Modulation of selenium tissue distribution and selenoproteins expression in Atlantic salmon (*Salmo salar* L.) fed diets with graded levels of plant ingredients

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Abbreviations: BHT, butylated hydroxytoluene; CAT; catalase; COF2, cofilin-2; DHA, docosahexaenoic acid; ELF1α, elongation factor 1 α; FAME, fatty acid methyl esters; FM, fish meal; FO, fish oil; GPX, glutathione peroxidase; LC-PUFA, long-chain polyunsaturated fatty acid; NQC, Norwegian quality cut; NTC, no-template control; PIn, peroxidation index; PP, plant protein; qPCR, quantitative PCR; SGR, specific growth rate; SEM, standard error of the mean; SECP43, tRNA selenocysteine 1 associated protein 1b; SEPK; selenoprotein K; SEPP, selenoprotein P; SOD; superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; VO, vegetable oil.

28 **Abstract**

29 Increased substitution of marine ingredients by terrestrial plant products in aquafeeds has
30 proved to be suitable for Atlantic salmon farming. However, a reduction in omega-3 long-
31 chain polyunsaturated fatty acids is a consequence of this substitution. In contrast, relatively
32 little attention has been paid to the effects of fish meal and oil substitution on levels of
33 micronutrients such as selenium (Se), considering fish are major sources of this mineral for
34 human consumers. To evaluate the effects of dietary marine ingredient substitution on tissue
35 Se distribution and the expression of Se metabolism and antioxidant enzymes genes, Atlantic
36 salmon were fed three feeds based on commercial formulations with increasing levels of
37 plant proteins (PP) and vegetable oil. Lipid content did not vary at any sampling point in
38 flesh, whereas was higher in fish fed higher PP in liver of 1 kg fish. Fatty acid content
39 reflected dietary input and was related to oxidation levels. Liver had the highest Se levels,
40 followed by head kidney whereas the lowest contents were found in brain and gill. The Se
41 concentration of flesh decreased considerably with high levels of substitution, reducing the
42 added value of fish consumption. Only brain showed significant differences in glutathione
43 peroxidase, tRNA selenocysteine associated protein 1 and superoxide dismutase expression,
44 whereas no significant regulation of Se related genes was found in liver. Although Se levels
45 in the diets satisfied essential requirements of salmon, high PP levels led to a reduction in the
46 supply of this essential micronutrient.

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Introduction

Fish are recognised as a healthy food as they are an excellent source of high quality protein and lipids in the human diet. Among lipids, dietary omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA) have a range of beneficial effects in several pathological conditions, including cardiovascular and neurological diseases, and some cancers[1-7]. The beneficial effects of fish have been generally attributed largely to the presence of n-3 LC-PUFA, however, fish provide significant levels of a number of other potentially protective components such as selenium (Se), a trace element essential for human health. Fish, especially marine species, are a major source of highly bioavailable dietary Se with average contents around 0.27 µg/g[8]. As a constituent of selenoproteins, Se has structural and enzymatic roles; for instance it is an essential component of the antioxidant enzyme glutathione peroxidase (GPX) and participates in the production of thyroid hormones. It is also necessary for the proper functioning of the immune system and has been shown to give protection from some cancers[9]. Recommendations for adequate intakes of Se are provided by the European Food Safety Authority[10] and indicate that an intake of Se of 70 µg/day is required for adults, which is increased by further 10 to 15 µg/day for pregnant and lactating women.

Feeds for carnivorous species such as salmonids have traditionally included high proportions of fish meal (FM) and fish oil (FO). However, the finite and limited supply of these marine ingredients as well as the continued increase in aquaculture production has resulted in a research for alternative raw materials for aquafeeds[11-14]. In this context, considerable research has focused on sustainable alternatives such as terrestrial plant sources[15-16], which have been demonstrated to be suitable for Atlantic salmon (*Salmo salar* L.) growth[11,17-20]. A considerable body of literature exists regarding the effect of FM/FO substitution on the n-3 LC-PUFA content of fish, although limited attention has so far been focused on the effect of this substitution on other beneficial nutrients such as Se. Se contents in plant proteins (PP) are highly variable depending on the soil composition where the crops are grown, which varies geographically[21]. For instance, in some areas of China, Finland and New Zealand, the levels of Se in the soil are very low (< 0.05 ppm) while higher concentrations (> 5 ppm) of this element are found in Canada, Ireland and some regions of the western USA[22]. In addition, Se incorporation into plants depends on other factors such as soil pH, rainfall, land contour and microbial activity[23]. Thus, the inclusion of dietary PP as substitutes for FM, which are naturally rich in Se, may lead to a reduction in the content of

85 this nutrient in the flesh of the farmed fish that, in turn, will reduce its nutritional value to
86 human consumers. In addition, Se is also an essential nutrient for fish as it is for human. Due
87 to the high contents in fish of n-3 LC-PUFA, which are highly susceptible to oxidation due to
88 their high degree of unsaturation, Se exerts a pivotal role as an antioxidant nutrient and
89 deficiency results in altered oxidative status[24-26].

90 Teleost fish possess the highest number of selenoproteins of any organisms, and a
91 total of thirty eight have been identified in zebrafish (*Danio rerio*)[27]. Among them,
92 selenoprotein (SEP) K (SEPK) plays a role in the immune system[28] while SEPN affects
93 calcium homeostasis[29] and SEPP has an antioxidant protection function[30]. Moreover, tRNA
94 selenocysteine - associated protein 1 (SECp43) plays a major role in selenoprotein synthesis
95 by inserting selenocysteine into selenoproteins[31]. The most studied selenoprotein family is
96 that of the antioxidant glutathione peroxidase (GPX) which has four members GPX1, GPX 2,
97 GPX 3 and GPX4[32]. GPX4 is a phospholipid hydroperoxidase with a unique catalytic
98 activity protecting cell membranes as it acts on hydroperoxides derived from oxidation of
99 phospholipids and cholesterol. This suggests that GPX4 may be an important antioxidant
100 enzyme in marine fish that accumulate high tissue levels of LC-PUFA that are highly
101 susceptible to oxidative damage[33]. In addition, other antioxidant enzymes found in most fish
102 species studied to date[34] include catalase (CAT) and superoxide dismutase (SOD), which
103 inhibit the lipid peroxidation catalytic cycle by preventing oxidation reactions, intercepting
104 and inactivating the reactive intermediates[35].

105 The overarching objective of the present study was to determine how sustainable
106 aquafeeds, with increased levels of plant ingredients, affect Se contents and metabolism in
107 order to better understand the potential effects of this substitution on fish as well as the
108 human consumer. To achieve this, a long-term feeding trial was performed on Atlantic
109 salmon from 100 g to 3000 g employing three increasing levels of PP and vegetable oil (VO)
110 substitution. Levels of Se in liver, muscle, kidney, anterior intestine, gill and brain were
111 determined. In addition, levels of thiobarbituric reactive substances (TBARS), indicators of
112 lipid peroxidation, were determined in muscle and liver. Analyses of the expression of genes
113 of several SEP and antioxidant enzymes in liver and brain provided new insights on the
114 potential effects of altered Se contents on SEP functions and synthesis, as well as their role in
115 the maintenance of oxidative homeostasis.

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117 **Materials and methods**

118 *Experimental diets and fish*

119 The trial was carried out in Atlantic salmon at Marine Harvest Scotland Feed Trial Unit
120 facilities in Ardnish (Scotland, UK). Three different isolipidic (increasing from
121 approximately 22 % at 100 g to 31% at 3000 g) and isoproteic (decreasing from
122 approximately 42 % at 100 g to 36% at 3000 g) feeds were formulated by including equal
123 amounts of FM/PP and FO/VOP. Feeds contained decreasing levels of FM and FO and
124 containing low (LV), medium (MV) or high (HV) levels of substitution of marine ingredients
125 with plant products were fed from smolt input (~100 g) to harvest (~3000 g) (Table 1). Soy
126 protein concentrate, corn and wheat gluten, pea protein and sunflower expeller were
127 employed as PP sources and rapeseed oil was used as the VO source. The experimental feeds
128 were formulated and produced by BioMar Ltd. (Brande, Denmark), and were based on
129 current commercial specifications (Table 1) to satisfy the nutritional requirements of
130 salmonid fish, using standard commercially available feed materials and without Se
131 supplementation. Diets were tested in triplicate over a period of 265 days. Replacement of
132 FM and FO with PP and VO resulted in increased proportions of oleic acid (OA; 18:1n-9)
133 and decreased percentages of EPA, DPA and DHA (Table 2). The PIn of the feeds was
134 related to the dietary content of LC-PUFA and decreased with increasing substitution with
135 plant products and was consistent in the different feed pellet sizes. The Se concentration was
136 higher in diets LV than in diets MV and HV. Dietary selenium content also decreased with
137 the progressive decline in FM levels in all feeds as pellet size increased throughout the
138 seawater production cycle. Thus, Se concentrations were 1.2 µg/g and 0.7 µg/g in diets LV
139 and HV, respectively, at the start of the trial whereas they decreased to 1.0 and 0.6 µg/g in
140 diets LV and HV, respectively, in the final pellet size prior to harvesting (Table 2).

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142 A total of 2250 Atlantic salmon with mean initial weight of 108.0 ± 1.5 g (average ± SD)
143 were randomly distributed into 9 sea pens, 5.5 × 5.5 m wide and 5 m deep. The seawater
144 temperature ranged from 7.7 to 14.7 °C during the experimental period. Fish were fed twice
145 daily to apparent satiation, using a combination of automatic feeders and hand feeding, with
146 monitoring. Feed waste was collected an hour after the end of each meal, and mortalities
147 were checked 3 times per week. Fish were treated in accordance with British national ethical
148 requirements and the experiment conducted under the UK Government Home Office Project

149 Licence number PPL 60/03969 in accordance with the amended Animals Scientific
150 Procedures Act 1986 implementing EU directive 2010/63.

151 *Growth and survival rate*

152 All fish were anesthetised before handling using 60 mg/L metacaine sulphonate (MS-222)
153 dissolved in sea water. Bulk weighing was conducted at the start of the trial when the fish
154 were small whereas individual fish were weighed on an electronic top loading balance at the
155 end of four feeding phases from 100 to 500 g, 500 to 1000 g, 1000 to 2000 g and 2000 to
156 3000 g to monitor growth performance. Final survival was determined by counting the total
157 fish at the beginning and end of the trial.

158 *Sample collection and management*

159 At the end of each feeding phase, eight fish per pen were collected and euthanised by an
160 overdose of MS-222. Flesh was sampled by collecting the Norwegian Quality Cut (NQC), the
161 region between the dorsal and ventral fins, the steaks skinned and boned, and homogenised in
162 a large blender (R23 Robotcoupe, Ultra, UK). The homogenate was immediately frozen and
163 stored at -20 °C prior to further analysis. In addition, at the final sampling (3000 g) samples
164 of flesh (NQC), brain, head kidney, gill, anterior intestine and liver were excised, pooled per
165 pen and frozen for total selenium determination (n = 3 per treatment) of total Se content.
166 Approximately 100 mg of liver and brain from two fish per pen (n = 6 per treatment) were
167 placed in RNA Later (Sigma - Aldrich, Dorset, UK) and frozen at -20 °C for total RNA
168 extraction.

169 *Proximate composition of diets*

170 The nutrient compositions of experimental diets were determined by proximate analysis
171 (Table 2). Moisture was determined by weighing three replicates of between 0.3 and 1.0 g
172 into pre-weighed crucibles using a microbalance (AC100, UK) and placing them in an oven
173 at 105 °C overnight. After cooling in a desiccator for 1 h, moisture was expressed as a
174 percentage of wet weight. Crude protein was determined by Kjeldahl analysis (nitrogen ×
175 6.25; Tecator Kjeltec Auto 1030 analyzer, Foss, Warrington, UK). Crude fat content was
176 determined after acid hydrolysis using a Soxhlet lipid extraction system (Tecator Soxtec 2050
177 Auto Extraction apparatus, Foss, Warrington, UK). Ash content was determined by heating in
178 a porcelain crucible in a muffle furnace at 600 °C overnight. All proximate analysis methods
179 were based on those of the Association of Official Analytical Chemists[36].

180 *Lipid extraction*

181 Total lipid of flesh and liver samples was extracted using a modified Folch method[37].
182 Samples of homogenised NQC of approximately 1 g were weighed in duplicates (0.5-1 g)
183 into 50 ml quickfit tubes and homogenised in 20 volumes of ice-cold chloroform/methanol
184 (2:1, v/v) using an Ultra Turrax tissue disrupter (Fisher Scientific, Loughborough, UK).
185 Potassium chloride (KCl, 0.88 %) was added to the homogenised samples, mixed and
186 centrifuged at 1500 rpm for 7 min to separate different layers. The upper aqueous phase was
187 removed by aspiration, the bottom layer containing the lipid extract was filtered (Whatman
188 No.1) and transferred to pre weighed tubes. The lower solvent phase was evaporated on a
189 nitrogen evaporator and desiccated overnight before reweighing. Chloroform/methanol (2:1,
190 v/v) containing 0.01 % (w/v) butylated hydroxytoluene (BHT) was used to re-suspend total
191 lipid at a concentration of 10 mg/ml. Total lipids were stored under nitrogen at -20 °C until
192 subsequent analysis. The accepted variance in measured lipid content between samples was ±
193 10 %.

194 *Fatty acid composition*

195 Fatty acid methyl esters (FAMEs) were prepared by acid - catalysed transesterification of
196 total lipid as described by Christie[38]. Approximately, 1 mg of dry total lipids were incubated
197 at 50 °C for 16 h following addition of 1 ml toluene and 2 ml of 1 % (v/v) sulphuric acid in
198 methanol. The methylation reaction was terminated using 2.5 ml of 2 % aqueous $KHCO_3$
199 (w/v) and FAME extraction was performed with 5 ml of iso-hexane/diethyl ether (1:1, v/v)
200 containing 0.01 % (w/v) BHT and again with 5 ml of iso-hexane/diethyl ether (1:1, v/v)
201 without BHT[39]. Methyl esters were purified by thin-layer chromatography (TLC) by loading
202 onto 20 × 20 cm plates (Merck, Germany) and that were fully developed with iso-
203 hexane/diethyl ether/acetic acid (90:10:1, v/v) as solvent system. Plates were sprayed with 1
204 % (w/v) iodine in chloroform to visualise FAMEs, bands marked and scraped into test tubes
205 and eluted from silica with 10ml of iso-hexane/diethyl ether (1:1, v/v) + 0.001 % (w/v) BHT
206 and re- suspended in isohexane + 0.01 % BHT at a concentration of 1 mg/ml. FAME were
207 separated and quantified by gas liquid chromatography using a Fisons GC-8160 (Thermo
208 Scientific, Milan, Italy) fitted with on-column injector and flame ionisation detector (FID),
209 and equipped with a 30 m x 0.32 mm i.d x 0.25 µm ZB- wax column (Phenomenex,
210 Cheshire, UK). Hydrogen was used as the carrier gas at a flow rate of 2.5mL/min, with an
211 initial oven thermal gradient from 50 °C to 150 °C at 40 °C min^{-1} and then to a final

212 temperature of 225 °C at 2 °C min^{-1}. Peak area was processed by using Chrom- Card for
213 Windows software (version 1.19; Thermoquest Italia S.p.A., Milan, Italy). Individual FAME
214 was identified by comparison of the retention time and known standards (Supelco 37-FAME
215 mix, Sigma – Aldrich Ltd., Poole, UK).

216 *Selenium determination*

217 Total selenium concentrations were measured in feeds, muscle, brain, head kidney, gill,
218 anterior intestine and liver according to the method established in Betancor et al.[(24)]. Dried
219 samples were weighed in three replicates between 0.04 and 0.1 g and digested in a
220 microwave digester (MarsXpress, CEM, USA) with 5 % of 69 % pure nitric acid in three
221 steps as follows; 21° C to 190° C for 10 min at 800 W then 190° C for 20 min at 800 W and
222 finally a 30 min cooling period. The digested solution was poured into 10 ml volumetric
223 flasks and made up to volume with distilled water. A total of 0.4 ml of this solution was
224 added to 10 ml tubes, 10 µl of the internal standard (Gallium and Scandium, 10 ppm, BDH,
225 UK) included and 0.2 ml of methanol added. The tube was made up to volume with distilled
226 water and total selenium was measured in a reaction cell by Inductively Coupled Plasma
227 Mass Spectrometry (Thermo Scientific, XSeries2 ICP-MS, USA) using argon and hydrogen
228 as carrier gas.

229 *Thiobarbituric acid reactive substances (TBARS)*

230 TBARS were determined in total lipid extracts (10 mg/ml) of liver and brain at each sampling
231 point according to a modification of the protocol of Burk et al.[(40)]. Briefly, 50 µl of 0.2 %
232 (w/v) BHT in ethanol was added to 200 mg of lipid samples followed by 0.5 ml of 1 % (w/v)
233 TBA and 0.5 ml 10 % (w/v) trichloroacetic acid, both solutions freshly prepared. The
234 reagents were mixed in a stoppered test tube and heated at 100 °C for 20 min. After cooling,
235 particulate matter was removed by centrifugation at 2000 *g*, and absorbance in the
236 supernatant determined in a spectrophotometer at 532 nm against a blank sample. The
237 concentration of TBARS, expressed as mmol malondialdehyde (MDA)/g lipid was calculated
238 using the absorption coefficient 0.156 μM^{-1} cm^{-1}.

239 *RNA extraction and quantitative real time PCR (qPCR)*

240 Liver and muscle from six individual fish per dietary treatment were homogenised in 1 ml of
241 TriReagent® (Sigma-Aldrich, Dorset, UK) RNA extraction buffer using a bead tissue
242 disruptor (Bio Spec, Bartlesville, Oklahoma, USA). These two tissues were chosen because

243 liver is the main metabolic tissue and muscle is the most abundant tissue in whole fish. Total
244 RNA was isolated following manufacturer's instructions and quantity and quality determined
245 by spectrophotometry using a Nanodrop ND-1000 (Labtech Int., East Sussex, UK) and
246 electrophoresis using 200 ng of total RNA in a 1 % agarose gel. Expression of genes of
247 interest (Table 3) was determined by quantitative PCR (qPCR) from fish fed all diets. Results
248 were normalised using reference genes, elongation factor 1α (*elf1α)* and cofilin 2 (*cof2*),
249 which were chosen as the most stable according to GeNorm. cDNA was synthesised using 2
250 μg of total RNA and random primers in 20 μl reactions and the high capacity reverse
251 transcription kit without RNase inhibiter according to the manufacturer's protocol (Applied
252 Biosystems, Warrington, UK). The resulting cDNA was diluted 20-fold with milliQ water.
253 The efficiency of the primers for each gene was previously evaluated by serial dilutions to
254 ensure that it was close to 100 %. qPCR was performed using a Biometra TOptical
255 Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates in duplicate 20 μl
256 reaction volumes containing 10 μl of Luminaris Color HiGreen qPCR Master Mix (Thermo
257 Scientific, Hemel Hempstead, UK), 1 μl of the primer corresponding to the analysed gene (10
258 pmol), 3 μl of molecular biology grade water and 5 μl of cDNA, with the exception of the
259 reference genes, which were determined using 2 μl of cDNA. In addition amplifications were
260 carried out with a systematic negative control (NTC-non template control) containing no
261 cDNA. Standard amplification parameters contained an UDG pre-treatment at 50 °C for 2
262 min, an initial denaturation step at 95 °C for 10 min, followed by 35 cycles: 15 s at 95 °C, 30
263 s at the annealing Tm and 30 s at 72 °C.

264 *Calculations*

265 The peroxidation index (PIn) was used as an estimate of susceptibility of lipids to oxidation
266 and was calculated using the formula: PIn= 0.025 × (percentage of monoenoics) + 1 ×
267 (percentage of dienoics) + 2 × (percentage of trienoics) + 4 × (percentage of tetraenoics) + 6
268 × (percentage of pentaenoics) + 8 × (percentage of hexaenoics)[41]. Specific growth rate
269 (SGR) = 100 × (lnWf – lnWo) / t, where Wo = initial weight (g) and Wf = final weight (g) at
270 time t (days). Thermal growth coefficient (TGC) = ($Wf^{1/3}$ – $Wo^{1/3)}$ × (1000/DD), where Wf
271 and Wo are as previously addressed for SGR, and DD is the thermal sum (feeding days ×
272 average temperature). Feed conversion ratio (FCR) = (wet feed intake; kg)/(wet weight gain;
273 kg).

274 *Statistical analysis*

275 All the data were presented as mean ± S.E. (standard error). Percentage data for survival rate, 276 total lipid content and fatty acid composition were all arcsin transformed prior to statistical 277 analysis. Data were tested for normality and homogeneity of the variances with Levene's test. 278 Normal distribution data were analysed by one-way analysis of variance (ANOVA) followed, 279 when appropriate, by a Tukey comparison of means test. Two-way ANOVA was used to 280 determine the interaction between dietary treatments and sampling point (weight). Significant 281 differences were accepted at $p \leq 0.05$. All statistical analysis was performed using Minitab 282 (version 16.1; Minitab Inc., State College, PA, USA). Gene expression results were analysed 283 using the relative expression software tool (REST, Qiagen, http://rest.gene- 284 quantification.info/) with efficiency correction[42] to determine the statistical significance of 285 expression ratios (gene expression fold changes) between the three treatments.

286 **Results**

287 *Growth and survival rate*

288 All experimental feeds were well accepted by the fish. The average survival rate of fish fed 289 the three dietary treatments throughout the experimental period was high (>98 %) and was 290 not affected by dietary treatments ($p > 0.05$). In terms of growth performance, specific 291 growth rate (SGR) and thermal growth coefficient (TGC) were not affected by dietary 292 treatment over the 265 day feeding period ($p > 0.05$). However, feed conversion ratios (FCR) 293 of fish fed the LV and MV diets were slightly lower than that of fish fed the HV diet ($p \leq$ 294 0.05; Table 4), although differences were only observed at the final sampling point (2.0 to 3.0 295 kg; Supplementary Fig. 1).

296 *Nutritional composition of muscle and liver*

297 Increasing inclusion of plant ingredients in the diets had no significant effect on the lipid 298 content of salmon muscle (flesh) within each sampling period ($p > 0.05$; Table 5). In 299 addition, flesh lipid levels of fish fed all three dietary treatments tended to increase with 300 increasing fish weight, although size did not influence total lipid deposition (Table 6). The 301 total lipid content in flesh was lowest at the 1000 g sampling point at 8.0 – 9.0 % (of wet 302 weight, w.w.), and was highest at 11.3 – 12.7 %, (w.w.) in harvest size (Table 5) albeit not 303 significant ($p > 0.05$). Fatty acid compositions of the flesh were influenced by those of the 304 diets, with OA increased whereas EPA and DHA were significantly reduced with increased 305 inclusion of PP and VO in the feeds ($p \leq 0.05$; Table 5). Furthermore, the n-3/n-6 PUFA ratio

306 of flesh in each feeding phase tended to decrease with increasing inclusion of plant
307 ingredients, although significant differences were only present for 2 and 3 kg fish ($p \leq 0.05$;
308 Table 5). The n-3/n-6 PUFA ratio was also related to weight gain, with a tendency to increase
309 with increasing weight in fish fed the LV diet whereas it decreased with increasing weight in
310 fish fed the MV and HV diets ($p \leq 0.05$; Table 5). Thus, the ratio increased in fish fed diet
311 LV as the dietary oil had high FO (high n-3 LC-PUFA), whereas the MV and HV diets had
312 higher n-6 PUFA and so the n-3/n-6 ratio declined as dietary lipid was accumulated in the
313 flesh.

314 In contrast to muscle, liver total lipid content did not increase with time, indeed a decrease in
315 the fat content was observed, particularly in fish fed diets with high levels of substitution,
316 although significant differences were only found in 2 kg fish (HV; $p \leq 0.05$; Table 7). No
317 differences in the fatty acid profile were found in 1 kg fish among the three different dietary
318 treatments, although clear differences were found at the other two sizes. In this sense, and
319 similar to flesh, inclusion of high levels of plant ingredients lead to increased levels of OA
320 and n-6 PUFA whereas n-3 LC-PUFA decreased ($p \leq 0.05$; Table 7). The factor "diet" had a
321 marked effect on both tissues fatty acid profile, affecting all the studied fatty acids (Table 6).
322 Additionally the factor "size" had a greater effect on liver than on flesh, affecting all of the
323 evaluated fatty acids except total saturated fatty acids and n-3/n-6 ratio (Table 6). An
324 interaction between size at sampling and dietary treatment on total lipids and fatty acid
325 profile only occurred for n-3/n-6 ratio for flesh and total n-6 PUFA and EPA for liver (Table
326 6).

327 *Tissue Se content*

328 Increased inclusion of plant ingredients had significant effects on Se concentrations in all the
329 studied tissues (Fig. 1A). Liver had the highest average Se content (3.09 μg/g), followed by
330 head kidney (0.99 μg/g) and intestine (0.49 μg/g), whereas brain and gill displayed the lowest
331 contents (0.26 and 0.16 μg/g respectively). There was a strong correlation between dietary Se
332 content and the concentration of this trace element in the different tissues. This was
333 particularly true for liver, head kidney and brain where differences were found in Se contents
334 between fish fed the MV and HV diets, whereas in gills and intestine these differences were
335 not as obvious (Fig. 1A). Both factors, size at sampling and diet influenced the flesh Se
336 content, although an interaction between both factors was also evident albeit not as
337 significant ($p = 0.025$). Se content of the flesh over the entire growth cycle reflected dietary

338 content of Se, with decreased Se content in fish fed diets with higher levels of terrestrial plant
339 ingredients. In addition, interaction was found between dietary treatment and duration of
340 feeding in tissue Se concentrations ($p \leq 0.05$). The amount of Se in flesh decreased ($p \leq 0.05$)
341 with increasing fish weight, which is in agreement with reduced Se levels in the diets, with
342 the average Se content in fish at the 1000 g sampling point being 0.45 µg/g in fish fed the LV
343 diet, whereas at harvest size the level was 0.29 µg/g in flesh of fish fed the lowest substitution
344 levels (LV; Fig. 1B).

345 *TBARS content and peroxidation index in flesh and liver*

346 Analysis of lipid peroxidation products in liver and muscle showed differences between the
347 two tissues and among dietary treatments. Muscle exhibited higher TBARS content than liver
348 for all diets and sampling points ($p \leq 0.05$; Tables 5 and 7). For both tissues, TBARS
349 increased from 1000 g to 2000 g fish, although TBARS values were reduced in 3000 g fish (p
350 ≤ 0.05; Tables 5 and 7). In liver, no differences were observed between fish fed the LV and
351 MV diets ($p = 0.061$) and 1000 g fish only displayed differences between fish fed the MV
352 and HV diets ($p \leq 0.05$; Table 7). Conversely, flesh of 1000 g fish fed LV feeds did not show
353 differences in TBARS content with HV-fed fish ($p \leq 0.01$; Table 5). Furthermore, fish fed the
354 LV diet had the highest TBARS contents at 2000 and 3000 g. In contrast, TBARS contents
355 were correlated with tissue PIn and DHA contents both in flesh and liver (Tables 5 and 7).
356 The higher dietary plant ingredient levels led to decreased contents of LC-PUFA that
357 translated to lower peroxidation risk and thus, lower levels of lipid peroxidation products
358 (TBARS). No interactions between fish size and diets were observed in either tissues for PIn
359 and TBARS, whereas diet did influence these values (Table 6). Fish size influenced PIn and
360 TBARS contents in liver but not in flesh (Table 6).

361 *Gene expression in liver and brain*

362 Differing levels of plant ingredients did not elicit a differential response in the studied genes
363 in liver ($p > 0.05$; Fig. 2) whereas significant differential expression of some of the genes was
364 observed in brain (Fig. 3). Generally, brain showed higher expression of both selenoprotein
365 and oxidative stress-related genes than liver. The general pattern of selenoprotein gene
366 expression in both tissues was up-regulation with increased dietary plant ingredients. Thus,
367 salmon fed diet LV showed the lowest expression of *gpx4b* ($p = 0.04$) in brain when
368 compared to fish fed MV and HV diets, whereas *gpx7*, *sepp* and *sepk* showed variable
369 expression with no clear dietary effects on the expression of these genes in either tissue ($p >$

370 0.05; Fig. 3). With oxidative stress-related genes, both *cat* and *sod* showed variable
371 expression in brain and liver and the only statistically significant difference was lower *sod*
372 gene expression in brain of fish fed diet LV ($p = 0.043$). In addition, *secp43* also showed the
373 lowest expression in brain when fish were fed diet LV ($p = 0.028$; Figs. 2 and 3).

374 **Discussion**

375 The current increasing substitution of high levels of dietary marine ingredients by terrestrial
376 plant products in aquafeeds due to sustainability and availability issues may lead to altered
377 nutritional value of the final farmed fish products. A large body of data is available regarding
378 the effect of substitution of FM and FO by plant products on the n-3 LC-PUFA content of
379 fish flesh in several species[43-45]. Nevertheless, fish provide many other key nutrients
380 components including vitamins D and B_{12}, iodine, taurine and Se, whose levels could be
381 affected by the origin of the raw materials employed in the formulation of aquafeeds. In the
382 present report we focussed on the effect of inclusion of plant ingredients on Se status in
383 Atlantic salmon because fish is an ideal package to deliver this essential micronutrient to
384 human consumers. In addition, a reduction in the intake in certain nutrients may lead to
385 adverse health status of the fish itself.

386 In the present study, increasing levels of plant ingredients in feeds led to decreased
387 concentrations of Se in all the studied tissues in salmon. Six tissues including liver, brain,
388 gill, head kidney, intestine and muscle were chosen in the present study based on the different
389 roles they play in the absorption and incorporation of Se, as well as biosynthesis of
390 selenoproteins. Fairweather et al.[46] indicated that liver is the primary organ involved in Se
391 metabolism, where SEPP is synthesised prior uptake by kidney and brain via the apoER2
392 receptor and megalin. The results of the present study showed that the highest Se content was
393 found in the liver (3.09 µg/g) of fish fed LV diet, slightly higher than levels reported in a
394 previous study (2.33 µg/g)[47]. Furthermore, the Se content of liver was three and seven times
395 higher in comparison to the kidney and muscle respectively, whereas the lowest Se
396 concentrations were found in brain and gill. This is in agreement with Burger et al.[48] who
397 indicated that levels of Se were similarly low in brain and gill of bluefish (*Pomatomus*
398 *saltatrix*) but in the previous study the highest Se content was found in head kidney.

399 It was noteworthy that increased inclusion of PP and VO in the diets dramatically reduced the
400 Se content in flesh of fish fed the HV diets at all three sampling points. Se concentration in
401 flesh of fish fed the HV diet was 2-fold lower than fish fed the LV diet after the 9 month

402 feeding period. The present study showed that a portion of fish (130 g) of the commercial
403 size (3 kg) fed the LV diet can supply 28.6 μg of Se, which covers 41 % of the recommended
404 daily intake of Se. In contrast, 130 g portion of fish fed the HV diet can supply only 22 % of
405 the recommended Se intake. Despite of the reduction in flesh Se content, salmon could still
406 contribute highly to the overall Se intake for humans. For instance, in the UK one hen egg
407 (60 g) provides only 6.6 μg of Se (9.4 % of the recommended intake) and 100 g of bread
408 contains only 9 μg of this essential micronutrient (12.8% of the recommended intake)[(49)].
409 This highlighted that substitution of marine ingredients by terrestrial plant products leads to a
410 substantial decrease in Se in flesh reducing the ability of farmed fish to supply an adequate Se
411 dose to human consumers. Therefore, it would be interesting to study the possible restoration
412 of Se content by returning fish to a "finishing" diet containing higher levels of FM and FO
413 for a period of time before harvest, as has been shown for n-3 LC-PUFA[(50-52)]. Alternatively,
414 the supplementation of aquafeeds with organic sources of Se (Se enriched yeast), less likely
415 to cause toxicity than inorganic sources could be used as a measure to maintain adequate Se
416 levels in salmon flesh as shown for several other fish species[(53-56)]. Additionally, the use of
417 processed animal proteins such as poultry by-product meal with an average Se content of
418 ~0.78 mg/kg[(57)] could supply Se of high digestibility for aquafeeds. In addition, it was
419 perhaps surprising to observe a reduction in Se contents in muscle as fish size increased.
420 However, previous studies have shown that in certain marine fish species such as yellowfin
421 tuna (*Thunnus albacares*) or windowpane flounder (*Scopthalmus aquosus*) a significant
422 negative correlation exists between muscle Se contents and fish size[(58)], similar to that found
423 in the present study. Although a slight reduction was observed in dietary Se content along
424 time, the feeds employed during the periods in which samplings were performed (from 500 to
425 3000 g) did not vary at all in LV feeds (1.0 μg/g), what indicates that the reduction in Se in
426 the different tissues is mainly related to fish size/age.

427 The use of high levels of terrestrial plant products did not cause any noticeable adverse
428 effects on fish physiology. Fish grew adequately on diets with high substitution levels and
429 only FCR was slightly increased in fish fed the HV diet. One of the first symptoms of Se
430 deficiency in fish is reduced growth, which was not observed in the present study with
431 increased dietary terrestrial plant ingredients. Similarly, a limited response was observed in
432 the expression of several selenoproteins and oxidative stress-related genes. Differences were
433 only found in the expression of *secp43* and antioxidant enzymes *gpx4b* and *sod* in brain,
434 whereas more stable expression was observed in liver. This different pattern of expression

435 could indicate that fish develop tissue-specific adaptive responses to protect cells against
436 oxidative stress as suggested previously[(59)]. In this sense, brain is a tissue rich in n-3 LC-
437 PUFA[(39,60-61)] with naturally low Se contents, as shown in this study and thus, is more prone
438 to suffering peroxidation. In agreement, a study in Manchurian trout (*Brachymystax lenok*)
439 showed that brain together with gills were the most sensitive tissues to oxidative damage[(62)].
440 Up-regulation of the expression of the antioxidant enzyme *sod* in fish fed the diets with
441 lowest Se contents may indicate an enhancement of enzymatic antioxidant protection to
442 compensate for the deficiency in this essential micronutrient. On the other hand, a recent
443 study in rainbow trout (*Oncorhynchus mykiss*) found an up-regulation of *gpx1a*, another *gpx*
444 isoform, in the liver and kidney of fish fed low dietary organic Se in comparison to fish fed
445 higher levels of this mineral[(63)]. An up-regulation of *gpx4* was also observed in chicken fed
446 low Se feeds[(64)]. The reason for an up-regulation of *gpx4* is not clear and has be found to be
447 tissue and species-specific[(64)].

448 On the other hand, tRNA[ser]sec (SECP43) is a key player in orchestrating the interactions and
449 localizations of factors involved in selenoprotein biosynthesis by increasing selenocysteine
450 incorporation and selenoprotein mRNA levels[(31)]. In the present study, higher expression of
451 this gene was observed in brain of fish fed diets with high substitution levels and thus, lower
452 Se contents, which could indicate an up-regulation in the biosynthesis of new selenoproteins.
453 Although in mammals transcriptional changes in the brain in response to Se are rare[(65)], some
454 studies in fish brain have found differential expression when Se was supplemented to the diet.
455 For instance, Benner et al.[(66)] found increased expression of *secp43* in brain with increased
456 dietary Se supplementation in zebrafish. This contrasted with the results obtained in the
457 present study, where the lowest Se levels induced increased expression in *secp43*. This
458 difference could be related to the different dietary Se levels employed in these studies, being
459 lower in the present study, which may indicate that both low and supranutritional levels
460 trigger activation of the synthesis of selenoproteins. Besides, the level of n-3 LC-PUFA and
461 PIn in feeds appears to have an effect on *secp43* expression. A recent study in zebrafish fed
462 diets of high and low oxidation risk, down-regulation was observed in hepatic *secp43*
463 expression when fish were fed a high DHA/high oxidation risk diet[(25)]. This is in agreement
464 with the present study, where down-regulation was observed in fish fed the feed with highest
465 DHA level. Therefore, transcriptional or post-transcriptional regulation of selenoproteins by
466 LC-PUFA is a possibility, as was shown *in vitro* for GPX1 and GPX4[(67-68)]. Furthermore, the
467 lack of effect in the expression of the other studied selenoproteins may indicate that not all

468 respond in the same way when Se levels are altered, with a hierarchy existing as described
469 previously in mammals[69]. In the case of brain, SECP43 appears to have priority in this tissue
470 over the other studied selenoproteins, resulting in them responding earlier to alterations in Se
471 levels. The lack of effect on selenoprotein expression observed in liver could be attributed to
472 variations among selenoproteins and tissues in response to Se which has been shown in
473 mammals[70].

474 Although antioxidant properties of Se are widely recognised both in mammals and fish[9], in
475 the present study, decreased levels of dietary Se did not lead to increased levels of TBARS,
476 an indicator of lipid oxidation, in liver or flesh. However, inclusion of terrestrial plant
477 ingredients in aquafeeds also leads to decreased contents of LC-PUFA, not allowing us to
478 check a direct effect of Se on TBARS. The reduction in these highly unsaturated fatty acids,
479 highly susceptible to oxidation, can increase the stability of the tissues to peroxidation, as
480 indicated by the PIn. These results are in agreement with previous studies in rainbow trout
481 and Atlantic salmon, where replacement of FO with VO reduced formation of primary
482 oxidation products such as lipid hydroperoxides[71] or TBARS[72]. Other studies have not
483 found differences in oxidative stability in salmon muscle when FO was substituted by VO[43],
484 or have found lower oxidation rates in VO-fed fish than in FO-fed ones[70], which indicates
485 that the presence of other antioxidant nutrients such as α-tocopherol or carotenoids may be
486 more critical for oxidative stability in flesh than the degree of unsaturation of the feeds or Se
487 levels in the flesh.

488 Overt Se deficiency in humans has been associated with dilated cardiomyopathy, skeletal
489 muscle myopathy, osteoarthropathy, cretinism, reduced immune function, some cancers and
490 viral diseases[74]. In the UK, the daily Se intake is estimated to be around 29-39 µg/day[75],
491 which is lower than the recommended dietary intake. Therefore, individuals are encouraged
492 to consume foods with high Se content such as salmon. The present study demonstrated that,
493 although reduced levels of FM/FO in feeds did not result in any difference in fish
494 performance or health, it did reduce the n-3 and n-6 LC-PUFA contents as well as Se levels
495 in Atlantic salmon, potentially affecting the nutritional value of farmed products. Therefore,
496 the present study highlights that even if fish Se requirements are satisfied in diets with high
497 levels of plant ingredients, the Se content will be reduced to human consumers such that their
498 Se intake can be almost halved when high levels of plant ingredients are used.

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501 some of the analysis and participated in the writing of the manuscript; D.T.M.C. performed
502 part of the analysis and participated in the writing of the manuscript; J.W. and T.M. designed
503 the study and participated in the writing; P.J.C. and D.R.T. were involved in data
504 interpretation and edited the manuscript. All authors read and approved the final manuscript.
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697 **Figure legends**

698 Fig. 1. Selenium content (µg/g) in liver, head kidney, brain, gill and anterior intestine of
699 Atlantic salmon fed diets with differing contents of PP/VO after 9 months of feeding (3000 g)
700 (A); in flesh of fish fed the three dietary treatments at each sampling point (B). Data are
701 presented as mean ± SEM. Different letter denote statistically significant differences between
702 the three dietary groups at the final time point of finishing feeding period identified by one-
703 way ANOVA. The inset Table presents p values for the effect of weight, diet and their
704 interaction of the selenium content of the flesh; $^*p<0.05$; $^{**}p<0.01$.

705 Fig. 2. Selenoprotein and other antioxidant stress genes expression measured by qPCR in
706 Atlantic salmon liver after 9 months feeding (3000 g). Diet LV to HV represents feeds with
707 increasing levels of PP/VO as described in Materials and Methods section. Data are
708 normalized expression ratios (mean ± SEM, n=6) of the expression of these genes fed
709 different diets in relation to fish fed diet LV. Different letter denote statistically significant
710 differences between the three dietary group identified by one- way ANOVA. *sepk*,
711 selenoprotein K; *sepp*, selenoprotein P ; *secp43*, tRNA selenocysteine associated protein 1;
712 *gpx4b*, glutathione peroxidase 4b; *gpx7*, glutathione peroxidase 7; *cat*, catalase; *sod*,
713 superoxide dismutase.

714 Fig. 3. Selenoprotein and other antioxidant stress genes expression measured by qPCR in
715 Atlantic salmon brain after 9 months feeding (3000 g). Diet LV to HV represents feeds with
716 increasing levels of PP/VO as described in Materials and Methods section. Data are
717 normalized expression ratios (mean ± SEM, n=6) of the expression of these genes fed
718 different diets in relation to fish fed diet LV. Different letter denote statistically significant
719 differences between the three dietary group identified by one- way ANOVA. *sepk*,
720 selenoprotein K; *sepp*, selenoprotein P ; *secp43*, tRNA selenocysteine associated protein 1;
721 *gpx4b*, glutathione peroxidase 4b; *gpx7*, glutathione peroxidase 7; *cat*, catalase; *sod*,
722 superoxide dismutase.

723

724 **Supplementary Information:**

725 Supplementary Fig. 1. Feed conversion ratio calculations of Atlantic salmon fed the three
726 dietary treatments with differing substitution levels at each sampling point. Diet LV to HV
727 represents feeds with increasing levels of PP/VO as described in Materials and Methods
728 section.

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732

733 Table 1.- Formulation of experimental diets (all values are g/kg)

	100 to 500 g (3 - 4.5 mm)			**500 to 1000 g (6 mm)**			**1000 to 2000 g (9 mm)**			**2000 to 3000 g (10 mm)**		
Ingredients	**LV**	**MV**	**HV**	**LV**	**MV**	**HV**	**LV**	**MV**	**HV**	**LV**	**MV**	**HV**
North Atlantic LT FM	175.0	100.0	50.0	150.0	100.0	50.0	125.0	75.0	50.0	125.0	75.0	50.0
South American Super Prime FM	175.0	100.0	50.0	150.0	100.0	50.0	125.0	75.0	50.0	125.0	75.0	50.0
Plant protein concentrates*	220.0	400.0	440.0	263.0	427.0	440.0	317.0	419.0	430.0	231.0	401.0	451.0
Wheat Gluten	9.0	33.0	82.0	0.0	0.0	73.8	0.0	25.7	54.0	0.0	0.0	16.9
Sunflower expeller	92.0	13.0	4.0	92.9	11.4	8.5	45.8	0.0	0.0	100.0	21.3	0.0
Purified wheat	120.0	120.0	120.0	120.0	120.0	120.0	120.0	120.0	120.0	120.0	120.0	120.0
Fish oil	131.0	98.0	60.0	164.0	121.0	72.2	192.0	140.0	84.0	210.0	151.0	90.0
Rapeseed oil	56.0	98.0	140.0	70.1	121	169	82.2	140.0	197.0	90.0	151.0	209.0
Yttrium	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Crystalline amino acids	4.4	11.3	19.0	2.4	5.7	12.9	2.5	6.8	9.8	1.2	4.2	6.5
Monocalcium phosphate	17.5	23.4	29.4	6.7	12.1	18.1	6.2	11.8	14.8	3.7	9.1	11.2
Lucanthin Pink 10% (BASF)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Vitamins and minerals**	5.2	6.4	7.7	5.3	6.3	8.3	5.6	7.0	8.5	11.2	12.2	13.8

734

735 *Plant protein concentrates comprised of soya protein concentrate, maize gluten and pea protein in
736 similar ratio across diets independent of fishmeal level.

737 **Proprietary blend of vitamins and minerals sufficient to meet nutrient requirement[57].

738 Diets LV to HV represent feeds with increasing levels of PP/VO in three feeding periods as described
739 in Materials and Methods section

740

Table 2.- Proximate and fatty acid composition (percentage of total fatty acids) of the experimental feeds employed in the present study

	100-200 g/3 mm			**200-500 g/4.5 mm**			**500-1000 g/6 mm**			**1000-2000 g/9 mm**			**2000-3000 g/10 mm**		
	LV	**MV**	**HV**	**LV**	**MV**	**HV**	**LV**	**MV**	**HV**	**LV**	**MV**	**HV**	**LV**	**MV**	**HV**
Proximate															
Dry matter	902	924	922	919	922	911	948	940	935	923	926	930	940	936	933
Protein	425	426	419	431	442	417	404	403	405	374	382	373	353	375	365
Fat	223	219	233	214	227	214	271	275	253	310	327	313	341	317	313
Ash	77	67	61	80	67	60	68	57	52	59	52	47	58	51	48
Energy	22	23	22	22	22	22	24	23	23	23	24	24	24	24	23
Fatty acid (%)															
ΣSAFA[1]	21.0	18.0	15.0	22.0	18.0	15.0	21.0	18.0	14.0	24.0	22.0	21.0	25.0	21.0	20.0
OA	25.0	34.0	42.0	25.0	34.0	42.0	25.0	34.0	42.0	20.0	23.0	25.0	23.0	26.0	32.0
ARA	0.5	0.3	0.2	0.5	0.3	0.2	0.4	0.3	0.2	0.7	0.4	0.2	0.3	0.1	0.1
Σn-6 PUFA[2]	13.0	16.0	19.0	13.0	16.0	19.0	12.4	14.0	18.0	16.0	24.0	32.0	12.0	21.0	26.0
EPA	5.6	3.7	2.1	5.4	3.8	2.0	5.8	4.2	2.8	7.4	5.1	3.0	6.1	4.9	2.7
DPA	0.9	0.6	0.3	0.9	0.6	0.3	0.7	0.5	0.4	1.1	0.7	0.4	0.9	0.7	0.4
DHA	6.9	4.7	2.6	6.9	4.9	2.5	6.5	4.6	3.2	5.8	4.2	2.6	5.3	4.2	2.5
Σn-3 PUFA[3]	19.0	16.0	12.0	19.0	16.0	12.0	19.4	16.0	14.0	20.0	16.0	11.0	17.0	16.0	12.0
n-3/n-6	1.5	1.0	0.6	1.5	1.0	0.6	1.6	1.1	0.8	1.2	0.7	0.3	1.4	0.8	0.5
PIn	126.3	98.0	72.4	124.5	101.2	59.0	115.6	98.1	77.4	124.3	118.1	83.5	119.1	102.8	73.5
Se	1.2	0.9	0.7	1.2	1.0	0.7	1.0	0.9	0.7	1.0	0.8	0.7	1.0	0.8	0.6

Dry matter (DM), g/kg DM; Protein, g/kg DM; Fat, g/kg DM; Ash g/kg DM; Energy, MJ/kg; Se, µg/g. Diets LV to HV represent feeds with increasing levels of PP/VO in three feeding periods as described in Materials and Methods section. [1]contains 14:0, 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0; [2]contains 18:2n-6, 18:3n-6, 20:2n-6, and 20:3n-6; [3]contains 18:3n-3, 18:4n-3, 20:3n-3 and 20:4n-3. SAFA, saturated fatty acids; OA, oleic acid; ARA, arachidonic acid; PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA; docosahexaenoic acid; PIn, peroxidation index.

752 Table 3.- Details of PCR primers used in the present study for real-time quantitative PCR
753 (qPCR). The data include sequences and annealing temperatures (Ta) for primers pairs,
754 amplicon size and accession number.

Transcript	Primer sequence (5'-3')	Amplicon (bp)	Ta (˚C)	Accession No.
gpx4b	F: GGGCAGGTGGTGAAGAGATA R: CACGCTAGGTTTCATCAGGC	217	59	BT044014.1[a]
secp43	F:CACTGGCAACCCTTGGAATC R:CAAGGACTGGGGATATGCC	185	59	BT050015.1[a]
gpx7	F:TGGGAAAGTCATGGATGCCT R:GCTCAGGGTGTTTTGTTGCA	196	59	NM_001140889.2[a]
sepk	F: CCCATCCTCTACAGCTGCTT R: GGGACAAGAAGAGGGGTCTG	122	59	NM_00114091.2[a]
sepp	F: CACCTTCACACCTTGCTGAG R: CAGTCCCCACAGATGCTTTG	233	59	BT072527.1[a]
cat	F: AGGCCCTACTCAACAATGCT R: TAGGATCTTGGACAGCAGGC	162	59	NM_001140302.1[a]
sod	F: GGACCCCACTCTATCATCGG R: GGGTAAGCTACGGTGGTCTT	215	59	NM_001123587.1[a]
cof2	F: AGCCTATGACCAACCCACTG R: TGTTCACAGCTCGTTTACCG	224	60	TC63899[b]
elf1α	F:CTGCCCCTCCAGGACGTTTACAA R: CACCGGGCATAGCCGATTCC	175	60	AF321836[a]

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756 *gpx4b*, glutathione peroxidase 4b; *gpx7*, glutathione peroxidase 7; *sepp*, selenoprotein P; *sepk*;
757 selenoprotein K; *secp43*; tRNA selenocysteine associated protein1; *cat*, catalase; *sod*, superoxide
758 dismutase; *cof2*, cofilin 2; *elf1α*, elongation factor 1α.

759 [a]GenBank (http://www.ncbi.nlm.nih.gov/)

760 [b]Atlantic salmon Gene Index (http://compbio.dfci.harvard.edu/tgi/)

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762 Table 4.- Growth performance at the end of the experimental period.

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	LV		MV		HV	
	Mean	SEM	Mean	SEM	Mean	SEM
Start weight (g)	107.70	0.46	109.10	1.74	106.90	0.35
End weight (g)	2737.60	30.71	2692.00	39.62	2573.00	44.51
SGR (%/day)	1.28	0.01	1.27	0.01	1.26	0.01
TGC (g (days/^{o} C))	3.12	0.02	3.09	0.03	3.03	0.03
FCR	1.01^{b}	0.01	1.01^{b}	0.01	1.09^{a}	0.02
Survival rate (%)	98.0	0.40	98.8	0.61	98.8	0.22

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765 Diets LV to HV represent feeds with increasing levels of PP/VO as described in Materials and
766 Methods section. Data are presented as mean ± SEM (n=3). Different superscript letters denote
767 statistical differences as determined by one-way ANOVA followed by Turkey's test ($p<0.05$). SGR,
768 Specific growth rate; TGC, Thermal Growth Coefficient; FCR, Feed conversion ratio.

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Table 5.- Selected fatty acid compositions, peroxidation index and TBARS content of the flesh (Norwegian quality cut, NQC) of Atlantic salmon at 1000 g, 2000 g and 3000 g fed three experimental feeds with reducing levels of fish meal and oil and increasing levels of vegetable oil and meal.

	LV		MV		HV	
	Mean	SEM	Mean	SEM	Mean	SEM
NQC 1000 g						
Lipids	9.0	0.7	8.0	0.4	8.4	0.7
ΣSAFA[1]	21.0^{a}	0.3	20.0^{a}	0.3	17.2^{b}	0.1
OA	28.2^{c}	0.9	33^{b}	0.2	38.2^{a}	0.6
ARA	0.3^{a}	0.0	0.3^{a}	0.0	0.2^{b}	0.0
Σn-6 PUFA[2]	14.6	0.9	15.2	0.4	17.4	0.6
EPA	3.5	0.5	2.9	0.0	2.3	0.1
DPA	2.2	0.6	1.0	0.2	0.9	0.0
DHA	6.2^{a}	0.1	5.3^{a}	0.1	3.9^{b}	0.2
Σn-3 PUFA[3]	16.6^{a}	1.0	15.3^{b}	0.2	13.6^{b}	0.5
n-3/n-6	1.1	0.1	1.0	0.0	0.8	0.0
PIn	119.0^{a}	4.2	110.8^{b}	2.1	87.3^{c}	2.1
TBARS	2305.2	172.1^{a}	1097.5	165.7^{b}	1393.5	291.1^{ab}
NQC 2000 g						
Lipids	10.2	0.1	10.7	0.1	11.3	0.3
ΣSAFA[1]	22.7^{a}	0.4	20.3^{b}	0.5	17.2^{c}	0.5
OA	26.6^{c}	1.0	30.4^{b}	0.2	37.0^{a}	1.5
ARA	0.4^{a}	0.0	0.2^{b}	0.0	0.2^{b}	0.0
Σn-6 PUFA[2]	14.3	0.9	18.8	0.2	21.5	2.3
EPA	3.4^{a}	0.5	2.9^{b}	0.1	2.0^{c}	0.1
DPA	1.3	0.2	1.3	0.1	0.8	0.0
DHA	6.3^{a}	0.4	4.6^{b}	0.2	3.5^{c}	0.3
Σn-3 PUFA[3]	16.3	0.8	14.4	0.6	12.1	0.6
n-3/n-6	1.1^{a}	0.0	0.8^{b}	0.0	0.6^{b}	0.1
PIn	112.4^{a}	6.5	99.6^{ab}	4.0	83.5^{b}	0.9
TBARS	2103.8	119.3^{a}	1715.8^{ab}	84.0	1332.3^{b}	125.7
NQC 3000 g						
Lipids	12.7	0.6	11.3	0.5	12.2	0.3
ΣSAFA[1]	21.2^{a}	0.4	18.9^{b}	0.2	17.6^{b}	0.3
OA	25.0^{c}	0.3	29.0^{b}	0.1	33.1^{a}	0.0
ARA	0.5^{a}	0.0	0.4^{b}	0.0	0.3^{c}	0.0
Σn-6 PUFA[2]	14.4^{c}	0.1	19.9^{b}	0.0	25.0^{a}	0.3
EPA	4.8^{a}	0.2	3.5^{b}	0.1	2.2^{c}	0.0
DPA	2.0^{a}	0.1	1.5^{b}	0.0	0.9^{c}	0.0
DHA	7.2^{a}	0.4	5.8^{b}	0.2	3.8^{c}	0.0
Σn-3 PUFA[3]	19.7^{a}	0.8	16.7^{b}	0.4	12.9^{c}	0.1
n-3/n-6	1.4^{a}	0.0	0.8^{b}	0.0	0.5^{c}	0.0
PIn	130.8^{a}	5.5	113.8^{b}	3.3	90.8^{c}	0.8
TBARS	2233.9^{a}	121.1	1802.9^{b}	122.9	1199.4^{c}	72.2

Lipids, %; Fatty acids, % of total fatty acids; TBARS, nmol MDA/ g lipid. Data expressed as means ± SEM (n=3 for fatty acids; n=6 for TBARS). Different superscript letters within a row denote significant differences among diets for a given sampling point. Statistical differences were determined by one-way ANOVA with Tukey's comparison test ($p<0.05$). Diets LV to HV represent feeds with increasing levels of PP/VO in three feeding periods as described in Materials and Methods section. [1]contains 14:0, 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0; [2]contains 18:2n-6, 18:3n-6, 20:2n-6, and 20:3n-6; [3]contains 18:3n-3, 18:4n-3, 20:3n-3 and 20:4n-3. SAFA, saturated fatty acids; OA, oleic acid; ARA, arachidonic acid; PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA; docosahexaenoic acid; PIn, peroxidation index; TBARS, thiobarbituric reactive substances.

804 Table 6.- Effects of the dietary treatment, fish weight and their interaction on flesh and liver
805 selected fatty acids, PIn and TBARS of Atlantic salmon fed increasing substitution levels of
806 vegetable oil and meal.

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	Two-way ANOVA p values					
	Flesh			Liver		
	Weight	**Diet**	**W*D**	**Weight**	**Diet**	**W*D**
Lipids	n.s.	n.s.	n.s.	n.s.	*	n.s.
ΣSAFA	*	***	n.s.	n.s.	***	n.s.
OA	n.s.	***	n.s.	***	***	n.s.
ARA	***	***	n.s.	***	***	n.s.
Σn-6 PUFA	***	***	n.s.	***	***	**
EPA	*	***	n.s.	***	***	**
DPA	n.s.	***	n.s.	***	**	n.s.
DHA	*	***	n.s.	**	***	n.s.
Σn-3 PUFA	*	***	n.s.	**	***	n.s.
n-3/n-6	**	***	*	n.s.	***	n.s.
PIn	n.s.	**	n.s.	**	***	n.s.
TBARS	n.s.	***	n.s.	***	**	n.s.

817 *p<0.05; **p<0.01; ***p<0.001; n.s., indicates no significant differences. W, weight; D, diet; SAFA,
818 saturated fatty acids; OA, oleic acid; ARA, arachidonic acid; PUFA, polyunsaturated fatty acids;
819 EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA; docosahexaenoic acid; PIn,
820 peroxidation index; TBARS, thiobarbituric reactive substances

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Table 7.- Selected fatty acid compositions, peroxidation index and TBARS content of the liver of Atlantic salmon at 1000 g, 2000 g and 3000 g fed the three experimental feeds with reducing levels of fish meal and oil and increasing levels of vegetable oil and meal.

	LV		**MV**		**HV**	
	Mean	SEM	Mean	SEM	Mean	SEM
Liver 1000 g						
Lipids	5.8	1.3	5.5	0.3	5.6	0.3
ΣSAFA[1]	19.5	2.2	20.7	0.4	17.9	0.6
OA	28.9	3.8	31.5	1.3	35.1	1.1
ARA	1.5	0.3	1.3	0.1	1.1	0.1
Σn-6 PUFA[2]	15.1	3.8	12.4	0.3	15.4	0.3
EPA	5.2	1.0	4.7	0.3	4.0	0.2
DPA	1.6	0.4	1.4	0.1	1.1	0.1
DHA	12.2	2.9	11.5	0.5	9.9	0.3
Σn-3 PUFA[3]	22.4	3.9	21.1	1.0	18.9	0.4
n-3/n-6	1.8	0.6	1.7	0.1	1.2	0.0
PIn	169.4	49.0	156.3	12.8	140.4	6.3
TBARS	846.4[ab]	33.3	1008.7[a]	61.4	831.1[b]	38.5
Liver 2000 g						
Lipids	3.9[b]	0.3	4.0[b]	0.1	5.6[a]	0.6
ΣSAFA[1]	22.9[a]	0.8	22.0[a]	0.7	17.4[b]	0.8
OA	19.3[b]	1.4	20.6[b]	0.4	27.2[a]	1.4
ARA	2.4[a]	0.1	2.3[a]	0.2	1.4[b]	0.2
Σn-6 PUFA[2]	13.4[c]	0.2	18.4[b]	0.2	27.1[a]	1.0
EPA	8.7[a]	0.4	7.3[a]	0.3	4.1[b]	0.4
DPA	2.6[a]	0.1	2.0[b]	0.1	1.1[b]	0.1
DHA	16.7[a]	1.2	16.5[a]	0.6	10.2[b]	1.4
Σn-3 PUFA[3]	30.8[a]	1.6	28.7[a]	0.8	18.9[b]	1.9
n-3/n-6	1.7[a]	1.2	1.6[b]	0.1	0.7[c]	0.0
PIn	323.8	22.1	223.6	12.0	155.2	27.3
TBARS	1113.2[a]	65.0	1101.0[a]	54.5	773.8[b]	73.8
Liver 3000 g						
Lipids	4.4	0.3	4.1	0.1	6.2	1.3
ΣSAFA[1]	22.7[a]	0.9	20.8[ab]	0.5	17.6[b]	1.2
OA	22.0[b]	1.6	20.9[b]	0.9	31.3[a]	2.4
ARA	2.5[a]	0.1	2.3[a]	0.1	1.3[b]	0.2
Σn-6 PUFA[2]	11.4[c]	0.5	16.2[b]	0.2	21.9[a]	0.5
EPA	6.4[a]	2.6	8.1[a]	0.4	4.3[b]	0.6
DPA	2.6[a]	0.0	2.1[b]	0.1	1.1[b]	0.1
DHA	17.8[a]	0.6	17.3[a]	0.8	9.4[b]	1.5
Σn-3 PUFA[3]	29.6[a]	2.9	30.7[a]	1.2	18.4[b]	2.2
n-3/n-6	2.6[a]	0.4	1.9[a]	0.1	0.8[b]	0.1
PIn	223.3	31.4	234.6	16.3	144.6	29.2
TBARS	600.9[a]	36.7	602.5[a]	31.9	418.3[b]	24.8

Lipids, %; Fatty acids, % of total fatty acids; TBARS, mmol MDA g/lipid. Data expressed as means ± SEM (n=3 for fatty acids; n=6 for TBARS). Different superscript letters within a row denote significant differences among diets for a given sampling point. Statistical differences were determined by one-way ANOVA with Tukey's comparison test ($p<0.05$). Diets LV to HV represent feeds with increasing levels of PP/VO in three feeding periods as described in Materials and Methods section. [1]contains 14:0, 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0; [2]contains 18:2n-6, 18:3n-6, 20:2n-6, and 20:3n-6; [3]contains 18:3n-3, 18:4n-3, 20:3n-3 and 20:4n-3. SAFA, saturated fatty acids; OA, oleic acid; ARA, arachidonic acid; PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA; docosahexaenoic acid; PIn, peroxidation index; TBARS, thiobarbituric reactive substances.

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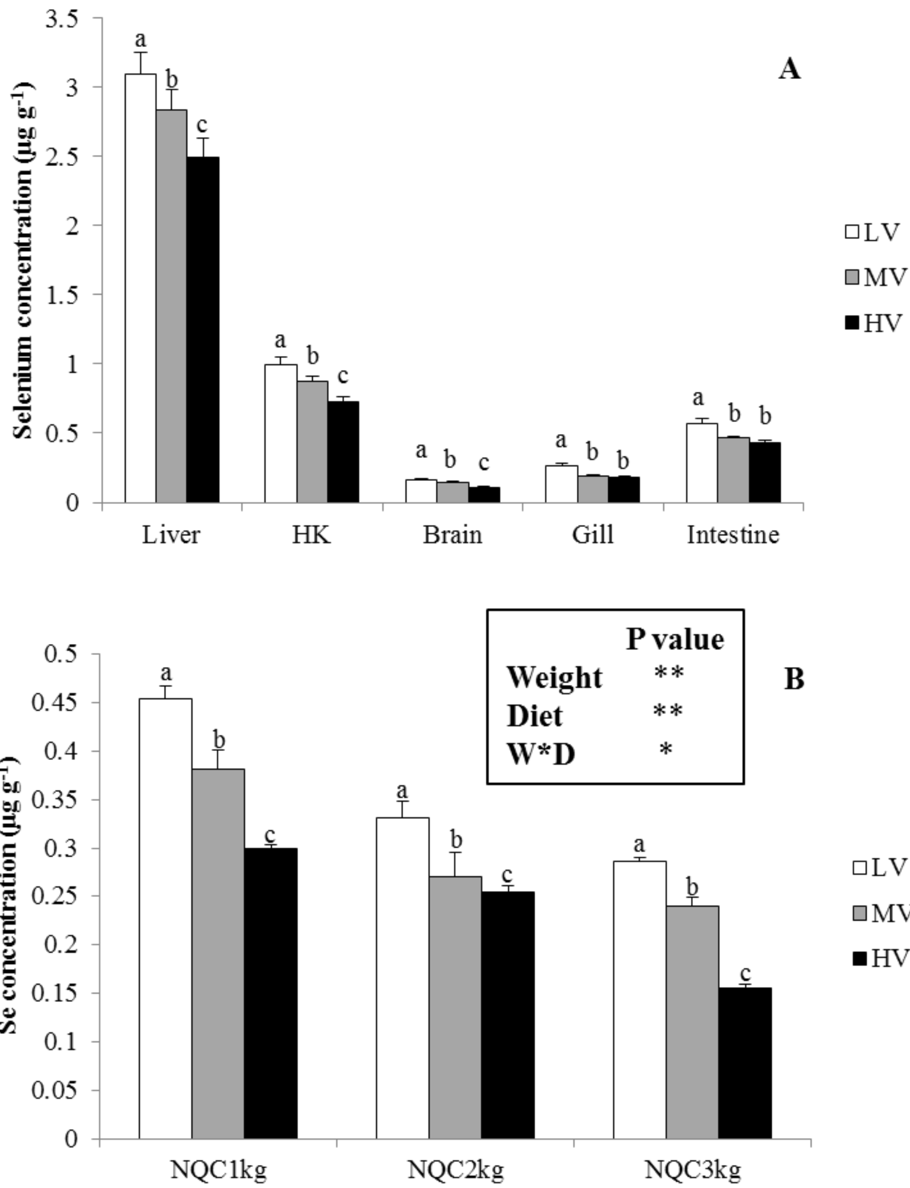
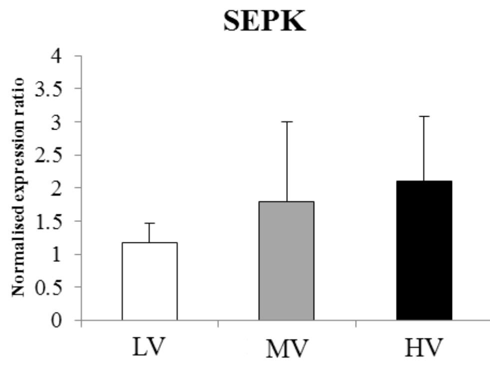
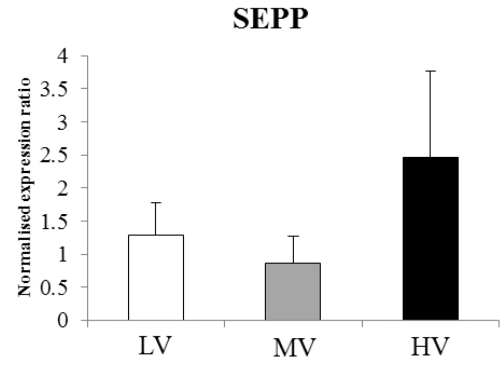
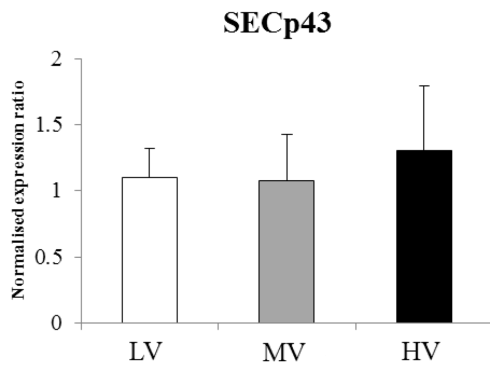
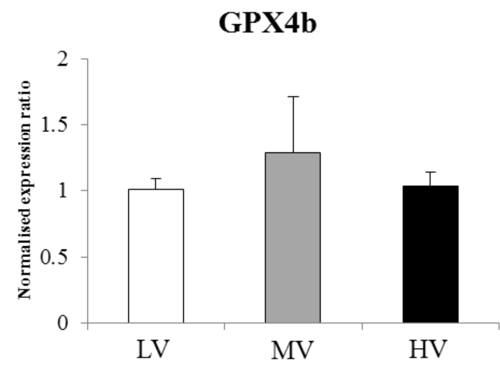


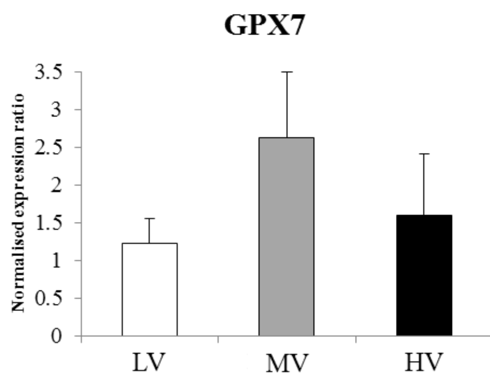
Figure 1

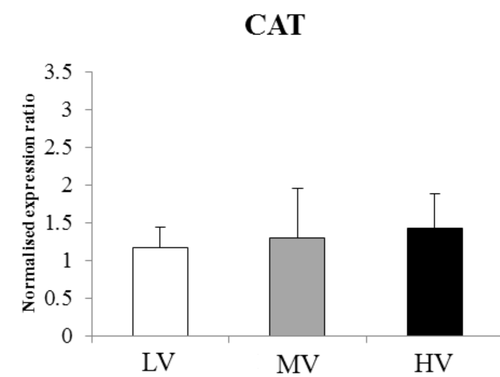












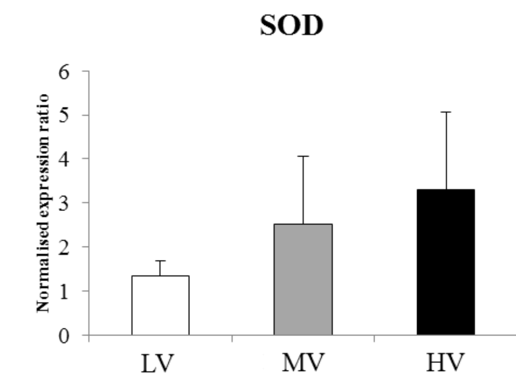


Figure 2

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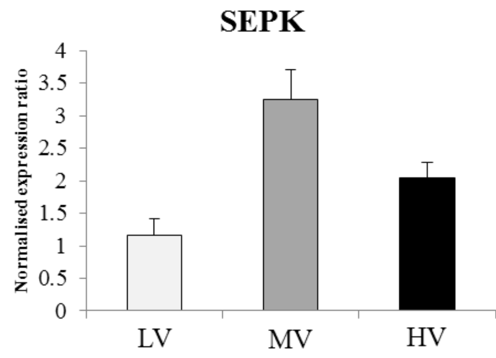

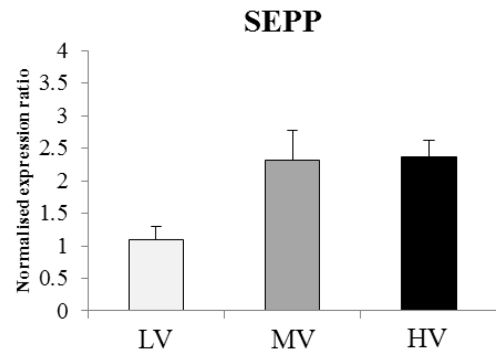

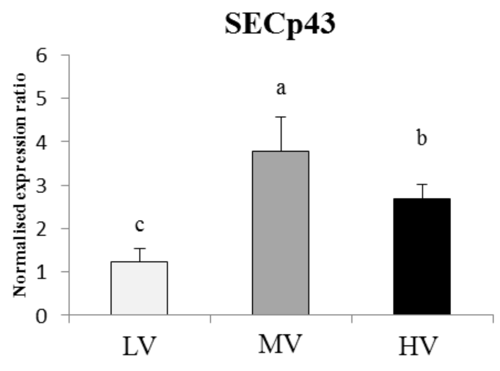

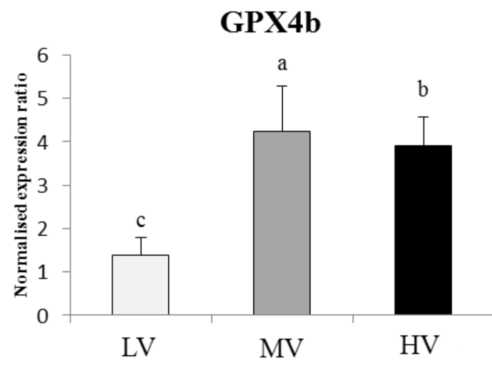

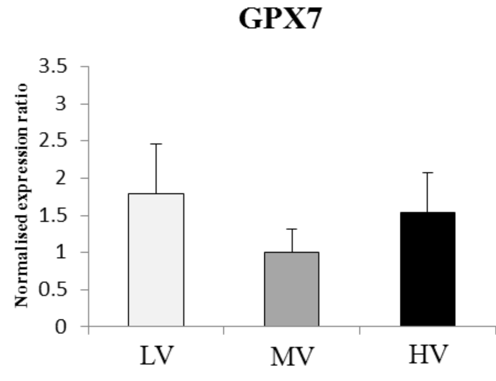

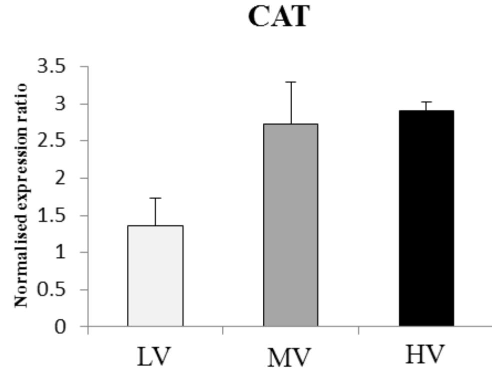

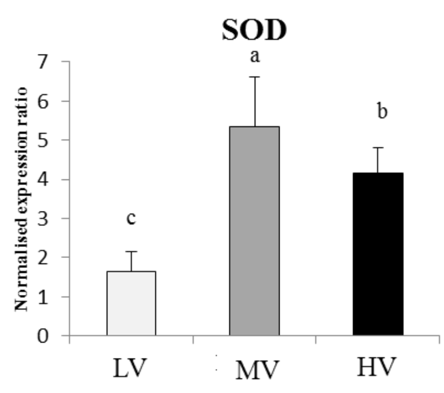

Figure 3

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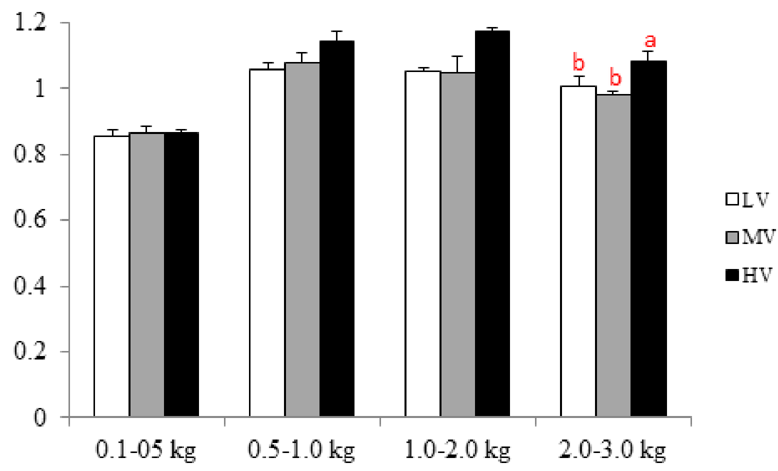

Supplementary Figure 1

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