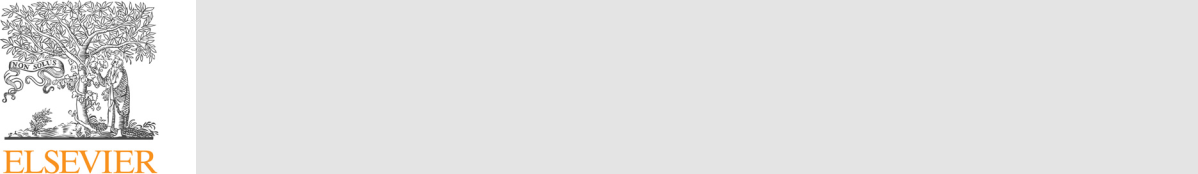
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Higher dietary micronutrients are required to maintain optimal performance T of Atlantic salmon (Salmo salar) fed a high plant material diet during the full  production cycle



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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 1×, 2× and 4×, where 1× 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 in-fluence 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 mi-cronutrient 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.

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](#page15); [Hardy, 2010](#page15); [Turchini](#page15) [et al., 2011](#page15)). 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](#page15)). Over this period, many studies investigated the eﬀects of the inclusion of plant-derived in-gredients in feeds. The majority of studies focussed on the impacts on growth performance and feed utilisation ([Torstensen et al., 2000](#page15); [Opstvedt et al., 2003](#page15); [Mundheim et al., 2004](#page15); [Espe et al., 2006](#page15); [Torstensen et al., 2005](#page15); [Pratoomyot et al., 2008](#page15); [Waagbø et al., 2013](#page15)), and also on product quality for human consumers ([Waagbø et al., 1993](#page15);



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[Mundheim et al., 2004](#page15); [Torstensen et al., 2005](#page15); [Menoyo et al., 2007](#page15)). 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 and Jackson, 2013](#page15)).

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](#page15)), including changing micronutrient concentrations and their chemical forms, as well as introducing compounds that in-teract with micronutrient uptake and metabolism ([Olsvik et al., 2013](#page15)). 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](#page15); [Seierstad et al., 2005](#page15); [Waagbø, 2006](#page15); [Hemre and Sandnes, 2008](#page15); [Waagbø, 2008](#page15); [Seierstad](#page15) [et al., 2009](#page15)). As a result, there has been increasing interest in improving

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knowledge of practical nutrient requirements of Atlantic salmon when fed modern, plant based feeds ([Hansen et al., 2015](#page15)). 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](#page15)). When feeding plant-based diets enriched with the micronutrients folate, vi-tamin B12, vitamin B6, methionine and choline to zebrafish (Danio rerio) over the entire life cycle, fish responded with increased growth, higher fecundity and altered metabolic profile of several N-metabolites, indicating an overall positive eﬀect of a micronutrient-enriched plant-based diet ([Skjaerven et al., 2016](#page15)). As a consequence, micronutrient and mineral requirements need careful re-evaluation when substituting raw materials, as the current established micronutrient supplements / premixes ([National Research Council (NRC), 2011](#page15)) may not be suﬃ-cient to satisfy requirements for Atlantic salmon, especially throughout the whole life cycle.

To investigate this, micronutrient requirements of Atlantic salmon fed diets formulated with high levels of plant meals and vegetable oil were studied in two regression experiments in parr in freshwater and post-smolts in seawater ([Hamre et al., 2016](#page15); [Hemre et al., 2016](#page15); [Prabhu](#page15) [et al., 2019a](#page15)). A nutrient package, consisting of vitamins B, C, D3, E, minerals, cholesterol, taurine and histidine was fed at various levels from below [NRC (2011)](#page15) recommended levels to well above NRC levels. Growth, health and welfare parameters responded to nutrient packages in parr, but not to the same extent in post-smolts ([Hemre et al., 2016](#page15)). Thus, parr fed diets containing the nutrient package supplying micro-nutrients above [NRC (2011)](#page15) recommended levels showed improved protein retention, and reduced liver and viscera indices, whereas the response was less pronounced in post-smolt ([Hemre et al., 2016](#page15)). Sig-nificant regressions were obtained in body compartments for vitamin C and several of the B-vitamins and, based on these results, the authors recommended that B-vitamin supplements should be adjusted in plant-based diets for Atlantic salmon ([Hamre et al., 2016](#page15); [Hemre et al., 2016](#page15)). There were no indications of vitamin E deficiency when vitamin E was not supplemented, indicating that the ingredients delivered suﬃcient amounts of vitamin E. Nevertheless, it was recommended to supple-ment 150 mg kg−1vitamin E to compensate in periods of oxidative stress ([Hamre et al., 2016](#page15)). Regarding the micro-minerals, requirements of Atlantic salmon fed the plant-based diet were met at NP inclusion of 100–150% and 150–200% in parr and post-smolt, respectively ([Prabhu](#page15) [et al., 2019a](#page15)).

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](#page15) [et al., 2019](#page15)). 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](#page15)). 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](#page15)). 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)](#page15) recommenda-tions for optimal growth and liver function of diploid Atlantic salmon fed plant-based diets in freshwater ([Taylor et al., 2019](#page15)). In addition, the above study also investigated the impact of NP level on skeletal de-formity and bone health including the prevalence, localisation and pathology of spinal malformation, and vertebral expression of bone biomarker genes ([Vera et al., 2019](#page15)). This study showed that prevalence of radiologically detectable spinal deformities decreased with

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increasing micronutrient supplementation when fed from parr to smolt. Concomitantly, expression of many osteogenic genes (bone morpho-genic protein, osteocalcin, alkaline phosphatase, matrix metallopepti-dase, 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 ex-pression (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](#page15); [Hemre et al., 2016](#page15); [Taylor et al., 2019](#page15); [Vera et al., 2019](#page15); [Prabhu et al.,](#page15) [2019a](#page15)). 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 re-tention, skeletal development and liver gene expression were de-termined.

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](#page15)). Feeds were supplemented with a nutrient package (NP, [Tables 3 and 4](#page15)) at one of three inclusion levels to produce 3 dietary treatments: L1, 100% NP; L2, 200% NP; L3, 400% NP ([Table 4](#page15)), 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](#page15)). Specifically, the NP contained 24 nutrients in total these being; vita-mins (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

Table 1

Formulation of the diets in terms of FM and FO inclusions used during the course of the study.

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

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Table 2

Raw material formulation (g.100 g diet−1) and proximate composition (ana-lysed) of experimental diets. ARRAINA Nutrient Package and Amino Acid Premix inclusion for L1/L2/L3 diets respectively.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Marine pellet size (mm) | |  |  |
|  |  |  |  |  |
|  | 3.5 | 5.0 / 7.0 | 9.0 |  |
|  |  |  |  |  |
| Ingredients |  |  |  |  |
| Fish meal[1](#page15) | 13.00 | 8.00 | 3.00 |  |
| Krill meal[2](#page15) | 2.00 | 2.00 | 2.00 |  |
| Soy protein concentrate[3](#page15) | 15.58 | 15.40 | 12.58 |  |
| Wheat gluten[4](#page15) | 12.46 | 13.09 | 10.70 |  |
| Maize gluten | 3.12 | 3.08 | 2.52 |  |
| Pea protein concentrate[5](#page15) | 12.46 | 13.09 | 10.70 |  |
| Wheat[6](#page15) | 17.86 | 10.00 | 13.33 |  |
| Sunflower expeller | – | 6.32 | 6.77 |  |
| Fish oil[7](#page15) | 5.00 | 4.00 | 3.00 |  |
| Rapeseed oil[4](#page15) | 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 | 0.75 / 1.50 / | 0.75 / 1.50 / | 0.75 / 1.50 / |  |
| package[8](#page15),[a](#page15),[b](#page15) | 3.00 | 3.00 | 3.00 |  |
| Monosodium phosphate | 2.03 | – | – |  |
| Monocalcium phosphate | – | 2.05 | 1.46 |  |
| Amino acid premix[9](#page15),[c](#page15) | 2.14 / 2.34 / | 2.07 / 2.11 / | 2.15 / 2.18 / |  |
|  | 2.32 | 2.16 | 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.
10. Added as components of the nutrient package (NP), and times requirement based on [NRC (2011)](#page15) minimum requirement for Atlantic salmon and modified according to [Hemre et al. (2016)](#page15), diet L1 achieving assumed 100% minimum requirement.
11. NP.
12. Balanced for lysine, methionine, threonine and valine. Contains anti-oxidant.

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 m3 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

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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.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Premix ingredients | NP |  |  |  |
|  |  |  |  |  |
|  | 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 D3, Cholecalciferol; Vit E, all-rac-alpha-tocopheryl acetate; Vit K, menadione nicotinamide bisul-phite; 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.

T2000, Arvotec, Finland) controlled by a PC system. Specific feeding rates (SFR; % tank biomass per day) were adjusted automatically ac-cording 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 re-servoir 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 tem-peratures 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.

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Table 4

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

* Current [NRC, 2011](#page15) 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.

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 (50 ppm Tricaine, PHARMAQ, UK), individual weights ( ± 0.1 g) and fork lengths ( ± 1.0 mm) mea-sured, 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: (eg−1) × 100, where g = (ln (Wf) − ln(Wi)) × (t2 − t1)−1 and TGC was calculated as: (Wf1/3-Wi1/3)

1. (∑Do)-1, where Wf is the final body weight, Wi is the initial body weight and Do is the cumulative sum of water temperature in degrees per day. FCR was calculated as: Fi / (Bf − Bi + Bm) where Fi is the fed intake (kg), Bf is the final biomass (kg), Bi is the initial biomass (kg) and Bm 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 (%) = (GW × 100)/ BW. Fish were classified as sexually recruited based on a threshold value of GSI > 0.20% or > 1.0% for males and females respectively ([Kadri](#page15) [et al., 1997](#page15)).

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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 prox-imate composition according to standard procedures ([AOAC, 2000](#page15)). 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)](#page15). The total amino acids in whole body and diets were analysed after being hydrolysed in 6 N HCl for 22 h as previously described ([Espe et al., 2014](#page15)) 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](#page15) [et al., 2000](#page15); [Feldsine et al., 2002](#page15)). Some of the B-vitamins were de-termined by HPLC by standard methods; thiamine ([CEN, 2003](#page15)), vitamin B6 ([CEN, 2006](#page15)) and riboflavin ([Brønstad et al., 2002](#page15)). Multi-element determination was done by ICP-MS (inductively coupled plasma mass spectrometry) ([Julshamn et al., 1999](#page15)). HPLC was used for determina-tion of ascorbic acid ([Mæland et al., 1999](#page15)) and tocopherols were ana-lysed according to standard methodology ([CEN, 1999](#page15)). Vitamins A and D were analysed according to [Moren et al. (2004)](#page15) and [Horvli and Lie](#page15) [(1994)](#page15), respectively.

2.4.3. 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 grav-imetrically ([Folch et al., 1957](#page15)). Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalysed transesterification at 50 °C for 16 h ([Christie, 2003](#page15)), and FAME extracted and purified as described previously ([Tocher and Harvie, 1988](#page15)). 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 pro-cessed 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).

2.4.4. 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

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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 volu-metric 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 ni-trogen 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 × 4.6 mm 3 μ 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 × 30 cm. Each radiograph was exposed for 32 mAs and 40 kV 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)](#page15) and deformity type cate-gorised according to [Witten et al. (2009)](#page15). Example pathologies were provided previously ([Vera et al., 2019](#page15)). 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 eu-thanised 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 mi-croarray (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 con-centration determined using an ND-1000 Nanodrop spectrophotometer (Labtech Int., East Sussex, UK), and RNA integrity assessed by elec-trophoresis.

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 mi-croarray targets. Amplified antisense RNA (aRNA) was produced from each RNA sample using TargetAmpTM 1-Round Aminoallyl-aRNA

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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)](#page15). 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 experi-mental biological replicate and Cy5-labelled reference pool were com-bined, following the protocol described by [Morais et al. (2012)](#page15).

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 °C for 2 min, 95 °C for 1 min, followed by 40 cycles at 95 °C for 15 s, annealing temperature (see Additional File

1. for 15 s and 72 °C for 30s. This was followed by a temperature ramp from 70 to 90 °C 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](#page15) [et al., 2012](#page15)). 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 Ct method ([Pfaﬄ, 2001](#page15)), 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](#page15)).

In liver, the genes investigated were involved in sterol metabolism (apolipoprotein B, acetyl-CoA carboxylase and cholesterol 7 alpha-hydro-xylase), 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 in-volved 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, micro-tubule-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 ne-cessary, the data were transformed (arcsine or ln) for normalisation before further statistical analysis. Statistical tests were performed using Minitab statistical software (Version 17©). 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 sig-nificant at p values < .05. Statistical analysis of microarray data was

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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 < .05, one-way ANOVA).

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | DIET |  |  |  |  |  | p values |  |
|  |  |  |  |  |  |  |  |  |
|  | L1 |  | L2 |  | L3 |  |  |  |
|  |  |  |  |  |  |  |  |  |
| 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 | ± 0.5c | 77.4 | ± 0.5a | 73.8 | ± 0.8b | 0.0001 |  |
| Condition factor (K) | 1.29 | ± 0.00c | 1.19 | ± 0.00b | 1.18 | ± 0.00a | 0.0001 |  |
| TGCwt | 1.29 | ± 0.01c | 1.59 | ± 0.01 a | 1.48 | ± 0.02b | 0.0001 |  |
| bFCR | 0.90 | ± 0.01a | 0.71 | ± 0.01c | 0.76 | ± 0.01b | 0.0001 |  |
| VSI (%) | 8.23 | ± 0.10 | 8.21 | ± 0.18 | 8.44 | ± 0.35 | 0.456 |  |
| HSI (%) | 1.28 | ± 0.06a | 1.04 | ± 0.01b | 1.15 | ± 0.06b | 0.003 |  |
| Mortality (%) | 0.2 ± 0.2 | | 0.3 ± 0.1 | | 0.0 ± 0.0 | | 0.646 |  |
| Seawater | 2127.0 ± 59.0b | | 2381.0 ± 82.0a | | 2385.0 ± 37.0a | |  |  |
| Final Wt. (g) | 0.041 |  |
| Condition factor (K) | 1.37 | ± 0.01b | 1.43 | ± 0.02ab | 1.46 | ± 0.03a | 0.049 |  |
| TGCwt | 2.87 | ± 0.04b | 2.98 | ± 0.05ab | 3.02 | ± 0.02a | 0.045 |  |
| bFCR | 1.11 | ± 0.01a | 1.08 | ± 0.01b | 1.08 | ± 0.01b | 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 |  |

Bold text indicates signifcant p values.

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](#page15)) for biological function analysis. The sig-nificance 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 < .05.

3. Results

3.1. Mortality, growth and maturation

There were no significant diﬀerences in cumulative mortality be-tween dietary treatments in freshwater or seawater ([Table 5](#page15)). Diet had

1. significant eﬀect on weight, growth rate (TGCwt) and bFCR in both freshwater and saltwater ([Fig. 1](#page15), [Table 5](#page15)). 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 TGCwt 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 be-tween each other. Moreover, a trend was clearly evident in TGCwt, which increased with increasing NP inclusion level ([Table 5](#page15)). In par-ticular, 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](#page15)). Final K factor also showed a significant increase with in-creasing 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 sig-nificantly diﬀerent at harvest between diets, and no statistical diﬀer-ences in maturation rates (predominantly males), were observed be-tween 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 de-formity was significantly aﬀected by diet ([Fig. 2](#page15)). There was a pro-gressive 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 af-fected 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 pre-dominant region aﬀected with spinal malformation was in the tail re-gion (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](#page15)). Retention of Cu decreased with in-creased NP inclusion, while Se retention was significantly higher in L3 than both L1 and L2. Remaining nutrient retentions did not diﬀer be-tween 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 per-centage 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

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|  |
| --- |
| **Weight (g)** |

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | ***15 / 8% FM/FO*** | | ***15 / 5% FM/FO*** | |  |  | ***10 / 4% FM/FO*** | | |  | ***5 / 3% FM/FO*** | | |  |  |
| **3000** |  | **Freshwater** | |  |  |  |  | **Seawater** | |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
| **2500** |  |  |  |  |  |  |  |  |  |  |  |  |  | **a a** |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  | **L1** |  |  |  |  |  |  |  |  |  |  | **b** |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| **2000** |  | **L2** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | **L3** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| **1500** |  |  |  |  |  |  |  |  |  |  |  | **a a** |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  | **b** |  |  |  |  |
| **1000** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| **500** |  |  |  |  |  |  |  |  | **b a a** |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  | **c a b** |  | **b a a** |  |  |  |  |  |  |  |  |  |  |
|  |  |  | **c a b** |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| **0** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| **Aug** | **Sep** | **Oct** | **Nov** | **Dec** | **Jan** | **Feb** | **Mar** | **Apr** | **May** | **Jun** | **Jul** | **Aug** | **Sep** | **Oct** | **Nov** |  |

**Date**

Fig. 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.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **50** |  |  |  |  |
|  |  | **Smolt** | **a** |  |  |
| **(%)** |  |  | **ab** |  |
| **40** | **Harvest** |  |  |
| **Deformed** |  |  |
| **30** | **ab** |  |  |  |
|  |  |  |  |
|  |  |  |  |  |
|  |  |  | **bc** |  |  |
| **Radiological** | **10** |  |  | **c** |  |
| **c** |  | **c** |  |
|  | **20** |  |  |
|  |  |  |  |
|  |  | **c** |  |  |  |
|  | **0** |  |  |  |  |
|  |  | **L1** | **L2** | **L3** |  |
|  |  |  | **Diet** |  |  |

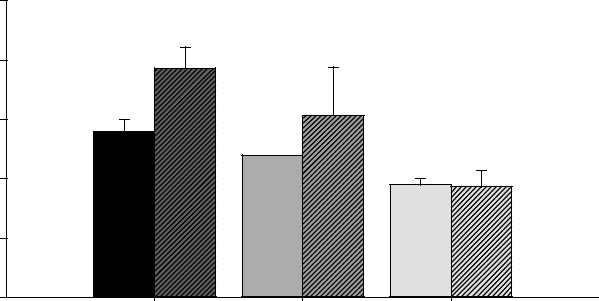


Fig. 2. Percentage fish showing at least one or more radiological spinal de-formities (≥ + 1dV) recorded at smolt (solid bars) and harvest (hatched bars) in the dietary treatments (25 fish per tank / pen, mean ± SD, n = 3). Superscripts denote significant diﬀerences between life stage and diet (Kruskall-Wallis, p < .05).

between fish fed the diﬀerent diets ([Table 7](#page15)). 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](#page15)).

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-vi-tamins in whole body were comparably independent of dietary NP in-clusion level ([Table 9](#page15)). 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-vita-mins 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 K1 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 in-clusion level ([Table 10](#page15)). 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 < .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](#page15)). Pathway analysis showed that the top significant diﬀerentially expressed path-ways 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 (Ad-ditional File 2). Moreover, the most represented metabolic pathways

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Table 6

Protein, lipid, ash (g.kg−1 wet wt.), mineral content (mg.kg−1 wet wt.), re-tention (%) 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 < .05, ANOVA), when non-homogenous variances: (mean ± SD, n = 3, p < .5, Kruskal-Wallis).

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | DIET | |  |  |  |  |  |  | p values |
|  |  |  |  |  |  |  |  |  |  |
|  | L1 |  |  | L2 |  | L3 |  |  |  |
|  |  |  |  |  |  |  |  |  |  |
| Whole body[‡](#page15) |  |  |  |  |  |  |  |  |  |
| 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 | ± 0.11b | | 1.5 ± 0.11b | | 1.7 | ± 0.11a | | 0.045 |
| Zn | 9.0 | ± 1.0c | | 18.0 | ± 3.0b | 29.0 | | ± 3.0a | 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 | | ± 0.01b | 0.22 | ± 0.02b | 0.31 | | ± 0.02a | 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 | | ± 0.5a | 16.9 | ± 1.0ab | 10.4 | | ± 1.5b | 0.0001 |
| Se | 12.5 | | ± 0.5b | 12.2 | ± 1.0b | 17.9 | | ± 1.5a | 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 | | ± 0.3b | 13.5 | ± 0.1b | 15.0 | | ± 0.5a | 0.004 |
| Total PUFA | 31.4 | | ± 0.4 | 31.4 | ± 0.3 | 30.8 | | ± 0.2 | 0.40 |
| n-6 LC PUFA | 17.5 | | ± 0.2a | 17.1 | ± 0.1ab | 16.9 | | ± 0.1b | 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 | | ± 0.05ab | 1.90 | ± 0.06a | 1.60 | | ± 0.07b | 0.036 |
| Total carotenoid | 4.27 | | ± 0.17 | 3.87 | ± 0.29 | 3.96 | | ± 0.33 | 0.59 |

Bold text indicates signifcant p values.

* 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).

suggested a downregulation of lipid metabolism (steroid biosynthesis, sphingolipid metabolism, glycerophospholipid metabolism, glycer-olipid metabolism and arachidonic acid metabolism) in fish fed diet L2 compared to the L1 diet ([Fig. 4](#page15)A, 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 me-tabolism were downregulated whereas oxidative phosphorylation and glycolysis were upregulated ([Fig. 4](#page15)B, Additional File 3).

In order to identify common functional categories aﬀected by mi-cronutrient 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

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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](#page15)). Regarding genes involved in fatty acid and LC-PUFA me-tabolism, 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 ex-pression 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](#page15)). 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 as-sociated 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 be-tween diets mirrored that previously reported in S1+ Atlantic salmon parr fed the same diet formulation ([Taylor et al., 2019](#page15)). 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 im-proved growth rate (TGCbw) and more eﬃcient FCR. In both studies, irrespective of smolt regime, diet L3 (4xNP) initially showed an in-creased growth rate relative to diet L1 (1xNP), but subsequently de-creased, 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](#page15)) and within 4 weeks of feeding under the S0+ regime in the current study. These results are in contrast to [Hemre et al. (2016)](#page15) and [Prabhu et al. (2019a, 2019b)](#page15), 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](#page15)) 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](#page15)), then there may be diﬀering dietary requirements and nutrient re-pro-filing 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 under-going PST. However, both the current study and that of [Taylor et al.](#page15) [(2019)](#page15) 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.

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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 < .05, ANOVA).

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Diet |  |  |  |  |  |  | p values |
|  |  |  |  |  |  |  |  |  |
|  | L1 |  | L2 |  | L3 |  |  |  |
|  |  |  |  |  |  |  | |  |
| His | 31.2 | ± 0.3a | 30.2 | ± 0.5a | 26.8 | ± 0.9b | | 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 | ± 1.4a | 49.3 | ± 0.4a | 45.7 | ± 0.2b | | 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 |

Bold text indicates signifcant p values.

Table 8

Non-protein N metabolites in muscle (μmol.g−1 wet wt.). Superscripts denote significant diﬀerences between diets (mean ± SD, n = 3, p < .05, ANOVA).

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | Diet |  |  |  |  |  | p values |
|  |  |  |  |  |  |  |  |  |
|  | L1 | |  | L2 |  | L3 |  |  |
|  |  | |  |  |  |  |  |  |
| 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 | | ± 0.02a | 0.25 | ± 0.01ab | 0.16 | ± 0.01b | 0.03 |
| Thr | 0.43 | | ± 0.02 | 0.40 | ± 0.01 | 0.43 | ± 0.02 | 0.62 |
| Ser | 0.14 | | ± 0.01a | 0.09 | ± 0.01b | 0.06 | ± 0.01b | 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 | | ± 0.10a | 1.46 | ± 0.08b | 0.98 | ± 0.06c | 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 | 0.04 | | ± 0.00 | 0.05 | ± 0.00 | 0.04 | ± 0.00 | 0.22 |
| butyric acid |  |  |  |  |  |  |  |  |
| 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 | | ± 0.20c | 2.33 | ± 0.07b | 4.10 | ± 0.09a | 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 |

Bold text indicates signifcant p values.

In seawater, growth rate (TGCbw) 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)](#page15) 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

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Table 9

Tissue concentrations (mg.kg−1 wet wt.) and retention (%) of water soluble vitamins. Superscripts denote significant diﬀerences between diets when var-iances were homogenous (mean ± SD, n = 3, p < .05, ANOVA), when non-homogenous variances: (mean ± SD, n = 3, p < .5, Kruskal-Wallis).

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Diet |  |  |  |  |  |  |  |  | p values |  |
|  |  |  |  |  |  |  |  |  |  |  |  |
|  | L1 |  |  | L2 |  |  | L3 |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |
| Whole body |  |  | ± 4.0b |  |  | ± 6.0a |  |  | ± 5.0a |  |  |
| Vitamin C | 45.0 | | 60.0 | | 68.0 | | 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 | ± 0.7b | | 12.0 | | ± 0.0a | 12.3 | | ± 0.6a | 6\*10−4 |  |
| Niacin | 32 ± 3c | | | 46 ± 2b | | | 56 ± 4a | | | 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 |  |

Bold text indicates signifcant p values.

Table 10

Tissue concentrations (mg.kg−1 wet wt.) and retention (%) of lipid soluble vitamins. Superscripts denote significant diﬀerences between diets when var-iances were homogenous (p < .05, ANOVA), when non-homogenous var-iances: (p < .5, Kruskal-Wallis) (mean ± SD, n = 3 tanks).

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Diet |  |  |  |  |  | p value |  |
|  |  |  |  |  |  |  |  |  |
|  | L1 |  | L2 |  | L3 |  |  |  |
|  |  |  |  |  |  |  |  |  |
| Whole body |  |  |  |  |  |  |  |  |
| Dry matter % | 41.0 | ± 1.0 | 40.0 | ± 1.0 | 41.0 | ± 1.0 | 0.40 |  |
| Vitamin A1 | 0.20 | ± 0.16 | 0.58 | ± 0.13 | 2.23 | ± 0.42 | 0.04 |  |
| Vitamin A2 | 0.39 | ± 0.06c | 0.83 | ± 0.12b | 1.77 | ± 0.25a | 0.0001 |  |
| Vitamin K | 30.0 | ± 6.0 | 32.0 | ± 1.0 | 30.0 | ± 0.0 | 0.25 |  |
| (K1) |  | ± 6.0b |  | ± 8.0 ab | 100.0 ± 7.0a | |  |  |
| Vitamin K | 73.0 | 84.0 | 0.008 |  |
| (MK4) |  | ± 5.0c |  | ± 5.0 b |  | ± 2.0a |  |  |
| Vitamin E (α- | 28.0 | 41.0 | 57.0 | 0.0005 |  |
| TOH) |  |  |  |  |  |  |  |  |
| Vitamin E (γ- | 12.5 | ± 3.8 | 12.1 | ± 1.4 | 11.1 | ± 0.5 | 0.56 |  |
| TOH) |  |  |  |  |  |  |  |  |
| Muscle | 0.023 ± 0.006c | | 0.030 ± 0.000b | | 0.043 ± 0.006a | |  |  |
| Vitamin D3 | 0.01 |  |
| Vitamin E (α- | 17 ± 2c | | 24 ± 1b | | 36 ± 3a | | 0.0001 |  |
| TOH) |  |  |  |  |  |  |  |  |
| Vitamin E (γ | 9.9 ± 1.7 | | 9.8 ± 0.8 | | 9.2 ± 0.5 | | ns |  |
| -TOH) |  |  |  |  |  |  |  |  |
| Whole body retention | |  |  |  |  |  |  |  |
| Vitamin E (α- | 8.8 ± 1.7a | | 9.2 ± 0.9ab | | 11.8 | ± 0.6b | 0.03 |  |
| TOH) |  |  |  |  |  |  |  |  |
| Vitamin E (γ | 34.0 | ± 10.0 | 30.0 | ± 3.0 | 30.0 | ± 1.0 | 0.60 |  |

-TOH)

Bold text indicates signifcant p values.

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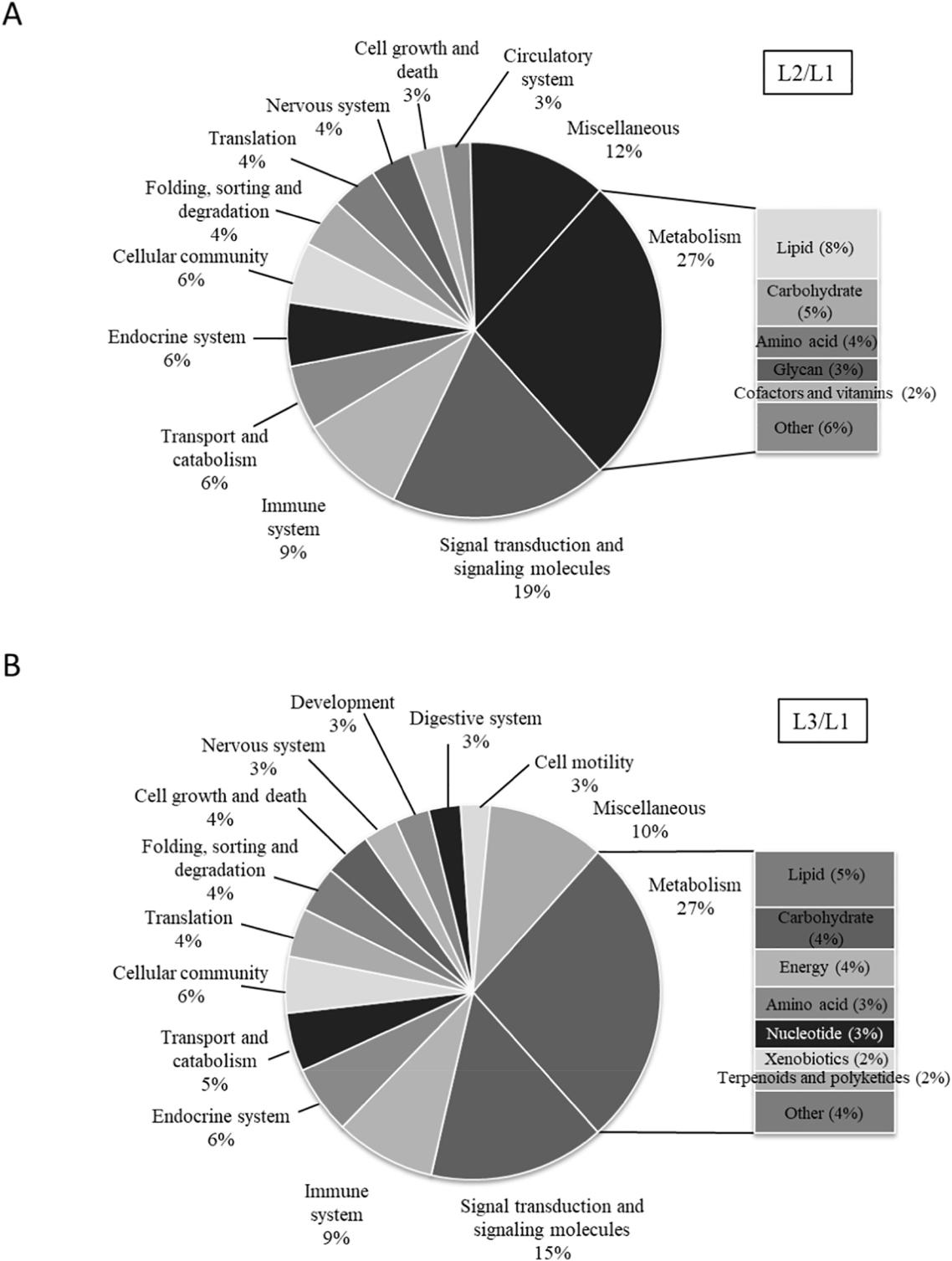


Fig. 3. Functional categories of genes diﬀerentially expressed in liver of Atlantic salmon fed diets L2 (A) and L3 (B) compared to fish fed diet L1. Non-annotated genes and features corresponding to the same gene are not represented.

current study: shorter duration (5 months vs. 12 months), cooler water temperatures experienced (4.1–10 °C vs. 6–16 °C) and subsequently lower TGCbw (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](#page15) [et al., 2016](#page15); [Hemre et al., 2016](#page15); [Taylor et al., 2019](#page15); [Prabhu et al.,](#page15) [2019a, 2019b](#page15)) that specific components of the NP are required to be supplemented at between 2× and 4× 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

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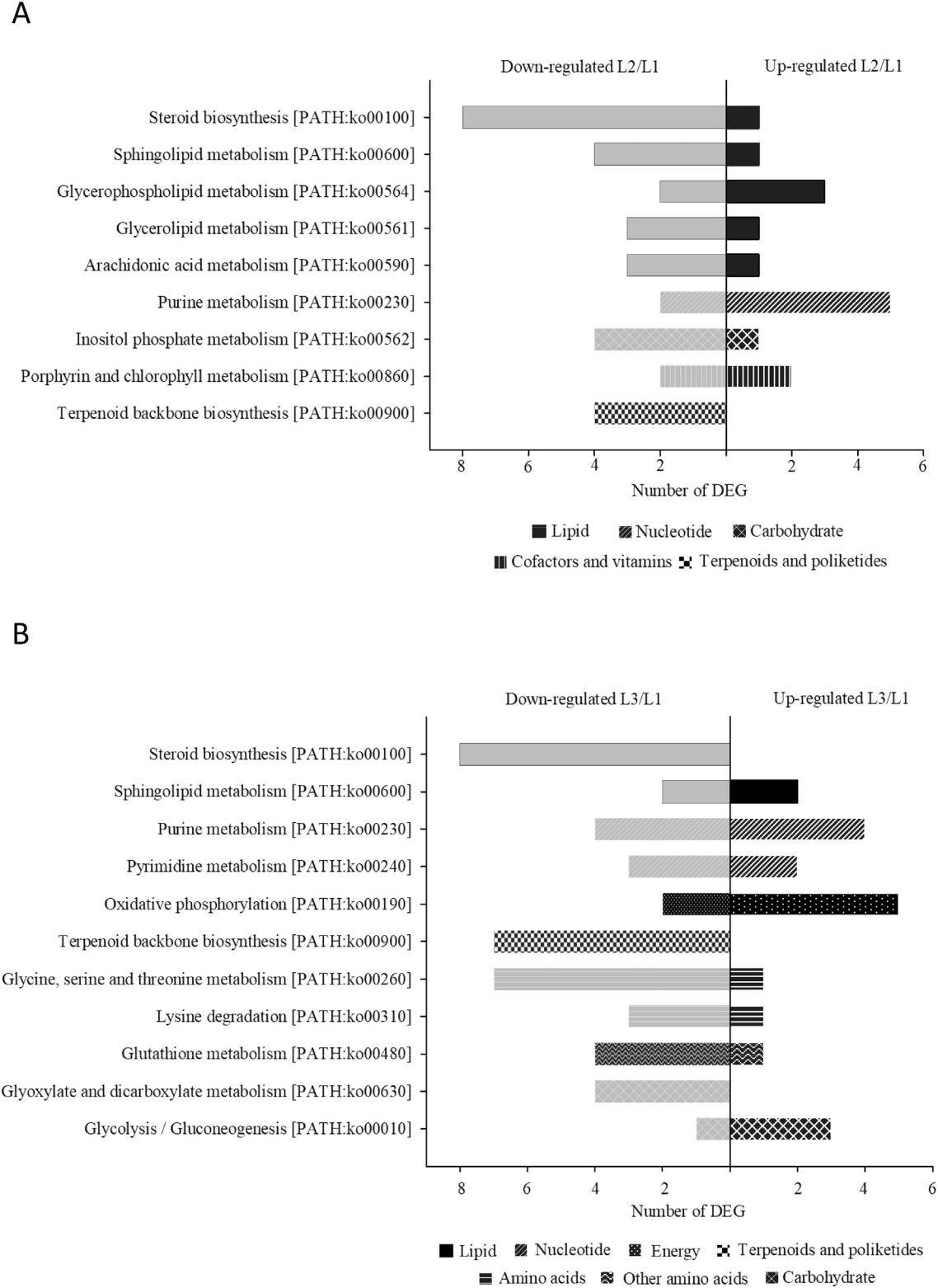


Fig. 4. KEGG pathway analysis of genes belonging to the metabolism category in liver of Atlantic salmon fed diets L2 (A) and L3 (B) compared to fish fed diet L1. Bars represent number of up- and down-regulated genes. Diﬀerent colours indicate diﬀerent nutrient groups.

radiological deformity with increasing NP inclusion in freshwater which concurs with results from our previous short-term feeding study ([Vera et al., 2019](#page15)). Further, in the current study, there was also a progression of spinal pathology in post-smolts when reassessed at har-vest compared to initial smolt in fish fed diets L1 and L2, while diet L3 showed no further increase in pathology. Concomitantly, spinal

deformity was also inversely correlated with final whole body ash concentration, a widely accepted indicator of body/bone mineralisation ([Baeverfjord et al., 2019](#page15)), indicating increasing NP level improved post-smolt mineral status. Previously, upregulation of bone gene expression associated with extracellular matrix (ECM) mineralisation (ALP, Mgp, Ostcn), and corresponding decrease in skeletal pathology was observed

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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 | | ± 0.26ab | 1.19 | ± 0.20a | 0.56 | ± 0.15b | 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 | | ± 0.34a | 0.36 | ± 0.09b | 0.44 | ± 0.12ab | 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 | | ± 0.40a | 0.36 | ± 0.11b | 0.79 | ± 0.32ab | 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 | | ± 0.08b | 1.04 | ± 0.05ab | 1.29 | ± 0.09a | 0.029 |
| GABA(A) receptor-associated protein (gabarap) | 1.00 | | ± 0.05b | 1.24 | ± 0.06ab | 1.47 | ± 0.09a | 0.001 |
| Muscle-specific RING finger protein 1a (murf1a) | 1.00 | | ± 0.04b | 1.11 | ± 0.17ab | 1.19 | ± 0.08a | 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 | | ± 0.06b | 1.20 | ± 0.09ab | 1.48 | ± 0.07a | 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 |

Bold text indicates signifcant p values.

following increased NP supplementation in parr ([Vera et al., 2019](#page15)). Eﬀects were hypothesised to be mediated through altered P-Ca home-ostasis pathways, as reflected by increased whole-body Ca:P ratios in smolts. Candidate components of the NP were suggested as vitamins D3 and K as cofactors of calcium sequestering. However, in the current study, improved mineralisation was not explained by diﬀerences in whole body macromineral concentration or retention in post-smolts which were similar across all diets and in the normal range for salmon ([Shearer et al., 1994](#page15)). Similarly, [Fjelldal et al. (2009)](#page15) reported a re-duction in vertebral deformity at 4.1 kg in S0+ post-smolts fed a high mineral diet for the first 8 weeks post sea entry, and while analysis of plasma Ca, P and D-vitamin metabolites reflected changes in P home-ostasis, these factors could not explain the preventive eﬀect of a high mineral diet on development of bone deformities. To this end, given that analysed dietary vitamin D3 and K in the current formulation were within established requirement ranges of 0.15–0.19 mg kg−1 ([NRC,](#page15) [2011](#page15)) and > 0.1 mg kg−1 ([Krossøy et al., 2009](#page15)) respectively, it is unlikely these components of the NP are directly attributable to the graded decrease in spinal pathology observed in post-smolts, and other components of the NP are worth consideration.

Whole body Zn and Se increased across diets with NP inclusion rate, with both being identified as the principal limiting micro-minerals in plant-based diets ([Prabhu et al., 2018](#page15); [Prabhu et al., 2019a, 2019b](#page15)). Selenium is involved in antioxidant mechanisms and redox reactions in liver and blood cells, yet the role of Se in bone metabolism remains unexplored in salmonids to date ([Baeverfjord et al., 2019](#page15)). Recent studies have shown that Se, in the form of selenoproteins, plays a vital role in bone metabolism in humans ([Pietschmann et al., 2014](#page15)), with plasma selenoprotein P concentrations found to positively correlate with bone mineral density in elderly women ([Zhang et al., 2014](#page15)). As

such, although whole body Se levels across all diets were within the normal reported range for Atlantic salmon ([Prabhu et al., 2016](#page15)), the role of Se in teleost bone development should be investigated.

It is well established that Zn is the most abundant micro-mineral in the vertebrae of many teleost fish species and has functional importance in the activity of ALP, and associated processes related to mineralisation of bone ([Prabhu et al., 2016](#page15)). Thus, improved Zn status and down-stream processes may be directly attributable to the observed reduction in spinal pathology and progression as post-smolts, particularly in fish fed diet L3 (4xNP). While studies on spinal development have often focused on dietary macro- and micro-minerals, the role of specific vi-tamins within the NP should not be overlooked as having a beneficial eﬀect on skeletal health. Whole body vitamin A levels suggested that diet L1 covered minimum requirement (> 0.75 mg kg−1, [Moren et al.,](#page15) [2004](#page15)), and diet L3 had significantly lower skeletal deformity. Diet L1-L3 in this study contained 3.7–12.2 mg kg−1 VA, well below the upper limits shown to induce skeletal pathology (> 37 mg kg−1) ([Ørnsrud](#page15) [et al., 2002](#page15); [Ørnsrud et al., 2013](#page15)). Therefore, it is unlikely that vitamin

1. deficiency or hypervitaminosis is attributable to the diﬀerences in spinal deformity rates observed. The specific role of B-vitamins on bone development in humans has been documented to a degree ([Dai and](#page15) [Koh, 2015](#page15)) but, as yet, has not been investigated in fish ([Lall and Lewis-McCrea, 2007](#page15)). Evidence from humans as reviewed by [Dai and Koh](#page15) [(2015)](#page15) has shown a variety of eﬀects of vitamin B deficiencies in-cluding: reduced dietary intake of B6 is associated with lower bone mineral density; synthesis of methionine depends on both folate and B12 for remethylation, and deficiency of either B vitamin results in megaloblastic changes in bone marrow; B vitamin deficiency aﬀects the anatomical/biochemical properties of bone if it is profound enough to cause hyperhomocysteinemia that is suﬃcient to induce an

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accumulation of homocysteine in the bone tissue. In light of the growing research in B vitamin involvement in bone metabolism in humans, greater consideration of B vitamin requirements in fish would also warrant consideration in relation to skeletal health.

4.3. Protein and amino acid status and retention

The present study increased dietary taurine and histidine keeping the methionine constant in high plant protein diets for Atlantic salmon growing from parr to approximately 2.5 kg BW. NRC requirement for methionine in Atlantic salmon is reported to be 7 g kg−1 ([NRC, 2011](#page15)). However, it was previously reported that dietary methionine of 7.9 vs 11.4 g kg−1 methionine improved growth and hepatosomatic index in salmon fed the latter diet ([Espe et al., 2014](#page15)). In another study, Atlantic salmon fed high plant protein diets containing either 8 or 11 g me-thionine per kg of diet, the weight gain in the latter diet did not diﬀer from the control group fed a high fishmeal diet (760 g kg−1 diet). Thus, in the current trial it was decided to maintain the dietary methionine constant at around 9 g kg−1 diet, while histidine and taurine were in-creased in the nutrient packages.

The retention of amino acids is known to be high when an amino acid is slightly deficient and then decrease as the requirement for amino acid deposition is met or increased above requirement for protein de-position ([Espe et al., 2017](#page15)). In the current study this is also true for histidine, where retention decreased as dietary histidine increased. In addition to histidine, lysine retention was also reduced in salmon fed the L3 diet as compared to fish fed the L1 and L2 diets. Lysine is mainly used for protein deposition ([Espe et al., 2007](#page15)) and the reduced retention of lysine in fish fed the highest nutrient package thus might indicate a lower protein gain in muscle of these fish. However, as protein reten-tion did not diﬀer in fish fed any of the diets, reduced lysine retention does not seem to be associated with a reduced muscle protein deposi-tion in the current trial. Thus, to address the reduced retention of lysine, interaction with other nutrients in the NP needs to be further explored. It is well known that both amino acid supply and the balance between dietary amino acids aﬀect growth hormone, IGF and GH ([Hevrøy et al.,](#page15) [2007](#page15); [Bower et al., 2008](#page15); [Valente et al., 2012](#page15)). In the current trial these were not aﬀected, which supports the fact that protein retention did not diﬀer between fish fed any of the diets in the current trial.

Any imbalanced supply of amino acids may increase the ubiquiti-nation and thereby degradation of proteins ([Bower et al., 2008](#page15); [Valente](#page15) [et al., 2012](#page15)). The gene expression level of murf, 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](#page15)). 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.,](#page15) [2012a, 2012b](#page15)). Both dietary histidine and taurine are more associated with oxidation and protection against ROS than with growth and me-tabolism ([Remø et al., 2014](#page15)) as well as survival of the cells ([Espe and](#page15) [Holen, 2013](#page15)), 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](#page15)), 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 micro-nutrients 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

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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 af-fected 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](#page15)), as well as some of the minerals ([Prabhu et al., 2019a, 2019b](#page15)). In an experiment with salmon parr, it was estimated that the 2xNP would cover requirement for several mi-cronutrients, especially when given with high plant raw material diets ([Hemre et al., 2016](#page15); [Hamre et al., 2016](#page15); [Prabhu et al., 2019a](#page15)). The present study, therefore, confirmed earlier conclusions that plant-based diets need new requirement estimates for several micronutrients. The suggested body-concentrations, health outcomes and growth response used as requirement markers were not suﬃcient in the former study ([Hemre et al., 2016](#page15)), and the present study confirmed this.

Pantothenic acid in whole body was higher in fish fed diets L2 and

L3 compared to diet L1, while niacin increased gradually from L1 to L3, significantly at each step. Pantothenic acid in gill tissue also increased but diﬀerences were not significant. There was also a tendency for higher B6 concentration in whole body in fish fed diets L2 and L3 compared to L1. This is in agreement with earlier findings for salmon post-smolt ([Hemre et al., 2016](#page15)). These results suggest that these three B-vitamin optimal levels should be raised when compared to [NRC](#page15) [(2011)](#page15), to the 2xNP level for pantothenic acid and B6 and to the 4xNP level for niacin.

Vitamin B6 interacts highly with protein metabolism ([Hansen et al.,](#page15) [2015](#page15)). Pyridoxine functions as phosphorylated coenzymes pyridoxal phosphate (PLP) in many reactions, such as transaminases in amino acid metabolism. Pyridoxine is a cofactor for the enzyme aromatic amino acid decarboxylase, which is required for the synthesis of neu-rotransmitters ([Rorsman et al., 1995](#page15)). Therefore, symptoms and con-sequences of vitamin B6 deficiency are many, diverse and severe, and include nervous disorders and abnormal behavior, none of which was registered in the present study. Tissues with mitochondria-rich cells and cells that undergo rapid cell divisions like gill tissue are especially sensitive to pantothenic acid deficiency. The 1xNP levels seemed to be suﬃcient for the other B vitamins (thiamine, riboflavin, folic acid and biotin), as found by [Hemre et al. (2016)](#page15). There was, however, a ten-dency for a decrease in retention of B-vitamins, significant only for ri-boflavin and folate. This may point to either an interaction and/or control factor at the gut-barrier level, or excretion of excess vitamin, since whole body levels were maintained or increased.

Vitamin C in whole body increased from diet L1 to L2 and was then unchanged in fish fed diet L3. This is in accordance with previous re-sults ([Hamre et al., 2016](#page15)). In the liver, changes in vitamin C con-centrations were not significant (p = .06), but there was a tendency for an increase in fish fed diet L2 compared to L1, and then a decrease in fish fed L3. The variation was very high in both the L2 and L3 groups. It is well known that vitamin C may act as a pro-oxidant at high con-centrations ([Hamre et al., 1997](#page15)), and this may indicate that diet L3, and perhaps L2, contained too much vitamin C, and an optimal vitamin C level at the 2xNP level or slightly less is needed.

4.5. Lipid soluble vitamins

Lipid soluble vitamins have a tendency to accumulate in the body in response to increased supplementation. Therefore, it is not possible to use body concentrations as an indication of requirement. This is true for α-tocopherol (TOH) which normally shows a linear relationship be-tween supplementation and whole body concentration ([Hamre et al.,](#page15) [1997](#page15); [Hamre et al., 2016](#page15)) as confirmed in the present study. [Hamre](#page15) [et al. (2016)](#page15) recommended supplementation with at least 150 mg kg−1

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α-TOH to compensate for variations in composition of feed ingredients and variation in oxidative stress, in response to handling, seasons and disease. TOH was not supplemented, but came from the feed in-gredients, and the feed and whole body concentrations did not vary among the dietary treatments.

For vitamin A, the situation is more complicated due to the presence of many diﬀerent forms. Most of the vitamin A is present in the storage form retinol-esters ([Moren et al., 2004](#page15)), and the method used here determined esters and free forms combined. However, both free retinol and retinol esters increased exponentially with increasing concentra-tion of vitamin A in the diet, and there was a large increase in body stores with an increase of dietary retinol equivalents from 0.75 to 2.5 mg kg−1 diet. Nevertheless, a requirement was found in Atlantic halibut of 2.5 mg kg−1 diet based on cell proliferation in the intestine as measured by DNA content, PCNA and maturity of the brush border membrane by enzyme activities ([Moren et al., 2004](#page15)). With reference to [Moren et al. (2004)](#page15) and the whole body levels found here, diet L1 had enough vitamin A (3.7 mg kg−1) to cover the requirement (≥0.75 mg kg−1[NRC, 2011](#page15)).

The diets were supplemented with the synthetic pro-vitamin K, menadione (vitamin K3). Menadione is very unstable during feed pro-cessing ([Krossøy et al., 2009](#page15)), and the analysed dietary 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.,](#page15) [2011](#page15)) 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)](#page15) 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 require-ments in other species range from 0.2 to 2 mg kg−1 ([NRC, 2011](#page15)).

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 D3. 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.,](#page15) [1998](#page15); [Graﬀ et al., 2002](#page15)). In other fish species estimated requirements vary from 0.006 to 0.060 mg kg−1 ([NRC, 2011](#page15)), well below the sup-plementation 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](#page15)). Deficient or sub-optimal supply of dietary micro-minerals will deplete body mineral status, especially over the long term ([Maage and Julshamn, 1993](#page15)). 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 sal-monid feeds, Zn and Se have been identified as the most limiting micro-minerals ([Prabhu et al., 2018, 2019a](#page15)). 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.,](#page15) [2019a](#page15)). 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 de-creased the Zn status to half and one-third in L2 and L3 fed-fish, re-spectively. Similar reductions in whole body Zn ranging from 1/3 to ½ were observed in Atlantic salmon parr fed sub-optimal Zn levels ([Maage](#page15) [and Julshamn, 1993](#page15)). The L2 diet with 156 mg Zn kg−1 was able to sustain the body Zn status on parr to the initial Zn status (18 mg kg−1 wet weight). However, the initial Zn status of the Atlantic salmon in this study was lower than values reported in the literature for similar size

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fish ([Maage and Julshamn, 1993](#page15); [Shearer et al., 1994](#page15)). The Zn status of Atlantic salmon fed the L2 diet (18 mg kg−1 wet weight, averaging 2.3 kg) was considerably lower than the Zn status of 27–29 mg kg−1 of 500 g post-smolt fed similar Zn levels in a previous trial ([Prabhu et al.,](#page15) [2019a](#page15)). Se levels in all three dietary groups were in the normal range observed in salmonids or Atlantic salmon specifically ([Prabhu et al.,](#page15) [2016](#page15)), mostly because the high background Se levels in basal diet already met the requirement. Although the NP composition did not change between the present and previous studies ([Hamre et al., 2016](#page15); [Prabhu et al., 2019a](#page15)), the total dietary Se levels were considerably diﬀerent for corresponding NP inclusions, thereby Se recommendations diﬀer between studies. Variation in Se content of feed ingredients have to be considered in feed formulations and regulative legislation. The supplementation of manganese of 12 mg/kg diet in diet L1 corre-sponding to a total dietary Mn of 42 mg/kg diet was suﬃcient to maintain normal body status. Recently, [Prabhu et al. (2019b)](#page15) also showed that Mn supplementation of 14 mg/kg as MnSO4 satisfied the minimal dietary requirement of Atlantic salmon post-smolt fed plant-based diets. The macro-mineral composition was within normal range and unaltered in Atlantic salmon fed the diﬀerent diets implying the diets met the macro-mineral needs of Atlantic salmon over the entire study period. Overall, Atlantic salmon post-smolt in seawater were able to maintain their body status in the long run with minimal dietary supply of most micro-minerals from the L1 diet, except for Zn which required L2 levels.

4.7. Gene expression

In general, the number of significantly diﬀerentially regulated functional pathways were greater for the L3 vs. L1 dietary comparison, than for the L2 vs. L1 comparison. In fact, not only the number of up- or down-regulated pathways was higher in L3/L1, but also the number of DEGs involved in each pathway was greater, suggesting a correlation between increased dietary micronutrient supplementation in low marine diets and changes in liver transcriptome. Regarding the eﬀects on specific pathways, in both diet comparisons (L3/L1 and L2/L1), the terpenoid backbone and steroid biosynthesis pathways were down-regulated. A micronutrient dependent epigenetic gene regulation of the upstream regulator (acetyl-CoA carboxylase alpha, acaca) that provides malonyl-CoA substrate for the biosynthesis of fatty acids has been de-monstrated (Saito et al., publication pending). The terpenoid backbone pathway underpins cholesterol (sterol), heme and vitamin (retinoids, retinol, retinoic acid, menadione) synthesis while both biosynthetic pathways are involved in steroid (e.g. testosterone) hormone produc-tion. The steroid and sterol biosynthetic pathways were also the most significantly aﬀected in zebrafish oﬀspring after feeding the parental generation a diet deficient in micronutrients (vitamin B12, vitamin B6, folic acid, methionine and choline) ([Skjaerven et al., 2018](#page15)). Further, the oﬀspring from deficient parents had accumulated higher levels of lipid inclusion in the hepatocytes and showed a downregulation in the steroid biosynthetic process and widespread epigenetic changes of gene regulation in the liver ([Skjaerven et al., 2018](#page15)). This suggests that mi-cronutrient supplementation may not only be aﬀecting nutrient reten-tion directly, but also via epigenetic mechanisms altering gene reg-ulation and downstream biochemical process.

These eﬀects on gene expression may be in response to the increased supplementation of the L2 and L3 diets with cholesterol, menadione (vitamin K) and retinol (vitamin A) as part of the micronutrient premix and therefore reflect an increased requirement of these compounds in fish fed diet L1 ([Kortner et al., 2014](#page15)). The lower level of dietary pan-tothenic acid could also have aﬀected the up-regulation of several metabolic pathways in fish fed diet L1. Pantothenic acid is required for synthesis of coenzyme A, which participates in a range of acyl transfer reactions in energy production, fatty acid oxidation, cholesterol and steroid synthesis, heme synthesis, amino acid catabolism, acetylcholine synthesis and many other acetylation and acylation reactions ([Olsvik](#page15)

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[et al., 2013](#page15)).

Increased expression of genes related to glyoxylate/dicarboxylate metabolism was observed in salmon fed diet L1 in comparison to fish fed L3, suggesting lower levels of glucose in liver of L1 salmon and therefore the need to synthesise it from fatty acids. Meanwhile, an ac-tivation of intermediary metabolism in fish fed diet L3 may indicate higher levels of glucose available for energy production, and an en-hancement of cellular energy levels as suggested by the increased number of up-regulated genes in glycolysis/gluconeogenesis, and oxi-dative phosphorylation pathways in fish fed diet L3. The diﬀerences in hepatic metabolic activities, energy generation and transport and growth performance between salmon fed diets L3 and L1 could be linked to the higher levels in L3 fish of B vitamins (riboflavin, niacin), which are involved in a range of cellular processes, most importantly energy generation and transport that enables fish fed diet L3 to sustain vital metabolic processes without compromising the amount of energy available for growth ([Hansen et al., 2015](#page15)).

The q-PCR results of genes involved in LC-PUFA biosynthesis showed down-regulation of biosynthesis with increasing micronutrient supplementation. A down-regulation of fads2d6a was found, indicating greater LC-PUFA biosynthesis capacity in the liver of L1 salmon. However, these diﬀerences were not fully reflected in hepatic n-3 LC-PUFA profiles in the present study, which showed no major diﬀerences between the dietary groups. In muscle, mlp3b, atg12 and gabarap genes were found to be upregulated in L3-fed salmon. These genes are in-volved in autophagosome mediated autophagy, playing an important role in autophagosome formation and sequestration of cytosolic cargo into double membrane vesicles, leading to subsequent degradation after fusion with lysosomes ([Mannack and Lane, 2014](#page15)).

5. Conclusions

The present study as part EU ARRAINA project, confirmed earlier studies ([Hamre et al., 2016](#page15); [Hemre et al., 2016](#page15); [Prabhu et al., 2019a,](#page15) [2019b](#page15); [Taylor et al., 2019](#page15); [Vera et al., 2019](#page15)) that plant-based diets for Atlantic salmon need new requirement estimates for several micro-nutrients. In particular, these optimal levels may diﬀer between fresh-water and seawater phases of the production cycle, and that require-ments for growth and skeletal health may also diﬀer. Based on the present results and conclusions from earlier studies ([Hamre et al., 2016](#page15); [Hemre et al., 2016](#page15); [Prabhu et al., 2019a](#page15); [Taylor et al., 2019](#page15); [Vera et al.,](#page15) [2019](#page15)), [NRC (2011)](#page15) 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)](#page15) 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.

Author statement

Author contributions were as follows. All: study design, data ana-lysis, article writing & reviewing; JFT, Lead UoS PDRA, trial manage-ment, sampling co-ordination; LMV, AJP: PDRA; DRT: UoS project leader; KH: IMR project leader; HM: UoS project Co-PI; GIH, EJL, ME, KS: IMR Co-I; DL: design and formulation of experimental feed; AJP, EJL: sampling, mineral analysis & data interpretation; KJ, ME: sam-pling, Amino Acid analysis & data interpretation; LMV: sampling, transcriptomics, qPCR analysis & data interpretation; KH, GIH: vitamin analysis & data interpretation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

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influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at [https://](https://doi.org/10.1016/j.aquaculture.2020.735551) [doi.org/10.1016/j.aquaculture.2020.735551](https://doi.org/10.1016/j.aquaculture.2020.735551).

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