A peer-reviewed version of this preprint was published in PeerJ on 8 November 2016.

Hamre K, Sissener NH, Lock E, Olsvik PA, Espe M, Torstensen BE, Silva J, Johansen J, Waagbø R, Hemre G. 2016. 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. PeerJ 4:e2688 https://doi.org/10.7717/peerj.2688

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

Kristin Hamre, Nini Sissner, Erik-Jan Lock, Pål A Olsvik, Marit Espe, Bente E Torstensen, Joana Silva, Johan Johansen, Rune Waagbø, Gro-Ingunn Hemre

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}.

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

K. Hamre[1,4], N.H. Sissener[1], E.J. Lock[1], P.A. Olsvik[1], M. Espe[1], B.E. Torstensen[1], J. Silva[2], J. Johansen[3], R. Waagbø[1,4], G.-I. Hemre[1].

[1]National Institute of Seafood and Nutrition Research (NIFES), Bergen, Norway

[2]Biomar AS, Trondheim, Norway

[3]GIFAS, Inndyr, Norway

[4]University of Bergen, Norway

Corresponding author:

K. Hamre

NIFES, Postboks 2029 Nordnes

5817 Bergen, Norway

e-mail: kha@nifes.no

phone: (+47) 48185034

Abstract

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, 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[sup]-1[sup].

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

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

In classical nutrition, vitamin C (ascorbic acid, Asc) has a well defined role as a cofactor for the enzymes catalyzing the hydroxylation of proline and lysine, necessary for formation of collagen and bone matrix (Barnes & Kodicek 1972; Gould et al. 1960; Terova et al. 1998). The function of Asc is to keep the iron present at the enzymes' active site in the reduced state (Meister 1994). More generally, Asc is a water-soluble antioxidant, it scavenges free radicals and probably participates in recycling oxidized vitamin E (Hamre et al. 1997; Tappel 1962). The resulting Asc radical or dehydroascorbic acid can in turn be reduced by glutathione (GSH) and ultimately by NADPH produced in energy metabolism (Mrtensson & Meister 1991). Crystalline Asc is very unstable, susceptible to leakage from feed pellets immersed in water and to oxidation. It was therefore not until stable and fully bioavailable forms of vitamin C were developed that meaningful requirement studies in fish could be designed (Woodward 1994). Sandnes et al. (1992) performed one of the first of such studies in Atlantic salmon using Ca ascorbate-monophosphate (AP). They found a minimum dietary requirement, based on growth, mortality and skin and backbone hydroxyproline concentration, of 10-20 mg kg^{-1}. Severe deficiency symptoms appeared first after 18 weeks of feeding the non-supplemented diet. Concentration of Asc in the liver was linearly related to dietary AP up to 160 mg kg^{-1} Asc equivalents. These findings were confirmed by Hamre *et al*. (1997), however, the appearance of vitamin C deficiency symptoms depended on the dietary concentration of vitamin E and is probably affected by other variations in the experimental conditions as well (Dabrowski et al. 2004; Gabaudan & Verlhac 2001; Sandnes et al. 1992). It is common for several fish species that dietary Asc required for maximum body or tissue storage surpasses the requirement levels for growth, survival and hydroxyproline concentrations (NRC 2011). Furthermore, immune response indicators are stimulated by Asc levels far above conventional requirements, which also protect against stress (Trichet 2010; Waagbø 1994; Waagbø 2006).

Selenium (Se) is an essential trace element inserted in selenocysteine (Sec), situated at the active site of Se dependent proteins, termed selenoproteins (Brigelius-Flohe 1999). The selenoproteins can be grouped in stress-related and housekeeping proteins, the first group responds readily to dietary Se and includes glutathione peroxidase (GPX) 1 and 3 (Penglase et al. 2014). In mammals, the dietary Se level at which GPX1 activity and gene expression level off, is commonly used as an indicator of the Se requirement (Sunde et al. 2009; Weiss et al. 1996; Weiss et al. 1997). GPX1 activity in rainbow trout plasma followed a similar trajectory to that in mammalian tissues and leveled off at dietary Se levels (given as Na_2SeO_3)

between 0.15 and 0.38 mg kg^{-1} (Hilton et al. 1980). However, in zebrafish, maximum growth correlated with minimum whole body GPX1 activity and mRNA expression at 0.3 mg kg^{-1} dietary Se. Dietary Se above this level reduced growth and increased GPX1 activity and expression (Se given as SelPlex, Se enriched yeast which contains mainly selenomethionine; Penglase et al., 2014). Likewise, in whole cod larvae, 3.0 compared to 0.7 mg kg^{-1} dietary Se had a negative effect on growth early in development, whereas GPX1 activity and expression were stimulated (Penglase et al. 2010). This may indicate that the relation between GPX1 and Se requirement is different in fish and mammals. There was a linear relationship between dietary Se in the range 0.1 to 1.0 mg kg^{-1} and whole body Se concentrations in the zebrafish study (Penglase et al. 2014).

The cellular redox environment affects the cell's fate because gene expression, protein function and molecular pathways are often redox sensitive (Go & Jones 2013; Jones & Sies 2015; Kirlin et al. 1999). The key mechanism is that protein cysteine residues switch between oxidized and reduced states corresponding to active and inactive states of the involved proteins (redox switches). An example is that a more oxidized cellular environment induces a redox switch that releases the nuclear factor-erythroid 2-related factor 2 (NFE2L2) transcription factor from a complex with another protein, KEAP1. Once released, NFE2L2 induces the transcription of at least 50 mammalian genes; many of which code for antioxidants, thiol oxidoreductases and glutathione synthesis/recycling genes; that are involved in maintaining the redox balance and/or are involved in redox signaling (reviewed byMa 2013). Cellular redox homeostasis is thought to be maintained by redox couples that act as electron buffers due to their ability to readily cycle between oxidized and reduced forms (Jones & Sies 2015). The major redox couples are reduced/oxidized glutathione (2GSH/GSSG), cysteine (2Cys/CySS) and thioredoxin ($Trx(SH)_2$/TrxSS) (Huseby et al. 2009) and the related redox potential (E) is proportional to the ratio between the $(reduced)^2$ and oxidized forms of the redox couples, according to the Nernst Equation. GSH/GSSG is present in cells in high concentrations. It may therefore be the most important cellular redox couple and is used as an indicator of tissue redox state in the present study.

Using a multivariate design, we studied the variation of the 2GSH/GSSG couple in juvenile Atlantic salmon (*Salmo salar*) supplemented with dietary vitamins C and E, astaxanthin, lipid, iron, copper and manganese at levels just above the requirement or just below anticipated toxicity/over supplementation. This resulted in variation of less than 10 % in the GSH/GSSH concentrations and more or less constant redox potentials (E_{GSH}) in muscle, liver and plasma, which were also similar after 14 and 23 weeks of feeding the experimental diets (Hamre et al. 2010b). In Atlantic salmon post-smolts, organ specific E_{GSH} therefore

seems to be strictly regulated and stable with regard to variation of the nutrients used in this study. A hypothesis of the present study is therefore that GSH/GSSG concentrations and E_{GSH} are stable in healthy fish and can be used as indicators of fish welfare during the growth phase in salmon. On the other hand, the 2GSH/GSSG redox couple and many genes coding for proteins that maintain the cellular redox system are dynamically regulated during fish embryonic and larval development (Hamre et al. 2014; Penglase et al. 2015; Skjærven et al. 2013; Timme-Laragy et al. 2013).

Here we present data on antioxidant nutrients and redox regulation of two feeding trials (freshwater and seawater) with graded NP levels. Tissue concentrations and retention of vitamin C, E and Se, traditionally viewed as the main antioxidant nutrients, as well as effects of a graded nutrient premix on tissue GSH/GSSG concentrations and the related redox potentials were analyzed. We have also measured the expression of some central genes for regulation of redox homeostasis in the liver; the transcription factor *nfe2l2*, *cuznsod*, *mnsod*, *cat*, *gpx1* and *gpx3* which metabolize superoxide anions and H_2O_2, *gclc* which translates into the rate limiting protein in GSH synthesis, and *g6pd* and *gr* involved in keeping glutathione in the reduced state.

Materials and methods

2.1. Experimental diets

The seven experimental diets were produced at Biomar Technology Centre (Denmark), as described in Hemre et al. (in prep). All diets contained the same basal mixture of ingredients (10.4% marine protein ingredients and 3.5% fish oil of the whole receipt, the rest plant ingredients, Table 1). A nutrient premix (NP) was added in graded amounts, replacing some of the field peas in the formulation. Phosphate, lysine, threonine and choline were added to all diets in equal amounts. An antioxidant mixture to protect the feed during production, and yttrium for later digestibility measurements were also added to all diets. Diet acronyms were as follows: 0NP had no addition of the micronutrient premix, then the NP was added in graded amounts to the six diets called 25NP, 50NP, 100NP, 150NP, 200NP and 400NP. The general idea was that the 100NP diets should contain 100% of the assumed requirement, achieved by addition of the nutrient premix to the basal diet (based on available data, primarily for rainbow trout (NRC 2011) for each nutrient). However, some nutrients such as α-tocopherol and Se were already present at or above expected minimum requirements in the basal diet, but these were added in the NP to achieve increasing levels in the 7 diets and to examine if higher requirements could be assessed. The 25NP diet contained 0.25 times the NP from the 100NP diet, while the 400NP contained 4 times the NP from the 100NP diet. As all nutrients were also present in the diet ingredients to some extent (not only contributed by the NP), the 400NP diet would never have 4 times higher content than the 100NP diet, and the fold difference would vary depending on the contribution from the NP versus the diet ingredients for each nutrient. The NP contained vitamin D_3, α-tocopheryl acetate, vitamin K_3, vitamin A_1, ascorbyl monophosphate, vitamin B_6, biotin, cobalamin, folate, pantothenic acid, riboflavin, thiamine, niacin, Se (as inorganic sodium selenite), iodine, copper, cobalt, manganese, zinc, crystalline DL-methionine, and taurine. Crystalline L-histidine and cholesterol were also added in graded amounts. The analyzed composition of the diets can be found in Table 2, including proximate composition and contents of the micronutrients focused in this presentation.

2.2 Feeding trials

Both feeding trials were conducted in accordance with Norwegian laws and regulations concerning experiments with live animals, which are overseen by the Norwegian Food Safety Authority.

Trial 1: The trial with parr in freshwater took place at the Institute of Marine Research (Matredal, 61°N, Western Norway). The salmon were hatched in February, and in June the salmon parr were randomly distributed in fifteen 400 litre (1x1x0.4m^3) experimental tanks and acclimated for 1 week while being fed a commercial feed (Ewos). The trial commenced on July 3rd, with duplicate tanks for each diet with the exception of NP100 that was run in triplicates. Each tank contained 100 fish with mean initial body weight of 18.3±2.2 g. The fish were fed *ad libitum* with continuous feeding from automated feeders during night and day. However, care was taken to limit overfeeding, due to uncertainties in the collection of uneaten feed at such a small pellet size. Collection and weighing of uneaten feed was conducted daily at 13:00, with the exception of weekends. The fish were exposed to continuous light, and oxygen saturation was monitored on a regular basis and was never below 75%. The fish were reared in freshwater, but with seawater added as a buffer, creating a salinity of 1.1 to 1.3 g L^{-1}.The temperature was kept constant throughout the experiment, at 12.4 °C (SD ±0.7). The total duration of the feeding trial was 12 weeks, e.g. 84 days.

Trial 2: Post-smolt Atlantic salmon were randomly distributed among fifteen sea cages (5m x 5m x 5m; 125 m^3; 150 fish per cage) at Gildeskål Research Station, GIFAS, Gildeskål kommune, Norway (67°N, Northern Norway). Prior to the start of the trial, fish were acclimated to the environmental conditions for two weeks, the feeding trial started in January 2013. At start, the average fish weight was 228 ± 5 g and during the 157 day feeding period the fish more than doubled in weight. As in standard aquaculture practice, fish were reared under 24h light regime before the start of the trial and during the first 3 months of the experiment. Cages were illuminated by four 400W IDEMA underwater lights that were positioned at the centre of each block of four cages at a depth of three meters. Fish were hand-fed to satiation two times daily and feed intake was recorded for each sea cage. Total feed intake and mortality were recorded daily. Water temperature, salinity and oxygen saturation over the course of the trial varied from 4.1 (January) to 10°C (June), 30-34.2 g L^{-1}, and 8.7-12.2 mg/L, respectively.

2.3 Sampling

Fish were anesthetized (Benzoak® VET, 0.2 ml/L, ACD Pharmaceuticals, Leknes, Norway) and killed by a blow to the head. In Trial 1 , the fish in each tank were bulk weighed for total biomass at each sampling point and body weight and length were measured on individual fish, 5-44 fish depending on sampling point (sexually maturing males were excluded). In Trial 2, individual fish were weighed at each sampling

(mid sampling 42 fish per cage, end sampling 34 fish per cage). In both trials, blood was drawn from the caudal vein (*Vena caudalis*) by means of a heparinized medical syringe from 8 fish per tank, before organs were dissected and kept as individual samples. Pooled organ samples based on 10 fish for each tank (liver, gills, muscle) were frozen on dry ice and later homogenized, while individual organ samples were flash frozen in liquid nitrogen. Liver weight and gutted weight were recorded (10 fish per tank).

2.4 Chemical analysis of diets, whole fish and organs

Multi-element determination in the feed and tissue samples was done by ICP-MS (inductively coupled plasma mass spectrometry) (Julshamn et al. 1999). HPLC was used for determination of ascorbic acid (Mæland & Waagbø 1998) and tocopherols analysed according to (CEN 1999). TBARS was analyzed according to (Hamre et al. 2001). For the analysis of total (tGSH) and oxidised (GSSG) glutathione, supernatants were prepared from samples using a commercial kit (Prod. No. GT40, Oxford Biomedical Research, Oxford, UK) before analysed at 405 nm in a microplate reader (iEMS Reader Ms, Labsystems, Finland) as described by (Hamre et al. 2014).

2.5 Gene expression analysis

Total RNA was purified from frozen liver using the EZ1 RNA Universal Tissue Kit on the BioRobot® EZ1 (Qiagen, Hilden, Germany), including the optional DNase treatment step in the protocol. Homogenisation in QIAzol lysis reagent from the kit was performed on the bead grinder homogeniser Precellys 24 (Bertin Technologies, Montigny-le-Bretonneux, France) for 3x10 sec at 6000rpm. Quantity and quality of RNA were assessed with the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and a selection of samples were evaluated on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), with the 6000 Nano LabChip® kit (Agilent Technologies, Palo Alto, CA, USA).). Average RNA integrity number (RIN) for the samples in Trial 1 (39 samples) was 9.4±0.1 (mean±SEM, n=24), in Trial 2, (90 samples): 9.5±01 (mean±SEM, n=11)

Reverse transcription (RT) was performed on a GeneAmp PCR 9700 (Applied Biosystems, AB) using the TaqMan® reverse transcriptase kit with oligo(dT) primers (Applied Biosystems). Primer sequences are given in Table 3. Samples were run in duplicate (500 ng, ±5%), in addition to a six point dilution curve in triplicate (1000 to 31.25 ng), non-template and non-amplification controls. Real-time PCR amplification and analysis were performed on a LightCycler 480 Real-time PCR system (Roche Applied Science, Basel,

Switzerland) with SYBR® Green I Mastermix (Roche Applied Science). Pipetting of plates was done using a Biomek®3000 Laboratory automation workstation (Beckman Coulter, Fullerton, USA). Thermal cycling was done for 45 cycles of 10 sec at each of 95°C, 60°C and 72°C (basic programme from Roche), followed by melting analysis to confirm that only one product is present.

Cycle threshold (Ct) -values were calculated using the second maximum derivative method in the Lightcycler® software. Amplification efficiency was determined using the dilution curves with the formula E=10^(-1/slope), with the slope of the linear curve of Ct-values plotted against the log-dilution (Higuchi et al. 1993). Normalization to the reference genes and data analysis was conducted with GenEx 4.3.5 (MultiD Analyses AB, Gothenburg, Sweden) or with the geNorm applet (Vandesompele et al., 2002).

2.7 Calculations

Nutrient retention= [(final biomass × final nutrient concentration) – (initial biomass × initial nutrient concentration)] × 100/ (Total feed intake × nutrient concentration in feed).

The two-electron half-cell reduction potential of the 2GSH/GSSG redox-couple was calculated according to the Nernst equation:

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

where the GSH and GSSG concentrations are in M and E_h is given in volts. $E^{0\prime}$ is the standard reduction potential at pH7 and 25 °C and was assumed to be -0.240 V (Kemp et al. 2008; Schafer & Buettner 2001). The measurements are the average of whole organs and do not take into account that the reduction potential varies between cell types, and between organelles within the cells (Kemp et al. 2008; Morgan et al. 2013; Schafer & Buettner 2001).

2.8 Statistical analyses

GraphPad Prism (ver. 6.05) was used for the regression analyses. For the individual nutrients, the x-axis represented analyzed dietary concentrations, while for glutathione and gene expression data the x-axis represented added NP. Since the nutrient concentrations in the 400%NP diet were outliers in Trial 2, the value of this diet was recalculated to 239%NP, the mean of the concentrations of α-TOH, Asc and Se. Data were fitted to second order polynomials and the fit was then compared to the fit of a first order

polynomial. In figures with first order polynomials, the second order polynomial was rejected ($p>0.05$). The p given in these figures indicates the probability that the slope is equal to 0. In figures with second order fits, p gives the probability that the first order equation fits the data better than the second order equation. None of these equations fitted the data on retention of vitamin C, where a logarithmic equation was applied and p indicates the probability that a second order polynomial has a better fit than the logarithmic function. GraphPad Prism has a function for overall comparison of slopes and intercepts of datasets fitted to linear equations, which was used for comparison of data between Trials 1 and 2. The software also has a function for detecting outliers which was used for the data on muscle GSH/GSSG in post-smolts and on the gene expression data.

Correlation analyses of gene expression was performed using the software Statistica (ver. 11, Statsoft Inc., Tulsa OK) and PCA plots were produced in Sirius (ver 8.1, Pattern Recognition Systems AS, Bergen Norway).

Results

Fish performance is reported in detail by Hemre et al. (in prep). Briefly, fish in Trial 1 grew from an initial 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 increased with increasing dietary NP, while lipid retention decreased, together with liver index and viscera-somatic index. In trial 2, initial fish size was 228 g (± 4.2) and average final weight was 482 g (± 17) There was no effect of the NP on growth or protein and lipid retention in this trial. Survival was high in both trials, close to 100%, and with no difference between diet groups.

The dietary concentrations of α-TOH, Asc and Se (Figure S1) were well fitted to first order polynomials in Trial 1 (R^2>0.98). In Trial 2, the diet designated 400% was an outlier, while the other diets showed a good fit to a linear equation (R^2>0.97). The diet planned to contain 400%NP in trial 2 was recalculated to contain 239%NP, based on the nutrient analyses of the diets. Gamma-TOH level was similar in all diets, as it was only derived from the feed ingredients (Figure S1). The dietary concentrations at 100% NP were 118 and 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, 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} for Asc and 0.42 and 0.47 mg kg^{-1} for Se (Table 2).

There was a linear relationship between dietary and whole body concentrations of α-TOH in Trial 1 (R^2=0.94; p (slope=0)<10^{-4}; Figure 1). In Trial 2, a second order polynomial fitted the data better than a first order polynomial (p=0.03). The range of whole body concentrations was between 20 and 60 mg kg^{-1} in Trial 1 and between 30 and 50 mg kg^{-1} in Trial 2. Whole body γ-TOH concentration varied between 5 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 1). Whole body Asc concentration followed a second order polynomial in both trials (Trial 1, R^2=0.92, p<0.0001; Trial 2, R^2=0.88, p=0.016). Whole body concentration plateaued at a dietary concentration of 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 reached at dietary and whole body concentrations of 100 and 14 mg kg^{-1}, respectively. Whole body Se showed a linear relationship with the dietary concentration in both trials (R^2>0.93, p(slope=0)<10^{-4}). The diet dependent whole body concentration was significantly higher in Trial 2 than in Trial 1 (p<10^{-4}).

The retention of α-TOH was not affected by diet in Trial 1 and followed a second order polynomial in Trial 2 (p=0.02) with the highest retentions at intermediate dietary α-TOH concentrations (Figure 2). Diet did not affect the retention of γ-TOH in Trial 1, while increasing the NP in Trial 2 had a negative effect on γ-TOH retention (p<0.004). The range of retention of both α- and γ-TOH was 20-30%. In Trial 1, retention of Asc ranged from 11-42 % and was negatively linearly related to dietary Asc (R^2=0.34, p(slope=0)=0.02). In

Trial 2, vitamin C retention was negative at dietary levels below 63 mg kg^{-1} and max retention was 16% at 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 retention of Se, which ranged from 20 to 30%. The retention of Se was higher in Trial 2 than in Trial 1 ($p<10^{-4}$).

GSH and GSSG concentrations were measured in muscle (Figure 3) and liver (Figure 4). In muscle in Trial 1, GSH concentrations ranged from 100 to 200 µmol kg^{-1}, GSSG ranged from 0 to 0.4 µmol kg^{-1} with no effect of diet. There was a tendency that the resulting glutathione based redox potential followed a second order polynomial at dietary NP below 150%, so that the tissue became transiently more reduced at supplementation of 25-100% NP than at 150-400% NP (p=0.058). The redox potential of muscle in Trial 1 ranged from -240 to -190 mV. In Trial 2, muscle GSH and GSSG concentrations ranged from 43 to 80 and 0 to 0.4 µmol kg^{-1}, respectively, and the redox potential from -170 to -220 mV, with no effect of diet. The muscle GSH/GSSG concentrations in these fish were close to quantification limits and some of the data points were removed as outliers. Muscle GSH concentration was higher (p<0.0001), the redox potential lower (p=0.002) and GSSG concentration similar in Trial 1 compared to Trial 2. In the liver, GSH, GSSG and the glutathione based redox potential were not affected by the diet, however there was a tendency towards a significant second order fit to the data on liver redox potential in Trial 1 (p=0.066), implicating a higher redox potential in fish fed diets with intermediate NP additions. Liver GSH concentration was higher (p<0.0001), while GSSG concentration (p=0.01) and the redox potential (p<0.0001) were lower in Trial 1 than in Trial 2.

Muscle TBARS (Figure 5) was negatively correlated to NP addition in both trials (p=0.004 in Trial 1, p=0.03 in Trial 2) and the absolute level was similar in the two trials (p=0.09).

Expression of redox dependent genes was measured in the liver of individual salmon in both trials. PCA plots showed that the dietary groups were not well separated according to expression of the redox related genes (Figure 6). However, in Trial 1 there was a tendency to grouping of samples related to dietary supplementation of NP, with NP supplementation correlating negatively to *g6pd*, *gclc*, *gpx1* and *gr* ($-0.38<R^2<-0.43$) and positively to *gpx3* (R^2=0.39). *Gr* showed the most covariation with other genes and 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, respectively. *Gr* was also correlated to *nfe2l2* (R^2=0.52), *cat* was not correlated to the other genes while *gpx3* was negatively correlated to the other genes, significant for *g6pd* (R^2=-0.33). In Trial 2, diet was correlated to *cuznsod*, *mnsod* and *cat* ($0.39<R^2<0.24$). The expression of *gr*, *g6pd*, *gpx1* and *gclc* comprised one distinct group and *mnsod*, *cat* and *cuznsod* another. *Gr* correlated with *g6pd*, *gpx1* and *gclc* at

352 0.67>R^2>0.37, while gr correlation to *mnsod*, *cat* and *cuznsod* was 0.32>R^2>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

given by (NRC 2011), which will protect the fish in periods with oxidative stress, such as high water temperatures (Waagbø et al., unpublished data) elevated water oxygen levels (Lygren et al. 2000), vaccination (Lygren et al. 2001) and disease (Trichet 2010; Waagbø 2006). Accordingly, the diets with 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} Se, respectively.

The most sensitive biomarker of vitamin E deficiency in salmonids is lowered hemoglobin (Hb) concentration (Hamre 2011). There was a slight positive correlation between NP addition and Hb concentration in fish from Trial 1 (p(slope=0)=0.03)(Hemre et al., in prep), however, the slope was not significantly different from 0 after omitting the 400% NP group (p=0.44), so fish fed the diets with low NP supplementation were clearly not vitamin E deficient. In Trial 2, there was no effect of diet on Hb (Hemre et al., in prep). Hamre and Lie (1995) found that mortality due to vitamin E deficiency commenced at whole body levels of α-TOH of 5 mg kg^{-1} wet weight, while the whole body concentration in fish fed the basal diet in this study were 18 and 32 mg kg^{-1} in Trials 1 and 2, respectively. Accordingly, none of the fish groups in this study experienced vitamin E deficiency, even though the low level of vitamin C in the diet without NP would make the fish more susceptible to low dietary vitamin E (Hamre et al. 1997).

The diets were fed for 12 and 22 weeks in Trials 1 and 2, respectively. The twelve weeks feeding period in Trial 1 should just be enough for the fish to adjust to the α-TOH concentrations of the diet according to previous reports (Hamre 2011; Hamre & Lie 1995). However, the slope of whole body concentration to dietary concentration was lower (0.13 vs 0.25) and the intercept with the y-axis was higher (15.6 vs 0) than previously reported, indicating that the steady state whole body α-TOH concentration had not yet been obtained (Hamre 1995). A relation of body to dietary concentrations of α-TOH has until now only been measured in very young fish and these relationships were always linear (Hamre et al. 1997; Hamre & Lie 1995). In post-smolts in the present study, the relationship followed a second order polynomial. It is possible that uptake of α-TOH is changing over development, moving from a strict linear relationship just after first-feeding to a saturable mechanism at later stages. If this is the case, the diet 100% NP with 141 mg kg^{-1} α-TOH would be sufficient to saturate the system, while supplementation above this gave a decreased retention. In summary, supplementing commercial diets with 150-200 mg kg^{-1} α-TOH is recommended to promote fish health and welfare. A fish feed surveillance program run by NIFES in 2015 reported 185-320 mg kg^{-1} α-TOH in 18 commercial salmon feeds, while two feeds contained as high as 570 mg kg^{-1} (unpublished results). A variable part of this α-TOH would have been derived from the feed ingredients. One have to take into account that α-TOH in the feed ingredients is in the free form, which is

easily broken down in salmon intestine and therefore has quite low retention (Hamre, unpublished results). It is therefore a risk involved in including ingredient contribution when deciding dietary α-TOH supplementation.

The non-α-TOHs cannot substitute for α-TOH, because of their lower biological activity (Hamre 2011; Kayden & Traber 1993), however they may have specific biological functions in cell signaling (Golli & Azzi 2010; Wallert et al. 2014). In both trials of this study, the retentions of α- and γ-TOH were similar at 20-30%. Due to lower biological activity, retention of γ-TOH should be lower than α-TOH, but the result can be explained by similar retention of α- and γ-TOH in muscle and adipose tissue, which are the main constituents of the whole body sample. Retention of γ-TOH in other vital organs of salmon, such as gonads, intestine, and gills, is less than 30% compared to α-TOH (Hamre & Lie 1997). The body concentration of γ-TOH increased in response to increased analyzed diet concentration in Trial 1, which showed a minor variation even though it was not supplemented in the NP. The effect of diet was driven by lowered levels of γ-TOH in fish fed the diets with 25 and 50% NP, which also had a non-significant tendency of reduced γ-TOH retention. This could indicate that the redox balance of these fish was affected by NP supplementation. Whole body γ-TOH was stable in Trial 2.

The whole body concentration of Asc followed a second order equation with respect to Asc supplementation in both trials. It is well known that tissue levels of Asc level off at dietary supplementation that is much higher than the levels where traditional deficiency symptoms are displayed (Gabaudan & Verlhac 2001; Waagbø & Sandnes 1996). Since Asc above this minimum requirement has positive effects on immune function, stress resistance and reproduction (Gabaudan & Verlhac 2001; Waagbø 1994; Waagbø 2006), it may be fruitful to supplement more of the vitamin. In previous studies, Asc concentration in the liver was commonly used as an indicator of Asc status. Whole body concentration was measured in the present study, due to limited amounts of liver sample, and is probably a good indicator of when the system is saturated with the vitamin. The whole body concentration levelled off at 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 concentration of Asc in fish from Trial 2 was approximately 30% of that in fish from Trial 1. While the data from Trial 1 follow the expected trajectory based on the literature, the fish in Trial 2 seems to have had an extraordinary high consumption of Asc, with negative retention for fish supplemented below 63 mg kg^{-1} and maximum retention of 16% at higher supplementation, compared to max 42% retention in Trial 1.

There is probably an optimal ratio between supplementation of α-TOH and Asc, based on the hypothesis that vitamin E is recycled by vitamin C. A buildup of tocopheroxyl radicals would occur if too little vitamin C is present to recycle it, which would have a pro-oxidant effect on the tissues (Hamre et al. 1997). An indication of this principle is the higher mortality due to vitamin C deficiency in fish fed 300 compared to 150 mg kg^{-1} vitamin E (Hamre et al. 1997). Another indication is that vitamin E and C in tissues from copepods and wild Ballan wrasse are 100 and 500 mg kg^{-1} dry matter and 9 and 18 mg kg^{-1} wet weight, respectively (Hamre et al. 2013; Hamre et al. 2008). Theoretically, one should supplement more Asc than vitamin E, although the exact optimal ratio has not yet been identified. Seventeen commercial salmon feeds analyzed in a fish feed surveillance program performed at NIFES in 2015, contained between 118 and 418 mg kg^{-1} Asc, one feed contained 800 and two feeds contained more than 1300 mg kg^{-1} Asc. The concentration of Asc in the feeds was frequently below the concentration of α-TOH (NIFES, unpublished results).

Whole body Se concentration followed a first order relationship with the dietary concentration both in parr and post-smolt, and the retention was not affected by NP supplementation. A similar relationship was found in zebrafish, but this species had higher whole body Se concentration at similar supplementation levels. The difference may be species dependent or perhaps due to the use of Se enriched yeast containing mainly selenomethionine as the Se source in the zebrafish (Penglase et al. 2014) and selenite (Na_2SeO_3) here. Different retention efficiencies of the two Se sources has been shown in Atlantic salmon (Lorentzen et al. 1994). The basal diet contained 0.4-0.5 mg kg^{-1} Se, while the requirement in rainbow trout is estimated at 0.15-0.30 mg kg^{-1} (Hilton et al. 1980). It is therefore unlikely that fish fed the diets used in this study would become Se deficient, even when the feed is given throughout the life cycle. Judged by retention efficiency, the Se present in the feed ingredients had similar bioavailability to the added selenite. If *gpx1* expression had been affected by low Se in this study, increased NP would probably have increased this response (Hilton et al. 1980; Penglase et al. 2014). However, *gpx1* was not expressed according to this hypothesis in either trial. Retention and body concentrations of Se were higher in seawater than in fresh water, possibly caused by differences in waterborne Se concentrations. Se toxicity was probably not encountered in the present study, since the diet with the highest NP inclusion contained 1.1 and 1.4 mg kg^{-1} Se, which is below levels reported to be toxic in fish (Penglase et al. 2014).

To examine whether the graded dietary NP affected redox regulation in the salmon, we measured GSH/GSSG concentrations in liver and muscle and calculated the resulting E_{GSH}. In Trial 1, there was a tendency (p=0.06-0.07) that NP supplementation below 150% gave more reduced fish, resulting from

higher concentrations of GSH. Considering the above discussion, it is unlikely that this effect would have been caused by low levels of vitamin C, E or Se. In a previous experiment, we also found that zebrafish fed graded levels of taurine and minimum requirement levels of methionine had higher levels of GSH and became more reduced (Guimares et al., unpublished). The diets fed in the present study had graded levels of taurine and methionine (Hemre et al., in prep.) and at low levels of these nutrients, GSH synthesis or recycling and a resulting lowered E_{GSH} could have been stimulated to protect against oxidation induced by low taurine (Jong et al. 2012; Penglase et al. 2015). The relatively higher liver *gclc*, *g6pd* and *gr* expressions at low NP inclusion may have stimulated GSH synthesis and recycling and contributed to make the livers more reduced. In Trial 2, there were no effects of diet on GSH/GSSG concentrations and no correlation between diet and expression of the genes coding for GSH metabolizing enzymes. The higher muscle TBARS at low NP inclusion in both trials confirm that fish fed low levels of micronutrients and selected amino acids may become oxidized. Overall, the results confirm that the tissue GSH/GSSG concentrations are relatively stable within experiments with salmon, even with large variations in dietary composition, as found by (Hamre et al. 2010b).

However, the GSH/GSSG concentrations and E_{GSH} were different in Trial 1 and 2, the fish in Trial 2 being more oxidized, with less GSH both in liver and muscle and more GSSG in the liver, than the fish in Trial 1. Expression of liver *gpx1* (cytosol and mitochondria) and *gpx3* (extracellular) was also higher in Trial 2, indicating excess activity in removal of H2O2 at the expense of GSH. This corresponds with an increased consumption of Asc in Trial 2. These results indicate that the fish in Trial 2 experienced some sort of oxidative stress. The fish did not seem to be diseased, since the growth and feed intake were good and mortalities were negligible (Hemre et al., in prep.). The feed was not analyzed for oxidation, however, oxidized feed most often leads to reduced vitamin E retention (Baker & Davies 1997; Hung et al. 1981), which was not encountered in the present study. The samples in Trial 2 were taken in June, with rapidly increasing water temperatures and longer day light, resulting in strong growth stimulation in salmon. In cod larvae fed copepods, a similar growth stimulation correlated with more oxidized fish having a higher whole body E_{GSH} (Karlsen et al. 2015; Penglase et al. 2015). Based on these observations, we hypothesize that redox regulation is involved in surges in growth.

Conclusion

This study gives no indications that diets rich in plant ingredients increase the vitamin C, E or Se requirements in Atlantic salmon. Feeds produced using the current feed ingredients, seem to have sufficient amounts of α-TOH and Se from the feed ingredients alone. However, due to possible variations in ingredient quality, feed processing and farming conditions, feeds should be supplemented with 150-200 mg kg^{-1} α-TOH equivalents. The Se in plant based feed ingredients may vary and Se supplementation may be warranted in some cases. However, the legal upper limit of Se in fish feeds is 0.5 mg kg^{-1} (Regulation (EC) No 1831/2003) and the law comes into force if the feeds are supplemented. Fish fed the highest concentration of Se (1.4 mg kg^{-1}) did not show any signs of toxicity. Based on body concentrations, Asc supplementation should be above 190 mg kg^{-1}, where the whole body Asc concentration leveled off in Trial 1. At this supplementation level, Asc deficiency can be avoided in periods with oxidative stress and optimal immune function and stress resistance can be obtained. Redox regulation, which includes differential consumption of antioxidant nutrients, seems to change during the production cycle of Atlantic salmon.

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

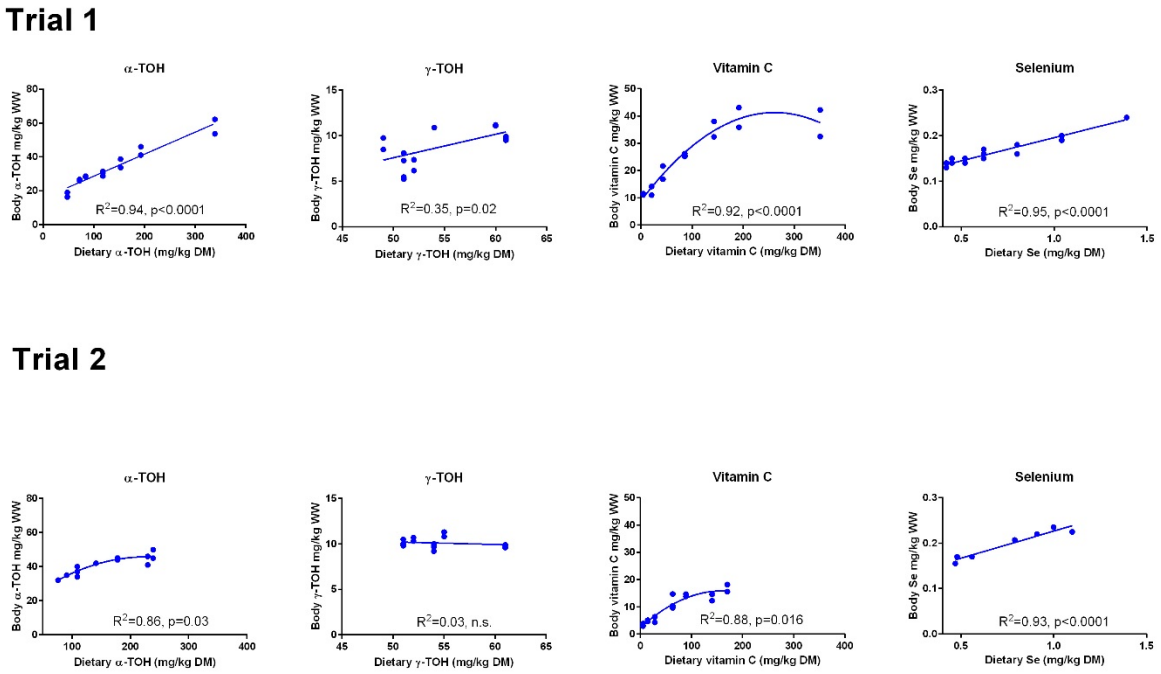
533 Table 3. PCR assays.

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	CCAGTGATGGCTTTTTTGAACTT	CCGGCCCCCACTATGAC	61	2.03/1.97
g6pd	Glucose-6-Phosphate Dehydrogenase	>Contig7869_Atlantic_salmon	CTTTGGGCCAATCTGGAACA	TCCCGGATGATTCCAAAGTC	114	2.08/2.00
nfe2l2	Nuclear factor (erythroid-derived 2)-like 2	BT044699	TCGCTGAAGGAGGAGAAGGA	GTCCCTCAGCAGACGGAAAA	120	1.83/2.05
gclc	Glutamate-Cysteine Ligase, Catalytic Subunit	>Contig16361_Atlantic_salmon	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

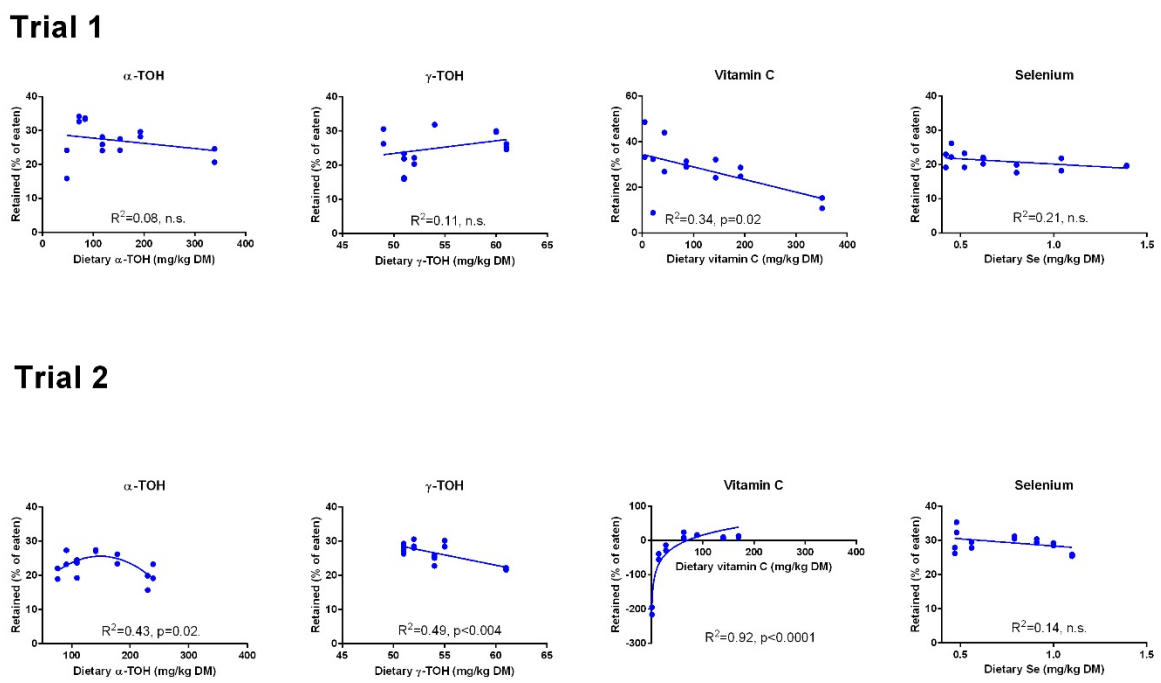
536



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

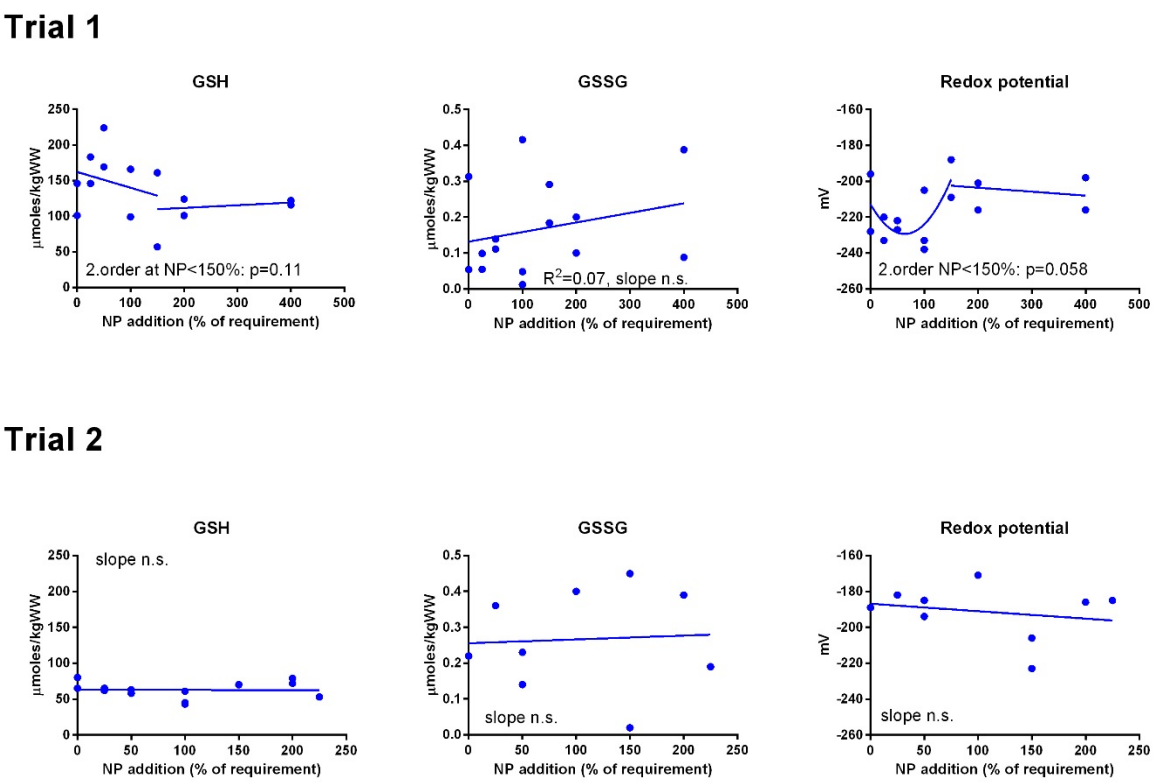
545



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)

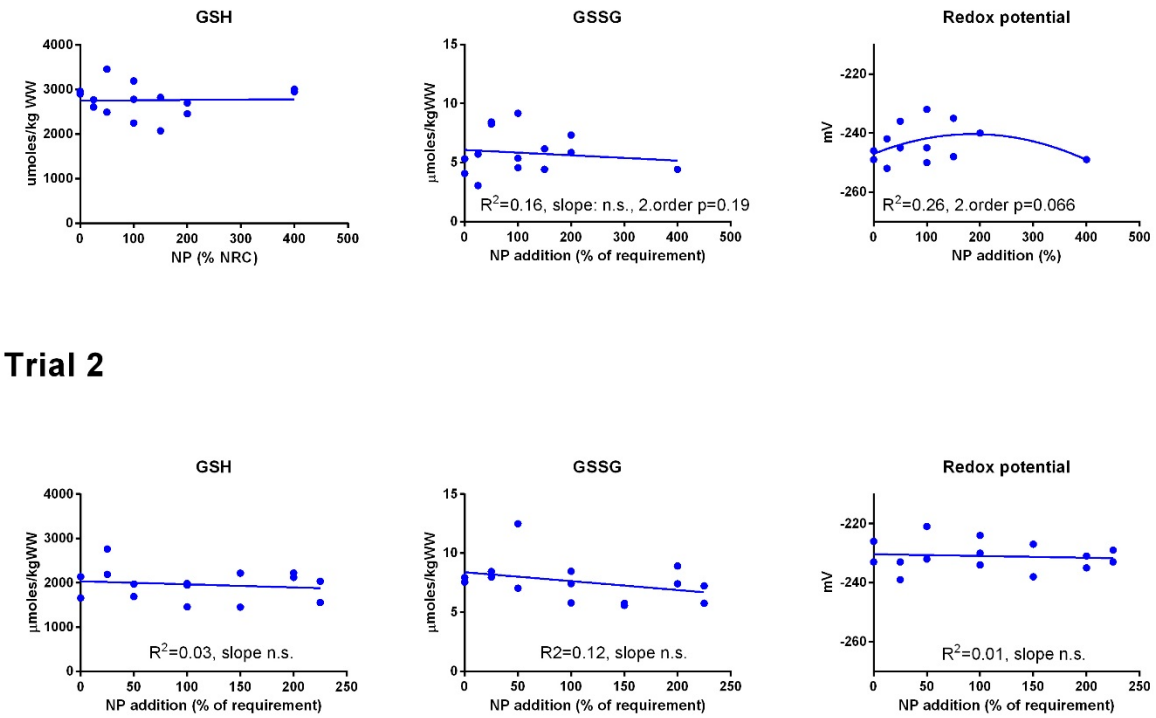


555

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

560



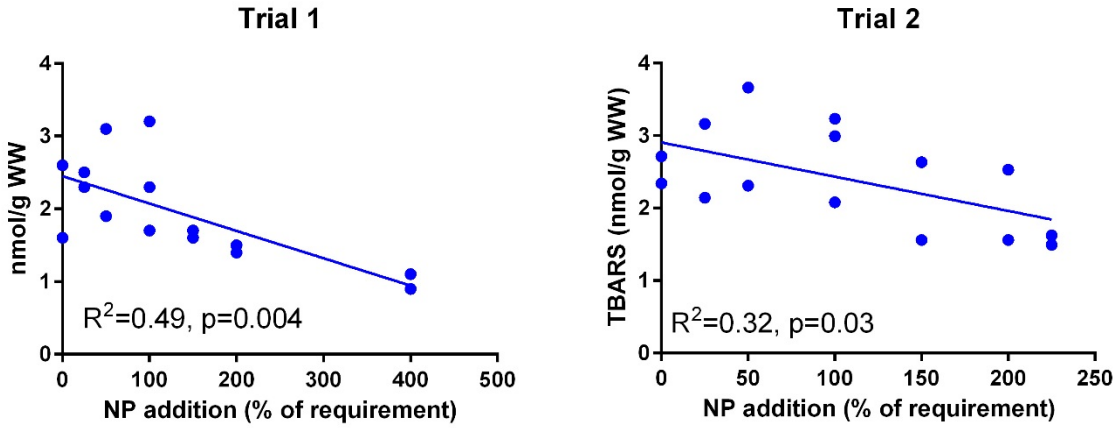
561

562

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

566

567



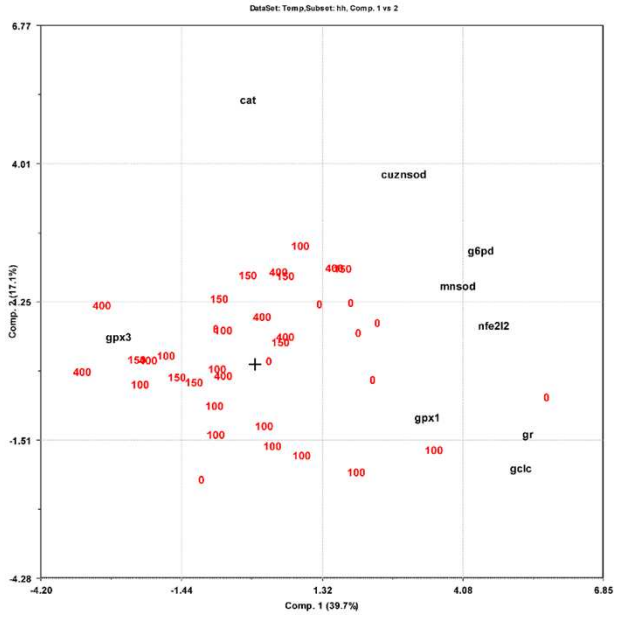
568

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

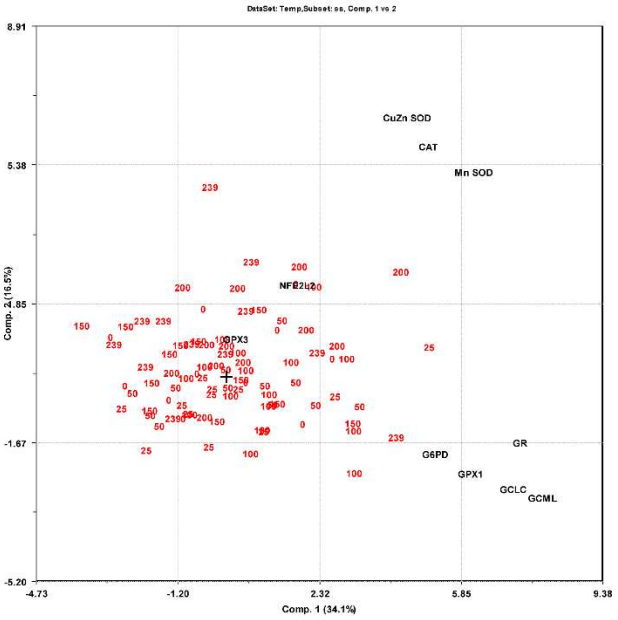
572

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.

Referernces

Alfthan G, Eurola M, Ekholm P, Venalainen ER, Root T, Korkalainen K, Hartikainen H, Salminen P, Hietaniemi V, Aspila P et al. . 2015. Effects of nationwide addition of selenium to fertilizers on foods, and animal and human health in Finland: From deficiency to optimal selenium status of the population. *Journal of Trace Elements in Medicine and Biology* 31:142-147.

Baker RTM, and Davies SJ. 1997. Modulation of tissue alpha-tocopherol in African catfish, Clarias gariepinus (Burchell), fed oxidized oils, and the compensatory effect of supplemental dietary vitamin E. *Aquaculture Nutrition* 3:91-97.

Barnes MJ, and Kodicek E. 1972. Biological hydroxylations and ascorbic acid with special regard to collagen metabolism. *Vitam Horm* 30:1-43.

Brigelius-Flohe R. 1999. Tissue-specific functions of individual glutathione peroxidases. *Free Radical Biology and Medicine* 27:951-965.

Buettner GR. 1993. The Pecking Order of Free-Radicals and Antioxidants - Lipid-Peroxidation, Alpha-Tocopherol, and Ascorbate. *Archives of Biochemistry and Biophysics* 300:535-543.

CEN. 1999. Foodstuffs – Determination of vitamin E by high performance liquid chromatography - Measurement of alpha-, beta-, gamma- and delta-tocopherols. *Comitè Europeèn de Normalisation* prEN 12822.

Dabrowski K, Lee KJ, Guz L, Verlhac V, and Gabaudan J. 2004. Effects of dietary ascorbic acid on oxygen stress (hypoxia or hyperoxia), growth and tissue vitamin concentrations in juvenile rainbow trout (Oncorhynchus mykiss). *Aquaculture* 233:383-392.

Espe M, Lemme A, Petri A, and El-Mowafi A. 2006. Can Atlantic salmon (*Salmo salar*) grow on diets devoid of fish meal? *Aquaculture* 255:255-262.

Espe M, Lemme A, Petri A, and El-Mowafi A. 2007. Assessment of lysine requirement for maximal protein accretion in Atlantic salmon using plant protein diets. *Aquaculture* 263:168-178.

Gabaudan J, and Verlhac V. 2001. Critical review of the requirements of ascorbic acid in cold and cool water fishes (salmonids, percids, plecoglossids and flatfishes). In: Dabrowski K, ed. *Ascorbic acid in aquatic organisms Status and perspectives*. London, New York, Washington D.C:: CRC press, 33-48.

Go Y, and Jones DJ. 2013. The Redox Proteome. *Journal of Biological Chemistry*.

Golli F, and Azzi A. 2010. Present trends in vitamin E research. *Biofactors* 36:33-42.

Gould BS, Manner G, Goldman HM, and Stolman JM. 1960. Some Aspects of Collagen Formation. *Annals of the New York Academy of Sciences* 85:385-398.

Hamre K. 1995. Metabolism, interactions and requirement of vitamin E in Atlantic salmon (*Salmo salar*, L.) Dr. scient thesis University of Bergen.

Hamre K. 2011. Metabolism, interactions, requirements and functions of vitamin E in fish. *Aquaculture Nutrition* 17:98-115.

Hamre K, Berge RK, and Lie Ø. 1998. Turnover of α-, γ-, and δ-tocopherol and distribution in subcellular and lipoprotein fractions indicate presence of an hepatic tocopherol binding protein in Atlantic salmon (*Salmo salar*, L.). *Fish Physiol Biochem* 18:71-83.

Hamre K, Berge RK, Waagbø R, and Lie Ø. 1997. Vitamins C and E interact in juvenile Atlantic salmon (*Salmo salar*, L.). *Free Rad Biol Med* 22:137-149.

Hamre K, Kolås K, and Sandnes K. 2010a. Protection of fish feed, made directly from marine raw materials, with natural antioxidants. *Food Chemistry* 119:270-278.

Hamre K, and Lie Ø. 1995. Minimum requirement of vitamin E for Atlantic salmon, *Salmo salar*, L., at first feeding. *Aquaculture Res* 26:175-184.

Hamre K, and Lie Ø. 1997. Retained levels of dietary α-, γ- and δ-tocopherol in tissues and body fluids of Atlantic salmon (*Salmo salar*, L.). *Aquaculture Nutr* 3:99-107.

Hamre K, Nordgreen A, Grøtan E, and Breck O. 2013. A holistic approach to development of diets for Ballan wrasse (*Labrus berggylta*) - a new species in aquaculture. *PeerJ* 1:e99: 10.7717/peerj.7799.

Hamre K, Næss T, Espe M, Holm JC, and Lie Ø. 2001. A formulated diet for Atlantic halibut (*Hippoglossus hippoglossus*, L.) larvae. *Aqua Nutr* 7:123–132.

Hamre K, Penglase SJ, Rasinger JD, Skjaerven KH, and Olsvik PA. 2014. Ontogeny of redox regulation in Atlantic cod (Gadus morhua) larvae. *Free Radical Biology and Medicine* 73:337-348.

Hamre K, Srivastava A, Rønnestad I, Mangor-Jensen A, and Stoss J. 2008. Several micronutrients in the rotifer *Brachionus* sp may not fulfil the nutritional requirements of marine fish larvae. *Aquaculture Nutr* 14:51-60.

Hamre K, Torstensen BE, Maage A, Waagbø R, Berge RK, and Albrektsen S. 2010b. Effects of dietary lipid, vitamins and minerals on total amounts and redox status of glutathione and ubiquinone in tissues of Atlantic salmon (*Salmo salar*): a multivariate approach. *Br J Nutr* 104:980-988.

Higuchi R, Fockler C, Dollinger G, and Watson R. 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Nat Biotech* 11:1026-1030.

Hilton JW, Hodson PV, and Slinger SJ. 1980. The requirement and toxicity of selenium in rainbow trout (*Salmo Gairdneri*). *J Nutr* 110.

Hung SSO, Cho CY, and Slinger SJ. 1981. Effect of Oxidized Fish Oil, Dl-Alpha-Tocopheryl Acetate and Ethoxyquin Supplementation on the Vitamin-E Nutrition of Rainbow-Trout (*Salmo-Gairdneri*) Fed Practical Diets. *Journal of Nutrition* 111:648-657.

Huseby NE, Sundkvist E, and Svineng G. 2009. Glutathione and sulfur containing amino acids: antioxidant and conjugation activities. In: Masella R, and Mazza G, eds. *Glutathione and Sulfur Containing Amino Acids in Human Health and Disease*. Hoboken, NJ: Wiley.

Jones DP, and Sies H. 2015. The Redox Code. *Antioxid Redox Signal* 23:734-746.

Jong CJ, Azuma J, and Schaffer S. 2012. Mechanism underlying the antioxidant activity of taurine: prevention of mitochondrial oxidant production. *Amino Acids* 42:2223-2232.

Julshamn K, Brenna J, Holland R, and Tanner S. 1999. Plasma source mass spectrometry- New developments and applications. *R Soc Chem* 241:167-172.

Karlsen Ø, van der Meeren T, Rønnestad I, Mangor-Jensen A, Galloway T, Kjørsvik E, and Hamre K. 2015. Copepods enhance nutritional status, growth, and development in Atlantic cod (*Gadus morhua* L.) larvae - can we identify the underlying factors? *PeerJ* 3:e902 https://dx.doi.org/10.7717/peerj.902.

Kayden HJ, and Traber MG. 1993. Absorption, lipoprotein transport, and regulation of plasma concentrations of vitamin E in humans. *Journal of Lipid Research* 34:343-358.

Kemp M, Go YM, and Jones DP. 2008. Nonequilibrium thermodynamics of thiol/disulfide redox systems: a perspective on redox systems biology. *Free Radic Biol Med* 44:921-937.

Kirlin WG, Cai J, Thompson SA, Diaz D, Kavanagh TJ, and Jones DP. 1999. Glutathione redox potential in response to differentiation and enzyme inducers. *Free radical biology & medicine* 27:1208-1218.

Krogdahl Å, Hemre G-I, and Mommsen TP. 2005. Carbohydrates in fish nutrition: digestion and absorption in postlarval stages. *Aquaculture Nutrition* 11:103-122.

Lorentzen M, Maage A, and Julshamn K. 1994. Effects of Dietary Selenite or Selenomethionine on Tissue Selenium Levels of Atlantic Salmon (*Salmo-Salar*). *Aquaculture* 121:359-367.

Lygren B, Hamre K, and Waagbø R. 2000. Effect of induced hyperoxia on the antioxidant status of Atlantic salmon, *Salmo salar*, L. fed three different levels of dietary vitamin E. *Aquaculture Research* 31:401-407.

Lygren B, Hjeltnes B, and Waagbø R. 2001. Immune response and disease resistance in Atlantic salmon (*Salmo salar*, L.) fed three levels of dietary vitamin E and the effect of vaccination on the liver status of antioxidant vitamins. *Aquaculture International* 9:401-411.

674 Ma Q. 2013. Role of Nrf2 in Oxidative Stress and Toxicity. *Pharmacology and Toxicology* 53:401-.
675 Meister A. 1994. Glutathione Ascorbic Acid Antioxidant System in Animals. *Journal of Biological*
676 *Chemistry* 269:9397-9400.
677 Morgan B, Ezerina D, Amoako TNE, Riemer J, Seedorf M, and Dick TP. 2013. Multiple glutathione
678 disulfide removal pathways mediate cytosolic redox homeostasis. *Nature Chemical Biology*
679 9:119-125.
680 Mrtensson J, and Meister A. 1991. Glutation deficiency decreases tissue ascorbate levels in newborn
681 rats: Ascorbate spares glutathion and protects. *Proceedings of the National Academy of Sciences*
682 *of the United States of America* 88:4656-4660.
683 Mæland A, and Waagbø R. 1998. Examination of the qualitative ability of some cold water marine
684 teleosts to synthesise ascorbic acid. *Comparative Biochemistry and Physiology Part A* 121:249-
685 255.
686 NRC. 2011. *Nutrient requirements of fish and shrimp*: The Natioanal Academic Press, Washington D.C.
687 Olsvik PA, Torstensen BE, Hemre GI, Sanden M, and Waagbø R. 2011. Hepatic oxidative stress in Atlantic
688 salmon (*Salmo salar* L.) transferred from a diet based on marine feed ingredients to a diet based
689 on plant ingredients. *Aquaculture Nutrition* 17:E424-E436.
690 Penglase S, Hamre K, Rasinger JD, and Ellingsen S. 2014. Selenium status affects selenoprotein
691 expression, reproduction, and F-1 generation locomotor activity in zebrafish (*Danio rerio*).
692 *British Journal of Nutrition* 111:1918-1931.
693 Penglase S, Nordgreen A, van der Meeren T, Olsvik PA, Sæle O, Sweetman JW, Bæverfjord G, Helland S,
694 and Hamre K. 2010. Increasing the level of selenium in rotifers (*Brachionus plicatilis* 'Cayman')
695 enhances the mRNA expression and activity of glutathione peroxidase in cod (*Gadus morhua* L.)
696 larvae. *Aquaculture* 306:259-269.
697 Penglase SJ, Edvardsen RB, Furmanek T, Rønnestad I, Karlsen Ø, Van der Meeren T, and Hamre K. 2015.
698 Diet affects the redox system in developing Atlantic cod (*Gadus morhua*) larvae *Redox Biology*
699 5:308-318.
700 Rigotti A. 2007. Absorption, transport, and tissue delivery of vitamin E. *Molecular Aspects of Medicine*
701 28:423-436.
702 Sandnes K, Torrissen O, and Waagbø R. 1992. The minimum dietary requirement of vitamin C in Atlantic
703 salmon (*Salmo salar*) fry using Ca-ascorbate-2-monophosphate as dietary source. *Fish Physiol*
704 *Biochem* 10:315-319.
705 Schafer FQ, and Buettner GR. 2001. Redox environment of the cell as viewed through the redox state of
706 the glutathione disulfide/glutathione couple. *Free Radical Biology and Medicine* 30:1191-1212.
707 Skjærven KH, Penglase S, Olsvik PA, and Hamre K. 2013. Redox regulation in Atlantic cod (*Gadus*
708 *morhua*) embryos developing under normal and heat-stressed conditions. *Free Radical Biology*
709 *and Medicine* 57:29-38.
710 Sunde RA, Raines AM, Barnes KM, and Evenson JK. 2009. Selenium status highly regulates selenoprotein
711 mRNA levels for only a subset of the selenoproteins in the selenoproteome. *Bioscience Reports*
712 29:329-338.
713 Tappel AL. 1962. Vitamin E as the biological lipid antioxidant. *Vitamins and Hormones* 20:493-510.
714 Terova G, Saroglia M, Papp ZG, and Cecchini S. 1998. Dynamics of collagen indicating amino acids, in
715 embryos and larvae of sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*),
716 originated from broodstocks fed with different vitamin C content in the diet. *Comparative*
717 *Biochemistry and Physiology a-Molecular and Integrative Physiology* 121:111-118.
718 Timme-Laragy A, Goldstone J, Imhoff B, Stegeman J, Hahn M, and Hansen J. 2013. Glutathione redox
719 dynamics and expression of glutathione-related genes in the developing embryo. *Free radical*
720 *biology & medicine* 65:89-101.
721 Trichet VV. 2010. Nutrition and immunity: an update. *Aquaculture Research* 41:356-372.

722 Waagbø R. 1994. The impact of nutritional factors on the immune system in Atlantic salmon, *Salmo*
723 *salar*, L.: A review. *Aquaculture and Fisheries Management* 25:175-197.
724 Waagbø R. 2006. Feeding and disease resistence in fish. In: Mosenthin R, Zentek J, and Zebrowska T,
725 eds. *Biology of growing animals*. London: Elsevier Ltd, 387-415.
726 Waagbø R, and Sandnes K. 1996. Effects of dietary vitamin C on growth and parr-smolt transformation in
727 Atlantic salmon, *Salmo salar*, L. *Aquaculture Nutrition* 2:65-69.
728 Wallert M, Schmolz L, Galli F, Birringer M, and Lorkowski S. 2014. Regulatory metabolites of vitamin E
729 and their putative relevance for atherogenesis. *Redox Biology* 2:495-503.
730 Weiss SL, Evenson JK, Thompson KM, and Sunde RA. 1996. The selenium requirement for glutathione
731 peroxidase mRNA level is half of the selenium requirement for glutathione peroxidase activity in
732 female rats. *Journal of Nutrition* 126:2260-2267.
733 Weiss SL, Evenson JK, Thompson KM, and Sunde RA. 1997. Dietary selenium regulation of glutathione
734 peroxidase mRNA and other selenium-dependent parameters in male rats. *Journal of Nutritional*
735 *Biochemistry* 8:85-91.
736 Woodward B. 1994. Dietary Vitamin Requirements of Cultured Young Fish, with Emphasis on
737 Quantitative Estimates for Salmonids. *Aquaculture* 124:133-168.
738 Yoshida H, Yusin M, Ren I, Kuhlenkamp J, Hirano T, Stolz A, and Kaplowitz N. 1992. Identification,
739 Purification, and Immunochemical Characterization of a Tocopherol-Binding Protein in Rat-Liver
740 Cytosol. *Journal of Lipid Research* 33:343-350.

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