Antioxidant nutrition in Atlantic salmon (*Salmo salar*) parr and post-smolt, fed diets with high inclusion of plant ingredients and graded levels of micronutrients and selected amino acids

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The shift from marine to plant based ingredients in fish feeds affects the dietary concentrations and bioavailability of micronutrients, amino acids and lipids and consequently warrants a re-evaluation of dietary nutrient recommendations. In the present study, an Atlantic salmon diet high in plant ingredients was supplemented with graded levels of nutrient premix (NP), containing selected amino acids, taurine, cholesterol, vitamins and minerals. This article presents the results on the antioxidant nutrients vitamin C, E and selenium (Se), and effects on tissue redox status. The feed ingredients appeared to contain sufficient levels of vitamin E and Se to cover the requirements to prevent clinical deficiency symptoms. The body levels of α-tocopherol (TOH) in parr and that of Se in parr and post-smolt showed a linear relationship with dietary concentration, while α-TOH in post-smolt seemed to be saturable with a breakpoint near 140 mg kg^{-1}. Ascorbic acid (Asc) concentration in the basal feed was below the expected minimum requirement, but the experimental period was probably too short for the fish to develop visible deficiency symptoms. Asc was saturable in both parr and post-smolt whole body at dietary concentrations of 190 and 63-89 mg kg^{-1}, respectively. Maximum whole body Asc concentration was approximately 40 mg kg^{-1} in parr and 14 mg kg^{-1} in post-smolt. Retention ranged from 41 to 10% in parr and from -206 to 12% in post-smolt with increasing NP supplementation. This indicates that the post-smolts had an extraordinarily high consumption of Asc. Analyses of glutathione (GSH) and glutathione disulphide (GSSG) concentrations and the calculated GSH based redox potentials in liver and muscle tissue, indicated only minor effects of diets on redox regulation. However, the post-smolt were more oxidized than the parr. This was supported by the high consumption of Asc and high expression of gpx1 and gpx3 in liver. Based on the present trials, the recommendations for supplementation of vitamin C and E in diets for Atlantic salmon are similar to current practices, e.g 150-200 mg kg^{-1} of α-TOH and 190 mg kg^{-1} Asc which was the saturating concentration in parr. Higher concentrations than what would prevent clinical deficiency symptoms are necessary to protect fish against incidents of oxidative stress and to

improve immune and stress responses. There were no indications that the Se requirement exceeded the current recommendation of 0.3 mg kg^{-1}.

1 **Antioxidant nutrition in Atlantic salmon ([i]*Salmo salar*[i]) parr and post-smolt, fed diets with high**
2 **inclusion of plant ingredients and graded levels of micronutrients and selected amino acids**

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20   **Abstract**

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23   dietary nutrient recommendations. In the present study, an Atlantic salmon diet high in plant

24   ingredients was supplemented with graded levels of nutrient premix (NP), containing selected amino

25   acids, taurine, cholesterol, vitamins and minerals. This article presents the results on the antioxidant

26   nutrients vitamin C, E and selenium (Se), and effects on tissue redox status. The feed ingredients

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28   deficiency symptoms. The body levels of α-tocopherol (TOH) in parr and that of Se in parr and post-

29   smolt showed a linear relationship with dietary concentration, while α-TOH in post-smolt seemed to be

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35   parr and from -206 to 12% in post-smolt with increasing NP supplementation. This indicates that the

36   post-smolts had an extraordinarily high consumption of Asc. Analyses of glutathione (GSH) and

37   glutathione disulphide (GSSG) concentrations and the calculated GSH based redox potentials in liver and

38   muscle tissue, indicated only minor effects of diets on redox regulation. However, the post-smolt were

39   more oxidized than the parr. This was supported by the high consumption of Asc and high expression of

40   *gpx1* and *gpx3* in liver. Based on the present trials, the recommendations for supplementation of

41   vitamin C and E in diets for Atlantic salmon are similar to current practices, e.g 150-200 mg kg^{-1} of α-TOH

42   and 190 mg kg^{-1} Asc which was the saturating concentration in parr. Higher concentrations than what

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44   stress and to improve immune and stress responses. There were no indications that the Se requirement

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Introduction

The shift from marine to plant based ingredients in fish feeds leads to changes in dietary concentrations and bioavailability of several nutrients. So far, research on plant based diets for Atlantic salmon has mainly focused on balancing the amino acid profile and reducing the amounts of antinutrients (Espe et al. 2006; Espe et al. 2007; Krogdahl et al. 2005), while micronutrients have received far less attention. In the present study, two feeding experiments were conducted on salmon, one with parr in freshwater and one with post-smolt in seawater, using a basic diet containing 10% fishmeal and 3.5% fish oil of the total recipe, in addition to plant based protein and lipid sources. A nutrient premix (NP), containing potentially critical micronutrients, amino acids, taurine and cholesterol, was added at graded levels to this diet and health indicators and biomarkers were used to estimate optimal supplementation of the different nutrients. Hemre et al. (in prep.) presents data on general fish performance, retention of macronutrients, B-vitamins and amino acid metabolism. Data on selected minerals and vitamin A, D and K are presented by Lock et al. (in prep). The present publication is related to antioxidant nutrients and redox regulation.

Vitamin E is the general term for a group of lipid soluble antioxidants, tocopherols and tocotrienols, where α-tocopherol (α-TOH) has the highest biological activity (United States Pharmacopeia 1993). α-TOH is selectively retained in the body of mammals (Kayden & Traber 1993; Rigotti 2007) and Atlantic salmon (Hamre 2011; Hamre et al. 1998), possibly due to a liver TOH binding protein, with high affinity for this isomer (Kayden & Traber 1993; Yoshida et al. 1992). Vitamin E breaks the chain of lipid peroxidation by reducing fatty acid peroxide radicals, preventing oxidation of new fatty acids (Buettner 1993). It takes approximately 3 months for Atlantic salmon parr to adjust their body concentration of α-TOH to the dietary concentration and there is a linear relation between dietary and whole body concentrations when dietary α-TOH ranges between 0 and 300 mg kg^{-1}. The slope is quite reproducible between experiments, at approximately 0.25 between fish concentration on wet weight and feed concentration on dry weight (Hamre et al. 1997; Hamre & Lie 1995). Vitamin E is added as α-tocopheryl acetate (α-TOAc) in fish feed, thereby protected against oxidation. The requirement is highly variable, in rainbow trout (*Oncorhynchus mykiss)* dietary requirements between 5 and 100 mg kg^{-1} have been reported, dependent on dietary composition and experimental conditions. Factors that affect the vitamin E requirement are dietary PUFA, vitamin C and Se concentrations and oxidized feed (Hamre 2011). Furthermore, the estimated requirement varies with the response variable used. Hemoglobin concentration is a sensitive indicator of overall health and growth performance while indicators of immune function respond at higher dietary levels of vitamin E and will often give very high requirement assessments (Hamre 2011). In the present

79   study, γ-TOH is used as a representative of the non-α-TOHs, since it is often present in plant oils at high

80   concentrations.

81   In classical nutrition, vitamin C (ascorbic acid, Asc) has a well defined role as a cofactor for the enzymes

82   catalyzing the hydroxylation of proline and lysine, necessary for formation of collagen and bone matrix

83   (Barnes & Kodicek 1972; Gould et al. 1960; Terova et al. 1998). The function of Asc is to keep the iron

84   present at the enzymes' active site in the reduced state (Meister 1994). More generally, Asc is a water-

85   soluble antioxidant, it scavenges free radicals and probably participates in recycling oxidized vitamin E

86   (Hamre et al. 1997; Tappel 1962). The resulting Asc radical or dehydroascorbic acid can in turn be reduced

87   by glutathione (GSH) and ultimately by NADPH produced in energy metabolism (Mrtensson & Meister

88   1991). Crystalline Asc is very unstable, susceptible to leakage from feed pellets immersed in water and to

89   oxidation. It was therefore not until stable and fully bioavailable forms of vitamin C were developed that

90   meaningful requirement studies in fish could be designed (Woodward 1994). Sandnes et al. (1992)

91   performed one of the first of such studies in Atlantic salmon using Ca ascorbate-monophosphate (AP).

92   They found a minimum dietary requirement, based on growth, mortality and skin and backbone

93   hydroxyproline concentration, of 10-20 mg kg^{-1}. Severe deficiency symptoms appeared first after 18 weeks

94   of feeding the non-supplemented diet. Concentration of Asc in the liver was linearly related to dietary AP

95   up to 160 mg kg^{-1} Asc equivalents. These findings were confirmed by Hamre *et al*. (1997), however, the

96   appearance of vitamin C deficiency symptoms depended on the dietary concentration of vitamin E and is

97   probably affected by other variations in the experimental conditions as well (Dabrowski et al. 2004;

98   Gabaudan & Verlhac 2001; Sandnes et al. 1992). It is common for several fish species that dietary Asc

99   required for maximum body or tissue storage surpasses the requirement levels for growth, survival and

100   hydroxyproline concentrations (NRC 2011). Furthermore, immune response indicators are stimulated by

101   Asc levels far above conventional requirements, which also protect against stress (Trichet 2010; Waagbø

102   1994; Waagbø 2006).

103   Selenium (Se) is an essential trace element inserted in selenocysteine (Sec), situated at the active site of

104   Se dependent proteins, termed selenoproteins (Brigelius-Flohe 1999). The selenoproteins can be grouped

105   in stress-related and housekeeping proteins, the first group responds readily to dietary Se and includes

106   glutathione peroxidase (GPX) 1 and 3 (Penglase et al. 2014). In mammals, the dietary Se level at which

107   GPX1 activity and gene expression level off, is commonly used as an indicator of the Se requirement

108   (Sunde et al. 2009; Weiss et al. 1996; Weiss et al. 1997). GPX1 activity in rainbow trout plasma followed a

109   similar trajectory to that in mammalian tissues and leveled off at dietary Se levels (given as Na_2SeO_3)

110   between 0.15 and 0.38 mg kg^{-1} (Hilton et al. 1980). However, in zebrafish, maximum growth correlated

111   with minimum whole body GPX1 activity and mRNA expression at 0.3 mg kg^{-1} dietary Se. Dietary Se above

112   this level reduced growth and increased GPX1 activity and expression (Se given as SelPlex, Se enriched

113   yeast which contains mainly selenomethionine; Penglase et al., 2014). Likewise, in whole cod larvae, 3.0

114   compared to 0.7 mg kg^{-1} dietary Se had a negative effect on growth early in development, whereas GPX1

115   activity and expression were stimulated (Penglase et al. 2010). This may indicate that the relation between

116   GPX1 and Se requirement is different in fish and mammals. There was a linear relationship between

117   dietary Se in the range 0.1 to 1.0 mg kg^{-1} and whole body Se concentrations in the zebrafish study

118   (Penglase et al. 2014).

119   The cellular redox environment affects the cell's fate because gene expression, protein function and

120   molecular pathways are often redox sensitive (Go & Jones 2013; Jones & Sies 2015; Kirlin et al. 1999). The

121   key mechanism is that protein cysteine residues switch between oxidized and reduced states

122   corresponding to active and inactive states of the involved proteins (redox switches). An example is that

123   a more oxidized cellular environment induces a redox switch that releases the nuclear factor-erythroid 2-

124   related factor 2 (NFE2L2) transcription factor from a complex with another protein, KEAP1. Once released,

125   NFE2L2 induces the transcription of at least 50 mammalian genes; many of which code for antioxidants,

126   thiol oxidoreductases and glutathione synthesis/recycling genes; that are involved in maintaining the

127   redox balance and/or are involved in redox signaling (reviewed byMa 2013). Cellular redox homeostasis

128   is thought to be maintained by redox couples that act as electron buffers due to their ability to readily

129   cycle between oxidized and reduced forms (Jones & Sies 2015). The major redox couples are

130   reduced/oxidized glutathione (2GSH/GSSG), cysteine (2Cys/CySS) and thioredoxin (Trx(SH)$_2$/TrxSS)

131   (Huseby et al. 2009) and the related redox potential (E) is proportional to the ratio between the (reduced)^2

132   and oxidized forms of the redox couples, according to the Nernst Equation. GSH/GSSG is present in cells

133   in high concentrations. It may therefore be the most important cellular redox couple and is used as an

134   indicator of tissue redox state in the present study.

135   Using a multivariate design, we studied the variation of the 2GSH/GSSG couple in juvenile Atlantic salmon

136   (*Salmo salar*) supplemented with dietary vitamins C and E, astaxanthin, lipid, iron, copper and manganese

137   at levels just above the requirement or just below anticipated toxicity/over supplementation. This

138   resulted in variation of less than 10 % in the GSH/GSSH concentrations and more or less constant redox

139   potentials (E$_{GSH}$) in muscle, liver and plasma, which were also similar after 14 and 23 weeks of feeding the

140   experimental diets (Hamre et al. 2010b). In Atlantic salmon post-smolts, organ specific E$_{GSH}$ therefore

141 seems to be strictly regulated and stable with regard to variation of the nutrients used in this study. A

142 hypothesis of the present study is therefore that GSH/GSSG concentrations and E_{GSH} are stable in healthy

143 fish and can be used as indicators of fish welfare during the growth phase in salmon. On the other hand,

144 the 2GSH/GSSG redox couple and many genes coding for proteins that maintain the cellular redox system

145 are dynamically regulated during fish embryonic and larval development (Hamre et al. 2014; Penglase et

146 al. 2015; Skjærven et al. 2013; Timme-Laragy et al. 2013).

147 Here we present data on antioxidant nutrients and redox regulation of two feeding trials (freshwater and

148 seawater) with graded NP levels. Tissue concentrations and retention of vitamin C, E and Se, traditionally

149 viewed as the main antioxidant nutrients, as well as effects of a graded nutrient premix on tissue

150 GSH/GSSG concentrations and the related redox potentials were analyzed. We have also measured the

151 expression of some central genes for regulation of redox homeostasis in the liver; the transcription factor

152 *nfe2l2*, *cuznsod*, *mnsod*, *cat*, *gpx1* and *gpx3* which metabolize superoxide anions and H_2O_2, *gclc* which

153 translates into the rate limiting protein in GSH synthesis, and *g6pd* and *gr* involved in keeping glutathione

154 in the reduced state.

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158 **Materials and methods**

159

160 *2.1. Experimental diets*

161 The seven experimental diets were produced at Biomar Technology Centre (Denmark), as described in

162 Hemre et al. (in prep). All diets contained the same basal mixture of ingredients (10.4% marine protein

163 ingredients and 3.5% fish oil of the whole receipt, the rest plant ingredients, Table 1). A nutrient premix

164 (NP) was added in graded amounts, replacing some of the field peas in the formulation. Phosphate, lysine,

165 threonine and choline were added to all diets in equal amounts. An antioxidant mixture to protect the

166 feed during production, and yttrium for later digestibility measurements were also added to all diets. Diet

167 acronyms were as follows: 0NP had no addition of the micronutrient premix, then the NP was added in

168 graded amounts to the six diets called 25NP, 50NP, 100NP, 150NP, 200NP and 400NP. The general idea

169 was that the 100NP diets should contain 100% of the assumed requirement, achieved by addition of the

170 nutrient premix to the basal diet (based on available data, primarily for rainbow trout (NRC 2011) for each

171 nutrient). However, some nutrients such as α-tocopherol and Se were already present at or above

172 expected minimum requirements in the basal diet, but these were added in the NP to achieve increasing

173 levels in the 7 diets and to examine if higher requirements could be assessed. The 25NP diet contained

174 0.25 times the NP from the 100NP diet, while the 400NP contained 4 times the NP from the 100NP diet.

175 As all nutrients were also present in the diet ingredients to some extent (not only contributed by the NP),

176 the 400NP diet would never have 4 times higher content than the 100NP diet, and the fold difference

177 would vary depending on the contribution from the NP versus the diet ingredients for each nutrient. The

178 NP contained vitamin D_3, α-tocopheryl acetate, vitamin K_3, vitamin A_1, ascorbyl monophosphate, vitamin

179 B_6, biotin, cobalamin, folate, pantothenic acid, riboflavin, thiamine, niacin, Se (as inorganic sodium

180 selenite), iodine, copper, cobalt, manganese, zinc, crystalline DL-methionine, and taurine. Crystalline L-

181 histidine and cholesterol were also added in graded amounts. The analyzed composition of the diets can

182 be found in Table 2, including proximate composition and contents of the micronutrients focused in this

183 presentation.

184

185 *2.2 Feeding trials*

186 Both feeding trials were conducted in accordance with Norwegian laws and regulations concerning

187 experiments with live animals, which are overseen by the Norwegian Food Safety Authority.

188   Trial 1: The trial with parr in freshwater took place at the Institute of Marine Research (Matredal, 61°N,

189   Western Norway). The salmon were hatched in February, and in June the salmon parr were randomly

190   distributed in fifteen 400 litre (1x1x0.4m^3) experimental tanks and acclimated for 1 week while being fed

191   a commercial feed (Ewos). The trial commenced on July 3rd, with duplicate tanks for each diet with the

192   exception of NP100 that was run in triplicates. Each tank contained 100 fish with mean initial body weight

193   of 18.3±2.2 g. The fish were fed *ad libitum* with continuous feeding from automated feeders during night

194   and day. However, care was taken to limit overfeeding, due to uncertainties in the collection of uneaten

195   feed at such a small pellet size. Collection and weighing of uneaten feed was conducted daily at 13:00,

196   with the exception of weekends. The fish were exposed to continuous light, and oxygen saturation was

197   monitored on a regular basis and was never below 75%. The fish were reared in freshwater, but with

198   seawater added as a buffer, creating a salinity of 1.1 to 1.3 g L^{-1}.The temperature was kept constant

199   throughout the experiment, at 12.4 °C (SD ±0.7). The total duration of the feeding trial was 12 weeks, e.g.

200   84 days.

201   Trial 2: Post-smolt Atlantic salmon were randomly distributed among fifteen sea cages (5m x 5m x 5m;

202   125 m^3; 150 fish per cage) at Gildeskål Research Station, GIFAS, Gildeskål kommune, Norway (67°N,

203   Northern Norway). Prior to the start of the trial, fish were acclimated to the environmental conditions for

204   two weeks, the feeding trial started in January 2013. At start, the average fish weight was 228 ± 5 g and

205   during the 157 day feeding period the fish more than doubled in weight. As in standard aquaculture

206   practice, fish were reared under 24h light regime before the start of the trial and during the first 3 months

207   of the experiment. Cages were illuminated by four 400W IDEMA underwater lights that were positioned

208   at the centre of each block of four cages at a depth of three meters. Fish were hand-fed to satiation two

209   times daily and feed intake was recorded for each sea cage. Total feed intake and mortality were recorded

210   daily. Water temperature, salinity and oxygen saturation over the course of the trial varied from 4.1

211   (January) to 10°C (June), 30-34.2 g L^{-1}, and 8.7-12.2 mg/L, respectively.

212

213   *2.3 Sampling*

214   Fish were anesthetized (Benzoak® VET, 0.2 ml/L, ACD Pharmaceuticals, Leknes, Norway) and killed by a

215   blow to the head. In Trial 1 , the fish in each tank were bulk weighed for total biomass at each sampling

216   point and body weight and length were measured on individual fish, 5-44 fish depending on sampling

217   point (sexually maturing males were excluded). In Trial 2, individual fish were weighed at each sampling

218 (mid sampling 42 fish per cage, end sampling 34 fish per cage). In both trials, blood was drawn from the

219 caudal vein (*Vena caudalis*) by means of a heparinized medical syringe from 8 fish per tank, before organs

220 were dissected and kept as individual samples. Pooled organ samples based on 10 fish for each tank (liver,

221 gills, muscle) were frozen on dry ice and later homogenized, while individual organ samples were flash

222 frozen in liquid nitrogen. Liver weight and gutted weight were recorded (10 fish per tank).

223

224 *2.4 Chemical analysis of diets, whole fish and organs*

225 Multi-element determination in the feed and tissue samples was done by ICP-MS (inductively coupled

226 plasma mass spectrometry) (Julshamn et al. 1999). HPLC was used for determination of ascorbic acid

227 (Mæland & Waagbø 1998) and tocopherols analysed according to (CEN 1999). TBARS was analyzed

228 according to (Hamre et al. 2001). For the analysis of total (tGSH) and oxidised (GSSG) glutathione,

229 supernatants were prepared from samples using a commercial kit (Prod. No. GT40, Oxford Biomedical

230 Research, Oxford, UK) before analysed at 405 nm in a microplate reader (iEMS Reader Ms, Labsystems,

231 Finland) as described by (Hamre et al. 2014).

232

233 *2.5 Gene expression analysis*

234 Total RNA was purified from frozen liver using the EZ1 RNA Universal Tissue Kit on the BioRobot® EZ1

235 (Qiagen, Hilden, Germany), including the optional DNase treatment step in the protocol. Homogenisation

236 in QIAzol lysis reagent from the kit was performed on the bead grinder homogeniser Precellys 24 (Bertin

237 Technologies, Montigny-le-Bretonneux, France) for 3x10 sec at 6000rpm. Quantity and quality of RNA

238 were assessed with the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies,

239 Wilmington, DE, USA) and a selection of samples were evaluated on the Agilent 2100 Bioanalyzer (Agilent

240 Technologies, Palo Alto, CA, USA), with the 6000 Nano LabChip® kit (Agilent Technologies, Palo Alto, CA,

241 USA).). Average RNA integrity number (RIN) for the samples in Trial 1 (39 samples) was 9.4±0.1

242 (mean±SEM, n=24), in Trial 2, (90 samples): 9.5±01 (mean±SEM, n=11)

243 Reverse transcription (RT) was performed on a GeneAmp PCR 9700 (Applied Biosystems, AB) using the

244 TaqMan® reverse transcriptase kit with oligo(dT) primers (Applied Biosystems). Primer sequences are

245 given in Table 3. Samples were run in duplicate (500 ng, ±5%), in addition to a six point dilution curve in

246 triplicate (1000 to 31.25 ng), non-template and non-amplification controls. Real-time PCR amplification

247 and analysis were performed on a LightCycler 480 Real-time PCR system (Roche Applied Science, Basel,

248 Switzerland) with SYBR® Green I Mastermix (Roche Applied Science).   Pipetting of plates was done using

249 a Biomek®3000 Laboratory automation workstation (Beckman Coulter, Fullerton, USA). Thermal cycling

250 was done for 45 cycles of 10 sec at each of 95°C, 60°C and 72°C (basic programme from Roche), followed

251 by melting analysis to confirm that only one product is present.

252 Cycle threshold (Ct) -values were calculated using the second maximum derivative method in the

253 Lightcycler® software. Amplification efficiency was determined using the dilution curves with the formula

254 E=10^(-1/slope), with the slope of the linear curve of Ct-values plotted against the log-dilution (Higuchi et

255 al. 1993). Normalization to the reference genes and data analysis was conducted with GenEx 4.3.5 (MultiD

256 Analyses AB, Gothenburg, Sweden) or with the geNorm applet (Vandesompele et al., 2002).

257

258 *2.7 Calculations*

259 Nutrient retention= [(final biomass × final nutrient concentration) – (initial biomass × initial nutrient

260 concentration)] × 100/ (Total feed intake × nutrient concentration in feed).

261 The two-electron half-cell reduction potential of the 2GSH/GSSG redox-couple was calculated according

262 to the Nernst equation:

263 $E_h = E^{0'} - RT/nF \ln([GSH]^2/[GSSG])$

264 where the GSH and GSSG concentrations are in M and E_h is given in volts. $E^{0'}$ is the standard reduction

265 potential at pH7 and 25 °C and was assumed to be -0.240 V (Kemp et al. 2008; Schafer & Buettner 2001).

266 The measurements are the average of whole organs and do not take into account that the reduction

267 potential varies between cell types, and between organelles within the cells (Kemp et al. 2008; Morgan et

268 al. 2013; Schafer & Buettner 2001).

269

270 *2.8 Statistical analyses*

271 GraphPad Prism (ver. 6.05) was used for the regression analyses. For the individual nutrients, the x-axis

272 represented analyzed dietary concentrations, while for glutathione and gene expression data the x-axis

273 represented added NP. Since the nutrient concentrations in the 400%NP diet were outliers in Trial 2, the

274 value of this diet was recalculated to 239%NP, the mean of the concentrations of α-TOH, Asc and Se. Data

275 were fitted to second order polynomials and the fit was then compared to the fit of a first order

276   polynomial. In figures with first order polynomials, the second order polynomial was rejected (p>0.05).

277   The p given in these figures indicates the probability that the slope is equal to 0. In figures with second

278   order fits, p gives the probability that the first order equation fits the data better than the second order

279   equation. None of these equations fitted the data on retention of vitamin C, where a logarithmic equation

280   was applied and p indicates the probability that a second order polynomial has a better fit than the

281   logarithmic function. GraphPad Prism has a function for overall comparison of slopes and intercepts of

282   datasets fitted to linear equations, which was used for comparison of data between Trials 1 and 2. The

283   software also has a function for detecting outliers which was used for the data on muscle GSH/GSSG in

284   post-smolts and on the gene expression data.

285   Correlation analyses of gene expression was performed using the software Statistica (ver. 11, Statsoft Inc.,

286   Tulsa OK) and PCA plots were produced in Sirius (ver 8.1, Pattern Recognition Systems AS, Bergen

287   Norway).

288

290   **Results**

291   Fish performance is reported in detail by Hemre et al. (in prep). Briefly, fish in Trial 1 grew from an initial

292   weight of 18.3 g (± 2.2) to a range of 78.6 g (± 1.9) to 87.3 g (± 4.5). Both fish growth and protein retention

293   increased with increasing dietary NP, while lipid retention decreased, together with liver index and

294   viscera-somatic index. In trial 2, initial fish size was 228 g (± 4.2) and average final weight was 482 g (± 17)

295   There was no effect of the NP on growth or protein and lipid retention in this trial. Survival was high in

296   both trials, close to 100%, and with no difference between diet groups.

297   The dietary concentrations of α-TOH, Asc and Se (Figure S1) were well fitted to first order polynomials in

298   Trial 1 (R^2>0.98). In Trial 2, the diet designated 400% was an outlier, while the other diets showed a good

299   fit to a linear equation (R^2>0.97). The diet planned to contain 400%NP in trial 2 was recalculated to contain

300   239%NP, based on the nutrient analyses of the diets. Gamma-TOH level was similar in all diets, as it was

301   only derived from the feed ingredients (Figure S1). The dietary concentrations at 100% NP were 118 and

302   141 mg kg^{-1} for α-TOH, 67 and 63 mg kg^{-1} for Asc and 0.62 and 0.79 mg kg^{-1} for Se, in Trials 1 and 2,

303   respectively. At 0% NP the dietary concentrations were 48 and 76 mg kg^{-1} for α-TOH, 4.7 and <5.5 mg kg^{-1}

304   ^{1} for Asc and 0.42 and 0.47 mg kg^{-1} for Se (Table 2).

305   There was a linear relationship between dietary and whole body concentrations of α-TOH in Trial 1

306   (R^2=0.94; p (slope=0)<10^{-4}; Figure 1). In Trial 2, a second order polynomial fitted the data better than a

307   first order polynomial (p=0.03). The range of whole body concentrations was between 20 and 60 mg kg^{-1}

308   in Trial 1 and between 30 and 50 mg kg^{-1} in Trial 2. Whole body γ-TOH concentration varied between 5

309   and 10 mg kg^{-1}, with a significant positive slope in Trial 1 (p=0.02) and no effect of diet in Trial 2 (Figure

310   1). Whole body Asc concentration followed a second order polynomial in both trials (Trial 1, R^2=0.92,

311   p<0.0001; Trial 2, R^2=0.88, p=0.016). Whole body concentration plateaued at a dietary concentration of

312   Asc of mg kg^{-1} DM and a whole body concentration of 39.5 mg kg^{-1} in Trial 1. In Trial 2, the plateau was

313   reached at dietary and whole body concentrations of 100 and 14 mg kg^{-1}, respectively. Whole body Se

314   showed a linear relationship with the dietary concentration in both trials (R^2>0.93, p(slope=0)<10^{-4}). The

315   diet dependent whole body concentration was significantly higher in Trial 2 than in Trial 1 (p<10^{-4}).

316   The retention of α-TOH was not affected by diet in Trial 1 and followed a second order polynomial in Trial

317   2 (p=0.02) with the highest retentions at intermediate dietary α-TOH concentrations (Figure 2). Diet did

318   not affect the retention of γ-TOH in Trial 1, while increasing the NP in Trial 2 had a negative effect on γ-

319   TOH retention (p<0.004). The range of retention of both α- and γ-TOH was 20-30%. In Trial 1, retention of

320   Asc ranged from 11-42 % and was negatively linearly related to dietary Asc (R^2=0.34, p(slope=0)=0.02). In

321   Trial 2, vitamin C retention was negative at dietary levels below 63 mg kg^{-1} and max retention was 16% at

322   89 mg kg^{-1}. A logarithmic function was found to have the best fit to the data (R^2=0.92). Diet did not affect

323   retention of Se, which ranged from 20 to 30%. The retention of Se was higher in Trial 2 than in Trial 1

324   ($p<10^{-4}$).

325   GSH and GSSG concentrations were measured in muscle (Figure 3) and liver (Figure 4). In muscle in Trial

326   1, GSH concentrations ranged from 100 to 200 µmol kg^{-1}, GSSG ranged from 0 to 0.4 µmol kg^{-1} with no

327   effect of diet. There was a tendency that the resulting glutathione based redox potential followed a

328   second order polynomial at dietary NP below 150%, so that the tissue became transiently more reduced

329   at supplementation of 25-100% NP than at 150-400% NP (p=0.058). The redox potential of muscle in Trial

330   1 ranged from -240 to -190 mV. In Trial 2, muscle GSH and GSSG concentrations ranged from 43 to 80 and

331   0 to 0.4 µmol kg^{-1}, respectively, and the redox potential from -170 to -220 mV, with no effect of diet. The

332   muscle GSH/GSSG concentrations in these fish were close to quantification limits and some of the data

333   points were removed as outliers. Muscle GSH concentration was higher (p<0.0001), the redox potential

334   lower (p=0.002) and GSSG concentration similar in Trial 1 compared to Trial 2. In the liver, GSH, GSSG and

335   the glutathione based redox potential were not affected by the diet, however there was a tendency

336   towards a significant second order fit to the data on liver redox potential in Trial 1 (p=0.066), implicating

337   a higher redox potential in fish fed diets with intermediate NP additions. Liver GSH concentration was

338   higher (p<0.0001), while GSSG concentration (p=0.01) and the redox potential (p<0.0001) were lower in

339   Trial 1 than in Trial 2.

340   Muscle TBARS (Figure 5) was negatively correlated to NP addition in both trials (p=0.004 in Trial 1, p=0.03

341   in Trial 2) and the absolute level was similar in the two trials (p=0.09).

342   Expression of redox dependent genes was measured in the liver of individual salmon in both trials. PCA

343   plots showed that the dietary groups were not well separated according to expression of the redox related

344   genes (Figure 6). However, in Trial 1 there was a tendency to grouping of samples related to dietary

345   supplementation of NP, with NP supplementation correlating negatively to *g6pd*, *gclc*, *gpx1* and *gr* (-

346   0.38<R^2<-0.43) and positively to *gpx3* (R^2=0.39). *Gr* showed the most covariation with other genes and

347   correlated to *g6pd*, *gpx1* and *gclc* at 0.79>R^2>0.49 and to *mnsod* and *cuznsod* at R^2= 0.39 and 0.34,

348   respectively. *Gr* was also correlated to *nfe2l2* (R^2=0.52), *cat* was not correlated to the other genes while

349   *gpx3* was negatively correlated to the other genes, significant for *g6pd* (R^2=-0.33). In Trial 2, diet was

350   correlated to *cuznsod*, *mnsod* and *cat* (0.39<R^2<0.24). The expression of *gr*, *g6pd*, *gpx1* and *gclc* comprised

351   one distinct group and *mnsod*, *cat* and *cuznsod* another. *Gr* correlated with *g6pd*, *gpx1* and *gclc* at

352 0.67>R²>0.37, while gr correlation to *mnsod*, *cat* and *cuznsod* was 0.32>R²>0.25. *Gpx3* and *nfe2l2* were

353 not significantly correlated to the other genes.

354 Relations between diet and gene expression are given in Figure S1. In Trial 1, increasing supplementation

355 of NP gave a reduction in *gclc*, *g6pd*, *gpx1*, *gr* and *mnsod* expression, while *nfe2l2*, *cat* and *cuznsod*

356 expression were unaffected by diet. In Trial 2, data on *nfe2l2* and *gclc* were fitted to second order

357 polynomials and had higher expression at intermediate NP supplementation. *Gpx1* showed the best fit

358 when using a third order polynomial with peak expression at 50 and 100% NP inclusion. *Gpx3* had higher

359 expression at low NP inclusion, while *mnsod* expression increased with increasing NP. *Cuznsod* expression

360 was slightly lower at intermediate NP inclusion than at high and low inclusion, while *G6pd* and *gr*

361 expression were unaffected by the diet. Average expression of *gpx1, gpx3* and *mnsod* was higher in Trial

362 2 than in Trial 1 (p<0.0002). Increasing NP inclusion had opposite effects on *mnsod* expression in the two

363 trials, which decreased in Trial 1 (p=0.04) and increased in Trial 2 (p=0.002).

364

365 **Discussion**

366 The present study showed differences in redox status between parr and post-smolts and effects of

367 supplementation of the nutrient premix (NP) on redox regulation. However, there were no clear

368 deficiencies of the antioxidant nutrients, vitamin C, vitamin E and Se, even in fish fed the unsupplemented

369 diet. The reason may be that the plant based feed ingredients contained relatively high levels of vitamin

370 E and Se and that the experimental periods were too short for fish of these body sizes to develop vitamin

371 C deficiency symptoms. The basal feed contained less than 5.5 mg kg^{-1} Asc, which is below the minimum

372 requirement in Atlantic salmon of 10-20 mg kg^{-1}, however it took 18 weeks to deplete first-feeding Atlantic

373 salmon for Asc (Sandnes et al. 1992) and a longer period of time may be needed to deplete larger fish

374 sizes as the post-smolts. The final concentrations of α-TOH and Se in the basal feed were at or above

375 minimum requirements at approximately 50 and 0.3 mg kg^{-1} diet, respectively (NRC 2011). Plant oils often

376 have high concentration of tocopherols, however tocopherols are unstable during feed processing and

377 storage and concentrations could vary considerably in the finished feeds (unpublished observations;

378 (Hamre et al. 2010a; Olsvik et al. 2011)). Se concentrations in plant ingredients vary according to Se

379 concentration in soil (Alfthan et al. 2015). So even if α-TOH and Se may be sufficient in some plant

380 ingredients, they should be added to fish feeds to ensure safe supplementation. Commercial fish feeds

381 are commonly supplemented with higher levels of antioxidant nutrients than the minimum requirements

382   given by (NRC 2011), which will protect the fish in periods with oxidative stress, such as high water

383   temperatures (Waagbø et al., unpublished data) elevated water oxygen levels (Lygren et al. 2000),

384   vaccination (Lygren et al. 2001) and disease (Trichet 2010; Waagbø 2006). Accordingly, the diets with

385   100% NP used in this study, contained 118-141 mg kg^{-1} α-TOH, 63-67 mg kg^{-1} Asc and 0.62-0.79 mg kg^{-1}

386   Se, respectively.

387   The most sensitive biomarker of vitamin E deficiency in salmonids is lowered hemoglobin (Hb)

388   concentration (Hamre 2011). There was a slight positive correlation between NP addition and Hb

389   concentration in fish from Trial 1 (p(slope=0)=0.03)(Hemre et al., in prep), however, the slope was not

390   significantly different from 0 after omitting the 400% NP group (p=0.44), so fish fed the diets with low NP

391   supplementation were clearly not vitamin E deficient. In Trial 2, there was no effect of diet on Hb (Hemre

392   et al., in prep). Hamre and Lie (1995) found that mortality due to vitamin E deficiency commenced at

393   whole body levels of α-TOH of 5 mg kg^{-1} wet weight, while the whole body concentration in fish fed the

394   basal diet in this study were 18 and 32 mg kg^{-1} in Trials 1 and 2, respectively. Accordingly, none of the fish

395   groups in this study experienced vitamin E deficiency, even though the low level of vitamin C in the diet

396   without NP would make the fish more susceptible to low dietary vitamin E (Hamre et al. 1997).

397   The diets were fed for 12 and 22 weeks in Trials 1 and 2, respectively. The twelve weeks feeding period in

398   Trial 1 should just be enough for the fish to adjust to the α-TOH concentrations of the diet according to

399   previous reports (Hamre 2011; Hamre & Lie 1995). However, the slope of whole body concentration to

400   dietary concentration was lower (0.13 vs 0.25) and the intercept with the y-axis was higher (15.6 vs 0)

401   than previously reported, indicating that the steady state whole body α-TOH concentration had not yet

402   been obtained (Hamre 1995). A relation of body to dietary concentrations of α-TOH has until now only

403   been measured in very young fish and these relationships were always linear (Hamre et al. 1997; Hamre

404   & Lie 1995). In post-smolts in the present study, the relationship followed a second order polynomial. It

405   is possible that uptake of α-TOH is changing over development, moving from a strict linear relationship

406   just after first-feeding to a saturable mechanism at later stages. If this is the case, the diet 100% NP with

407   141 mg kg^{-1} α-TOH would be sufficient to saturate the system, while supplementation above this gave a

408   decreased retention. In summary, supplementing commercial diets with 150-200 mg kg^{-1} α-TOH is

409   recommended to promote fish health and welfare. A fish feed surveillance program run by NIFES in 2015

410   reported 185-320 mg kg^{-1} α-TOH in 18 commercial salmon feeds, while two feeds contained as high as

411   570 mg kg^{-1} (unpublished results). A variable part of this α-TOH would have been derived from the feed

412   ingredients. One have to take into account that α-TOH in the feed ingredients is in the free form, which is

413 easily broken down in salmon intestine and therefore has quite low retention (Hamre, unpublished

414 results). It is therefore a risk involved in including ingredient contribution when deciding dietary α-TOH

415 supplementation.

416 The non-α-TOHs cannot substitute for α-TOH, because of their lower biological activity (Hamre 2011;

417 Kayden & Traber 1993), however they may have specific biological functions in cell signaling (Golli & Azzi

418 2010; Wallert et al. 2014). In both trials of this study, the retentions of α- and γ-TOH were similar at 20-

419 30%. Due to lower biological activity, retention of γ-TOH should be lower than α-TOH, but the result can

420 be explained by similar retention of α- and γ-TOH in muscle and adipose tissue, which are the main

421 constituents of the whole body sample. Retention of γ-TOH in other vital organs of salmon, such as

422 gonads, intestine, and gills, is less than 30% compared to α-TOH (Hamre & Lie 1997). The body

423 concentration of γ-TOH increased in response to increased analyzed diet concentration in Trial 1, which

424 showed a minor variation even though it was not supplemented in the NP. The effect of diet was driven

425 by lowered levels of γ-TOH in fish fed the diets with 25 and 50% NP, which also had a non-significant

426 tendency of reduced γ-TOH retention. This could indicate that the redox balance of these fish was affected

427 by NP supplementation. Whole body γ-TOH was stable in Trial 2.

428 The whole body concentration of Asc followed a second order equation with respect to Asc

429 supplementation in both trials. It is well known that tissue levels of Asc level off at dietary

430 supplementation that is much higher than the levels where traditional deficiency symptoms are displayed

431 (Gabaudan & Verlhac 2001; Waagbø & Sandnes 1996). Since Asc above this minimum requirement has

432 positive effects on immune function, stress resistance and reproduction (Gabaudan & Verlhac 2001;

433 Waagbø 1994; Waagbø 2006), it may be fruitful to supplement more of the vitamin. In previous studies,

434 Asc concentration in the liver was commonly used as an indicator of Asc status. Whole body concentration

435 was measured in the present study, due to limited amounts of liver sample, and is probably a good

436 indicator of when the system is saturated with the vitamin. The whole body concentration levelled off at

437 a dietary Asc concentration of 190 mg kg^{-1} in Trial 1 and at 63-89 mg kg^{-1} in Trial 2. The maximum body

438 concentration of Asc in fish from Trial 2 was approximately 30% of that in fish from Trial 1. While the data

439 from Trial 1 follow the expected trajectory based on the literature, the fish in Trial 2 seems to have had

440 an extraordinary high consumption of Asc, with negative retention for fish supplemented below 63 mg

441 kg^{-1} and maximum retention of 16% at higher supplementation, compared to max 42% retention in Trial

442 1.

443 There is probably an optimal ratio between supplementation of α-TOH and Asc, based on the hypothesis

444 that vitamin E is recycled by vitamin C. A buildup of tocopheroxyl radicals would occur if too little vitamin

445 C is present to recycle it, which would have a pro-oxidant effect on the tissues (Hamre et al. 1997). An

446 indication of this principle is the higher mortality due to vitamin C deficiency in fish fed 300 compared to

447 150 mg kg^{-1} vitamin E (Hamre et al. 1997). Another indication is that vitamin E and C in tissues from

448 copepods and wild Ballan wrasse are 100 and 500 mg kg^{-1} dry matter and 9 and 18 mg kg^{-1} wet weight,

449 respectively (Hamre et al. 2013; Hamre et al. 2008). Theoretically, one should supplement more Asc than

450 vitamin E, although the exact optimal ratio has not yet been identified. Seventeen commercial salmon

451 feeds analyzed in a fish feed surveillance program performed at NIFES in 2015, contained between 118

452 and 418 mg kg^{-1} Asc, one feed contained 800 and two feeds contained more than 1300 mg kg^{-1} Asc. The

453 concentration of Asc in the feeds was frequently below the concentration of α-TOH (NIFES, unpublished

454 results).

455 Whole body Se concentration followed a first order relationship with the dietary concentration both in

456 parr and post-smolt, and the retention was not affected by NP supplementation. A similar relationship

457 was found in zebrafish, but this species had higher whole body Se concentration at similar

458 supplementation levels. The difference may be species dependent or perhaps due to the use of Se

459 enriched yeast containing mainly selenomethionine as the Se source in the zebrafish (Penglase et al. 2014)

460 and selenite (Na$_2$SeO$_3$) here. Different retention efficiencies of the two Se sources has been shown in

461 Atlantic salmon (Lorentzen et al. 1994). The basal diet contained 0.4-0.5 mg kg^{-1} Se, while the requirement

462 in rainbow trout is estimated at 0.15-0.30 mg kg^{-1} (Hilton et al. 1980). It is therefore unlikely that fish fed

463 the diets used in this study would become Se deficient, even when the feed is given throughout the life

464 cycle. Judged by retention efficiency, the Se present in the feed ingredients had similar bioavailability to

465 the added selenite. If *gpx1* expression had been affected by low Se in this study, increased NP would

466 probably have increased this response (Hilton et al. 1980; Penglase et al. 2014). However, *gpx1* was not

467 expressed according to this hypothesis in either trial. Retention and body concentrations of Se were higher

468 in seawater than in fresh water, possibly caused by differences in waterborne Se concentrations. Se

469 toxicity was probably not encountered in the present study, since the diet with the highest NP inclusion

470 contained 1.1 and 1.4 mg kg^{-1} Se, which is below levels reported to be toxic in fish (Penglase et al. 2014).

471 To examine whether the graded dietary NP affected redox regulation in the salmon, we measured

472 GSH/GSSG concentrations in liver and muscle and calculated the resulting E$_{GSH}$. In Trial 1, there was a

473 tendency (p=0.06-0.07) that NP supplementation below 150% gave more reduced fish, resulting from

474   higher concentrations of GSH. Considering the above discussion, it is unlikely that this effect would have

475   been caused by low levels of vitamin C, E or Se. In a previous experiment, we also found that zebrafish fed

476   graded levels of taurine and minimum requirement levels of methionine had higher levels of GSH and

477   became more reduced (Guimares et al., unpublished). The diets fed in the present study had graded levels

478   of taurine and methionine (Hemre et al., in prep.) and at low levels of these nutrients, GSH synthesis or

479   recycling and a resulting lowered E_{GSH} could have been stimulated to protect against oxidation induced by

480   low taurine (Jong et al. 2012; Penglase et al. 2015). The relatively higher liver *gclc*, *g6pd* and *gr* expressions

481   at low NP inclusion may have stimulated GSH synthesis and recycling and contributed to make the livers

482   more reduced. In Trial 2, there were no effects of diet on GSH/GSSG concentrations and no correlation

483   between diet and expression of the genes coding for GSH metabolizing enzymes. The higher muscle TBARS

484   at low NP inclusion in both trials confirm that fish fed low levels of micronutrients and selected amino

485   acids may become oxidized. Overall, the results confirm that the tissue GSH/GSSG concentrations are

486   relatively stable within experiments with salmon, even with large variations in dietary composition, as

487   found by (Hamre et al. 2010b).

488   However, the GSH/GSSG concentrations and E_{GSH} were different in Trial 1 and 2, the fish in Trial 2 being

489   more oxidized, with less GSH both in liver and muscle and more GSSG in the liver, than the fish in Trial 1.

490   Expression of liver *gpx1* (cytosol and mitochondria) and *gpx3* (extracellular) was also higher in Trial 2,

491   indicating excess activity in removal of H2O2 at the expense of GSH. This corresponds with an increased

492   consumption of Asc in Trial 2. These results indicate that the fish in Trial 2 experienced some sort of

493   oxidative stress. The fish did not seem to be diseased, since the growth and feed intake were good and

494   mortalities were negligible (Hemre et al., in prep.). The feed was not analyzed for oxidation, however,

495   oxidized feed most often leads to reduced vitamin E retention (Baker & Davies 1997; Hung et al. 1981),

496   which was not encountered in the present study. The samples in Trial 2 were taken in June, with rapidly

497   increasing water temperatures and longer day light, resulting in strong growth stimulation in salmon. In

498   cod larvae fed copepods, a similar growth stimulation correlated with more oxidized fish having a higher

499   whole body E_{GSH} (Karlsen et al. 2015; Penglase et al. 2015). Based on these observations, we hypothesize

500   that redox regulation is involved in surges in growth.

501

502   **Conclusion**

503 This study gives no indications that diets rich in plant ingredients increase the vitamin C, E or Se

504 requirements in Atlantic salmon. Feeds produced using the current feed ingredients, seem to have

505 sufficient amounts of α-TOH and Se from the feed ingredients alone. However, due to possible variations

506 in ingredient quality, feed processing and farming conditions, feeds should be supplemented with 150-

507 200 mg kg^{-1} α-TOH equivalents. The Se in plant based feed ingredients may vary and Se supplementation

508 may be warranted in some cases. However, the legal upper limit of Se in fish feeds is 0.5 mg kg^{-1}

509 (Regulation (EC) No 1831/2003) and the law comes into force if the feeds are supplemented. Fish fed the

510 highest concentration of Se (1.4 mg kg^{-1}) did not show any signs of toxicity. Based on body concentrations,

511 Asc supplementation should be above 190 mg kg^{-1}, where the whole body Asc concentration leveled off

512 in Trial 1. At this supplementation level, Asc deficiency can be avoided in periods with oxidative stress and

513 optimal immune function and stress resistance can be obtained. Redox regulation, which includes

514 differential consumption of antioxidant nutrients, seems to change during the production cycle of Atlantic

515 salmon.

516

517

518 **Table 1**. Feed formulation Trial 1, and with slight difference given for Trial 2 in parenthesis (Trial 2).

519 Nutrient premix, methionine, taurine and cholesterol were added to the diets in graded amounts, and

520 balanced by reducing the content of field peas in the diets, all other ingredients were used in equal

521 amounts in all diets. Numbers are in g kg^{-1}.

Composition	0NP	25NP	50NP	100NP	150NP	200NP	400NP
Fish meal SA 68 superprime	80	80	80	80	80	80	80
Krill meal	24.2	24.2	24.2	24.2	24.2	24.2	24.2
Soy Prot. Conc. 60%	180	180	180	180	180	180	180
Corn gluten 60	40	40	40	40	40	40	40
Pea protein 75	124 (130)	124 (130)	124 (130)	124 (130)	124 (130)	124 (130)	124 (130)
Wheat gluten	180 (150)	180 (150)	180 (150)	180 (150)	180 (150)	180 (150)	180 (150)
Wheat	61 (60)	61 (60)	61 (60)	61 (60)	61 (60)	61 (60)	61 (60)
Field peas	100	98	95	90	85	80	60
Fish oil, capelin	35 (44)	35 (44)	35 (44)	35 (44)	35 (44)	35 (44)	35 (44)
Rapeseed oil	79 (88)	79 (88)	79 (88)	79 (88)	79 (88)	79 (88)	79 (88)
Linseed oil	22	22	22	22	22	22	22
Palm kernel oil	44 (48)	44 (48)	44 (48)	44 (48)	44 (48)	44 (48)	44 (48)
Nutrient premix *	0	0.25	0.5	1.0	1.5	2.0	4.0
Histidine	0.00	0.34	0.68	1.36	2.04	2.72	5.44
Cholesterol	0.00	0.28	0.56	1.13	1.69	2.25	4.50

522 *Times requirement. All diets were added 38 g kg^{-1} monosodium phosphate, mineral additions were

523 adjusted to each micronutrient premix (Lock et al. in preparation). All diets were added 9.3 g kg^{-1}

524 lysine, 1.8 g kg^{-1} threonine, 8g kg^{-1} choline (50%), 0.25 g kg^{-1} barox becp dry, 0.5g kg^{-1} yttrium oxide.

525

526

527 **Table 2**. Analyzed feed composition. All results are the mean of two analytical parallels. Protein, lipid,
528 starch ash and dry matter are given in g kg^{-1}, while all other diet components are given as mg kg^{-1}. Slight
529 difference in macronutrient composition; Trial 2 is given in parenthesis. For the micronutrients close to
530 similar levels existed in both experiments.

	0NP	25NP	50NP	100NP	150NP	200NP	400NP
Proximate composition, g kg^{-1}							
Protein	453 (480)	469 (472)	449 (440)	456 (480)	462 (480)	470 (480)	461 (480)
Lipid	213 (220)	203 (220)	219 (210)	211(230)	208 (220)	197 (240)	195 (220)
Starch	112	112	109	104	106	107	94
Ash	66	68	66	67	69	60	75
Dry matter	910 (950)	930 (940)	920 (930)	920 (930)	930	920 (930)	920
Energy, joule kg^{-1}	22.8()	22.7	22.6	22.7	22.4	22.5	22.0
Micronutrients involved in redox regulation mg kg^{-1}							
Trial1:							
α-TOH	48	72	84	118	153	193	339
γ-TOH	49	54	60	61	51	52	51
Ascorbic acid	4.7[1]	21	43	86	143	192	351
Selenium	0.42	0.45	0.52	0.62	0.80	1.04	1.39
Trial2:							
α-TOH	76	91	109	141	178	230	239
γ-TOH	54	51	51	52	54	61	55
Ascorbic acid	<5.5	14	28	63	89	140	170
Selenium	0.47	0.48	0.56	0.79	0.91	1.0	1.1

531 [1]Below the limit of quantification, uncertain value.

532

533   Table 3. PCR assays.

| Gene	Full gene name	Accession no.	Forward primer	Reverse primer	Amplicon size (bp)	PCR efficiency a/b*
cuznsod	CuZn superoxide dismutase	BG936553	CCACGTCCATGCCTTTGG	TCAGCTGCTGCAGTCACGTT	141	1.94/1.95
mnsod	Mn superoxide dismutase	DY718412	GTTTCTCTCCAGCCTGCTCTAAG	CCGCTCTCCTTGTCGAAGC	227	1.96/2.06
cat	Catalase	BG935638	CCCAAGTCTTCATCCAGAAACG	CGTGGGCTCAGTGTTGTTGA	101	1.90/1.97
gpx1	Glutathione peroxidase 1	DW566563	GCCCACCCCTTGTTTGTGTA	AGACAGGGCTCCACATGATGA	103	1.85/2.05
gpx3	Glutathione peroxidase 3	DW561212	TTCCCCTCCAATCAGTTTGG	ATCCCCCCTCTGGAATAGCA	123	1.97/2.05
gr	Glutathione reductase	BG934480 >Contig7869_Atlantic_salmon	CCAGTGATGGCTTTTTTGAACTT	CCGGCCCCCACTATGAC	61	2.03/1.97
g6pd	Glucose-6-Phosphate Dehydrogenase		CTTTGGGCCAATCTGGAACA	TCCCGGATGATTCCAAAGTC	114	2.08/2.00
nfe2l2	Nuclear factor (erythroid-derived 2)-like 2	BT044699 >Contig16361_Atlantic_salmon	TCGCTGAAGGAGGAGAAGGA	GTCCCTCAGCAGACGGAAAA	120	1.83/2.05
gclc	Glutamate-Cysteine Ligase, Catalytic Subunit		CGTCCTGCTCACCAGGGTTA	GCCCTCTTGGACTGCATTTC	112	1.93/2.00
actb	Beta-actin	BG933897	CCAAAGCCAACAGGGAGAA	AGGGACAACACTGCCTGGAT	92	1.93/20.00
ee1ab	Eukaryotic translation elongation factor 1 alpha B	BG933853	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACTG	57	2.04/2.03
uba52	Ubiquitin A-52 Residue Ribosomal Protein Fusion Product 1	NM_001141291	CCAATGTACAGCGCCTGAAA	CGTGGCCATCTTGAGTTCCT	110	2.04/-

* a=Trial 1, b=Trial 2
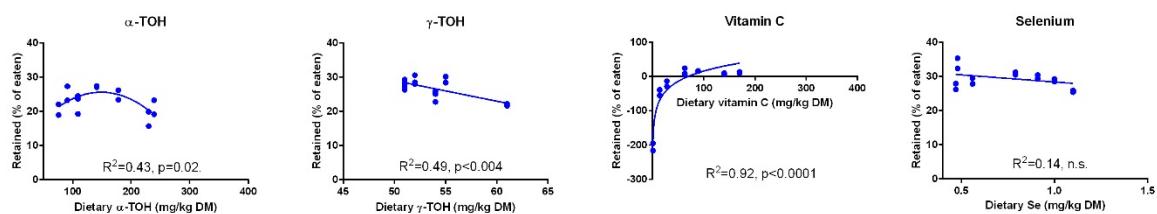
535

536
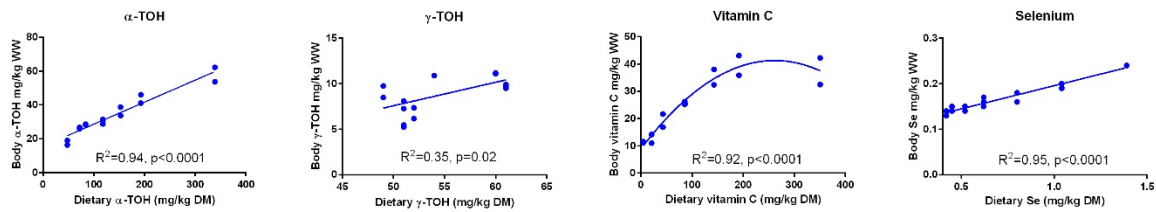
Trial 1

Trial 2
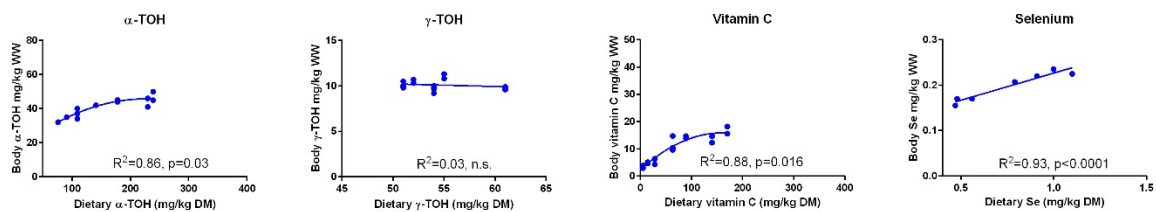
537

538 Figure 1. Whole body concentrations of redox dependent micronutrients in Atlantic salmon parr (Trial 1)
539 and post-smolt (Trial 2) in response to dietary supplementation of micronutrients and selected amino
540 acids at supplementation of 0-400% NP. Data were first fitted to second order polynomials and the fit was
541 then compared to a first order polynomial fit, which was chosen when p>0.05 for the second order
542 equation. The p given in figures with first order and second order fits indicate that the slope is significantly
543 different from 0 or that second order fits are significantly better than first order fits, respectively. n.s., not
544 significant

545

Trial 1
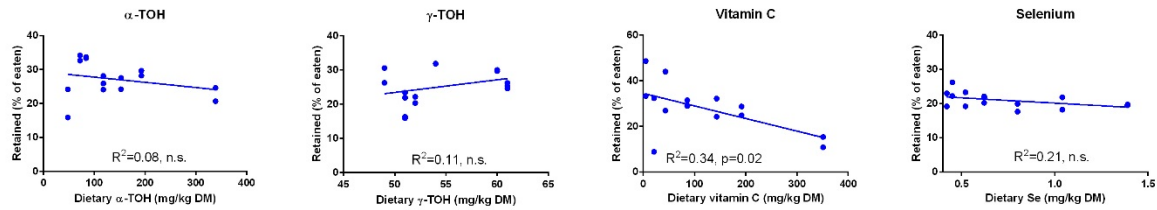
Trial 2

546

547

548 Figure 2. Retentions of redox dependent micronutrients in Atlantic salmon parr (Trial 1) and smolt (Trial
549 2) in response to dietary supplementation of micronutrients and selected amino acids at 0-400% NP. Data
550 were first fitted to second order polynomials and the fit was then compared to a first order polynomial
551 equation fit. α-Tocopherol in Trial 2 had significantly best fit to the second order equation(p<0.05).
552 Vitamin C retention fitted a logarithmic equation better than a second order polynomial (p<0.001). The p
553 given in figures with first order fits indicates that the slope is significantly different from 0. (n.s.; not
554 significant)
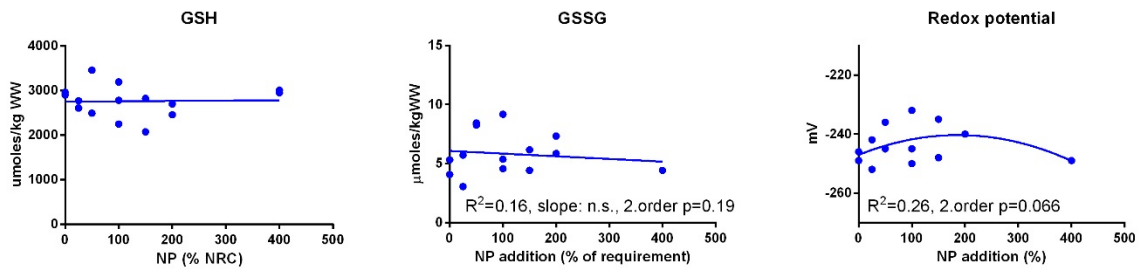
Trial 1
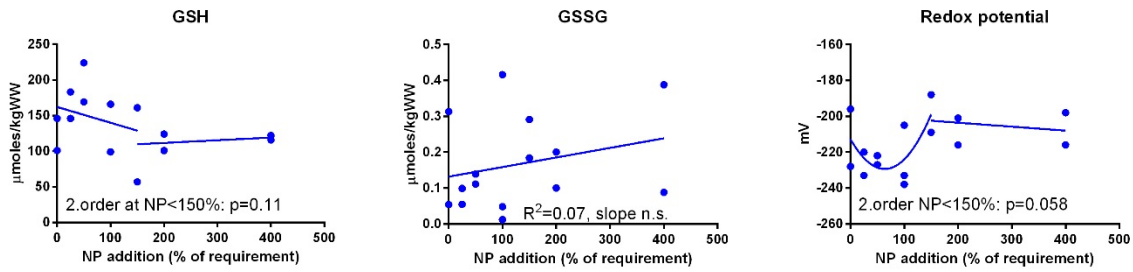
Trial 2
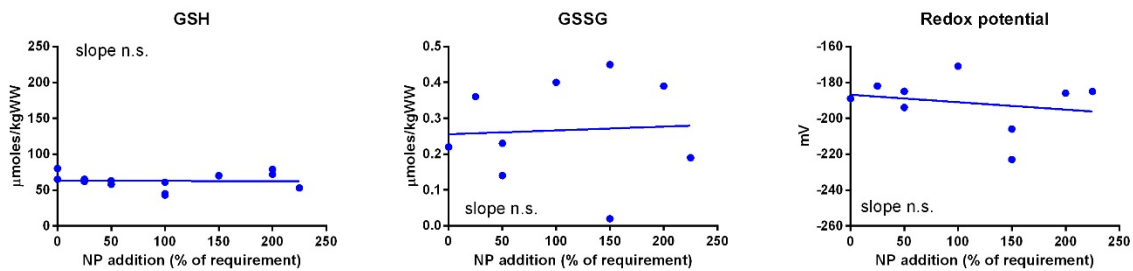
555

556 Figure 3. Muscle reduced and oxidized glutathione (GSH and GSSG) and the GSH based redox potential in
557 Atlantic salmon parr (Trial 1) and post-smolt (Trial 2) in response to dietary supplementation of
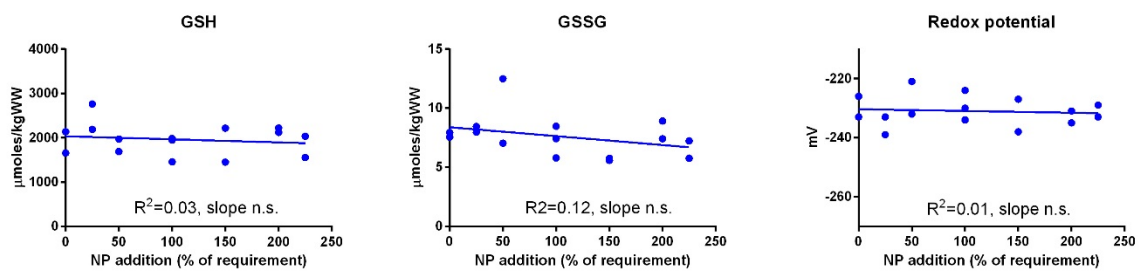558 micronutrients and selected amino acids at 0-400% NP.
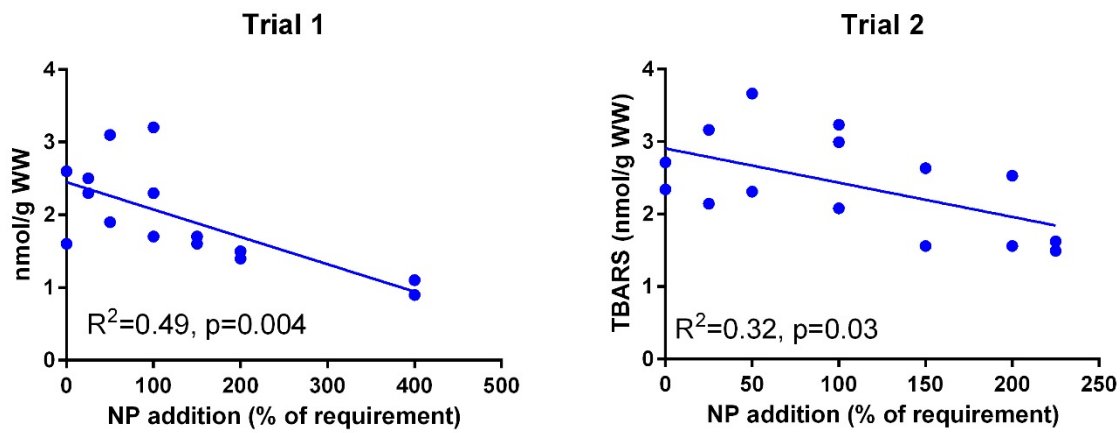
559

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Trial 1

Trial 2

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Figure 4. Liver reduced and oxidized glutathione (GSH and GSSG) and the GSH based redox potential in Atlantic salmon parr (Trial 1) and post-smolt (Trial 2) in response to dietary supplementation of micronutrients and selected amino acids at 0-400% NP.
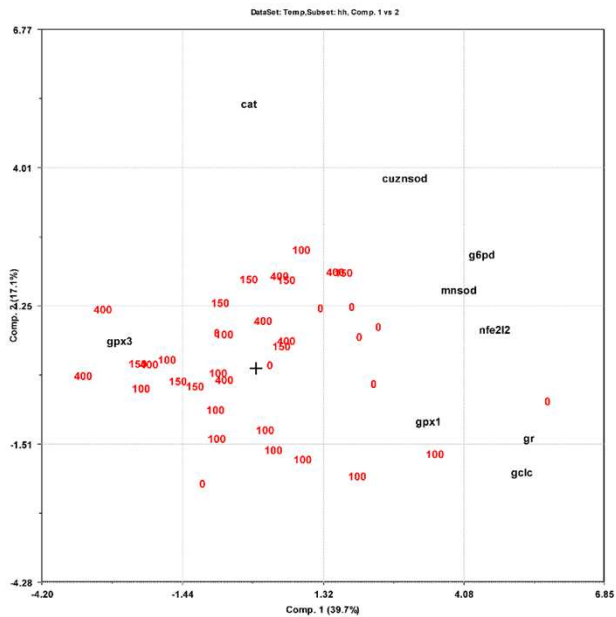
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568

569 Figure 5. Muscle TBARS (nmoles g^{-1} ww) in Atlantic salmon parr (Trial 1) and post-smolt (Trial 2) in response
570 to dietary supplementation of micronutrients and selected amino acids at 0-400% NP.
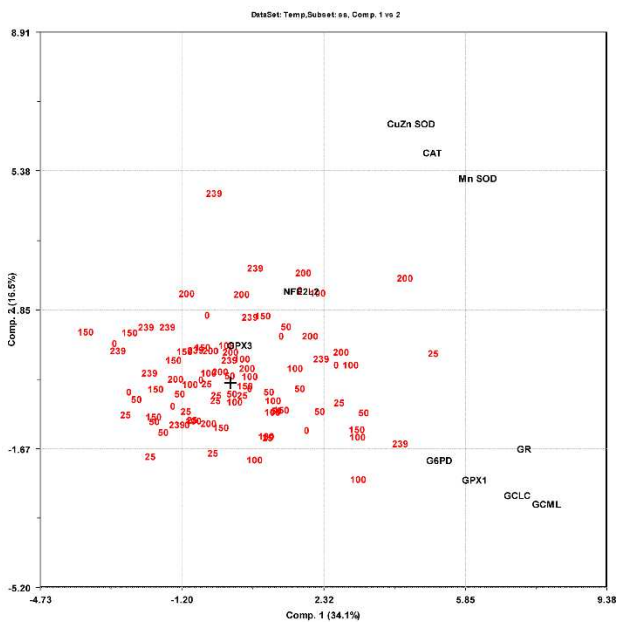
571

572

573   Trial 1

574

575   Trial 2

576

577   Figure 6. PCA bi-plots on expression of redox dependent genes related to dietary supplementation of
578   micronutrients and selected amino acids at 0-400% NP.

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