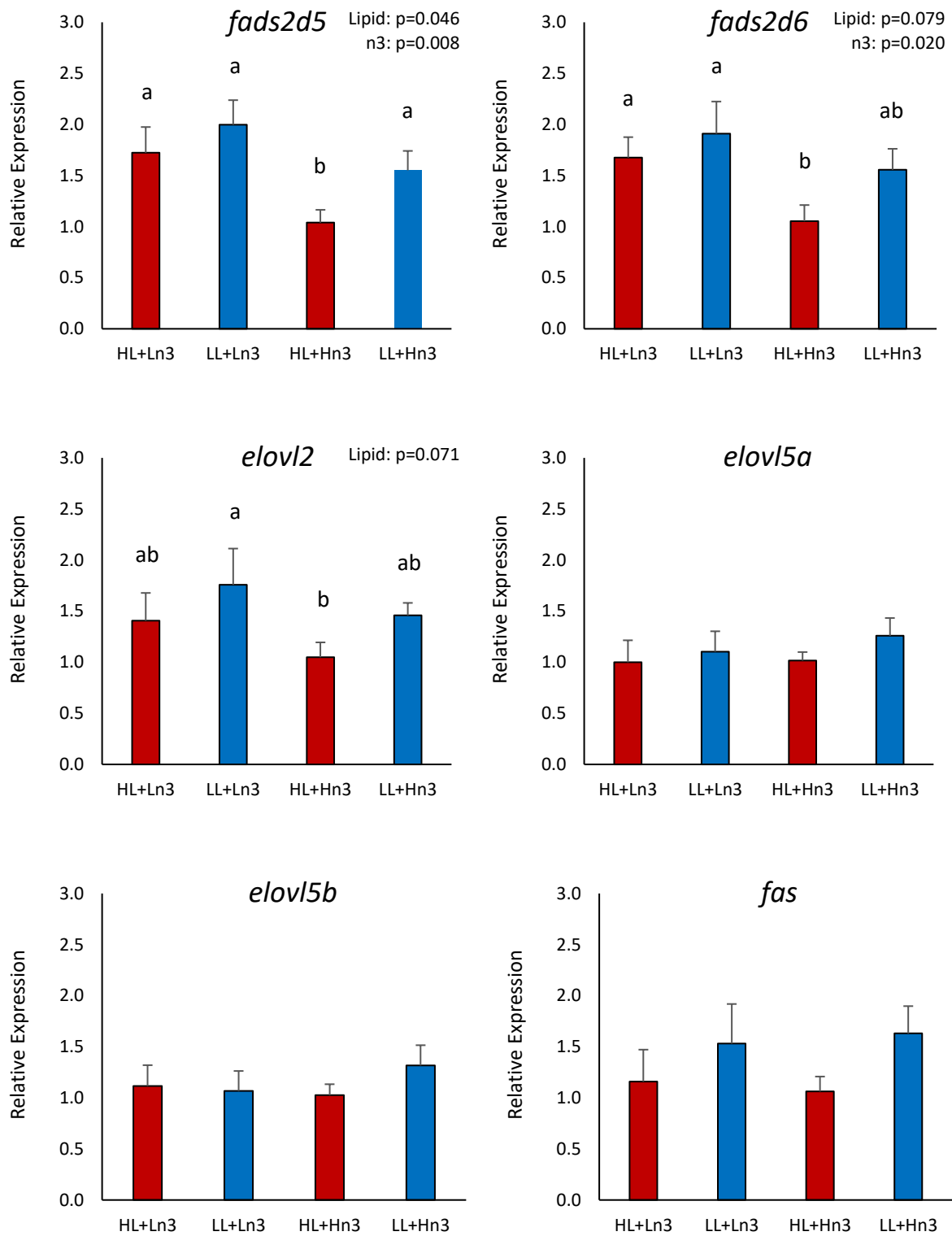


Figure3. Expression of genes (mean \pm SE, n=6/treatment) relative to the geometric mean of two reference genes (*hprt* and *rps5*) involved in fatty acid synthesis in the liver of Atlantic salmon fed low and high levels of lipids and/or n-3 LC-PUFA (i.e. EPA and DHA).

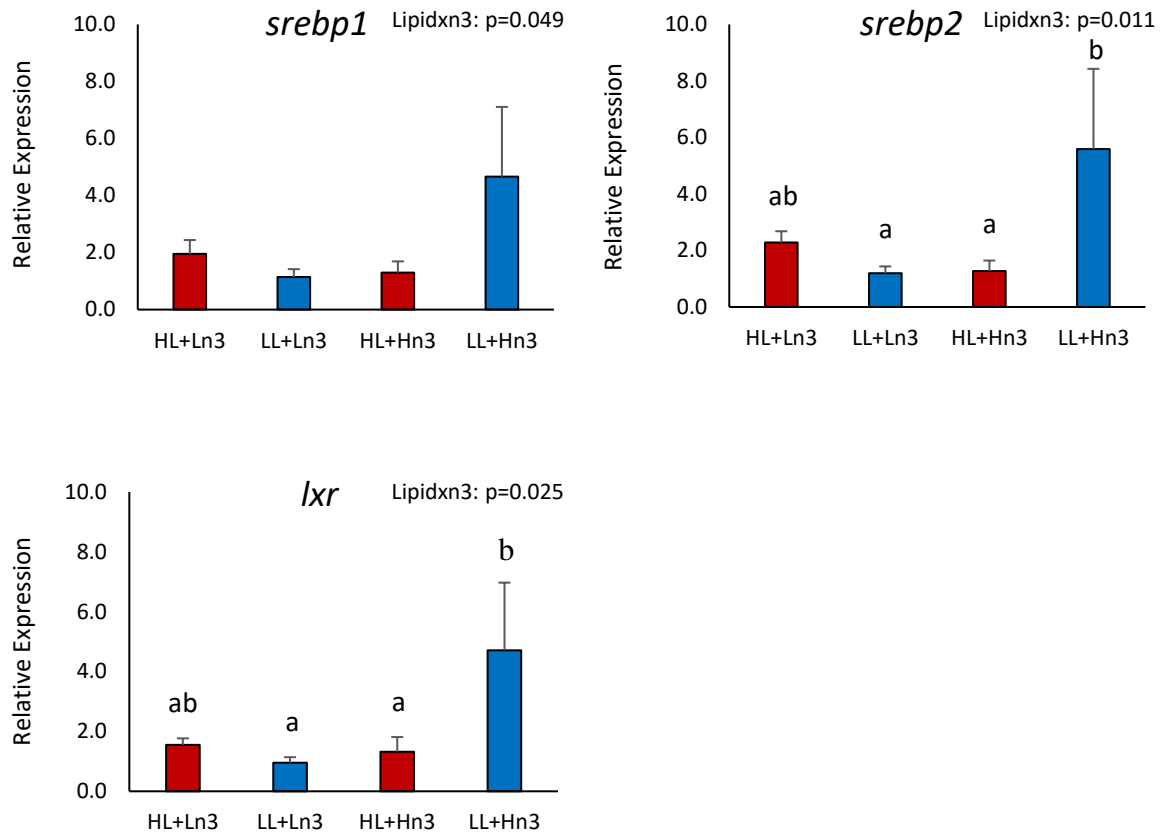


Figure 4. Expression of genes relative to the geometric mean of two reference genes (*hprt* and *rps5*) involved in transcription factors in lipid regulation in the liver of Atlantic salmon fed low and high levels of lipids and/or n-3 LC-PUFA (i.e. EPA and DHA).

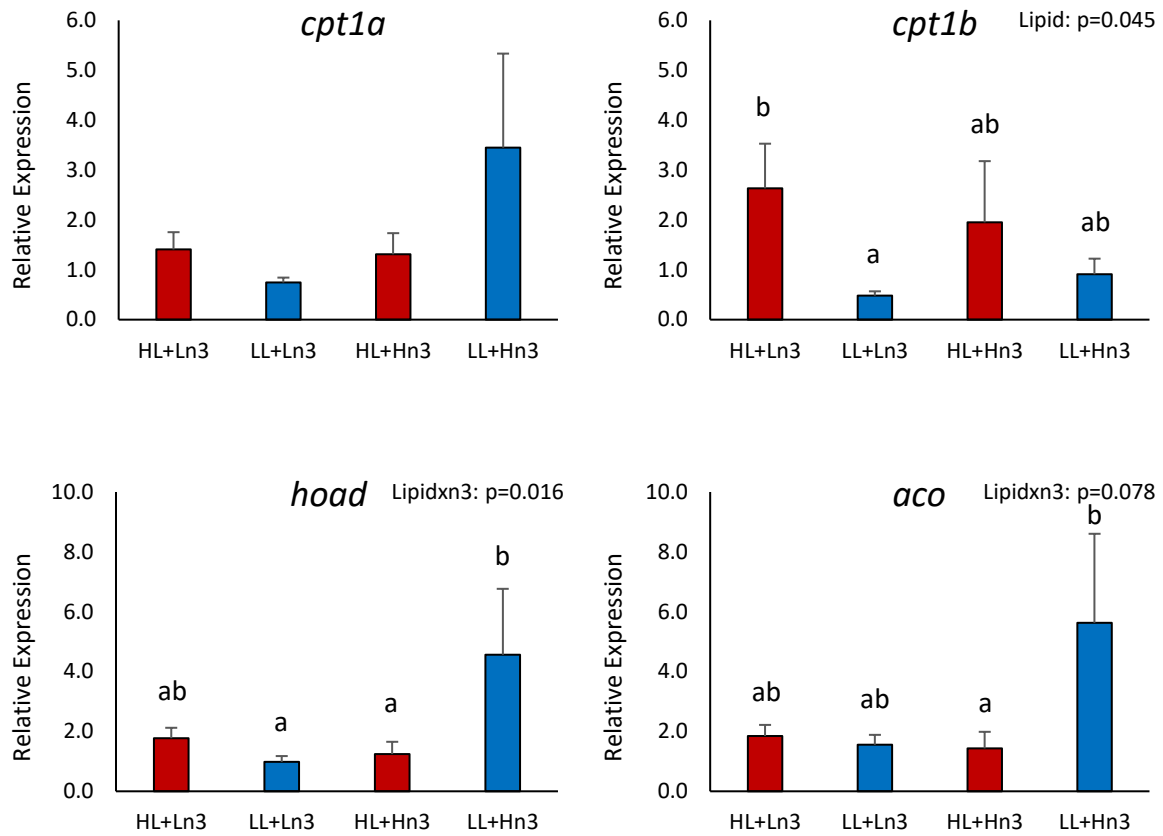


Figure 5. Expression of genes relative to the geometric mean of two reference genes (*hprt* and *rps5*) involved in beta oxidation of fatty acids in the liver of Atlantic salmon fed low and high levels of lipids and/or n-3 LC-PUFA (i.e. EPA and DHA).

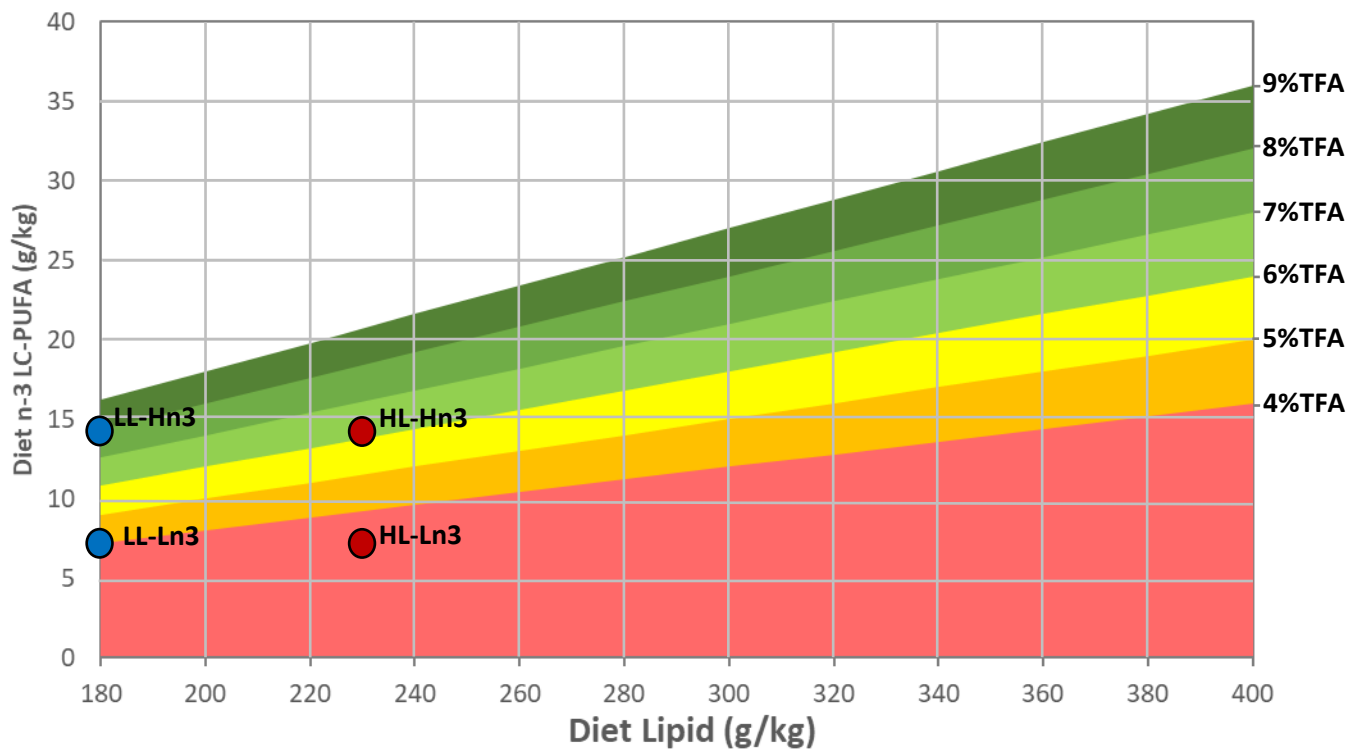


Figure 6. Expression of the relativity of n-3 LC-PUFA requirements by Atlantic salmon overlaid on to variable absolute dietary lipid levels (x-axis), absolute n-3 levels (left y-axis) and n-3 levels relative to lipid level (right y-axis). The four diets (i.e. LL-Ln3, HL-Ln3, LL-Hn3 and HL-Hn3) from this study are overlaid and show the n-3 level in relation to low (blue) and high (red) lipid level. The lower boundary between the orange and red is commensurate with an n-3 level at 4% of total fatty acids (TFA; marginal level), followed by the yellow and orange boundary at 5% TFA (threshold level), light-green and yellow boundary at 6% TFA (optimal level) and so on up to a 9%TFA level. Notable is how the actual (g/kg) level of n-3 in the diet needs to increase as dietary lipid level increases. An optimal level of 10 g/kg of n-3 in a diet with 200 g/kg of lipid being equivalent to an optimal level of 15 g/kg of n-3 in a diet with 300 g/kg of lipid.