Higher dietary micronutrients are required to maintain optimal performance of Atlantic salmon (*Salmo salar*) fed a high plant material diet during the full production cycle

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Abstract

A full life cycle (parr to harvest) study of growth and performance was conducted in Atlantic salmon fed diets high in plant ingredients supplemented with graded levels of a nutrient premix (NP), containing selected amino acids, taurine, cholesterol, vitamins and minerals to re-evaluate current nutrient recommendations. Triplicate groups were fed one of three NP levels included at 1x, 2x and 4x, where 1x corresponds to recommendations of NRC (2011). Whole body and specific tissue concentrations of nutrients were monitored throughout the experiment as requirement markers. Growth in parr was significantly enhanced in 2xNP, but restricted in 4xNP, while in post-smolts growth was positively correlated with NP level. Spinal deformity decreased linearly with increased NP level in both smolt and post-smolts. When fishmeal and fish oil are present at very low levels, as in the present study, we found beneficial effects with moderate increased levels of the B-vitamins niacin, riboflavin and cobalamin. Further, vitamin C should be increased, based on metabolic responses, although it did not influence growth. Increased Zn and Se affected fish metabolism in a positive manner. Alterations in hepatic transcriptome profiles and expression of specific genes of metabolic pathways were evident in response to micronutrient supplementation level. Collectively, increasing the levels of the micro-nutrient package to a too high level, showed a negative effect and cannot be recommended. NRC (2011) recommendations should therefore be revised for diets in which plant ingredients form the major part of the formulation.

Keywords: Fishmeal, fish oil, plant proteins, vegetable oil, vitamins, minerals, skeletal deformity, sustainable feeds.

1. Introduction

Over the last decade and a half, feed formulations for farmed Atlantic salmon (*Salmo salar*) have changed with marine raw materials, fish oil (FO) and fishmeal (FM), being increasingly replaced by high levels of plant ingredients (Gatlin III et al., 2007; Hardy, 2010; Turchini et al., 2011). Levels of marine ingredients, FM and FO, in salmon feeds are now approximately 20 % of total feed inclusion compared with 90 % around 20 years ago (Ytrestøyl et al., 2015). Over this period, many studies investigated the effects of the inclusion of plant-derived ingredients in feeds. The majority of studies focussed on the impacts on growth performance and feed utilisation (Torstensen et al., 2000; Opstvedt et al., 2003; Mundheim et al., 2004; Espe et al., 2006; Torstensen et al., 2005; Pratoomyot et al., 2008; Waagbø et al., 2013), and also on product quality for human consumers (Waagbø et al., 1993; Mundheim et al., 2004; Torstensen et al., 2005; Menoyo et al., 2007). However, overall, the development and application of modern, plant-based feed formulations has been largely successful enabling salmon production to be maintained and/or increased despite the finite and limited supplies of FM and FO (Shepherd & Jackson, 2013).

Despite the above, formulating salmon feeds with high levels of plant meals and vegetable oils significantly affects a range of nutrients (Sissener et al., 2013), including changing micronutrient concentrations and their chemical forms, as well as introducing compounds that interact with micronutrient uptake and metabolism (Olsvik et al., 2013). Importantly, in addition to affecting fish growth, feed efficiency and product quality, these changes in nutrient composition could also affect fish health and welfare (Oxley et al., 2005; Seierstad et al., 2005; Waagbø, 2006; Hemre & Sandnes, 2008; Waagbø, 2008; Seierstad et al., 2009). As a result, there has been increasing interest in improving knowledge of practical nutrient requirements of Atlantic salmon when fed modern, plant based feeds (Hansen et al., 2015). In particular, there has been a focus on micronutrients (minerals, vitamins etc.) as it is believed that these were likely supplied in surplus in feeds containing high levels of FM and FO, and that levels of some micronutrients in terrestrial raw materials can be lower (Sissener et al., 2013). When feeding plant-based diets enriched with the micronutrients folate,

63 vitamin B12, vitamin B6, methionine and choline to zebrafish (*Danio rerio*) over the entire life cycle,
64 fish responded with increased growth, higher fecundity and altered metabolic profile of several N-
65 metabolites, indicating an overall positive effect of a micronutrient-enriched plant-based diet
66 (Skjaerven et al., 2016). As a consequence, micronutrient and mineral requirements need careful re-
67 evaluation when substituting raw materials, as the current established micronutrient supplements /
68 premixes (National Research Council, NRC 2011) may not be sufficient to satisfy requirements for
69 Atlantic salmon, especially throughout the whole life cycle.

70 To investigate this, micronutrient requirements of Atlantic salmon fed diets formulated with
71 high levels of plant meals and vegetable oil were studied in two regression experiments in parr in
72 freshwater and post-smolts in seawater (Hamre et al., 2016; Hemre et al., 2016; Prabhu et al. 2019a).
73 A nutrient package, consisting of vitamins B, C, D_3, E, minerals, cholesterol, taurine and histidine
74 was fed at various levels from below NRC (2011) recommended levels to well above NRC levels.
75 Growth, health and welfare parameters responded to nutrient packages in parr, but not to the same
76 extent in post-smolts (Hemre et al., 2016). Thus, parr fed diets containing the nutrient package
77 supplying micronutrients above NRC (2011) recommended levels showed improved protein retention,
78 and reduced liver and viscera indices, whereas the response was less pronounced in post-smolt
79 (Hemre et al., 2016). Significant regressions were obtained in body compartments for vitamin C and
80 several of the B-vitamins and, based on these results, the authors recommended that B-vitamin
81 supplements should be adjusted in plant-based diets for Atlantic salmon (Hamre et al., 2016; Hemre
82 et al., 2016). There were no indications of vitamin E deficiency when vitamin E was not supplemented,
83 indicating that the ingredients delivered sufficient amounts of vitamin E. Nevertheless, it was
84 recommended to supplement 150 mg kg^{-1}vitamin E to compensate in periods of oxidative stress
85 (Hamre et al., 2016). Regarding the micro-minerals, requirements of Atlantic salmon fed the plant-
86 based diet were met at NP inclusion of 100-150 % and 150-200 % in parr and post-smolt, respectively
87 (Prabhu et al., 2019a).

More recently, the impact of micronutrients was investigated in both diploid and triploid Atlantic salmon parr fed graded levels of a nutrient package (NP) from around 30 g to seawater transfer (Taylor et al., 2019). The diets were formulated with very low levels of marine ingredients and supplemented with three levels of NP (L1, 100 %; L2, 200 % and L3, 400 % NP), which contained 24 micronutrients including selected amino acids, vitamins, minerals, taurine and cholesterol. The NP was based on the minimum nutrient recommendations for Atlantic salmon (NRC, 2011). Diploid parr fed L2 showed significantly improved growth and reduced liver (HSI) and viscera (VSI) indices, and hepatic steatosis, compared to fish fed L1, while diploids fed L3 also showed improved growth in the first 14 weeks, although growth rate was subsequently reduced (Taylor et al., 2019). In contrast, dietary NP level had less effect on triploid growth rate, VSI or HSI, and smoltification was not affected by NP in either ploidy. Overall, results suggested that, while micromineral requirements were met, other micronutrients may require to be supplemented above current NRC (2011) recommendations for optimal growth and liver function of diploid Atlantic salmon fed plant-based diets in freshwater (Taylor et al., 2019). In addition, the above study also investigated the impact of NP level on skeletal deformity and bone health including the prevalence, localisation and pathology of spinal malformation, and vertebral expression of bone biomarker genes (Vera et al., 2019). This study showed that prevalence of radiologically detectable spinal deformities decreased with increasing micronutrient supplementation when fed from parr to smolt. Concomitantly, expression of many osteogenic genes (bone morphogenic protein, osteocalcin, alkaline phosphatase, matrix metallopeptidase, insulin-like growth factor) increased with increasing NP inclusion. The observations suggested that components thereof, or interactions between micronutrients within the NP may be affecting downstream processes involved in bone formation and remodelling, which also occur through active epigenetic regulatory mechanisms of gene expression (Saito et al., *unpublished data*).

The previous trials investigating dietary micronutrients in salmon were all carried out during the freshwater phase other than one short-term (5-month) trial in post-smolt in seawater (Hamre et

al., 2016; Hemre *et al*., 2016; Taylor et al., 2019; Vera et al., 2019; Prabhu, et al., 2019a). Therefore, the aim of the present study was to determine the influence of micronutrients in Atlantic salmon fed diets formulated with very low levels of marine ingredients over the entire life cycle. To this end, diploid Atlantic salmon were fed from around 30 g to 3 Kg with plant-based feeds supplemented with the same NP as described above to produce three experimental diets (L1, 100 %; L2, 200 % and L3, 400 % NP). The impacts of NP levels on growth, feed efficiency, nutrient retention, skeletal development and liver gene expression were determined.

2. Methods & Materials

2.1 Fish and feeds

The trial was carried out with Atlantic salmon obtained from SalmoBreed AS (Norway). All experimental procedures and husbandry practices were conducted in compliance with the Animals Scientific Procedures Act 1986 (Home Office Code of Practice) in accordance with EU regulation (EC Directive 86/609/EEC) and approved by the Animal Welfare and Ethical Review Board (AWERB) of the University of Stirling. All fish were monitored daily by the Named Animal Care and Welfare Officer (NACWO).

Feeds were formulated to reflect standard practice in commercial salmon feeds in terms of protein, oil and energy contents. Thus, feeds were initially formulated to contain 48 % protein and 20 % lipid (~22 MJ), with protein content decreasing and lipid content increasing with increasing pellet size to reach 36 % protein and 34 % lipid (~24 MJ) in the largest pellet size in seawater. The experimental feeds were a low FM/FO formulation (initially 15 % and 8 % in freshwater, decreasing to 5% and 3 % respectively in seawater, Tables 1 & 2). Feeds were supplemented with a nutrient package (NP, Tables 3 and 4) at one of three inclusion levels to produce 3 dietary treatments: L1, 100 % NP; L2, 200 % NP; L3, 400 % NP (Table 4), the assumption being that the 100 % NP package should contain 100 % of assumed requirement based on the levels reported for Atlantic salmon at the time (NRC, 2011). Specifically, the NP contained 24 nutrients in total these being; vitamins (A, D3,

E, K3, C, thiamine, riboflavin, B6, B12, niacin, pantothenic acid, folic acid and biotin), minerals (Ca, Co, I, Se, Fe, Mn, Cu and Zn), crystalline amino acids (L-histidine and taurine) and cholesterol. Total and available phosphorus were fixed in all diets at 12.0 and 9.0 g kg^{-1} respectively, and magnesium at 1.5 g kg^{-1}, and were not part of the NP. Pellet size (2 mm, 3.5 mm, 5 mm, 7 mm, 9 mm) was adjusted according to fish weight. All non-oil ingredients were mixed and pellets produced by extrusion to produce three base pellets that had oil added by vacuum coating. All feeds were produced at the BioMar Tech-Centre (Brande, Denmark).

2.2 Feeding trial

The freshwater phase was carried out at the Niall Bromage Freshwater Research Facility (Stirlingshire, UK). Initially, 500 diploid salmon pre-smolt (initial mean weight, 38.2 ± 0.6 g) were stocked (2 Sept-14) into nine x 1.6 m^3 circular fibreglass tanks (3 tanks / diet, initial stocking density 12.0 ± 0.1 kg m^{-3}). Fish were acclimated to the experimental tanks for 2 weeks (Fed BioMar Inico Plus) before being fed the experimental diets. Fish were fed continuously during the light period of the light:dark cycle by automatic feeders (Arvotec T2000, Arvotec, Finland) controlled by a PC system. Specific feeding rates (SFR; % tank biomass per day) were adjusted automatically according to predicted growth and daily temperature. An out-of-season photoperiod (LL – 400 °days LD14:10 – 400 °days LL) and ambient water temperature (12 - 16 ˚C) was applied to produce S0+ smolts (final stocking density 20.2 – 22.8 kg m^{-3}), with lighting provided by two 28 W fluorescent daylight bulbs (4000 °K; RS Components, UK) mounted centrally within the tank lid. Water was supplied by an upstream reservoir under flow-through conditions (10 L min^{-1}). Oxygen levels were always higher than 8 mg L^{-1}. Uneaten feed recovery was not feasible during the freshwater phase.

Smolts were transferred (4 Nov-14) to the Marine Harvest (Mowi) Feed Trial Unit (Ardnish, Scotland) and on-grown for 11 months in nine 5x5x5 m sea pens under natural photoperiod and ambient water temperatures ranging from 6 to 16 ˚C. Triplicate groups of 250 post-smolts from respective tanks were stocked per pen (Initial stocking density 0.17 – 0.19 kg m^{-3}) and on-grown to

a final size of ~2.5 Kg (15 Sept-15, Final stocking density 4.52 – 5.18 kg m^{-3}). Fish were fed continuously during daylight by automatic feeders (Arvotec T2000) controlled by a PC system. Specific feeding rates (SFR; % pen biomass per day) were adjusted weekly according to predicted growth and water temperature. Waste feed was collected per pen by means of air uplifts following meal delivery ensuring satiation and allowing calculation of total daily feed intake.

2.3 Growth and feed efficiency

Fish were sampled at start and end of the freshwater phase, and then at approximately 250 g, 500 g, 1 kg, and ~2.5 kg in seawater prior to dietary pellet size/formulation changes. In freshwater at each sampling point, 50 fish / tank were anaesthetised (50ppm Tricaine, PHARMAQ, UK), individual weights (± 0.1 g) and fork lengths (± 1.0 mm) measured, while in seawater, all fish per pen were counted and individually measured. Following measurement, all fish were allowed to recover in aerated water before returning to their original experimental tanks/pens. Fulton's condition factor (K) was calculated using: $K = (WL^{-3})100$; where W is body weight (g) and L is fork length (mm). Weight data were used to calculate specific growth rate (SGRwt), thermal growth coefficient (TGC) and feed conversion rate (FCR) for each sampling period where SGRwt was calculated as: $(e^{g-1})\times100$, where $g = (\ln(W_f) - \ln(W_i)) \times (t_2 - t_1)^{-1}$ and TGC was calculated as: $(W_{f1/3}\text{-}W_{i1/3}) \times (\sum D_o)\text{-}1$, where W_f is the final body weight, W_i is the initial body weight and D_o is the cumulative sum of water temperature in degrees per day. FCR was calculated as: $Fi / (B_f - B_i + B_m)$ where F_i is the fed intake (kg), B_f is the final biomass (kg), B_i is the initial biomass (kg) and B_m is the mortality biomass for the period (kg). Maturation at harvest was determined by assessment of external appearance of secondary sexual characteristics and gonad development (n = 30 / pen), where gonadosomatic-index (GSI) was calculated as $GSI\ (\%) = (G_W\times100)/B_W$. Fish were classified as sexually recruited based on a threshold value of GSI >0.20 % or >1.0 % for males and females respectively (Kadri et al., 1997).

2.4 Biochemical composition and nutrient retention

At the termination of the trial, 10 fish per pen (30 / diet) were euthanised using lethal anaesthesia (>200 mg L^{-1} Tricaine, PHARMAQ UK) to provide samples for biochemical analyses. Five whole fish per pen (15 / diet, in 3 pen pools, n = 3) were taken for the determination of proximate composition (MOPA), minerals, and fatty acid composition. In addition, muscle/flesh (Norwegian Quality Cut, NQC) were collected from 5 additional fish per pen (15 fish/diet in 3 pen pools, n = 3) for MOPA, minerals, carotenoid (flesh), fatty acid composition, and total amino acid.

2.4.1. Proximate composition

Whole fish and diets were ground before determination of proximate composition according to standard procedures (AOAC, 2000). Five fish were pooled per pen and three technical replicates for the single batch feeds were analysed. Moisture contents were obtained after drying in an oven at 110 °C for 24 h and ash content determined after incineration at 600 °C for 16 h. Crude protein content was measured by determining nitrogen content (N x 6.25) using automated Kjeldahl analysis (Tecator Kjeltec Auto 1030 analyzer, Foss, Warrington, UK) and crude lipid content determined gravimetrically after Soxhlet lipid extraction (Tecator Soxtec system 2050 Auto Extraction apparatus).

2.4.2. Amino acid, mineral and vitamin analyses

Free amino acids and nitrogen metabolites were analysed using the Biochrome with post-column derivatisation with ninhydrin as described by Espe et al. (2014). The total amino acids in whole body and diets were analysed after being hydrolysed in 6N HCl for 22 h as previously described (Espe et al., 2014) and used for calculation of amino acid retention (% deposited of consumed amino acid). The B-vitamins biotin, niacin, folate, pantothenic acid and cobalamin were all determined by microbiological methods as described in detail previously (Mæland et al., 2000; Feldsine et al., 2002). Some of the B-vitamins were determined by HPLC by standard methods; thiamine (CEN, 2003), vitamin B6 (CEN, 2006) and riboflavin (Brønstad et al., 2002). Multi-element determination was done by ICP-MS (inductively coupled plasma mass spectrometry) (Julshamn et al., 1999). HPLC was

used for determination of ascorbic acid (Mæland and Waagbø, 1999) and tocopherols were analysed according to standard methodology (CEN, 1999). Vitamins A and D were analysed according to Moren et al. (2004) and Horvli and Lie (1994), respectively.

2.4.4. Lipid content and fatty acid composition

Samples of whole bodies and NQC from five fish per tank were prepared as pooled pen homogenates (n = 3 per diet). Total lipid was extracted from approximately 1 g of sample by homogenising in chloroform/methanol (2:1, v/v) using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, UK), and content determined gravimetrically (Folch et al., 1957). Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalysed transesterification at 50 °C for 16 h (Christie, 2003), and FAME extracted and purified as described previously (Tocher and Harvie, 1988). FAME were separated and quantified by gas-liquid chromatography using a Fisons GC-8160 (Thermo Scientific, Milan, Italy) equipped with a 30 m × 0.32 mm i.d. × 0.25 μm ZB-wax column (Phenomenex, Cheshire, UK), on-column injector and a flame ionisation detector. Data were collected and processed using Chromcard for Windows (version 2.01; Thermoquest Italia S.p. A., Milan, Italy). Individual FAME were identified by comparison to known standards and published data (Tocher and Harvie, 1988).

2.4.5. Pigment analysis

Flesh carotenoid levels were determined using a modified version of the JX Nippon Oil Corporation's Analytical methods for Panaferd-AX. Briefly, three grams of homogenised flesh samples were transferred to a 50 ml Quickfit tube. Measurements of the samples were done to two decimal places. One mL of deionised water was added to the tubes. Subsequently, 5 ml of tetrahydrofuran (THF)/methanol (20:1, v/v) were added and the mixture shaken vigorously for 1 min and then mixed using a shaker for 5 min. Ten millilitres of iso-hexane were then added and the contents mixed thoroughly before being centrifuged at 340 g for 10 min. The supernatant was transferred to a 50 ml

volumetric flask. Five mL of tetrahydrofuran (THF)/methanol (20:1, v/v) were added to the initial tube and the process repeated three times, making a pool of four extractions in the volumetric flask. Then, iso-hexane/THF/methanol (40:20:1, v/v/v) was added to the flask making up the volume of the pooled extractions to 50 ml. Subsequently, 10 ml of the samples were removed and evaporated to dryness under a nitrogen stream at room temperature, before being re-suspended in 2 ml isohexane prior to HPLC analysis. Samples were injected on a Thermo Scientific Ultimate 300 HPLC system equipped with a 150 x 4.6 mm 3µ Phenomenex silica column with detection at a wavelength of 474 nm and a column temperature of 25 ˚C. The carotenoids were quantified using an external standard of astaxanthin obtained from DSM (Heerlen, Netherlands).

2.5 Skeletal development and spinal deformity

Skeletal development and spinal malformation in smolt and harvest fish was assessed by x-ray radiography. Twenty-five fish per tank/pen (n = 3) were euthanised using lethal anaesthesia (>200 mg L^{-1} Tricaine, PHARMAQ UK), placed on flat trays and frozen at -20 ˚C prior to x-ray radiography. Frozen fish were x-radiographed using a portable x-ray unit (Celtic SMR PX40 HF) with an extremities plate measuring 24 x 30 cm. Each radiograph was exposed for 32 mAs and 40kV and the image digitised (AGFA CR-35X). Radiographs were analysed using Adobe Photoshop CS 6 (version 13.0.1, Adobe system Incorporated, California, USA) with the spine divided into four regions, R1, 2, 3, and 4 as per Kacem et al. (1998) and deformity type categorised according to Witten et al. (2009). Example pathologies were provided previously (Vera et al., 2019). Observations were made in the anterior-caudal direction and the total number of vertebra was recorded for each fish.

2.6 Gene expression

At the termination of the trial, a further 5 fish per pen were euthanised using lethal anaesthesia (>200 mg L^{-1} Tricaine, PHARMAQ UK) to provide samples for molecular analyses. Liver and muscle samples were aseptically dissected and placed in microcentrifuge tubes containing RNA-later for the

assessment of gene expression using microarray (liver transcriptome) and targeted gene expression through the application of real-time qPCR analysis of liver and muscle. The tissue samples were homogenised in 1 mL of TRIzol® (Invitrogen, UK) and total RNA extracted in accordance with the manufacturer's instructions. RNA pellets were rehydrated in MilliQ water, and total RNA concentration determined using an ND-1000 Nanodrop spectrophotometer (Labtech Int., East Sussex, UK), and RNA integrity assessed by electrophoresis.

2.6.1. Transcriptomic (microarray) analysis

Transcriptome analysis of liver (male only) was performed using an Atlantic salmon custom-made oligoarray with 44 k features per array in a four-array-per-slide format (Agilent Technologies UK Ltd., Wokingham, UK). A dual-label experimental design was used for the microarray hybridisations with Cy3-labelled test samples competitively hybridised to a common Cy5-labelled pooled-reference per array. A total of 15 arrays were employed, one array per individual fish. The common reference was a pool of equal amounts of amplified RNA from all test samples. Indirect labelling was employed in preparing the microarray targets. Amplified antisense RNA (aRNA) was produced from each RNA sample using TargetAmpTM 1-Round Aminoallyl-aRNA Amplification Kit 101 (Epicentre, Madison, Wisconsin, USA), as per manufacturer's methodology, followed by Cy3 or Cy5 fluor (PA23001 or PA25001, GE HealthCare) incorporation through a dye-coupling reaction, as described by Betancor et al. (2016). The hybridisations were performed using SureHyb hybridisation chambers (Agilent) in a DNA Microarray Hybridisation Oven (Agilent). Sample order was semi-randomised, with one replicate per experimental group being loaded into each slide. For each hybridisation, 825 ng of Cy3-labelled experimental biological replicate and Cy5-labelled reference pool were combined, following the protocol described by Morais et al. (2012).

2.6.2. Quantitative Real Time PCR

cDNA was reverse transcribed from 1 µg of total liver or muscle RNA using QuantiTect Reverse Transcription kit (Qiagen Ltd., Manchester, UK). The resulting cDNA was diluted 20-fold with milliQ water. Real-time PCR was performed using Luminaris color Higreen qPCR Master mix (Thermo Fisher Scientific, MA, USA) and Mastercycler RealPlex 2 thermocycler (Eppendorf, UK) which was programmed to perform the following protocol: 50 ºC for 2 min, 95 ºC for 1 min, followed by 40 cycles at 95 ºC for 15 s, annealing temperature (see Additional File 1) for 15 s and 72 ºC for 30s. This was followed by a temperature ramp from 70 to 90 ºC for melt-curve analysis to verify that no primer-dimer artefacts were present and only one product was generated from each qPCR assay. qPCR was performed in 96-well plates in duplicates per sample. The final volume of the PCR reaction was 10 µL: 2.5 µL of cDNA, 5 µL of the qPCR Master Mix and 2.5 µL of forward and reverse primers. The efficiency of the primers was verified and validated by doing standard curves for all genes investigated. The new primers used in this study were designed using the software PRIMER3 (Untergasser et al., 2012). Target specificity was checked in silico using Blast (NCBI). Only primer pairs with no unintended targets were selected (Additional File 1). The relative expression of target genes in liver and muscle of salmon post-smolts was calculated by the ΔΔCt method (Pfaffl, 2001), using *β-actin*, *ef1a*, *polr2f* and *rpl1* as the reference genes, which were chosen as the most stable according to RefFinder (Xie et al., 2012).

In liver, the genes investigated were involved in sterol metabolism (*apolipoprotein B, acetyl-CoA carboxylase* and *cholesterol 7 alpha-hydroxylase*), long-chain polyunsaturated fatty acid (LC-PUFA) metabolism (*fatty acyl elongase 2*, *fatty acyl elongase 5 isoform b*, *fatty acyl elongase 6* and *delta-6 fatty acyl desaturase isoform a*), immune system (*interleukin 8, interleukin 1 beta, major histocompatibility complex I* and *fas cell surface death receptor*), 1-carbon metabolism (*cystathionine-β-synthase*, *betaine-homocysteine S-methyltransferase, methionine adenosyltransferase1*) and *growth hormone receptor*. In muscle, the expression levels of genes involved in protein and energy metabolism (*glucose transporter type 4*, *muscle-specific RING finger protein 1a*, *muscle-specific RING finger protein 1b*, *autophagy-related protein 4 homolog B*,

autophagy related 12, *microtubule-associated proteins 1A/1B light chain 3B precursor* and *GABA(A) receptor-associated protein*), immune function (*interleukin 1 beta*) and growth (*growth hormone receptor* and *insulin-like growth factor*) were studied.

2.7 Statistical analysis

Growth and biochemical data were presented as means ± SD and analysed using one-way analysis of variance (ANOVA), general linear model, and pairwise comparison (Tukey) of means. Data were checked for homogeneity of the variances by the Bartlett test and, where necessary, the data were transformed (arcsine or ln) for normalisation before further statistical analysis. Statistical tests were performed using Minitab statistical software (Version 17©, University of Stirling, 2016). Data on vitamins, minerals, free and hydrolysed amino acids were analysed using Statistica (ver13.4, Sibco software inc.) by one way ANOVA and Tukey's post-hoc test when variances were homogenous, otherwise with the Kruskal-Wallis ANOVA by Ranks. Differences were considered statistically significant at p values < 0.05. Statistical analysis of microarray data was performed in GeneSpring GX (Agilent Technologies, Wokingham, Berkshire, UK) using a Moderated t-test, at 0.05 significance. Data were submitted to the Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa and Goto, 2000) for biological function analysis. The significance of differences in RT-qPCR data between dietary groups was determined using a one-way ANOVA followed by Tukey's test, using SPSS v.19 software (IBM, Armonk, NY), with significance level fixed at $p < 0.05$.

3. Results

3.1 Mortality, growth and maturation

There were no significant differences in cumulative mortality between dietary treatments in freshwater or seawater (Table 5). Diet had a significant effect on weight, growth rate (TGC_{wt}) and bFCR in both freshwater and saltwater (Fig. 1, Table 5). Fish fed diet L2 achieved the highest final smolt weight, with fish fed diet L1 significantly lower, and diet L3 intermediary to both. This pattern

was mirrored by similar significant dietary effects on TGC_{wt} and bFCR. Final smolt K factor decreased with increasing NP inclusion level. Diet did not significantly affect VSI, while HSI was significantly lower in salmon fed diets L2 and L3 than fish fed diet L1 at smolt.

In seawater, fish fed diets L2 and L3 achieved a significantly higher final weight than those fed diet L1, but did not differ significantly between each other. Moreover, a trend was clearly evident in TGC_{wt}, which increased with increasing NP inclusion level (Table 5). In particular, salmon fed diet L3 were transferred to sea at a significantly lower smolt weight than those fed diet L2, but achieved a comparable body weight to fish fed diet L2 within 4 months of seawater transfer (Fig. 1). Final K factor also showed a significant increase with increasing dietary NP inclusion level, and bFCR was significantly lower in fish fed diets L2 and L3 than diet L1. Both VSI and HSI were not significantly different at harvest between diets, and no statistical differences in maturation rates (predominantly males), were observed between fish fed the different diets.

3.2 Radiological deformity

Externally visible malformation was <1 % at the end of both freshwater and seawater phases, but radiologically detected spinal deformity was significantly affected by diet (Fig. 2). There was a progressive reduction in detectable malformation in fish with increasing NP inclusion level. At smolt, fish fed diet L3 had significantly lower detectable deformity than fish fed diet L1, and diet L2 was intermediary to both. The predominant region affected with spinal malformation was in the caudal trunk region (R2), with v24-28 the most commonly affected vertebral bodies. At the end of the seawater phase, there was an increase, albeit non-significant, in detectable deformity in fish fed diets L1 and L2 to that observed at smolt, and the pattern was maintained whereby salmon fed diet L3 had significantly lower deformity than those fed diet L1, and diet L2 was intermediary to both. The predominant region affected with spinal malformation was in the tail region (R3), posterior to the dorsal fin, with v38 - 44 the most commonly affected vertebral bodies. Most deformities presented themselves as compressive type pathologies (~53 % of total pathology) followed by reduction in

intervertebral spacing (~30 %) and, finally, more severe, fusion-type lesions (~15 %), and was generally similar across all diets.

3.3 Whole body composition and nutrient retention

Post-smolts had similar whole body composition independent of dietary NP inclusion level, with the exception of whole body ash that was significantly higher in salmon fed diet L3 than those fed diets L1 or L2. Whole body Zn and Se concentration increased significantly with increasing NP inclusion (Table 6). Retention of Cu decreased with increased NP inclusion, while Se retention was significantly higher in L3 than both L1 and L2. Remaining nutrient retentions did not differ between fish fed the different diets. NQC composition was generally comparable between diets, however there was a significantly higher total lipid in fish fed diet L3 than those fed diets L1 or L2. As a percentage of total fatty acids, n-6 long-chain polyunsaturated fatty acids (LC-PUFA) and docosahexaenoic acid (DHA; 22:6n-3) decreased with increasing NP inclusion level

3.4 Total amino acid concentration in whole body and free amino acids and N-metabolites in white muscle tissue

Whole body histidine and lysine retention decreased with increasing NP inclusion level, while all other amino acid retentions were similar between fish fed the different diets (Table 7). Muscle content of free OH-Pro, serine and glycine significantly decreased with increasing NP inclusion, while free histidine, increased significantly with NP inclusion level (Table 8).

3.5 Tissue water soluble vitamin status and retention

Vitamin C showed higher concentrations in whole body of fish fed L2 and L3 than in those fed L1. Vitamin C concentration in liver was not significantly different between fish fed the three diets, but the trend was that vitamin C increased from L1 to L2 and then decreased again in fish fed L3. Whole body pantothenic acid and niacin increased significantly with increasing NP inclusion, while

concentrations of the other B-vitamins in whole body were comparably independent of dietary NP inclusion level (Table 9). Riboflavin retention and the retention of folate decreased significantly with increasing NP inclusion, while thiamine, niacin and biotin showed no significant effect of NP inclusion. Tissue specific concentrations (liver, gill and muscle) of the measured B-vitamins were comparable and independent of dietary NP inclusion level, although there was a trend for increasing gill pantothenic acid and muscle vitamin B6 concentration with increasing NP inclusion.

3.6 Tissue lipid soluble vitamin status and retention

With the exception of vitamin K_1 and γ-TOH (tocopherol), all other lipid soluble vitamins in whole body and tissue (liver and muscle) showed a significant increase in concentration with increasing NP inclusion level (Table 10). Whole body α-TOH retention increased with NP inclusion level.

3.7 Gene expression

3.7.1 Liver transcriptome

Transcriptome analysis was performed on liver tissue in individual male fish (n = 5). Statistical analysis of the microarray data returned a list of 306 and 360 annotated differentially expressed genes (DEG) in liver of salmon fed the L2 and L3 diets, respectively, when compared to fish fed diet L1 (moderated t-test, $p < 0.05$ and fold-change > 1.3). In addition, a total of 396 genes were differentially expressed (fold-change > 1.3) in the liver of salmon fed diet L3 when compared to fish fed diet L2. Functional analysis for all contrasts (Kyoto Encyclopedia of Genes and Genomes, [KEGG] Orthology) showed a similar distribution by categories of DEG, with the major categories being metabolism, signal transduction and immune system. Within metabolism, the most represented category was lipid metabolism in all cases (Fig. 3). Pathway analysis showed that the top significant differentially expressed pathways in the L2 vs. L1 comparison were: PI3K-Akt signalling pathway, protein processing in endoplasmic reticulum, steroid biosynthesis, sphingolipid signalling pathway, phagosome and focal adhesion (Additional File 2). Moreover, the most represented metabolic

pathways suggested a downregulation of lipid metabolism (steroid biosynthesis, sphingolipid metabolism, glycerophospholipid metabolism, glycerolipid metabolism and arachidonic acid metabolism) in fish fed diet L2 compared to the L1 diet (Fig. 4A, Additional File 3). Regarding the L3/L1 contrast, the top significant differentially expressed pathways were PI3K-Akt signalling pathway, regulation of actin cytoskeleton, MAPK signalling pathway, Rap1 signalling pathway, NF-kappa B signalling pathway, focal adhesion and chemokine signalling pathway (Additional File 4). In this case, most genes involved in lipid and amino acid metabolism were downregulated whereas oxidative phosphorylation and glycolysis were upregulated (Fig. 4B, Additional File 3).

In order to identify common functional categories affected by micronutrient supplementation results from each pairwise contrast were intersected, obtaining a list of 138 common features corresponding to a total of 58 annotated genes. The most affected biological categories for L3/L1 were metabolism (27 %), signal transduction (15 %), immune system (9 %) and endocrine system (8 %). Within metabolism the most represented category was lipid metabolism at 11 % (Additional File 5).

3.7.2 Gene expression by qPCR

In liver, the expression of genes involved in sterol metabolism (*apoB, cyp7a1*) did not differ significantly between diets, although there was a trend for increased *apoB* expression with NP inclusion level (Table 11). Regarding genes involved in fatty acid and LC-PUFA metabolism, *fads2d6a* showed a significant decrease in fish fed diet L2 relative to fish fed diet L1, and diet L3 intermediary to both. Expression of *elovl2* was significantly higher in fish fed diet L2 than those fed diet L3, with diet L1 intermediary to both. Other genes (*acc, elovl5, elovl6, fas*) showed no significant effect of diet, although there was a trend for increased *acc* expression with increasing NP inclusion. Genes involved in 1-carbon metabolism (*cbs, bhmt, mat1*) and immune functions (*il8, il1b*) did not differ between fish fed the different diets. Finally, *ghr* expression was significantly higher in salmon fed diet L1 than those diet L2, with diet L3 intermediary to both.

In muscle, the expression of some genes involved in autophagy and protein turnover (*atg12, gabarap, murf1a, mlp3b*) showed significantly increased expression in salmon fed diet L3 relative to those fed diet L1, and diet L2 intermediary to both (Table 11). In addition, *atg4b*, and *murf1b* showed the same trend although differences between groups were not statistically significant. Differences between dietary groups for genes involved in growth and glucose transport (*ghr, igf, glut4*) were not significant. However, the pattern of *ghr* expression in muscle mirrored that of *ghr* in the liver, with fish fed diet L2 having lowest expression and those fed diet L1 highest, while *igf* expression showed a trend of increased expression with increasing NP inclusion. Finally, genes associated with immune function (*il1bm*) were not statistically different in muscle of post-smolt salmon.

4. Discussion

4.1 Growth

In the current long-term study utilising S0+ Atlantic salmon parr, growth in freshwater and the pattern of final smolt body weight between diets mirrored that previously reported in S1+ Atlantic salmon parr fed the same diet formulation (Taylor et al., 2019). In both studies, fish fed diet L2 (2xNP) achieved a significantly higher final smolt weight, than fish fed diets L1 and L3, which was reflected in an improved growth rate (TGC_{bw}) and more efficient FCR. In both studies, irrespective of smolt regime, diet L3 (4xNP) initially showed an increased growth rate relative to diet L1 (1xNP), but subsequently decreased, resulting in a final smolt weight intermediary to diets L1 and L2. This growth reduction occurred around 14 weeks post-feeding in the S1 salmon (Taylor et al., 2019) and within 4 weeks of feeding under the S0+ regime in the current study. These results are in contrast to Hemre et al. (2016) and Prabhu et al. (2019), where parr were fed similar dietary formulations for 12 weeks but a decrease in growth rate beyond 2xNP was not observed. However, parr were not taken through parr-smolt transformation (PST) in that short-term study. Of note, in both S1+ (Taylor et al., 2019) and the current S0+ parr study, the occurrence of growth reduction commenced at the start of the rising day length in spring (ambient in S1+; and following switch from LD 14:10 to LL in S0+). Given that

there are significant morphological, physiological and metabolic transformations during PST (Hoar, 1988), then there may be differing dietary requirements and nutrient re-profiling during this transition. As such, the current study suggested certain components in NP could be in "excess" or detrimental to growth when supplemented at >2xNP levels, particularly in Atlantic salmon undergoing PST. However, both the current study and that of Taylor et al. (2019) have not been able to conclusively ascertain which components and/or interactions within the NP were detrimental, and further studies are clearly warranted to elucidate this apparent phenomenon.

In seawater, growth rate (TGC_{bw}) improved linearly with increasing NP, and bFCR was more efficient in fish fed diets L2 and L3 than L1, such that fish fed diet L3 while initially stocked at a significantly smaller body weight at smolt, attained a body weight comparable to that of fish fed diet L2 at harvest. By contrast, Hemre et al., (2016) did not find a strong effect of graded NP on growth rate in post-smolts, but this may in part be related to differences between the former and current study: shorter duration (5 months *vs*. 12 months), cooler water temperatures experienced (4.1-10 ˚C *vs.* 6-16 ˚C) and subsequently lower TGC_{bw} (2.37-2.55 vs. 2.9-3.1). Nonetheless, with the progressive reduction of FM and FO down to 5 % and 3 % during the current trial, results do confirm and support the earlier shorter-term studies (Hamre et al., 2016; Hemre et al., 2016; Taylor et al., 2019; Prabhu et al., 2019) that specific components of the NP are required to be supplemented at between 2x and 4x current recommendation when utilising high plant-based diets for the duration of the life cycle. The specific components of the NP that are associated with improvements in growth and performance will be discussed in subsequent nutrient status sections.

4.2 Skeletal Health

In contrast to growth, we observed a clear linear decrease in radiological deformity with increasing NP inclusion in freshwater which concurs with results from our previous short-term feeding study (Vera et al 2019). Further, in the current study, there was also a progression of spinal pathology in post-smolts when reassessed at harvest compared to initial smolt in fish fed diets L1 and L2, while

503 diet L3 showed no further increase in pathology. Concomitantly, spinal deformity was also inversely
504 correlated with final whole body ash concentration, a widely accepted indicator of body/bone
505 mineralisation (Baeverfjord et al., 2019), indicating increasing NP level improved post-smolt mineral
506 status. Previously, upregulation of bone gene expression associated with extracellular matrix (ECM)
507 mineralisation (*ALP, Mgp, Ostcn*), and corresponding decrease in skeletal pathology was observed
508 following increased NP supplementation in parr (Vera et al., 2019). Effects were hypothesised to be
509 mediated through altered P-Ca homeostasis pathways, as reflected by increased whole-body Ca:P
510 ratios in smolts. Candidate components of the NP were suggested as vitamins D_3 and K as cofactors
511 of calcium sequestering. However, in the current study, improved mineralisation was not explained
512 by differences in whole body macromineral concentration or retention in post-smolts which were
513 similar across all diets and in the normal range for salmon (Shearer et al., 1994). Similarly, Fjelldal
514 et al. (2009) reported a reduction in vertebral deformity at 4.1 kg in S0+ post-smolts fed a high mineral
515 diet for the first 8 weeks post sea entry, and while analysis of plasma Ca, P and D-vitamin metabolites
516 reflected changes in P homeostasis, these factors could not explain the preventive effect of a high
517 mineral diet on development of bone deformities. To this end, given that analysed dietary vitamin D_3
518 and K in the current formulation were within established requirement ranges of 0.15-0.19 mg kg^{-1}
519 (NRC, 2011) and >0.1 mg kg^{-1} (Krossøy et al., 2009) respectively, it is unlikely these components of
520 the NP are directly attributable to the graded decrease in spinal pathology observed in post-smolts,
521 and other components of the NP are worth consideration.
522 Whole body Zn and Se increased across diets with NP inclusion rate, with both being
523 identified as the principal limiting micro-minerals in plant-based diets (Prabhu et al., 2018; Prabhu et
524 al., 2019). Selenium is involved in antioxidant mechanisms and redox reactions in liver and blood
525 cells, yet the role of Se in bone metabolism remains unexplored in salmonids to date (Baeverfjord et
526 al., 2019). Recent studies have shown that Se, in the form of selenoproteins, plays a vital role in bone
527 metabolism in humans (Pietschmann et al., 2014), with plasma selenoprotein P concentrations found
528 to positively correlate with bone mineral density in elderly women (Zhang et al., 2014). As such,

529 although whole body Se levels across all diets were within the normal reported range for Atlantic
530 salmon (Prabhu et al., 2016), the role of Se in teleost bone development should be investigated.

531 It is well established that Zn is the most abundant micro-mineral in the vertebrae of many
532 teleost fish species and has functional importance in the activity of ALP, and associated processes
533 related to mineralisation of bone (Prabhu et al., 2016). Thus, improved Zn status and downstream
534 processes may be directly attributable to the observed reduction in spinal pathology and progression
535 as post-smolts, particularly in fish fed diet L3 (4xNP). While studies on spinal development have
536 often focused on dietary macro- and micro-minerals, the role of specific vitamins within the NP
537 should not be overlooked as having a beneficial effect on skeletal health. Whole body vitamin A
538 levels suggested that diet L1 covered minimum requirement (>0.75mg kg^{-1} , Moren et al., 2004), and
539 diet L3 had significantly lower skeletal deformity. Diet L1-L3 in this study contained 3.7-12.2mg
540 kg^{-1} VA, well below the upper limits shown to induce skeletal pathology (>37 mg kg^{-1}) (Ørnsrud
541 et al., 2002; Ørnsrud et al., 2013). Therefore, it is unlikely that vitamin A deficiency or
542 hypervitaminosis is attributable to the differences in spinal deformity rates observed. The specific
543 role of B-vitamins on bone development in humans has been documented to a degree (Dai and Koh,
544 2015) but, as yet, has not been investigated in fish (Lall & McCrea, 2007). Evidence from humans as
545 reviewed by Dai and Koh (2015) has shown a variety of effects of vitamin B deficiencies including:
546 reduced dietary intake of B6 is associated with lower bone mineral density; synthesis of methionine
547 depends on both folate and B12 for remethylation, and deficiency of either B vitamin results in
548 megaloblastic changes in bone marrow; B vitamin deficiency affects the anatomical/biochemical
549 properties of bone if it is profound enough to cause hyperhomocysteinemia that is sufficient to induce
550 an accumulation of homocysteine in the bone tissue. In light of the growing research in B vitamin
551 involvement in bone metabolism in humans, greater consideration of B vitamin requirements in fish
552 would also warrant consideration in relation to skeletal health.
553

554 **4.3 Protein and amino acid status and retention**

555 The present study increased dietary taurine and histidine keeping the methionine constant in high
556 plant protein diets for Atlantic salmon growing from parr to approximately 2.5 kg BW. NRC
557 requirement for methionine in Atlantic salmon is reported to be 7 g kg^{-1} (NRC 2011). However, it
558 was previously reported that dietary methionine of 7.9 vs 11.4 g kg^{-1} methionine improved growth
559 and hepatosomatic index in salmon fed the latter diet (Espe et al., 2014). In another study, Atlantic
560 salmon fed high plant protein diets containing either 8 or 11 g methionine per kg of diet, the weight
561 gain in the latter diet did not differ from the control group fed a high fishmeal diet (760 g kg^{-1} diet).
562 Thus, in the current trial it was decided to maintain the dietary methionine constant at around 9 g kg^{-1}
563 diet, while histidine and taurine were increased in the nutrient packages.

564 The retention of amino acids is known to be high when an amino acid is slightly deficient and
565 then decrease as the requirement for amino acid deposition is met or increased above requirement for
566 protein deposition (Espe et al., 2017). In the current study this is also true for histidine, where
567 retention decreased as dietary histidine increased. In addition to histidine, lysine retention was also
568 reduced in salmon fed the L3 diet as compared to fish fed the L1 and L2 diets. Lysine is mainly used
569 for protein deposition (Espe et al., 2007) and the reduced retention of lysine in fish fed the highest
570 nutrient package thus might indicate a lower protein gain in muscle of these fish. However, as protein
571 retention did not differ in fish fed any of the diets, reduced lysine retention does not seem to be
572 associated with a reduced muscle protein deposition in the current trial. Thus, to address the reduced
573 retention of lysine, interaction with other nutrients in the NP needs to be further explored. It is well
574 known that both amino acid supply and the balance between dietary amino acids affect growth
575 hormone, IGF and GH (Hevrøy et al., 2007; Bower et al., 2008; Valente et al., 2012). In the current
576 trial these were not affected, which supports the fact that protein retention did not differ between fish
577 fed any of the diets in the current trial.

578 Any imbalanced supply of amino acids may increase the ubiquitination and thereby
579 degradation of proteins (Bower et al., 2008; Valente et al., 2012). The gene expression level of *murf,*
580 *gabarab* and *atg12* were higher in the higher nutrient package in the current trial, but this is more

likely due to a higher degradation of soluble protein than the degradation of muscle protein. However, this also needs to be addressed in new trials using fewer variables.

As expected, free histidine in muscle increased as dietary histidine increased simply reflecting the higher dietary histidine content in line with previous results (Remø et al., 2014). Taurine in muscle on the other hand did not increase following increased dietary taurine. This is most likely is due to increased excretion of excess taurine or that the taurine fed is directed to other tissues such as the liver (Espe et al., 2012a, 2012b). Both dietary histidine and taurine are more associated with oxidation and protection against ROS than with growth and metabolism (Remø et al., 2014) as well as survival of the cells (Espe and Holen, 2013), but these aspects were not addressed in the current trial. In addition, dietary taurine has been reported to reduce the visceral fat in salmon (Espe et al., 2012a, 2012b), but as neither HSI nor VSI were affected by increased inclusion of NP, the L1 diet appears to contain sufficient taurine to not significantly affect adiposity in salmon in the current trial.

Finally, free OH-proline decreased with increased nutrient package which might be due to an interaction with some of the other micronutrients fed, e.g. vitamin C. It is, however, unlikely that the lower OH-pro in fish muscle of fish fed the L3 diet is associated with any increased muscle degradation as deposition and growth actually was greatest in this group, although protein retention did not differ between the groups.

4.4 Water-soluble vitamins

Since growth was improved when increasing the nutrient package, without increasing methionine, the other components must have affected growth positively without the present level of methionine being critical, e.g. some of the B-vitamins will be critical for salmon to obtain maximum growth (Hemre et al., 2016), as well as some of the minerals (Prabhu et al., 2019a, b). In an experiment with salmon parr, it was estimated that the 2xNP would cover requirement for several micronutrients, especially when given with high plant raw material diets (Hemre et al., 2016, Hamre et al., 2016, Prabhu et al., 2019a). The present study, therefore, confirmed earlier conclusions that plant-based

607 diets need new requirement estimates for several micronutrients. The suggested body-concentrations,
608 health outcomes and growth response used as requirement markers were not sufficient in the former
609 study (Hemre et al., 2016), and the present study confirmed this.

610 Pantothenic acid in whole body was higher in fish fed diets L2 and L3 compared to diet L1,
611 while niacin increased gradually from L1 to L3, significantly at each step. Pantothenic acid in gill
612 tissue also increased but differences were not significant. There was also a tendency for higher B6
613 concentration in whole body in fish fed diets L2 and L3 compared to L1. This is in agreement with
614 earlier findings for salmon post-smolt (Hemre et al., 2016). These results suggest that these three B-
615 vitamin optimal levels should be raised when compared to NRC (2011), to the 2xNP level for
616 pantothenic acid and B6 and to the 4xNP level for niacin.

617 Vitamin B6 interacts highly with protein metabolism (Hansen et al., 2015). Pyridoxine
618 functions as phosphorylated coenzymes pyridoxal phosphate (PLP) in many reactions, such as
619 transaminases in amino acid metabolism. Pyridoxine is a cofactor for the enzyme aromatic amino
620 acid decarboxylase, which is required for the synthesis of neurotransmitters (Rorsman et al., 1995).
621 Therefore, symptoms and consequences of vitamin B6 deficiency are many, diverse and severe, and
622 include nervous disorders and abnormal behavior, none of which was registered in the present study.
623 Tissues with mitochondria-rich cells and cells that undergo rapid cell divisions like gill tissue are
624 especially sensitive to pantothenic acid deficiency. The 1xNP levels seemed to be sufficient for the
625 other B vitamins (thiamine, riboflavin, folic acid and biotin), as found by Hemre et al. (2016). There
626 was, however, a tendency for a decrease in retention of B-vitamins, significant only for riboflavin
627 and folate. This may point to either an interaction and/or control factor at the gut-barrier level, or
628 excretion of excess vitamin, since whole body levels were maintained or increased.

629 Vitamin C in whole body increased from diet L1 to L2 and was then unchanged in fish fed
630 diet L3. This is in accordance with previous results (Hamre et al., 2016). In the liver, changes in
631 vitamin C concentrations were not significant ($p = 0.06$), but there was a tendency for an increase in
632 fish fed diet L2 compared to L1, and then a decrease in fish fed L3. The variation was very high in

633 both the L2 and L3 groups. It is well known that vitamin C may act as a pro-oxidant at high
634 concentrations (Hamre et al., 1997), and this may indicate that diet L3, and perhaps L2, contained too
635 much vitamin C, and an optimal vitamin C level at the 2xNP level or slightly less is needed.
636
637 **4.5 Lipid soluble vitamins**
638 Lipid soluble vitamins have a tendency to accumulate in the body in response to increased
639 supplementation. Therefore, it is not possible to use body concentrations as an indication of
640 requirement. This is true for α-tocopherol (TOH) which normally shows a linear relationship between
641 supplementation and whole body concentration (Hamre et al., 1997; Hamre et al., 2016) as confirmed
642 in the present study. Hamre et al. (2016) recommended supplementation with at least 150 mg kg^{-1} α-
643 TOH to compensate for variations in composition of feed ingredients and variation in oxidative stress,
644 in response to handling, seasons and disease. TOH was not supplemented, but came from the feed
645 ingredients, and the feed and whole body concentrations did not vary among the dietary treatments.
646 For vitamin A, the situation is more complicated due to the presence of many different forms.
647 Most of the vitamin A is present in the storage form retinol-esters (Moren et al., 2004), and the method
648 used here determined esters and free forms combined. However, both free retinol and retinol esters
649 increased exponentially with increasing concentration of vitamin A in the diet, and there was a large
650 increase in body stores with an increase of dietary retinol equivalents from 0.75 to 2.5 mg kg^{-1} diet.
651 Nevertheless, a requirement was found in Atlantic halibut of 2.5 mg kg^{-1} diet based on cell
652 proliferation in the intestine as measured by DNA content, PCNA and maturity of the brush border
653 membrane by enzyme activities (Moren et al., 2004). With reference to Moren et al. (2004) and the
654 whole body levels found here, diet L1 had enough vitamin A (3.7mg kg^{-1}) to cover the requirement
655 (≥0.75 mg kg^{-1}NRC, 2011).
656 The diets were supplemented with the synthetic pro-vitamin K, menadione (vitamin K3).
657 Menadione is very unstable during feed processing (Krossøy et al., 2009), and the analysed dietary
658 levels of K3 were only 1/10 of the supplemented levels. Menadione is metabolised to menakinones

(MK4-13, vitamin K2) in the body (Krossøy et al., 2011) and therefore, MK4 increased in response to increase in dietary vitamin K3, while whole body vitamin K1 was similar in fish fed the different treatments. Krossøy et al. (2009) fed Atlantic salmon eight diets supplemented with 0 to 50 mg kg^{-1} vitamin K3 and found no effects on fish performance, or blood coagulation time and vertebrae stiffness. They concluded that the feed ingredients probably contained enough vitamin K (0.1 mg kg^{-1}) to cover the requirement, which is consistent with the findings in the present study. Estimated requirements in other species range from 0.2 to 2 mg kg^{-1} (NRC 2011).

Vitamin D was supplemented at 0.05, 0.10 and 0.20 mg kg^{-1}, but the analysed levels in muscle were 0.15-0.19 mg kg^{-1}, reflecting that the ingredients contained significant amounts of vitamin D_3. Marine feed ingredients usually contain ample amounts of vitamin D and this may be the reason that vitamin D requirements in Atlantic salmon have been difficult to define. Furthermore, high doses of vitamin D up to 57 mg kg^{-1} seem to be well tolerated by Atlantic salmon (Horvli et al., 1998; Graff et al., 2002). In other fish species estimated requirements vary from 0.006 to 0.060 mg kg^{-1} (NRC 2011), well below the supplementation levels in the present study.

4.6 Minerals

Optimal supply of dietary micro-minerals is essential to maintain body mineral homeostasis (Watanabe et al., 1997). Deficient or sub-optimal supply of dietary micro-minerals will deplete body mineral status, especially over the long term (Maage & Julshman, 1993). The NPs had graded levels of micro-minerals of which Zn and Se increased with increasing NP inclusions from diets L1 to L3. In plant-based salmonid feeds, Zn and Se have been identified as the most limiting micro-minerals (Prabhu et al., 2018, 2019a). In Atlantic salmon parr and post-smolt, dietary Zn levels of 101-132 mg kg^{-1} and 140-177 mg kg^{-1} respectively supplied by NP inclusion of 100-150 and 150-200 were able to meet the Zn requirement over the short-term (Prabhu et al., 2019a). Herein, on a long-term basis with an almost 30-fold increase in weight during the trial period, the L1 diet with 94 mg Zn kg^{-1} decreased the Zn status to half and one-third in L2 and L3 fed-fish, respectively. Similar reductions

685 in whole body Zn ranging from $^{1}/_{3}$ to ½ were observed in Atlantic salmon parr fed sub-optimal Zn
686 levels (Maage & Julshman, 1993). The L2 diet with 156 mg Zn kg^{-1} was able to sustain the body Zn
687 status on parr to the initial Zn status (18 mg kg^{-1} wet weight). However, the initial Zn status of the
688 Atlantic salmon in this study was lower than values reported in the literature for similar size fish
689 (Maage & Julshman, 1993; Shearer et al., 1994). The Zn status of Atlantic salmon fed the L2 diet (18
690 mg kg^{-1} wet weight, averaging 2.3 kg) was considerably lower than the Zn status of 27-29 mg kg^{-1} of
691 500 g post-smolt fed similar Zn levels in a previous trial (Prabhu et al. 2019a). Se levels in all three
692 dietary groups were in the normal range observed in salmonids or Atlantic salmon specifically
693 (Prabhu et al., 2016), mostly because the high background Se levels in basal diet already met the
694 requirement. Although the NP composition did not change between the present and previous studies
695 (Hamre et al., 2016; Prabhu et al., 2019a), the total dietary Se levels were considerably different for
696 corresponding NP inclusions, thereby Se recommendations differ between studies. Variation in Se
697 content of feed ingredients have to be considered in feed formulations and regulative legislation. The
698 supplementation of manganese of 12 mg/kg diet in diet L1 corresponding to a total dietary Mn of 42
699 mg/kg diet was sufficient to maintain normal body status. Recently, Prabhu et al. (2019b) also showed
700 that Mn supplementation of 14 mg/kg as $MnSO_4$ satisfied the minimal dietary requirement of Atlantic
701 salmon post-smolt fed plant-based diets. The macro-mineral composition was within normal range
702 and unaltered in Atlantic salmon fed the different diets implying the diets met the macro-mineral
703 needs of Atlantic salmon over the entire study period. Overall, Atlantic salmon post-smolt in seawater
704 were able to maintain their body status in the long run with minimal dietary supply of most micro-
705 minerals from the L1 diet, except for Zn which required L2 levels.
706
707 **4.7 Gene Expression**
708 In general, the number of significantly differentially regulated functional pathways were greater for
709 the L3 vs. L1 dietary comparison, than for the L2 vs. L1 comparison. In fact, not only the number of
710 up- or down-regulated pathways was higher in L3/L1, but also the number of DEGs involved in each

711 pathway was greater, suggesting a correlation between increased dietary micronutrient
712 supplementation in low marine diets and changes in liver transcriptome. Regarding the effects on
713 specific pathways, in both diet comparisons (L3/L1 and L2/L1), the terpenoid backbone and steroid
714 biosynthesis pathways were down-regulated. A micronutrient dependent epigenetic gene regulation
715 of the upstream regulator (acetyl-CoA carboxylase alpha, *acaca*) that provides malonyl-CoA
716 substrate for the biosynthesis of fatty acids has been demonstrated (Saito et al, *publication pending*).
717 The terpenoid backbone pathway underpins cholesterol (sterol), heme and vitamin (retinoids, retinol,
718 retinoic acid, menadione) synthesis while both biosynthetic pathways are involved in steroid (e.g.
719 testosterone) hormone production. The steroid and sterol biosynthetic pathways were also the most
720 significantly affected in zebrafish offspring after feeding the parental generation a diet deficient in
721 micronutrients (vitamin B12, vitamin B6, folic acid, methionine and choline) (Skjaerven et al., 2018).
722 Further, the offspring from deficient parents had accumulated higher levels of lipid inclusion in the
723 hepatocytes and showed a downregulation in the steroid biosynthetic process and widespread
724 epigenetic changes of gene regulation in the liver (Skjaerven et al., 2018). This suggests that
725 micronutrient supplementation may not only be affecting nutrient retention directly, but also via
726 epigenetic mechanisms altering gene regulation and downstream biochemical process.

727 These effects on gene expression may be in response to the increased supplementation of the
728 L2 and L3 diets with cholesterol, menadione (vitamin K) and retinol (vitamin A) as part of the
729 micronutrient premix and therefore reflect an increased requirement of these compounds in fish fed
730 diet L1 (Kortner et al., 2014). The lower level of dietary pantothenic acid could also have affected
731 the up-regulation of several metabolic pathways in fish fed diet L1. Pantothenic acid is required for
732 synthesis of coenzyme A, which participates in a range of acyl transfer reactions in energy production,
733 fatty acid oxidation, cholesterol and steroid synthesis, heme synthesis, amino acid catabolism,
734 acetylcholine synthesis and many other acetylation and acylation reactions (Olsvik et al., 2013).

735 Increased expression of genes related to glyoxylate/dicarboxylate metabolism was observed
736 in salmon fed diet L1 in comparison to fish fed L3, suggesting lower levels of glucose in liver of L1

737 salmon and therefore the need to synthesise it from fatty acids. Meanwhile, an activation of
738 intermediary metabolism in fish fed diet L3 may indicate higher levels of glucose available for energy
739 production, and an enhancement of cellular energy levels as suggested by the increased number of
740 up-regulated genes in glycolysis/gluconeogenesis, and oxidative phosphorylation pathways in fish
741 fed diet L3. The differences in hepatic metabolic activities, energy generation and transport and
742 growth performance between salmon fed diets L3 and L1 could be linked to the higher levels in L3
743 fish of B vitamins (riboflavin, niacin), which are involved in a range of cellular processes, most
744 importantly energy generation and transport that enables fish fed diet L3 to sustain vital metabolic
745 processes without compromising the amount of energy available for growth (Hansen et al., 2015).
746 The q-PCR results of genes involved in LC-PUFA biosynthesis showed down-regulation of
747 biosynthesis with increasing micronutrient supplementation. A down-regulation of *fads2d6a* was
748 found, indicating greater LC-PUFA biosynthesis capacity in the liver of L1 salmon. However, these
749 differences were not fully reflected in hepatic n-3 LC-PUFA profiles in the present study, which
750 showed no major differences between the dietary groups. In muscle, *mlp3b*, *atg12* and *gabarap* genes
751 were found to be upregulated in L3-fed salmon. These genes are involved in autophagosome mediated
752 autophagy, playing an important role in autophagosome formation and sequestration of cytosolic
753 cargo into double membrane vesicles, leading to subsequent degradation after fusion with lysosomes
754 (Mannackand Lane, 2014).
755
756 **5. Conclusions**
757 The present study as part EU ARRAINA project, confirmed earlier studies (Hamre et al., 2016;
758 Hemre et al., 2016; Prahbu et al., 2019; Taylor et al., 2019; Vera et al., 2019) that plant-based diets
759 for Atlantic salmon need new requirement estimates for several micronutrients. In particular, these
760 optimal levels may differ between freshwater and seawater phases of the production cycle, and that
761 requirements for growth and skeletal health may also differ. Based on the present results and
762 conclusions from earlier studies (Hamre et al., 2016; Hemre et al., 2016; Prahbu et al., 2019a; Taylor

et al., 2019; Vera et al., 2019), NRC (2011) should be revised for diets in which plants form the major part of the ingredient mix. For these plant-based diets most B-vitamin optimal levels were met at the NRC (2011) recommendations, but beneficial effects were found with moderately increased levels (L2) of niacin, riboflavin and cobalamin, vitamin C, and the minerals Zn and Se. However, as indicated in all previous studies, too high levels of the micro-nutrient package (L3) may reduce these beneficial effects.

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Figure Legends

Figure 1. Evolution of growth (increase in weight) though freshwater and seawater phases of Atlantic salmon fed the three experimental diets (mean ± SD, n=3). The feeding period for each dietary formulation (% FM/FO) is shown in the blocks at the top of the figure. Superscripts denote significant differences between diets at each respective time point.

Figure 2. Percentage fish showing at least one or more radiological spinal deformities (≥+1dV) recorded at smolt (solid bars) and harvest (hatched bars) in the dietary treatments (25 fish per tank / pen, mean ± SD, n=3). Superscripts denote significant differences between life stage and diet (Kruskall-Wallis, $p < 0.05$).

Figure 3. Functional categories of genes differentially expressed in liver of Atlantic salmon fed diets L2 (A) and L3 (B) compared to fish fed diet L1. Non-annotated genes and features corresponding to the same gene are not represented.

Figure 4. KEGG pathway analysis of genes belonging to the metabolism category in liver of Atlantic salmon fed diets L2 (**A**) and L3 (**B**) compared to fish fed diet L1. Bars represent number of up- and down-regulated genes. Different colours indicate different nutrient groups.

996 **Table 1.** Formulation of the diets in terms of FM and FO inclusions used during the course of the
997 study
998

Pellet Size	Fish Size	L1	L2	L3	
2.0 mm	20-75g	FM-15% FO-8%	FM-15% FO-8%	FM-15% FO-8%	FW
3.5 mm	75-250g	FM-15% FO-5%	FM-15% FO-5%	FM-15% FO-5%	SW
5.0 / 7.0 mm	250-1000g	FM-10% FO-4%	FM-10% FO-4%	FM-10% FO-4%	
9.0 mm	1000-2500g	FM-5% FO-3%	FM-5% FO-3%	FM-5% FO-3%	

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1000

Table 2. Raw material formulation (g.100g diet^{-1}) and proximate composition (analysed) of experimental diets. ARRAINA Nutrient Package and Amino Acid Premix inclusion for L1/L2/L3 diets respectively.

	MARINE PELLET SIZE (mm)		
INGREDIENTS	**3.5**	**5.0 / 7.0**	**9.0**
Fish Meal[1]	13.00	8.00	3.00
Krill Meal[2]	2.00	2.00	2.00
Soy Protein Concentrate[3]	15.58	15.40	12.58
Wheat Gluten[4]	12.46	13.09	10.70
Maize Gluten	3.12	3.08	2.52
Pea Protein Concentrate[5]	12.46	13.09	10.70
Wheat[6]	17.86	10.00	13.33
Sunflower Expeller	-	6.32	6.77
Fish Oil[7]	5.00	4.00	3.00
Rapeseed oil[4]	7.65	10.61	16.17
Linseed oil	1.91	2.55	3.84
Palm kernel oil	4.78	6.38	9.61
ARRAINA Nutrient Package[8]†‡	0.75 / 1.50 / 3.00	0.75 / 1.50 / 3.00	0.75 / 1.50 / 3.00
Monosodium Phosphate	2.03	-	-
Monocalcium phosphate	-	2.05	1.46
Amino acid Premix[9,]*	2.14 / 2.34 / 2.32	2.07 / 2.11 / 2.16	2.15 / 2.18 / 2.24
Yttrium	0.05	0.05	0.05
Lucantin Pink	0.06	0.06	0.06
Water change	-1.98	-0.52	0.28
Proximate Composition (Analysed)			
Moisture (%)	5.6	7.2	6.7
Crude lipid (%)	24.6	25.7	31.5
Crude protein (%)	43.8	41.4	41.2
Ash (%)	6.0	5.3	5.3
Energy (MJ / kg)	23.2	23.9	25.4

[1]Feed Services, Bremen, Germany; [2]Aker Biomarine, Norway; [3]Caramuru, Brazil; [4]Cargill, Germany; [5]Agrident, Germany; [6]WN Lindsey, UK; [7]ED & F Man, Germany; [8]DSM, Netherlands; [9]Evonik, Germany; †Added as components of the nutrient package (NP), and times requirement based on NRC (2011) minimum requirement for Atlantic salmon and modified according to Hamre et al., (2016), diet L1 achieving assumed 100 % minimum requirement; ‡ NP *Balanced for lysine, methionine, threonine and valine. Contains antioxidant.

Table 3. Analysed micronutrient contents ($mg.kg^{-1}$) within the nutrient package (NP): selected amino acids (histidine and taurine), minerals, vitamins and cholesterol. * Footnote denotes vitamin and mineral sources.

	NP		
PREMIX INGREDIENTS	**100%**	**200%**	**400%**
Vitamin A	3.79	7.58	15.16
Vitamin D3	0.05	0.10	0.20
Vitamin E	102.44	204.88	409.76
Vitamin K3	9.82	19.64	39.28
Thiamine	2.67	5.34	10.68
Riboflavin	8.30	16.60	33.20
B6	4.77	9.54	19.08
B12	0.25	0.50	1.00
Niacin	24.80	49.60	99.20
Pantothenic Acid	17.15	34.30	68.60
Folic Acid	2.82	5.64	11.28
Biotin	0.14	0.28	0.56
Vitamin C	80	160	320
Calcium	0.4	0.8	1.6
Cobalt	0.94	1.88	3.76
Iodine	0.67	1.34	2.68
Selenium	0.23	0.46	0.92
Iron	32.64	65.28	130.56
Manganese	12.03	24.06	48.12
Copper	3.24	6.48	12.96
Zinc	66.92	133.84	267.68
Taurine	2450	4900	9800
Histidine	1400	2800	5600
Cholesterol	1100	2200	4400

*__Vitamin & Mineral Source__: **Vit A**, Retinyl acetate; **Vit D_3**, Cholecalciferol; **Vit E**, all-rac-alpha-tocopheryl acetate; **Vit K**, menadione nicotinamide bisulphite; **Thiamine**, thiamine mononitrate; **Riboflavin**, Riboflavin; **B6**, Vitamin B6 / pyridoxine hydrochloride; **B12**, cyanocobalamin; **Niacin**, nicotinic acid; **Pantothenic acid**, Calcium-D-pantothenate; **Folic acid**, Folic acid; **Biotin**, Biotin; **Vit C**, L-ascorbic acid; **Co**: Cobalt carbonate; **I**, Calcium iodate anhydrous; **Se**, Sodium selenite; **Fe**, ferrous sulphate monohydrate; **Mn**, Manganous oxide; **Cu**, Cupric sulphate pentahydrate; **Zn**, Zinc oxide.

Table 4. Analysed concentrations of selected amino acids (taurine, histidine and methionine $g.kg^{-1}$) macro-minerals (calcium, magnesium and phosphorous, $g.kg^{-1}$) micro-minerals and vitamins ($mg.kg^{-1}$) of the experimental diets. Nutrients added at graded levels to the feeds are shown with an asterisk.

	DIET			
	L1	**L2**	**L3**	**NRC 2011‡**
Vitamin A*	3.7	5.2	12.2	0.75[a]
Vitamin D3*	0.15	0.19	0.19	0.04[a]
Vitamin E*	241.5	364.0	436.5	60[b]
Vitamin K3*	0.71	1.51	2.70	<10[b]
Thiamin*	4.5	7.1	8.8	1[a],
Riboflavin*	17.2	27.8	33.5	4[a],
Vitamin B6*	12.8	16.8	21.3	5[b]
Vitamin B12*	0.18	0.35	0.67	NT
Niacin*	73.0	112.0	148.0	10[a],
Pantothenic acid*	24.0	58.0	44.0	20[a,]
Folic acid*	6.53	9.69	11.67	1[a]
Biotin*	0.51	0.72	0.74	0.15[a]
Vitamin C*	183.0	251.0	409.0	20[b]
Cobalt*	0.18	0.22	0.32	NT
Iodine*	n.a	n.a	n.a	1.1[a]
Selenium*	1.13	1.48	1.65	0.15[a]
Iron*	330.0	358.0	403.0	30-60[b]
Manganese*	42.0	53.0	86.0	10[b]
Copper*	11.8	14.8	22.8	5[b]
Zinc*	94.0	156.0	330.0	37[b]
Taurine*	2.6	4.4	10.1	NR[b]
Methionine	9.7	9.9	10.3	7.0[b]
Histidine*	11.4	13.1	17.1	8.0[b]
Calcium*	6.7	7.1	8.2	NR[b]*
Magnesium	1.7	1.7	1.7	0.4[b]
Phosphorus	12.7	12.5	12.5	8.0[b]
Cholesterol*	n.a	n.a	n.a	NR

‡Current NRC, 2011 minimum requirement recommendations determined in [a] rainbow trout, [b] Atlantic salmon are shown for comparison. n.a. not analysed; NR* no requirement freshwater; NT, not tested.

Table 5. Growth and morphometric indices recorded at the end of the fresh and seawater phases of Atlantic salmon fed low marine ingredient diets (FM 15% / FO 8%) with differing micronutrient supplementation levels (L1 = 100 %, L2 = 200 % and L3 = 400 % NRC Premix). Superscripts denote significant differences between diets (mean ± SD, n=3, $p < 0.05$, one-way ANOVA)

	DIET			
	L1	**L2**	**L3**	***p* values**
FRESHWATER				
Initial Parr Wt. (g)	38.2 ± 0.6	38.0 ± 0.6	38.1 ± 0.5	0.946
Final Smolt Wt. (g)	68.0 ± 0.5^{c}	77.4 ± 0.5^{a}	73.8 ± 0.8^{b}	**0.0001**
Condition Factor (K)	1.29 ± 0.00^{c}	1.19 ± 0.00^{b}	1.18 ± 0.00^{a}	**0.0001**
TGC_{wt}	1.29 ± 0.01^{c}	1.59 ± 0.01^{a}	1.48 ± 0.02^{b}	**0.0001**
bFCR	0.90 ± 0.01^{a}	0.71 ± 0.01^{c}	0.76 ± 0.01^{b}	**0.0001**
VSI (%)	8.23 ± 0.10	8.21 ± 0.18	8.44 ± 0.35	0.456
HSI (%)	1.28 ± 0.06^{a}	1.04 ± 0.01^{b}	1.15 ± 0.06^{b}	**0.003**
Mortality (%)	0.2 ± 0.2	0.3 ± 0.1	0.0 ± 0.0	0.646
SEAWATER				
Final Wt. (g)	2127.0 ± 59.0^{b}	2381.0 ± 82.0^{a}	2385.0 ± 37.0^{a}	**0.041**
Condition Factor (K)	1.37 ± 0.01^{b}	1.43 ± 0.02^{ab}	1.46 ± 0.03^{a}	**0.049**
TGC_{wt}	2.87 ± 0.04^{b}	2.98 ± 0.05^{ab}	3.02 ± 0.02^{a}	**0.045**
bFCR	1.11 ± 0.01^{a}	1.08 ± 0.01^{b}	1.08 ± 0.01^{b}	**0.049**
VSI (%)	8.66 ± 0.10	8.98 ± 0.11	8.75 ± 0.35	0.106
HSI (%)	1.08 ± 0.06	1.02 ± 0.01	1.10 ± 0.04	0.426
% Mortality	0.95 ± 0.76	2.64 ± 2.25	0.54 ± 0.13	0.594
% Maturation	9.99 ± 1.64	5.94 ± 0.48	9.84 ± 0.74	0.063

Table 6. Protein, lipid, ash (g.kg^{-1} wet wt.), mineral content (mg.kg^{-1} wet wt.), retention (%) in whole body, and protein, lipid and fatty acid composition (%) in muscle (Norwegian Quality Cut, NQC) of Atlantic salmon at end of trial. Superscripts denote significant differences between diets when variances were homogenous (mean ± SD, n=3, p<0.05, ANOVA), when non-homogenous variances: (mean ± SD, n=3, $p < 0.5$, Kruskal-Wallis).

	DIET			
	L1	**L2**	**L3**	***p* values**
WHOLE BODY‡				
Protein	16.6 ± 0.3	16.8 ± 0.2	16.6 ± 0.5	0.61
Lipids	19.1 ± 1.4	20.0 ± 0.7	19.7 ± 0.8	0.59
Ash	1.5 ± 0.11^{b}	1.5 ± 0.11^{b}	1.7 ± 0.11^{a}	**0.045**
Zn	9.0 ± 1.0^{c}	18.0 ± 3.0^{b}	29.0 ± 3.0^{a}	**0.0003**
Mn	1.5 ± 0.5	1.5 ± 0.3	1.3 ± 0.8	0.89
Fe	7.1 ± 2.5	8.1 ± 0.4	8.9 ± 1.9	0.53
Cu	2.7 ± 0.1	2.8 ± 0.2	2.8 ± 0.4	0.81
Se	0.18 ± 0.01^{b}	0.22 ± 0.02^{b}	0.31 ± 0.02^{a}	**0.0003**
Mg	273.0 ± 15.0	273.0 ± 6.0	273.0 ± 15.0	1.00
Ca	2500 ± 1000	2800 ± 400	2600 ±1700	0.50
P	3000 ± 200	3400 ± 200	3400 ± 800	0.94
WHOLE BODY RETENTION				
Protein	37.0 ± 1.0	37.0 ± 1.0	37.0 ± 2.0	0.63
Lipids	62.0 ± 5.0	68.0 ± 2.0	67.0 ± 2.0	0.12
Ash	28.0 ± 1.6	28.7 ± 1.3	29.1 ± 1.4	0.83
Zn	8.3 ± 0.7	11.3 ± 2.2	7.7 ± 1.0	0.18
Mn	2.8 ± 0.9	2.6 ± 0.6	1.3 ± 0.8	0.11
Fe	1.8 ± 0.6	1.8 ± 0.1	2.0 ± 0.4	0.77
Cu	19.7 ± 0.5^{a}	16.9 ± 1.0^{ab}	10.4 ± 1.5^{b}	**0.0001**
Se	12.5 ± 0.5^{b}	12.2 ± 1.0^{b}	17.9 ± 1.5^{a}	**0.001**
Mg	14.0 ± 1.0	14.9 ± 0.4	15.2 ± 1.1	0.29
P	25.0 ± 7.0	26 ± 1.0	26.0 ± 6.0	1.00
Ca	38.3 ± 23.0	33.4 ± 6.0	27.4 ± 19.0	0.92
NQC				
Protein	19.8 ± 0.5	20.2 ± 0.6	20.0 ± 0.4	0.62
Lipid	13.9 ± 0.3^{b}	13.5 ± 0.1^{b}	15.0 ± 0.5^{a}	**0.004**
Total PUFA	31.4 ± 0.4	31.4 ± 0.3	30.8 ± 0.2	0.40
n-6 LC PUFA	17.5 ± 0.2^{a}	17.1 ± 0.1^{ab}	16.9 ± 0.1^{b}	**0.050**
n-3 LC PUFA	13.7 ± 0.2	14.0 ± 0.2	13.7 ± 0.1	0.45
EPA	1.5 ± 0.00	1.6 ± 0.0	1.6 ± 0.0	0.056
DHA	1.82 ± 0.05^{ab}	1.90 ± 0.06^{a}	1.60 ± 0.07^{b}	**0.036**
Total Carotenoid	4.27 ± 0.17	3.87 ± 0.29	3.96 ± 0.33	0.59

‡**Initial Body compositions at smolt sea transfer (L1 / L2 / L3):**
Protein: (16.24±0.16 / 16.49±0.19 / 16.7±0.11**); Lipids:** (11.30±0.24 / 11.8±0.35 / 11.6±0.46)**; Ash:** (2.23±0.12 / 2.25±0.14 / 2.15±0.07) **; Zn: (**25±4.5 / 30±0.2 / 44±4.5**); Mn: (**2.2±0.2 / 2.1±0.6 / 1.9±0.4**); Fe: (**10±0.4 / 14±5.9 / 11±0.1**); Cu: (**1.2±0.1 / 1.4±0.1 / 1.5±0.1**); Se: (**0.29±0.01 / 0.32±0.02 / 0.37±0.02**); Mg: (**355±21 / 345±21 / 350±0**); Ca: (**3300±707 / 3050±1061 / 2900±849**); P: (**4750±354 / 4650±566 / 4600±354**).**

Table 7. Amino acid retention (%) in Atlantic salmon fed the three plant-based diets. Superscripts denote significant differences between diets (mean ± SD, n=3, $p<0.05$, ANOVA).

	DIET			
	L1	**L2**	**L3**	***p* values**
His	31.2 ± 0.3[a]	30.2 ± 0.5[a]	26.8 ± 0.9[b]	**0.005**
OH-pro	87.5 ± 10.7	75.6 ± 1.9	101.2 ± 18.8	0.41
Arg	33.0 ± 0.4	33.4 ± 0.3	33.4 ± 1.4	0.94
Ser	31.5 ± 0.3	32.2 ± 0.4	32.6 ± 1.1	0.61
Gly	46.8 ± 1.1	44.4 ± 0.7	46.5 ± 2.6	0.56
Asp	43.5 ± 1.1	44.1 ± 0.8	43.0 ± 1.0	0.74
Glu	22.7 ± 0.2	22.8 ± 0.5	22.1 ± 0.9	0.71
Thr	42.1 ± 0.2	43.0 ± 0.2	42.8 ± 1.1	0.68
Ala	50.2 ± 1.1	50.6 ± 0.2	50.5 ± 1.4	0.95
Pro	20.7 ± 0.4	20.4 ± 0.1	20.8 ± 0.9	0.86
Tyr	36.6 ± 0.4	37.7 ± 0.3	36.4 ± 1.1	0.55
Met	43.6 ± 0.3	44.9 ± 0.3	42.0 ± 1.6	0.19
Lys	49.4 ± 1.4[a]	49.3 ± 0.4[a]	45.7 ± 0.2[b]	**0.04**
Val	41.0 ± 0.5	41.7 ± 1.1	39.4 ± 0.7	0.18
Ile	38.4 ± 0.5	39.2 ± 1.1	37.0 ± 0.6	0.21
Leu	35.5 ± 0.1	36.0 ± 0.1	35.5 ± 0.6	0.53
Phe	27.6 ± 0.3	28.5 ± 0.3	28.3 ± 1.2	0.71

Table 8. Non-protein N metabolites in muscle (µmol.g^{-1} wet wt.). Superscripts denote significant differences between diets (mean ± SD, n=3, $p<0.05$, ANOVA).

	DIET			
	L1	**L2**	**L3**	***p* values**
Taurine	0.94 ± 0.01	1.13 ± 0.22	1.00 ± 0.02	0.58
Urea	0.53 ± 0.02	0.52 ± 0.02	0.55 ± 0.06	0.88
Asp	0.09 ± 0.00	0.10 ± 0.00	0.12 ± 0.00	0.15
OH-pro	0.27 ± 0.02^{a}	0.25 ± 0.01^{ab}	0.16 ± 0.01^{b}	**0.03**
Thr	0.43 ± 0.02	0.40 ± 0.01	0.43 ± 0.02	0.62
Ser	0.14 ± 0.01^{a}	0.09 ± 0.01^{b}	0.06 ± 0.01^{b}	**0.0005**
Glu	0.99 ± 0.06	0.93 ± 0.02	0.87 ± 0.11	0.51
Gln	0.14 ± 0.02	0.23 ± 0.03	0.20 ± 0.02	0.08
Sarcosine	0.08 ± 0.02	0.11 ± 0.02	0.09 ± 0.02	0.64
Pro	0.53 ± 0.02	0.48 ± 0.06	0.29 ± 0.02	0.06
Gly	2.37 ± 0.10^{a}	1.46 ± 0.08^{b}	0.98 ± 0.06^{c}	**0.0006**
Ala	2.83 ± 0.08	2.78 ± 0.11	2.47 ± 0.12	0.11
Citruline	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.79
Alpha amino butyric acid	0.04 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	0.22
Val	0.28 ± 0.00	0.29 ± 0.01	0.29 ± 0.01	0.81
Met	0.10 ± 0.00	0.11 ± 0.00	0.10 ± 0.00	0.18
Cystathionine	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.42
Ile	0.13 ± 0.00	0.14 ± 0.00	0.13 ± 0.00	0.73
Leu	0.23 ± 0.01	0.25 ± 0.01	0.24 ± 0.01	0.30
Tyr	0.17 ± 0.02	0.23 ± 0.03	0.18 ± 0.02	0.35
Beta Ala	0.35 ± 0.02	0.39 ± 0.04	0.30 ± 0.01	0.10
Phe	0.07 ± 0.00	0.09 ± 0.00	0.07 ± 0.00	0.05
Ammonia	4.94 ± 0.15	4.80 ± 0.07	4.66 ± 0.05	0.22
Ornithine	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.30
Lys	0.81 ± 0.12	0.86 ± 0.04	0.95 ± 0.13	0.62
1-methyl His	0.05 ± 0.00	0.06 ± 0.00	0.06 ± 0.01	0.42
His	1.53 ± 0.20^{c}	2.33 ± 0.07^{b}	4.10 ± 0.09^{a}	**0.0001**
Anserine	26.58 ± 0.21	27.77 ± 0.20	27.46 ± 0.72	0.23
Carnosine	0.37 ± 0.05	0.57 ± 0.08	0.42 ± 0.05	0.12
Arg	0.16 ± 0.01	0.17 ± 0.00	0.18 ± 0.01	0.51

Table 9. Tissue concentrations (mg.kg^{-1} wet wt.) and retention (%) of water soluble vitamins. Superscripts denote significant differences between diets when variances were homogenous (mean ± SD, n=3, p<0.05, ANOVA), when non-homogenous variances: (mean ± SD, n=3, $p < 0.5$, Kruskal-Wallis).

	DIET			
	L1	**L2**	**L3**	***p* values**
WHOLE BODY				
Vitamin C	45.0 ± 4.0^{b}	60.0 ± 6.0^{a}	68.0 ± 5.0^{a}	**0.004**
Thiamin	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	0.3
Riboflavin	2.3 ± 0.1	2.4 ± 0.21	2.3 ± 0.2	0.57
Pantothenic acid	9.3 ± 0.7^{b}	12.0 ± 0.0^{a}	12.3 ± 0.6^{a}	**6*10^{-4}**
Niacin	32 ± 3^{c}	46 ± 2^{b}	56 ± 4^{a}	**4*10^{-4}**
Folic acid	0.2 ± 0.0	0.3 ± 0.04	0.3 ± 0.02	0.25
Biotin	0.1 ± 0.0	0.2 ± 0.2	0.1 ± 0.01	0.25
Vitamin B6	0.3 ± 0.02	0.5 ± 0.03	0.5 ± 0.03	0.05
LIVER				
Vitamin C	53.0 ± 1.0	91 ± 28	44 ± 24	0.06
Biotin	2.8 ± 0.6	2.7 ± 0.5	3.1 ± 0.6	0.37
Vitamin B12	0.6 ± 0.0	0.5 ± 0.1	0.6 ± 0.1	0.28
Folic acid	16.8 ± 0.3	14.8 ± 1.2	15.9 ± 0.7	0.06
Niacin	70.0 ± 3.0	72.0 ± 6.0	79.0 ± 12.0	0.42
Riboflavine	12.7 ± 0.6	12.3 ± 0.6	12.3 ± 0.6	0.73
GILL				
Pantothenic acid	8.5 ± 1.3	10.9 ± 1.9	12.0 ± 2.1	0.17
MUSCLE				
Vitamin B6	5.8 ± 0.2	6.9 ± 0.8	6.9 ± 1.0	0.17
RETENTION IN WHOLE BODY (%)				
Thiamin	21.2 ± 2.2	18.3 ± 1.0	18.6 ± 1.0	0.113
Riboflavin	9.8 ± 0.1	7.1 ± 0.5	6.5 ± 0.5	**0.051**
Niacin	37.4 ± 3.3	39.2 ± 2.8	33.1 ± 2.2	0.079
Biotin	11.4 ± 0.0	9.0 ± 0.3	10.1 ± 1.5	0.148
Folic acid	3.5 ± 0.1	2.3 ± 0.4	1.6 ± 0.1	**0.027**

Table 10. Tissue concentrations (mg.kg^{-1} wet wt.) and retention (%) of lipid soluble vitamins. Superscripts denote significant differences between diets when variances were homogenous (p<0.05, ANOVA), when non-homogenous variances: ($p < 0.5$, Kruskal-Wallis) (mean ± SD, n=3 tanks).

	DIET			
	L1	**L2**	**L3**	***p* value**
WHOLE BODY				
Dry matter %	41.0 ± 1.0	40.0 ± 1.0	41.0 ± 1.0	0.40
Vitamin A_1	0.20 ± 0.16	0.58 ± 0.13	2.23 ± 0.42	**0.04**
Vitamin A_2	0.39 ± 0.06^{c}	0.83 ± 0.12^{b}	1.77 ± 0.25^{a}	**0.0001**
Vitamin K (K_1)	30.0 ± 6.0	32.0 ± 1.0	30.0 ± 0.0	0.25
Vitamin K (MK_4)	73.0 ± 6.0^{b}	84.0 ± 8.0^{ab}	100.0 ± 7.0^{a}	**0.008**
Vitamin E (α-TOH)	28.0 ± 5.0^{c}	41.0 ± 5.0^{b}	57.0 ± 2.0^{a}	**0.0005**
Vitamin E (γ-TOH)	12.5 ± 3.8	12.1 ± 1.4	11.1 ± 0.5	0.56
MUSCLE				
Vitamin D_3	0.023 ± 0.006^{c}	0.030 ± 0.000^{b}	0.043 ± 0.006^{a}	**0.01**
Vitamin E (α-TOH)	17 ± 2^{c}	24 ± 1^{b}	36 ± 3^{a}	**0.0001**
Vitamin E (γ -TOH)	9.9 ± 1.7	9.8 ± 0.8	9.2 ± 0.5	ns
WHOLE BODY RETENTION				
Vitamin E (α-TOH)	8.8 ± 1.7^{a}	9.2 ± 0.9^{ab}	11.8 ± 0.6^{b}	**0.03**
Vitamin E (γ -TOH)	34.0 ± 10.0	30.0 ± 3.0	30.0 ± 1.0	0.60

Table 11. Relative expression of diets L2 and L3 to L1 for genes involved in lipid and fatty acid (FA) metabolism (liver) and protein turnover (muscle) in Atlantic salmon fed the experimental diets (mean ± SEM, n=3). Superscripts denote significant differences between diets.

GENE	DIET			*p* value
	L1	L2	L3	
LIPID AND FA METABOLISM (Liver)				
Apolipoprotein B-100 (*apoB*)	1.00 ± 0.09	1.68 ± 0.10	1.68 ± 0.31	0.890
Acetyl-CoA carboxylase (*acc*)	1.00 ± 0.13	1.16 ± 0.22	1.23 ± 0.18	0.520
Cholesterol 7 alpha-hydroxylase (*Cyp7a1*)	1.00 ± 0.40	0.64 ± 0.15	1.14 ± 0.55	0.572
Fatty acyl elongase 2 (*elovl2*)	1.00 ± 0.26^{ab}	1.19 ± 0.20^{a}	0.56 ± 0.15^{b}	**0.032**
Fatty acyl elongase 5 isoform b (*elovl5*)	1.00 ± 0.07	1.07 ± 0.14	0.84 ± 0.16	0.454
Fatty acyl elongase 6 (*elovl6*)	1.00 ± 0.50	1.17 ± 0.78	0.91 ± 0.39	0.577
Delta-6 fatty acyl desaturase isoform a (*fads2d6a*)	1.00 ± 0.34^{a}	0.36 ± 0.09^{b}	0.44 ± 0.12^{ab}	**0.035**
S-acyl fatty acid synthase thioesterase (*fas*)	1.00 ± 0.14	0.90 ± 0.09	1.00 ± 0.10	0.671
1-CARBON METABOLISM (Liver)				
Cystathionine-*β*-synthase (*cbs*)	1.00 ± 0.26	0.91 ± 0.31	0.86 ± 0.26	0.854
Betaine-homocysteine S-methyltransferase (*bhmt*)	1.00 ± 0.13	0.91 ± 0.13	1.09 ± 0.13	0.534
Methionine adenosyltransferase1 (*mat1*)	1.00 ± 0.15	0.70 ± 0.15	1.10 ± 0.13	0.271
Growth hormone receptor (*ghr*)	1.00 ± 0.40^{a}	0.36 ± 0.11^{b}	0.79 ± 0.32^{ab}	**0.043**
IMMUNE FUNCTION (Liver)				
Interleukin 8 (*il8*)	1.00 ± 0.20	0.74 ± 0.45	0.94 ± 0.17	0.640
Interleukin 1 beta (*il1b*)	1.00 ± 0.20	1.10 ± 0.17	1.14 ± 0.12	0.663
PROTEIN TURNOVER (Muscle)				
Autophagy-related protein 4 homolog B (*atg4b*)	1.00 ± 0.09	1.28 ± 0.20	1.24 ± 0.11	0.401
Autophagy related 12 (*atg12*)	1.00 ± 0.08^{b}	1.04 ± 0.05^{ab}	1.29 ± 0.09^{a}	**0.029**
GABA(A) receptor-associated protein (*gabarap*)	1.00 ± 0.05^{b}	1.24 ± 0.06^{ab}	1.47 ± 0.09^{a}	**0.001**
Muscle-specific RING finger protein 1a (*murf1a*)	1.00 ± 0.04^{b}	1.11 ± 0.17^{ab}	1.19 ± 0.08^{a}	**0.049**
Muscle-specific RING finger protein 1b (*murf1b*)	1.00 ± 0.09	1.01 ± 0.18	1.51 ± 0.25	0.057
Microtubule-associated proteins 1A/1B light chain 3B precursor (*mlp3b*)	1.00 ± 0.06^{b}	1.20 ± 0.09^{ab}	1.48 ± 0.07^{a}	**0.002**
GROWTH & GLUCOSE TRANSPORT (Muscle)				
Growth hormone receptor (*ghr*)	1.00 ± 0.12	0.73 ± 0.16	0.98 ± 0.15	0.427
Insulin-like growth factor 1 (*igf1*)	1.00 ± 0.27	1.17 ± 0.33	1.43 ± 0.38	0.251
Glucose transporter type 4 (*glut4*)	1.00 ± 0.06	0.95 ± 0.14	0.98 ± 0.09	0.824
IMMUNE FUNCTION (Muscle)				
Interleukin 1 beta (*il1b*)	1.00 ± 0.33	1.04 ± 0.64	0.80 ± 0.11	0.488

1088 **Fig.1.**

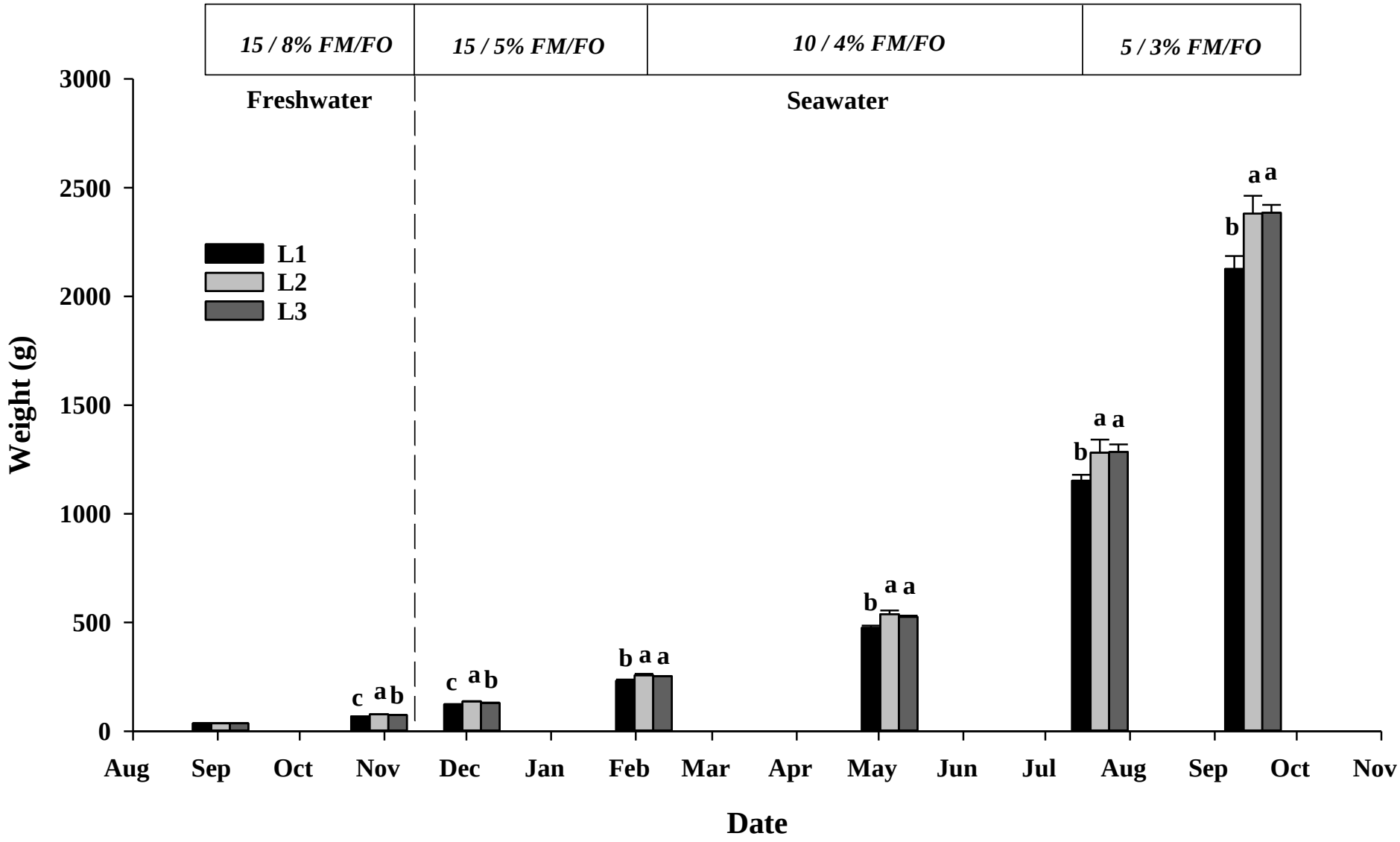

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Fig. 2.

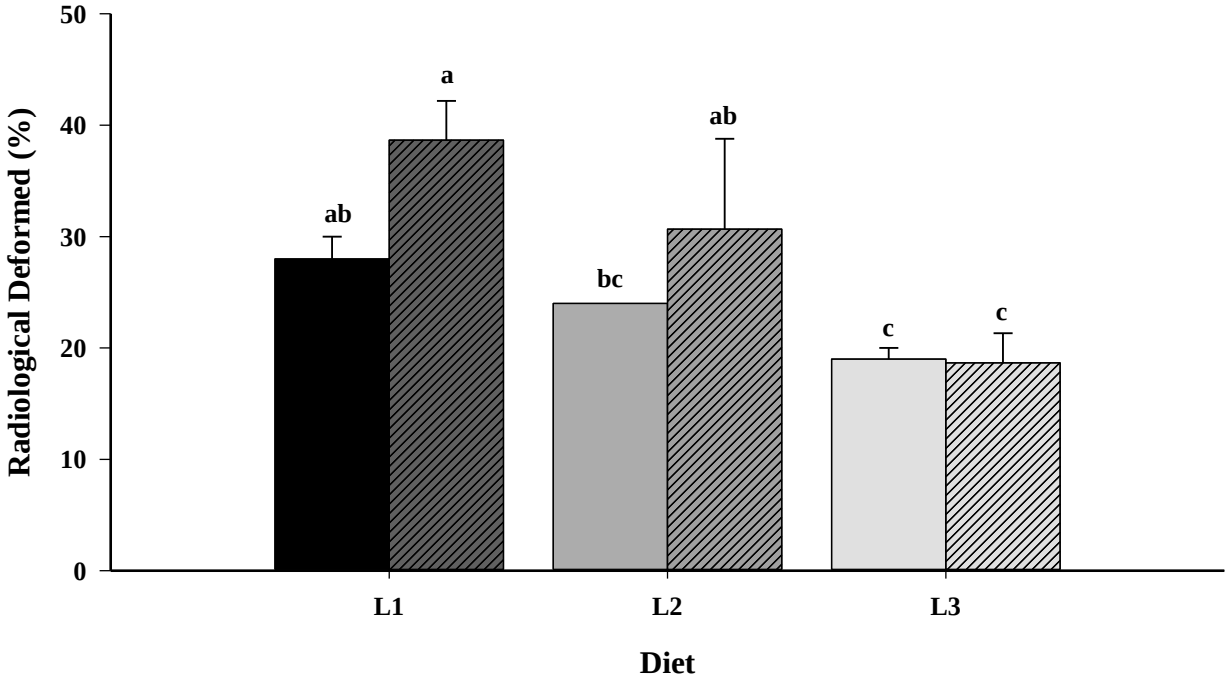

Radiological Deformed (%)
50
40
30
20
10
0
a
ab
ab
bc
c
c
L1
L2
L3
Diet

Fig 3.

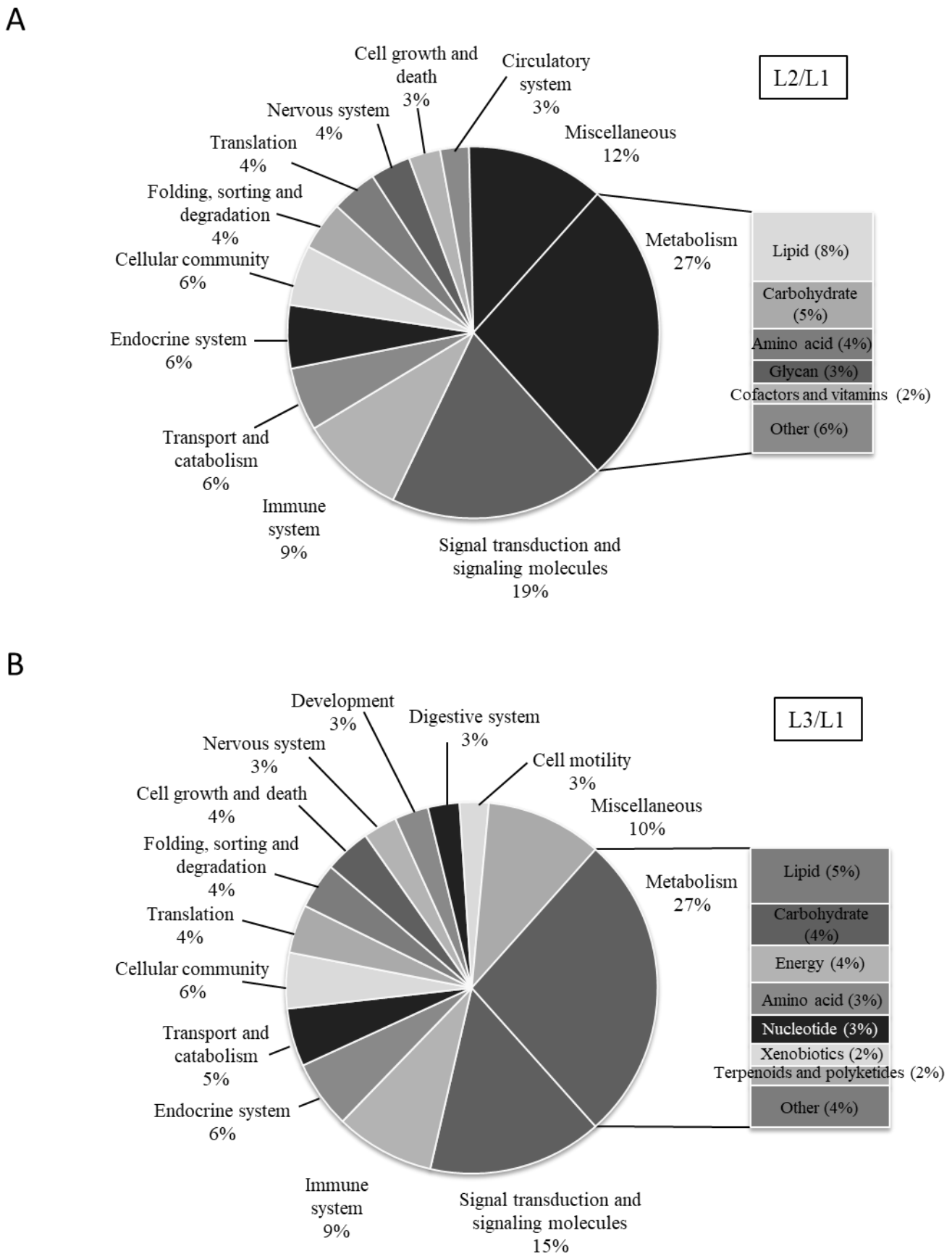
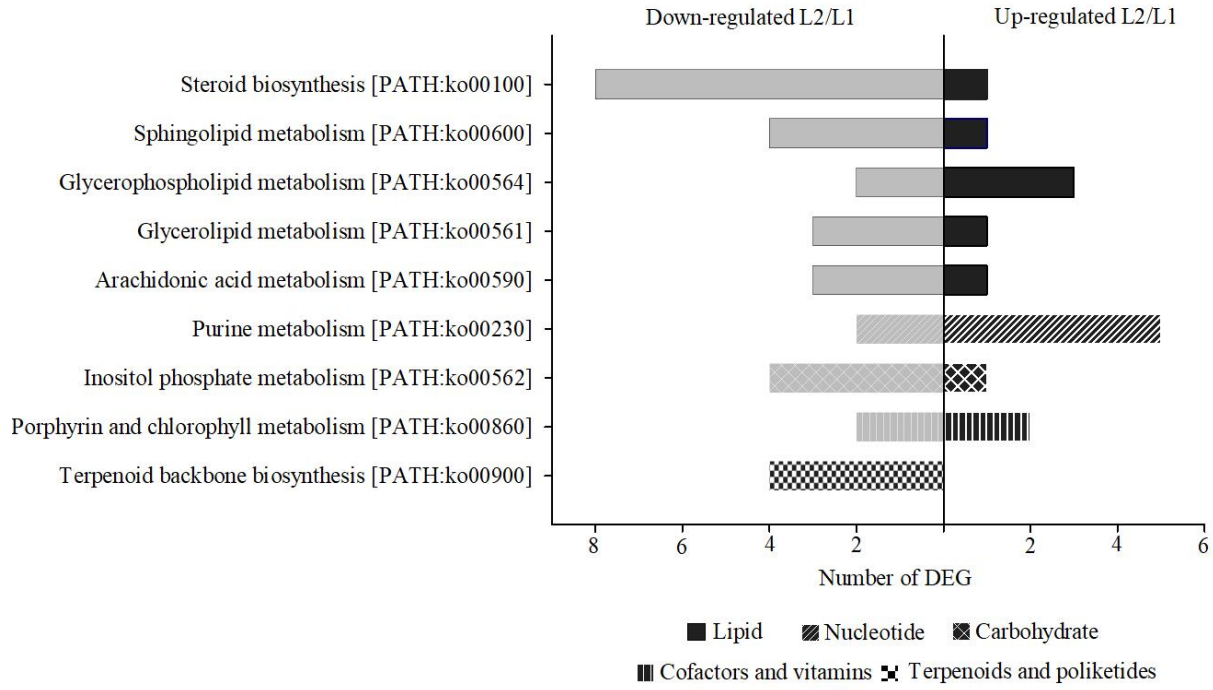
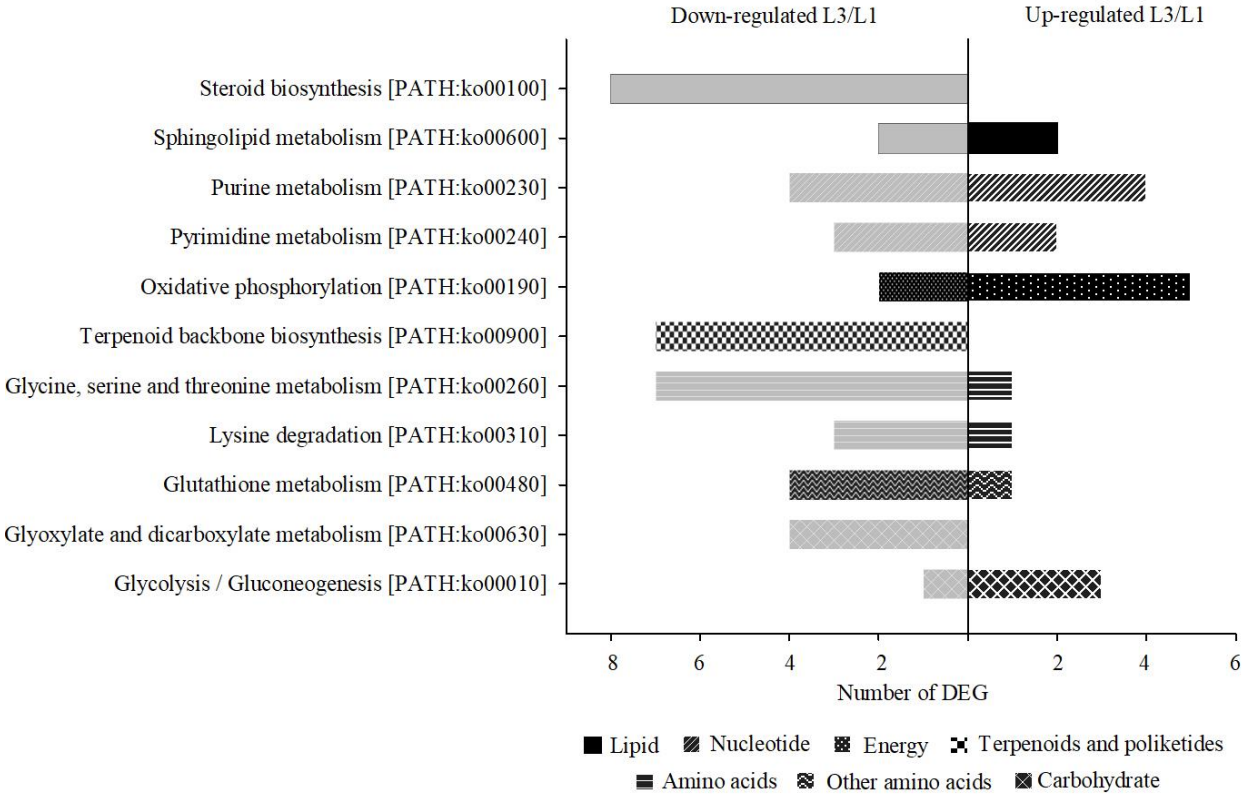

A
L2/L1
Cell growth and death 3%
Circulatory system 3%
Nervous system 4%
Miscellaneous 12%
Translation 4%
Folding, sorting and degradation 4%
Metabolism 27%
Cellular community 6%
Endocrine system 6%
Transport and catabolism 6%
Immune system 9%
Signal transduction and signaling molecules 19%
Lipid (8%)
Carbohydrate (5%)
Amino acid (4%)
Glycan (3%)
Cofactors and vitamins (2%)
Other (6%)
B
L3/L1
Development 3%
Digestive system 3%
Nervous system 3%
Cell motility 3%
Cell growth and death 4%
Miscellaneous 10%
Folding, sorting and degradation 4%
Metabolism 27%
Translation 4%
Cellular community 6%
Transport and catabolism 5%
Endocrine system 6%
Immune system 9%
Signal transduction and signaling molecules 15%
Lipid (5%)
Carbohydrate (4%)
Energy (4%)
Amino acid (3%)
Nucleotide (3%)
Xenobiotics (2%)
Terpenoids and polyketides (2%)
Other (4%)

Fig 4.

A


Down-regulated L2/L1
Up-regulated L2/L1
Steroid biosynthesis [PATH:ko00100]
Sphingolipid metabolism [PATH:ko00600]
Glycerophospholipid metabolism [PATH:ko00564]
Glycerolipid metabolism [PATH:ko00561]
Arachidonic acid metabolism [PATH:ko00590]
Purine metabolism [PATH:ko00230]
Inositol phosphate metabolism [PATH:ko00562]
Porphyrin and chlorophyll metabolism [PATH:ko00860]
Terpenoid backbone biosynthesis [PATH:ko00900]
8
6
4
2
2
4
6
Number of DEG
Lipid
Nucleotide
Carbohydrate
Cofactors and vitamins
Terpenoids and poliketides

B


Down-regulated L3/L1
Up-regulated L3/L1
Steroid biosynthesis [PATH:ko00100]
Sphingolipid metabolism [PATH:ko00600]
Purine metabolism [PATH:ko00230]
Pyrimidine metabolism [PATH:ko00240]
Oxidative phosphorylation [PATH:ko00190]
Terpenoid backbone biosynthesis [PATH:ko00900]
Glycine, serine and threonine metabolism [PATH:ko00260]
Lysine degradation [PATH:ko00310]
Glutathione metabolism [PATH:ko00480]
Glyoxylate and dicarboxylate metabolism [PATH:ko00630]
Glycolysis / Gluconeogenesis [PATH:ko00010]
8
6
4
2
2
4
6
Number of DEG
Lipid
Nucleotide
Energy
Terpenoids and poliketides
Amino acids
Other amino acids
Carbohydrate