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Enhanced micronutrient supplementation in low marine diets reduced vertebral malformation in diploid and triploid Atlantic salmon (*Salmo salar*) parr, and increased vertebral expression of bone biomarker genes in diploids

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Abstract

Previously we showed that, for optimum growth, micronutrient levels should be supplemented above current National Research Council (2011) recommendations for Atlantic salmon when they are fed diets formulated with low levels of marine ingredients. In the present study, the impact of graded levels (100, 200, 400%) of a micronutrient package (NP) on vertebral deformities and bone gene expression were determined in diploid and triploid salmon parr fed low marine diets. The prevalence of radiologically detectable spinal deformities decreased with increasing micronutrient supplementation in both ploidy. On average, triploids had a higher incidence of spinal deformity than diploids within a given diet. Micronutrient supplementation particularly reduced prevalence of fusion deformities in diploids and compression and reduced spacing deformities in triploids. Prevalence of affected vertebrae within each spinal region (cranial, caudal, tail and tail fin) varied significantly between diet and ploidy, and there was interaction. Prevalence of deformities was greatest in the caudal region of triploids and the impact of graded micronutrient supplementation in reducing deformities also greatest in triploids. Diet affected vertebral morphology with length:height (L:H) ratio generally increasing with level of micronutrient supplementation in both ploidy with no difference between ploidy. Increased dietary micronutrients level in diploid salmon increased the vertebral expression of several bone biomarker genes including bone morphogenetic protein 2 (*bmp2*), osteocalcin (*ostcn*), alkaline phosphatase (*alp*), matrix metallopeptidase 13 (*mmp13*), osteopontin (*opn*) and insulin-like growth factor 1 receptor (*igf1r*). In contrast, although some genes showed similar trends in triploids, vertebral gene expression was not significantly affected by dietary micronutrients level. The study confirmed earlier indications that dietary micronutrient levels should be increased in salmon fed diets with low marine ingredients and that there are differences in nutritional requirements between ploidies.

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

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1. Introduction

Farmed Atlantic salmon (*Salmo salar*), as all animals, have specific nutrient requirements. Most of these nutrients were historically supplied in surplus by the marine raw materials, fish oil (FO) and fishmeal (FM), traditionally used as the major feed ingredients. As a result of the limited supply of these marine products, plant meals and vegetable oils have increasingly replaced FM and FO in salmon feeds (Gatlin III *et al.*, 2007; Hardy, 2010; Turchini *et al.*, 2011) with these plant ingredients now constituting up to 70 % of feeds (Ytrestøyl *et al.*, 2015). As a consequence there are significant changes to the composition and contents of a range of nutrients and other components in farmed salmon diets (Sissener *et al.*, 2013). Specifically, challenges when replacing large proportions of dietary FM and FO with plant ingredients include alterations in concentrations of inherent micronutrients, changes in their chemical forms, as well as the presence of compounds interacting with micronutrient uptake and metabolism (Olsvik *et al.*, 2013). Therefore, increased and improved knowledge of practical nutrient requirements of Atlantic salmon when fed plant-based diets is critical (Hansen *et al.*, 2015).

The impacts of plant-derived feed ingredients on growth (Espe *et al.*, 2006; Foroutani *et al.*, 2018; Mundheim *et al.*, 2004; Pratoomyot *et al.*, 2008; Torstensen *et al.*, 2000; Torstensen *et al.*, 2005; Waagbø *et al.*, 2013), feed utilization (Opstvedt *et al.*, 2003), product quality (Menoyo *et al.*, 2007; Mundheim *et al.*, 2004; Tocher et al., 2019; Torstensen *et al.*, 2005; Waagbø *et al.*, 1993), and health (Caballero-Solares et al., 2017; Hemre and Sandnes, 2008; Oxley *et al.*, 2005; Seierstad *et al.*, 2005; Seierstad *et al.*, 2009; Waagbo, 2008; Waagbø, 2006) have been extensively studied in Atlantic salmon. In contrast, however, almost no studies have examined the consequences of plant-derived diets on long-term bone health. Plant ingredients not only contain less phosphorus (P) than FM, but may also contain high levels of indigestible phytic acid that may interfere with mineral bioavailability and reduce vertebral mineral content (Helland *et al.*, 2005; Helland *et al.*, 2006). While an early study reported that the combination of high inclusion levels of both plant proteins and lipids appeared to have a positive effect on bone health in diploid Atlantic salmon post-smolts

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compared to lower inclusion levels (Fjelldal *et al.* 2010), that may simply be a consequence of the slower growth rate in the high inclusion group. While several studies have investigated the effects of supplementing dietary nutrients (Åsgård and Shearer, 1997; Baeverfjord *et al.*, 1998), and vitamins and minerals (see Lall and Lewis-McCrea, 2007) on bone development in salmon in freshwater, no such studies have examined the role of micronutrient supplementation in relation to low marine raw material diets. This is particularly surprising given that certain pathologies including bone/skeletal deformities that are evident in saltwater may be of freshwater origin (Fjelldal *et al.*, 2012).

In salmon, the vertebral body comprises four mineralized or ossified layers, and Ca and P are the main constituents of the mineral fraction in bone (Toppe *et al.*, 2007), and vitamin D_3 being involved in the regulation of their homeostasis (Lock *et al.*, 2007). Formation of the different layers involves the balanced and highly regulated formation of bone and cartilaginous structures through patterns of mineralization and matrix deposition (Nordvik *et al.*, 2005). Salmonids are different to most teleosts in that they have three cell types that are actively involved in bone formation and remodelling: osteoblasts (bone forming cells), osteocytes (entrapped inside the bone matrix), and osteoclasts (multinucleated bone resorbing cells) (Lall and Lewis-McCrea, 2007). Subsequent development and growth of the vertebral column is under control of regulatory mechanisms involving transcription factors, signaling molecules and extracellular matrix proteins, and the interconnected pathways of chondrocyte and osteoblast differentiation during vertebral formation must be coordinated (Ytteborg *et al.*, 2010). Many of the micronutrients act as important cofactors in enzymatic pathways and other metabolic processes in vertebral formation. Several studies in diploid salmon have demonstrated a key role of nutrients, particularly P and Ca (Åsgård and Shearer, 1997; Baeverfjord *et al.*, 1998), vitamin A (Green *et al.*, 2016) and vitamin K (Krossøy *et al.*, 2010), in skeletal development and either their deficiency or toxicity leads to pathogenesis of skeletal deformities. Vitamin A regulates skeletogenesis and cartilage development by controlling chondrocyte function, maturation and proliferation of cells (Koyama *et al.*, 1999), while vitamin K

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acts a cofactor of the enzyme γ-glutamyl carboxylase (Ggcx), which catalyses the carboxylation of glutamyl (Glu) residues present in the vitamin K-dependent proteins, an essential reaction involved in the regulation of Ca homeostasis (Oldenburg *et al.*, 2008). In addition, oxidative stress affects bone turnover whereas vitamin E plays an important role in combating endogenous and exogenous free radicals. Previous research in mammals has shown a positive correlation between dietary supplementation with vitamin E and improved bone health (Kasai *et al.*, 2015).

There is growing interest in triploid Atlantic salmon within the commercial farming sector due to the fact that triploids are reproductively sterile, offering advantages such as reproductive containment of escapees, and also have potential for faster growth with subsequently reduced production cycle length (Benfey, 2016). Note that, while salmonids are considered to be pseudotetraploid due to whole genome duplication (Lien et al., 2016), the pseudotetraploid genome is considered "diploid" in the present paper to avoid confusion and improve readability. While specific dietary requirement trials in triploids Atlantic salmon are limited, it appears that differences between ploidies exist (Fjelldal & Hansen, 2010), with energy and nitrogen retention efficiencies possibly higher in triploids than diploids (Burke *et al.*, 2010). In addition, requirement for the essential amino acid histidine is higher in triploid salmon in order to prevent cataract formation in post-smolts and, possibly, improve feed conversion efficiency (Taylor *et al.*, 2015). Furthermore, triploids appear to have higher dietary requirements for certain macro-minerals including phosphorous, to prevent the onset of skeletal deformities (Fjelldal *et al.*, 2015). A recent study investigating graded levels of a micronutrient package in Atlantic salmon confirmed that there are likely qualitative and quantitative differences in nutritional requirements between ploidies (Taylor *et al.*, 2019).

In the latter most study, both diploid and triploid Atlantic salmon parr were fed graded levels of a nutrient package (NP) containing 24 micronutrients supplemented to feeds formulated with low levels of marine ingredients (Taylor *et al.*, 2019). Specifically, fish were fed a diet supplemented with one of three inclusion levels of the NP (100 %, 200 % and 400% NP), which

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was based on the current minimum nutrient recommendations for Atlantic salmon (NRC, 2011) modified according to recent studies investigating nutrient requirements of salmon fed low marine feeds (Hamre *et al.*, 2016; Hemre *et al.*, 2016). Previously we reported the effects on growth performance, biochemical composition, liver histology, hepatic gene expression (transcriptome) and smoltification efficiency (Taylor *et al.*, 2019). In the present study, the impact of NP level on skeletal deformity and bone health was investigated in diploid and triploid parr fed the three experimental diets from around 30 g to seawater transfer. Specifically, the prevalence, localization and pathology of spinal malformation, and vertebral expression of bone biomarker genes were determined.

2. Materials and Methods

2.1 Fish stock

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 Ethics and Welfare Committee of the University of Stirling. All fish were monitored daily by the Named Animal Care and Welfare Officer (NACWO).

The feeding trial was carried out at the Niall Bromage Freshwater Research Facility, University of Stirling (Buckieburn, Scotland) using fish obtained from a commercial Atlantic salmon stock (Landcatch Natural Selection, Ormsary, UK). Briefly, ova and milt were collected from a total of 20 unrelated dams and 5 sires. Per dam, ova were fertilized as one batch and a 1 L sub-sample was removed to induce triploidy using hydrostatic pressure shock (9500 PSI applied 300 degree minutes post-fertilization at 8 ˚C for a duration of 6.25 min, Fjelldal & Hansen, 2010). This procedure was repeated for each dam x sire cross (1 sire / 4 dams) creating 20 diploid incubators and 20 triploid incubators, and reared in constant darkness at 8.0 ± 0.5 ˚C. Eyed ova (380 °days post fertilisation) were shipped to University of Stirling facilities and ova were pooled (equal

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numbers of ova / family, ~250) per ploidy and reared in 6 x 250 L tanks in complete darkness until first feeding (4 March 2013, ~850 °dpf). At first feeding, diploids were fed a standard commercial salmon fry feed (Inicio Plus, BioMar, UK; 1.6 % total phosphorus) whereas triploids were fed the same standard commercial formulation but with a boosted phosphorous level (2.3 %) based on data obtained in previous studies on triploid salmon (Smedley *et al.*, 2016). All other dietary components were comparable between starter feeds. Fry were reared under constant light, and feed was supplied throughout the 24 h by belt feeders according to manufacturer's tables (specific feeding rate [SFR], 2-3 % body weight day^{-1}) in a flow-through system O_2 sat > 96%, 12.2 ± 1.2°C, SD 3.1 kg m^{-3}). To verify ploidy status, smears were prepared from blood collected following overdose with Tricaine (> 200 mg.L^{-1}, PHARMAQ, UK) and severance of the caudal peduncle from euthanized fish at 5 g (100 / ploidy). After air drying, slides were fixed in 100 % methanol and then placed into Giemsa stain for 10 min. Erythrocyte nuclear diameter were measured at 100× magnification using image capture software (Image-Pro Premier, MediaCybernetics, Rockville, USA). A total of 20 randomly chosen nuclei per slide were measured to the nearest 0.01 µm. Diploid control groups had significantly smaller erythrocyte nuclear diameter with no overlaps with the pressure shock triploid groups (2N 6.8–7.7 µm; 3N 9.0–10.2 µm) confirming that all 100 fish subjected to hydrostatic pressure shock were triploids. Cumulative mortality from first-feeding to start of the feeding trial was 2.8 ± 0.02 % and 3.5 ± 0.01 % for diploids and triploids, respectively.

2.2 Feeding trial

Two groups of Atlantic salmon parr of mean weight 37.5 ± 2.2 g (diploid) and 27.4 ± 0.7 g (triploid) were stocked into 12 x 1.8 m^3 circular fibreglass tanks (6 tanks / ploidy, n = 1000 / tank). Fish were acclimatized to the experimental conditions for 2 weeks before being fed the experimental diets for a period of 31 weeks. Duplicate groups were fed low FM / FO diets (15% FM / 8% FO) formulated to have identical protein / oil contents (480 / 215 g.kg^{-1}, respectively, of which 72 / 17 g.kg^{-1} were of marine origin) and supplemented with a nutrient package (NP) at

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graded inclusion levels. The NP contained 24 nutrients in total these being; vitamins (A, D3, E, K3, C, thiamin, 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. Specifically, the NP was added at three inclusion levels to produce 3 dietary treatments: L1, 100 % NP; L2, 200 % NP; L3, 400 % NP, with the 100% NP package formulated to contain 100 % of assumed requirement based on the reported requirement levels for diploid Atlantic salmon at the time (NRC, 2011) and modified according to an earlier study of the EU-funded ARRAINA project (Hemre *et al.*, 2016). Total and available phosphorus were fixed in all diets at 13.0 and 9.0 $g.kg^{-1}$ respectively, and magnesium at 1.5 $g.kg^{-1}$, and were not part of the NP. Pellet size was adjusted according to fish weight, with a 2 mm pellet fed for 23 weeks and a 3 mm pellet fed for the final 8 weeks. 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). Feed formulations, added micronutrient concentrations within the nutrient package and analyzed micronutrient concentration are provided in Tables 1, 2 and 3 respectively. With the exception of histidine, there was generally a positive relationship between added and analyzed nutrients in the 2 mm pellet (Table 3). In the 3 mm pellet, vitamin A, vitamin K3, pantothenic and folic acid, vitamin C, iron and manganese deviated from the linear relationship.

Fish were fed continuously during the light period of the light-dark cycle by automatic feeders (Arvotec T2000, Arvotec, Finland) controlled by a PC system. Although feed collection was not possible due to system constraints, presence of waste feed was ensured each day prior to tank flushing. Specific feeding rates (SFR; % tank biomass per day) were adjusted automatically according to predicted growth and daily temperature. A simulated natural photoperiod (SNP) was applied to produce S1+ smolts, with lighting provided by two 28 W fluorescent daylight bulbs (4000 °K, RS Components, UK) mounted centrally within the tank lid. Water was supplied by an upstream reservoir under flow-through conditions (10 L min^{-1}), with ambient temperatures

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decreasing from 15.5 ˚C (September) to 2.0 ˚C (February), and increasing to 9 ˚C by April. Oxygen levels were consistently ≥95% saturation.

2.3 Sampling procedures

Fish were sampled for growth at 3, 7, 14 and 31 weeks post application of the experimental feeds. At each time point, 50 fish / tank were anaesthetized (Tricaine, 50mg L^{-1} PHARMAQ, UK, buffered with bicarbonate, 100 mg L^{-1}), individual weights (± 0.1 g) and fork lengths (± 1.0 mm) measured, and fish allowed to recover in aerated water before returning to experimental tanks. 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 determine specific growth rate (SGR_{wt}), calculated as: $(e^{g}-1) \times 100$, where $g = (\ln(W_f) - \ln(W_i)) \times (t_2-t_1)^{-1}$ and thermal growth coefficient (TGC), calculated as W °C.d^{-1}. Feed conversion ratio (FCR) was calculated as feed given (g) / ($W_f - W_i$).

At smolt (week 31 post diet application) 100 fish per diet (50 / tank) were euthanized using lethal anaesthesia (> 200 mg.L^{-1} Tricaine), placed on flat trays and frozen at -20˚C prior to x-ray radiography. A further 8 fish per treatment (4 / tank) without any specific pathology of the vertebral column were identified, euthanized, and an ~1cm x 3mm section of the vertebral column carefully dissected, muscle scraped off, and placed into 1.5ml RNALater® (Sigma, Poole, UK) for subsequent gene analyses.

2.4 Spinal deformity – X ray radiography

Frozen fish (50 / tank, n = 2) were x-radiographed using a portable x-ray unit (Celtic SMR PX40 HF) with an extremities plate measuring 24 x 30 cm. Each radiograph was exposed for 32 mAs and 40kV and the image digitized (AGFA CR-35X, Agfa UK Ltd., London). The radiographs were observed 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)

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and deformity type categorized according to Witten *et al.* (2009). Example pathologies are shown in Supplementary file 1. Observations were made in the anterior-caudal direction and the total number of vertebrae was recorded for each fish. In addition, vertebral morphology was examined in 20 fish / diet (n = 10 / tank) showing no radiological spinal pathology, where each individual was measured using Image J (Image J 1.46r, NIH, USA) to calculate individual vertebral length : dorso-ventral diameter ratios (Supplementary file 2). Mean regional vertebra L: H values were then calculated for each spinal region defined by Kacem *et al.* (1998) prior to statistical analysis.

2.5 Micronutrient analysis

Micronutrient analyses were performed as described previously (Taylor *et al.*, 2019). Briefly, B-vitamins, biotin, niacin, folate, pantothenic acid and cobalamin were all determined by microbiological methods (Feldsine *et al.*, 2002; Mæland *et al.*, 2000). Other B-vitamins including thiamine (CEN, 2003), pyridoxine (CEN, 2006) and riboflavin (Brønstad *et al.*, 2002), as well as vitamins C (Mæland and Waagbø, 1998), E and K (CEN, 1999) were determined by high-performance liquid chromatography (HPLC). Multi-element determination of macro- and microminerals was performed by inductively coupled plasma mass spectrometry (ICP-MS) (Julshamn *et al.*, 1999).

2.6 Gene expression

Vertebrae samples were homogenized in 1 mL of TRIzol® (Invitrogen, UK) per 100 mg of sample and total RNA extracted, as indicated in the manufacturer's instructions. Samples were placed into tubes containing TRIzol® and glass beads and left to rest at room temperature for 30 min. Then samples were homogenised in a beadBeater (Biospec, Thiestles Scientific, UK) for 30 s before proceeding with RNA extractions. RNA pellets were rehydrated in MilliQ water and total RNA concentration was determined using an ND-1000 Nanodrop spectrophotometer (Labtech Int., East Sussex, UK). For all samples, A260/280 ratios were above 1.91 and A260/230 ratios were above

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2.00. RNA integrity was assessed by electrophoresis. Quantitec Reverse Transcription kit (Qiagen, Manchester, UK) was used in order to eliminate any genomic DNA contamination and synthesise cDNA from 1 µg of total RNA. Real-time PCR was performed using Luminaris color Higreen qPCR Master mix (Thermo Fisher Scientific, MA, USA) and Mastercycler RealPlex 2 thermocycler (Eppendorf, UK) which was programmed to perform the following protocol: 50 ºC for 2 min, 95 ºC for 1 min, followed by 40 cycles at 95 ºC for 15 s, X ºC for 15 s and 72 ºC for 30 s (see Table 4 for target specific annealing temperature). This was followed by a temperature ramp with 0.5 ºC increments ranging from 70 to 90 ºC for melt-curve analysis, to verify that no primer-dimer artefacts were present and only one product was generated from each qPCR assay. The final volume of the PCR reaction was 10 µL: 2.5 µL of cDNA (1:20 dilution), 5 µL of the qPCR Master Mix and 2.5 µL of forward and reverse primers (Table 4). All samples were run in duplicate. Amplifications were carried out including systematic negative controls containing no cDNA (NTC, no template control) and omitting reverse transcriptase enzyme (-RT) to check for DNA contamination. No primer–dimer

occurred in the NTC. The primers used to amplify *mgp*, *bmp2*, *ostcn*, *alp*, *mmp13*, *col1a1*, *opn* and *igf1r* were designed using PRIMER3 software (Untergasser *et al.*, 2012). Target specificity was checked *in silico* using Blast (NCBI). Only primer pairs with no unintended targets were selected. The relative expression of all genes was calculated by the ΔΔCt method (Pfaffl, 2001) using *β-actin* (McStay *et al.*, 2014), *ef1a* (Wargelius *et al.*, 2005) and *gapdh* (Takle *et al.*, 2005) as the reference genes, for which the geometric mean was calculated. The stability of references genes was evaluated using BestKeeper software tool (Pfaffl et al., 2004), which calculated the standard deviation (SD) and variation coefficient (CV) values between the Ct values of each reference gene. SD values were below 0.95 for the three genes whereas CV (% Ct) was below 3.76. In addition, to determine the PCR efficiencies, standard curves for both target and reference genes were included in each plate using a series of 5 cDNA dilutions (1:5, 1:10, 1:25, 1:50, 1:100) as a template.

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The target genes investigated in the present study were selected based on previous studies that have linked gene expression to the presence and risk of vertebral malformations in Atlantic salmon (Ytebborg, 2010; Wargelius et al., 2005; 2010).

2.7 Statistical analysis

Results are reported as mean ± standard error of the mean (SEM). Statistical analysis was carried out using Minitab (Version 17. Minitab Inc, Pennsylvania, USA). Differences between mortality, weight, SGR_{wt}, TGC, FCR and radiological deformity were assessed using a general linear model (GLM) and two-way ANOVA with replicate tank nested within ploidy and dietary treatment. All percentage data were transformed using arcsine square root transformation and data were checked for normality using a Kolmogorov-Smirnov test and homogeneity of variance using Levene's test and observations of residual plots. Post hoc tests were carried out using Tukey's multiple comparisons. A three-way ANOVA manipulated through GLM was used to analyse regional effects on % spinal deformity and mean vertebral L:H ratios such that ploidy, region and diet were considered independent variables, and tank as a random factor. In all instances, statistically significant differences were considered as $p < 0.05$. Gene expression results were analyzed using the relative expression software tool (REST 2008; http://www.gene-quantification.info/), which employs a pairwise fixed reallocation randomization test (10,000 randomizations) with efficiency correction (Pfaffl *et al.*, 2002) to determine the statistical significance of expression ratios (gene expression fold changes) between two treatments.

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3. Results

3.1 Growth and survival

There were no significant differences in cumulative mortality between dietary treatments or between ploidy during the period of study (Table 5). Diploids achieved significantly higher final smolt weights than triploids (Table 5). Within diploids, fish fed diet L2 achieved a significantly

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higher final weight than fish fed diet L1 or L3. By contrast, no dietary effect on final smolt weight was observed in triploids. Differences in final smolt weight were reflected in growth rates, where diploids fed L2 had significantly higher SGR_{wt} and TGC than all other treatments irrespective of ploidy. No significant differences in SGR_{wt} or TGC were evident between diploids fed diet L1 and L3 and any triploid dietary treatment. Within ploidy feed conversion ratio was not significantly affected by diet, however, feed conversion ratio was significantly poorer in triploids fed diet L2 compared to diploids fed L2.

3.2 Deformity

3.2.1 Prevalence of spinal malformation

Externally visible spinal deformity was < 1 % in fish fed all diets and ploidy at the end of the freshwater phase. X-ray radiography revealed a higher incidence of spinal malformation than external examination. In both ploidies there was a significant decrease in prevalence of radiologically detectable spinal deformities (L1 > L2 > L3) with increasing micronutrient supplementation (Fig. 1). Triploids had on average a higher incidence of spinal deformity than diploids within a given diet. However, triploids fed the highest supplementation had comparable prevalence of spinal deformity to diploids fed diet L1 and L2. Within deformed fish the average number of deformed vertebrae was significantly higher in triploids fed the lowest supplemented diet (L1) than any other diet (5.3 ± 1.0 vs. 3.0 ± 0.4 deformed vertebrae, data not shown). There was no significant difference in number of deformed vertebrae between any other diet or ploidy.

3.2.2 Localization and pathology of spinal malformation

Both ploidies exhibited radiological vertebral deformities in all four defined localities (R1 - R4) of the spinal region (Fig. 2, Table 6). Prevalence of affected vertebrae within each region varied significantly between diet, ploidies, and there was an interaction between ploidy and diet. Within diploids, overall prevalence of affected vertebra irrespective of diet was such that R2 = R4 = R3 >

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R1 ($p < 0.001$), while in triploids overall effects were such that R2 > R1 = R3 = R4 ($p < 0.001$). Irrespective of diet, triploids had significantly more affected vertebrae in R1 and R2 than diploids, but not in R3 and R4 (Fig 2).

In diploids, the cranial trunk (R1) was associated with the least pathology and number of deformed vertebrae. Diet L1 showed significantly greater numbers of deformed vertebra compared to L2 but not L3 (Fig. 2), accounting for 13 % of total deformity compared to only 0 – 4 % within fish fed diet L2 and L3 (Table 6). Vertebral compression (Type 2 and 5) accounted for over 50 % of pathology in diploids diet L1 within the cranial trunk, with v4 the most affected vertebra. In triploids, all three diets had comparable levels of deformed vertebrae in the cranial trunk, with peak prevalence at v5-6 (Fig. 2). Fusion (Type 6-7) pathologies were predominant in fish fed diet L1 (42 % of pathology types), compression (Type 5) in fish fed diet L2 (42 % of pathology types), while less severe, symmetry deviations (Type19) were the dominant pathology type in fish fed diet L3 (51 % of pathology types).

The caudal trunk (R2) was generally associated with the highest prevalence of deformed vertebra (42 – 58 % of total pathology) in all diets and both ploidy (Table 6). Only diploids fed diet L1 showed a difference in this pattern of deformity localization, accounting for only 29 % of all spinal deformity associated with this region rather than 42 - 44 % recorded in fish fed diets L2 and L3 (Table 5). Peak prevalence occurred in v26-29, with fusion pathologies (Type 6-7) being the dominant pathology accounting for 37 – 52 % of all deformity types recorded. In triploids, the caudal trunk was associated with the highest prevalence of deformed vertebra with fish fed diets L1, L2 and L3 having 58 %, 49 % and 45 %, respectively, of all spinal pathology recorded associated with this region (Table 6). Fish fed diet L1 had significantly greater prevalence than diet L3, with diet L2 intermediate to both (Fig. 2). Peak prevalence for deformed vertebrae occurred at v23-28. Dominant pathologies were compression pathologies (27 - 30 %, either Type 3 or Type 5) or fusion in all diets (24 – 31 %).

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Within the tail region (R3), no significant differences in affected vertebra were observed between ploidy or diet (Fig 2). Within diploids, peak prevalence for deformed vertebrae occurred at v38 - v40, with Type 6 and 7 fusions being the dominant pathology within this region. In triploids, as with R2, fish fed the L1 diet had a higher relative prevalence of deformed vertebrae peaked between v35 - v39. Dominant pathology within this region varied by diet within triploids with Type 1 accounting for 39 % in fish fed diet L1; Type 5 and Type 3 compressions accounting for 48 % in diet L2; and 51 % Type 6 fusions in diet L3.

In diploids, deformed vertebra within the tail fin (R4) showed a peak between v53 - v55, and fish fed diet L1 had an increased prevalence than fish fed both higher supplement diets. Deformities in this region accounted for 20 – 27 % of all spinal pathology (Table 6). Type 19 pathologies were the predominant deformities (30 - 65 %) within this region. In triploids, prevalence of deformed vertebrae were comparable between dietary treatments (Fig. 2). The most readily affected vertebra within this region was v54 in fish fed all three diets, and was affected in 9, 5, and 3 % of all individuals examined in fish fed diet L1, L2 and L3, respectively. Dominant pathologies were Type 19 in L1 and L3 diets (45 – 63 %) and single fusions Type 6 or 7 fusions (75 %) in diet L2.

3.3 Vertebra morphology

Ploidy did not have a significant effect on vertebral morphology (L:H ratio) in any of the spinal regions (R1-R4), whereas diet had a significant effect on L:H ratio in all four regions (R1: L3 > L1 = L2, $p = 0.002$; R2: L3 > L2 > L1, $p = 0.0001$; R3: L3 > L2 > L1, $p = 0.0001$; R4: L3 > L2 = L1, $p = 0.0001$) (Fig. 3). Only R3 showed a significant interaction between diet and ploidy ($P = 0.002$). Within diploids, fish fed diet L3 had a significantly higher L:H ratio in the cranial trunk (R1) and tail fin (R4) than diet L1, while diet L2 had an intermediate L:H within the same region. In the caudal trunk (R2) and tail region (R3) fish fed both diet L2 and L3 had significantly higher L:H ratios than fish fed diet L1. In triploids, there was no significant effect of diet on L:H ratio in the cranial trunk (R1), whereas in the caudal trunk, tail region and tail fin (R2-R4), fish fed diet L3 had

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a significantly higher L:H ratio than fish fed diet L1 and L2. Finally, in triploids, fish fed diet L3 had comparable L:H ratios within each region to that of diploids fed diet L2 and L3.

3.4 Whole body and liver compositions

Whole body Ca and P concentrations were above minimum recommended levels for juvenile Atlantic salmon (≥ 4000 $mg.kg^{-1}$) in diploids fed diet L2 and L3, but not in those fed diet L1, corresponding with a decrease in total deformity prevalence for fish fed those diets (Table 7). In triploids, average Ca levels were slightly above the minimum recommended level only when salmon were fed diet L1, whereas P levels were above 4000 $mg.kg^{-1}$ in fish fed diet L1 and L3 (Table 7). In diploids, as whole body Ca:P ratio increased there was a corresponding decrease in total deformity. By contrast, in triploids, whole body Ca:P ratio in all dietary treatments was < 1.0, and there was no apparent correlation with spinal deformity. Regarding other minerals, a slight effect of diet was observed for whole body concentration of Se, which increased with increasing micronutrient supplementation levels in both ploidies (L3 > L2 > L1; $p = 0.09$) (Table 7). With vitamins, a significant effect of ploidy ($p < 0.0001$), diet ($p < 0.0001$) and the interaction between these two factors ($p = 0.037$) was found for α-tocopherol (α-TOH) concentrations, with higher levels in diploids than in triploids and increasing body levels with increasing levels of supplementation coinciding with decreasing deformity prevalence in these dietary groups (Table 7). In liver, the concentrations of vitamins A1, A2, C and MK4 increased with supplementation level, while vitamin K1 decreased in both ploidies (L3 > L2 > L1) (Table 7). In addition, there was a significant effect of ploidy for vitamins K1 and MK4 ($p = 0.001$ and $p < 0.001$, respectively), with higher levels in diploid salmon (Table 7).

3.5 Gene expression

In diploids, expression of most bone biomarker genes showed an increasing trend related to micronutrient inclusion levels (Fig. 4). Thus, differences in *igf1r* expression were statistically

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significant between fish fed all the experimental diets (L1 < L2 < L3) whereas *bmp2*, *alp* and *ostcn* expression was significantly higher in fish fed diet L2 and L3 than in those fed diet L1 (L1 < L2 = L3), and expression of *opn* and *mmp13* genes showed statistical differences only between fish fed diet L1 and L3 (L1 < L3). Finally in diploids, *col1a1* and *mgp* expression showed no statistically significant differences between dietary groups although *mgp* showed the same trend observed for the other biomarkers. In triploids, differences in gene expression between treatments were only significant for *igf1r* (L1 > L3), although these differences were opposite to those observed in diploid salmon (Fig. 5).

4. Discussion

In the present study, the incidence of malformation was higher in triploid salmon than in diploids. However, there was a reduction in the prevalence of spinal deformity with increasing micronutrient supplementation (L3<L2<L1) in both ploidies, where triploid fish fed diet L3 showed comparable levels of spinal deformity to diploids fed diets L1 and L2. The higher prevalence of malformation in triploids supports the hypothesis that triploid salmon have specific dietary requirements different from and generally higher than diploids (Fjelldal and Hansen, 2010; Taylor *et al.*, 2015; Fjelldal *et al.*, 2016; Sambraus *et al.*, 2017; Smedley *et al.*, 2018).

In addition to general prevalence, the present study also showed that diet had significant impacts on the type of vertebral deformity and the spinal regions more affected, and this also varied with ploidy. Thus, increased micronutrient supplementation reduced the prevalence of fusion deformities in diploids, while in triploids diet had more significant effects on compression and reduced spacing deformities. While, irrespective of ploidy, many of vertebral deformities are most likely a consequence of effects on mineralization possibly related to P levels, it is less obvious how ploidy would affect the outcome (Witten *et al.*, 2009). Similarly, the prevalence of affected vertebrae within each spinal region (cranial, caudal, tail and tail fin) varied significantly between diet and ploidies, and there was interaction as has been reported previously (Kacem *et al.*, 1998; Fjelldal and

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Hansen, 2010). However, there was a difference with ploidy, as prevalence of deformities was significantly higher in the caudal region in triploids, whereas this was not evident with diploids. Similarly, the impact of graded micronutrient supplementation in reducing deformities was also more pronounced in triploids compared to diploids in the present study. This may possibly just reflect the greater incidence and extent of deformities in triploids (Smedley *et al.*, 2018). In the present study, diet affected vertebral morphology with L:H ratio generally increasing with level of micronutrient supplementation in both ploidy with no difference between ploidies. Previously it was shown that irrespective of ploidy, salmon parr fed low dietary P inclusion showed lower (shorter) L:H ratio in vertebrae (Fjelldal and Hansen, 2010; Fjelldal *et al.*, 2016). However, changes in L:H ratio may not be expressing a pathology, although they do suggest that morphological changes are occurring in vertebral body construction (Fraser *et al.*, 2014).

The above clearly indicated correlations between the supplemented level of the whole nutrient package and effects on skeletal development. However, while the above effects of diet on vertebral morphology, and type and location of deformities are undoubtedly significant, they do not give major insight to the impact of specific micronutrients on the underlying mechanisms of vertebral deformity. Therefore, the present study focussed more on the impact of diet on some key bone biomarker genes in an attempt to gain further understanding of the interaction between diet and skeletal development. Thus, although the multiplex-type design of the study using graded levels of a nutrient package rather than regressions of individual nutrients has some limitations, knowledge of the roles of individual nutrients in the function of specific genes/pathways enables some deeper analysis of the role of these nutrients on bone metabolism.

Fish bone is a metabolically active tissue composed of calcium hydroxyapatite and a matrix of type I collagen under continuous remodelling, a process comprising the coupled actions of osteoclasts and osteoblasts. Osteoclasts are involved in bone resorption, dissolving mineral crystals and digesting the organic matrix of bone, whereas osteoblasts deposit new organic collagenous matrix and regulate the mineralization process. In addition, some osteoblasts differentiate into

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osteocytes, which remain entrapped in the bone matrix (Doherty *et al.*, 2015). Several micronutrient deficiencies and toxicities have been linked to skeletal disorders in fish. Thus, there is wide evidence about the role played by certain vitamins (A, C, E and K) and minerals (calcium, phosphorus, boron, zinc, copper, silicon, vanadium, selenium, manganese, strontium and fluoride) in bone formation, mineralization and reabsorption (Lall and Lewis-McCrea, 2007). In the present study, concentrations of Se (selenium) and α-TOC (vitamin E) in fish whole body, and vitamins A1, A2, C and MK4 in liver increased with increasing levels of NP inclusion, and were inversely proportional to the prevalence of spinal malformations. In addition, in diploid salmon the expression of many of the bone biomarker genes investigated increased with increasing NP inclusion, which might suggest that some of the minerals and vitamins included in the NP regulate the expression of genes involved in bone formation and remodelling. In contrast, NP level did not significantly affect our chosen bone marker gene expression in triploid salmon, although some genes showed similar trends to the profiles observed in diploids (e.g. *ostcn*, *opn*, *mgp*), which may reflect different nutritional requirements reported for triploid salmon.

Vitamin E is a lipid-soluble antioxidant that plays a key role in inhibiting tissue peroxidation and, in bone, it is involved in skeletal health by protecting osteoblasts from free radical damage (Lewis-McCrea and Lall, 2007). Here, the percentage of malformations decreased with increasing whole body concentrations of vitamin E, and there was a lower prevalence of deformities in diploids, which presented higher vitamin E tissue levels than triploids. In sea bream (*Sparus aurata*), increased dietary α-TOC prevented the negative effects of docosahexaenoic acid (DHA) on the appearance of skeletal deformities, in particular jaw, haemal and neural spine malformations (Izquierdo *et al.*, 2013). While DHA is an essential fatty acid in marine fish, dietary excess can induce free radical damage. Similarly, dietary vitamin C reduced the incidence of cranial deformities when fish were fed high levels of DHA in sea bass (*Dicentrarchus labrax*) larvae (Betancor et al., 2012). Vitamin C (ascorbic acid) is a powerful water-soluble antioxidant that acts as an essential cofactor for collagen formation, bone matrix and connective tissue. Therefore, a lack

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of vitamin C causes poor bone calcification and metabolism and, consequently, this can result in the appearance of bone deformities in fish (Lall and Lewis-McCrea, 2007). In addition, there is an interaction between vitamins E and C in Atlantic salmon with vitamin C able to regenerate vitamin E from the α-tocopheroxyl radical. Indeed, vitamin C protected salmon against vitamin E deficiency in a dose-dependent manner as reflected in mortality, morphometric data and physiological parameters, although tissue concentrations of vitamin E were not affected by vitamin C supplementation, except when vitamin C was deficient, which caused a reduction of vitamin E levels (Hamre *et al.*, 1997). In the present study, while interaction between vitamins E and C cannot be discriminated, the percentage of malformations decreased with increasing tissue levels of both vitamins. In addition, key bone biomarkers were up-regulated in diploid salmon in parallel with increased dietary levels of vitamins E and C, which play roles in the regulation of these genes. Thus, vitamin C in fish is known to stimulate the expression of bone-specific osteocalcin (*ostcn*) gene (Darias *et al.*, 2011), which codes for the most abundant non-collagenous protein in teleosts' bone and is a key marker gene for the mineralization of the extracellular matrix (ECM). Similarly, other biomarkers for osteoblast activity and mineralization such as alkaline phosphatase (*alp*) and insulin-like growth factor 1 (*igf1*) are also affected by vitamin C, being down-regulated when there is a lack of this vitamin (Mahmoodian *et al.*, 1996; Mazurais *et al.*, 2008). This is consistent with data here in diploid salmon, which showed up-regulation of these two genes in parallel with higher concentrations of hepatic vitamin C.

Selenium is also an essential trace element in fish nutrition with antioxidant properties, acting as a free-radical scavenger thereby protecting tissues against oxidative damage through the activation of selenoenzymes and selenoproteins (Khan *et al.*, 2017). Previous studies indicated that Se levels in plant ingredients are lower than those in fishmeal and consequently plant-based diets for salmon must be supplemented with Se (Hamre et al., 2016). However, there is a narrow safe limit for dietary Se and, above this, Se is toxic for fish, causing skeletal deformities, in particular lordosis, cranio-facial abnormalities and fin malformations (Kupsco and Schlenk, 2016). In the present study,

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although Se concentration in whole body was higher in fish fed diet L3, the prevalence of skeletal anomalies was lower for both diploid and triploid salmon, suggesting that dietary Se concentrations were within the safe margins for Atlantic salmon. In liver of both diploids and triploids, vitamin A1 and A2 concentrations were positively correlated with increasing NP level and inversely correlated with percentage deformity. Vitamin A is involved in skeletogenesis, cartilage development and bone remodelling by regulating chondrocyte and osteoblast functions and stimulating osteoclasts (Lall and Lewis-MaCrea, 2007), and deficiency has been linked to higher prevalence of spinal deformities in fish (Yang *et al.*, 2008). However, although vitamin A is an essential micronutrient, high dietary intake can cause toxic effects in Atlantic salmon, including reduced growth, increased mortality, and spine deformities with increasing levels of dietary vitamin A during early life stages (Ørnsrud *et al.*, 2002). Similarly, chronic high dietary vitamin A had negative effects on growth and bone health in salmon post-smolts, and bone alkaline phosphatase activity increased with increasing vitamin A intake (Ørnsrud *et al.*, 2013), consistent with data in the present study showing higher expression of *alp* in diploids fed diets L2 and L3. Vitamin A also plays a role in the regulation of bone morphogenetic proteins (BMPs), which are growth factors involved in bone and cartilage formation. In the present study, *bmp2* expression was higher in diploid salmon fed diets L2 and L3, consistent with a previous study in Senegalese sole (*Solea senegalensis*) where *bmp2*, *bmp4* and *bmp16* genes were up-regulated by retinoic acid (a metabolite of vitamin A) (Marques *et al.*, 2014).

Vitamin K1 levels in liver were also affected by diet and ploidy, with concentration decreasing with increasing NP level (L1 > L2 > L3), and diploid salmon showing higher levels than triploids, whereas vitamin MK4 levels were only affected by ploidy (diploids > triploids). Vitamin K is involved in bone protein metabolism, stimulating bone formation and inhibiting resorption, decreasing bone loss (Lall and Lewis-MaCrea, 2007). In the present study, increasing vitamin K content in the NP package correlated with lower spinal malformations and up-regulation of *ostcn* expression in liver in diploids. However, this was not reflected in liver concentration of vitamin K1. The expression of *mgp* also showed an increasing (but non-significant) trend. Similarly, the

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expression of both *ostcn* and *mgp* was higher, but not significant, in triploids with higher vitamin K inclusion. Both osteocalcin and matrix Gla protein (MGP) are vitamin K-dependent proteins involved in producing extracellular matrix, accumulating in bone tissue and binding calcium and calcified matrices through interaction with Gla residues, which result from γ-carboxylation of glutamate residues, a vitamin K-dependent process (Cancela *et al.*, 2014).

Expression levels of *mmp13* (matrix metallopeptidase 13) and *opn* (osteopontin) were upregulated with higher NP level in diploid salmon. In general, MMPs are zinc-dependent extracellular proteinases and collagenases that cleave structural collagens during the remodelling of the ECM (Philips *et al.*, 2011). However, Zn concentrations in whole body showed no differences between dietary groups, irrespective of ploidy, in the present study. Therefore, although *mmp13* was up-regulated in diploids fed diets with higher NP level and showing lower levels of spinal deformities, these effects were not related to dietary Zn. The *opn* protein, osteopontin, is produced by osteoblasts, binds calcium, and plays a role in hard tissue mineralization in fish, modulating hydroxyapatite crystallization and is regulated by 1,25-dihydroxyvitamin D_3, the active form of vitamin D (Shen and Christakos, 2005; Fonseca *et al.*, 2007). Ca and P are key minerals affecting development and skeletal health in Atlantic salmon (Lall and Lewis-McCrea, 2007), and previous studies have showed that suboptimal P nutrition in juveniles is linked to higher prevalence of skeletal deformities (Fjelldal *et al.*, 2012), with triploid salmon showing higher P requirements than diploids (Smedley *et al.*, 2018). In fact, skeletogenesis is dependent on P and Ca homeostasis which is regulated by parathyroid hormone, vitamin D3 and fibroblast growth factor 23 (Martin *et al.*, 2012). However, Ca levels and Ca:P ratios were not affected by diet or ploidy in the present study.

In conclusion, the present study indicated that, for optimum growth and development, micronutrient levels should be supplemented above current NRC (2011) recommendations when Atlantic salmon are fed low marine diets. In particular, the present study showed that the prevalence of spinal deformities was reduced by increasing micronutrient supplementation in parr of both diploid and triploid salmon. The reduced vertebral deformity was associated with increased

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expression of a range of bone biomarker genes in diploids whereas, in triploids, some genes showed similar but non-significant trends. Overall the study confirmed earlier indications that there are differences in nutritional requirements between diploid and triploid salmon.

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Figure Legends

Fig. 1. Prevalence (Mean ± SEM) of radiologically deformed fish within juvenile diploid and triploid Atlantic salmon at end of freshwater phase having been fed a low marine diet (FM 15 % / FO 8 %) with differing micronutrient supplementation level (L1 = 100 %; L2 = 200 %; or L3 = 400 % NRC Premix). Fill patterns represent the vertebral pathology classification (% of deformed vertebrae) according to Witten *et al.* (2009). Upper case superscripts denote significant differences between diet and ploidy for mean prevalence (two-way ANOVA), while lower case superscripts denote significant differences between diet within ploidy for pathology classification (one-way ANOVA).

Fig. 2. Mean percentage of total deformed vertebra within each spinal region of juvenile diploid and triploid Atlantic salmon at end of freshwater phase having been fed a low marine diet (FM 15 % / FO 8 %) with differing micronutrient supplementation level (L1 = 100 %; L2 = 200 %; or L3 = 400 % NRC Premix). The vertebral column has been divided into four regions as defined by Kacem *et al.* (1998). Lower case superscripts denote significant differences between diet and ploidy for each region (two-way ANOVA).

Fig. 3. Mean vertebral length-height ratio (L:H) within each spinal region of juvenile diploid and triploid Atlantic salmon at end of freshwater phase having been fed a low marine diet (FM 15 % / FO 8 %) with differing micronutrient supplementation level (L1 = 100 %; L2 = 200 %; or L3 = 400 % NRC Premix). The vertebral column has been divided into four regions as defined by Kacem et al., (1998). Lower case superscripts denote significant differences between diet and ploidy for each region (two-way ANOVA).

Fig. 4. Relative expression of genes involved in bone formation and development in vertebrae of diploid Atlantic salmon smolts fed low marine feeds with differing levels of micronutrient supplementation. Superscript letters indicate statistically significant differences between treatments.

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Fig. 5. Relative expression of genes involved in bone formation and development in vertebrae of triploid Atlantic salmon smolts fed low marine feeds with differing levels of micronutrient supplementation. Superscript letters indicate statistically significant differences between treatments.

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Table 1. Formulations (g.100g diet^{-1}) and analyzed proximate compositions of experimental diets

	Diet		
Ingredients	L1	L2	L3
Fishmeal[1]	13.00	13.00	13.00
Krill Meal[2]	2.00	2.00	2.00
Soy protein concentrate[3]	17.94	18.00	17.65
Corn gluten[4]	4.49	3.00	3.00
Pea protein concentrate[5]	17.94	18.49	18.15
Wheat gluten[4]	14.36	14.79	14.52
Wheat[6]	8.63	8.26	7.46
Fish Oil[7]	8.00	8.00	8.00
Rapeseed oil[4]	5.25	5.32	5.47
Linseed oil	1.27	1.28	1.32
Palm kernel oil	3.17	3.21	3.30
ARRAINA Nutrient Package[8]†	0.75	1.50	3.00
Monosodium Phosphate	2.52	2.53	2.54
Amino acid Premix[9],*	0.68	0.62	0.59
Proximate Composition (Analyzed)			
Moisture (%)	6.3	6.8	6.1
Crude lipid (%)	20.8	21.1	22.7
Crude protein (%)	48.9	47.0	48.1
Ash (%)	6.5	6.8	7.4
Energy (MJ / kg)	23.4	23.4	23.4

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

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Table 2. Added micronutrient concentrations ($mg.kg^{-1}$) within the nutrient package (NP): selected amino acids (histidine and taurine), minerals, vitamins and cholesterol.

	Diet		
Premix Formulations	L1	L2	L3
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
Thiamin	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

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Table 3. Analyzed concentrations of selected amino acids (taurine, histidine and methionine $g.kg^{-1}$) macro-minerals (calcium, magnesium and phosphorous, $g.kg^{-1}$) micro-minerals and vitamins ($mg.kg^{-1}$) of the experimental diets for the 2 mm and 3 mm pellets.

Pellet Size	2mm			3mm			
	L1	L2	L3	L1	L2	L3	NRC 2011‡
Vitamin A*	5.2	7.2	14.2	6.2	5.1	7.2	0.75[a]
Vitamin D3*	0.17	0.19	0.29	0.17	0.18	0.26	0.04[a]
Vitamin E*	85	146	203	75	151	273	60[b]
Vitamin K3*	0.43	0.78	1.60	0.70	1.12	1.12	<10[b]
Thiamin*	3.3	5.8	9.3	3.7	20.2	29.9	1[a],
Riboflavin*	12.1	20.6	35.8	11.7	34.4	57.6	4[a],
Vitamin B6*	11.5	14.3	22.2	11.0	18.1	30.5	5[b]
Vitamin B12*	0.17	0.30	0.49	0.14	0.34	0.66	NT
Niacin*	75	107	163	80	221	434	10[a],
Pantothenic acid*	18.6	33.2	50.2	20.2	74.3	66.0	20[a,]
Folic acid*	2.82	n.a.	7.53	2.82	19.95	12.54	1[a]
Biotin*	0.44	0.71	1.03	0.47	1.10	1.91	0.15[a]
Vitamin C*	83	180	312	77	238	244	20[b]
Cobalt*	1.0	1.6	3.5	0.95	2.3	3.4	NT
Iodine*	n.a.	n.a.	n.a.	1.1	3.4	6.1	1.1[a]
Selenium*	1.2	1.5	2.2	1.3	1.6	2.5	0.15[a]
Iron*	300	330	510	330	310	410	30-60[b]
Manganese*	43	57	110	47	49	75	10[b]
Copper*	10	12	19	10	13	19	5[b]
Zinc*	160	190	300	100	200	350	37[b]
Taurine*	2.8	4.6	8.1	2.7	5.0	9.5	NR[b]
Methionine	8.7	8.8	8.6	9.0	8.8	9.1	7.0[b]
Histidine*	11.6	11.7	14.0	11.9	13.1	14.5	8.0[b]
Calcium*	6.6	6.9	8.6	6.3	6.7	7.5	NR[b]*
Magnesium	1.5	1.5	1.5	1.3	1.3	1.3	0.4[b]
Phosphorus	12.0	12.0	12.0	11.0	11.0	11.0	8.0[b]
Cholesterol*	n.a.	n.a.	n.a	n.a	n.a.	n.a.	NR

Nutrients added at graded levels to the feeds are shown with an asterisk. ‡Current NRC, 2011 minimum requirement recommendations determined in [a] rainbow trout, [b] Atlantic salmon are shown for comparison. n.a. not analyzed; NR* no requirement freshwater; NT, not tested.

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Table 4. Primers and conditions used for quantitative RT-PCR.

Genes	Primer sequence (5'-3')	Amplicon (bp)	Tm	Amplification Efficiency (%)	GenBank Accession number	Reference
mpg	F: TCATCAACCCACAGAGTCCA R: ACATGTCTCTGAGCGCCTTT	110	56 ºC	D: 95 T: 98	XM_014203686.1	New design
bmp2	F: TCTCATGGTACTGCTGCTGG R: TCCGAACATATTGAGGAGCC	160	56 ºC	D: 97 T: 95	NM_001173834.1	New design
ostcn	F: GACTCCTCTACCTCCACTGC R: AATGATCCCAGCTGTGTCCA	207	60 ºC	D: 97 T: 99	NM_001136551.1	New design
alp	F: CCAGGCAGCTCTTTGAGAAC R: TCCACTCCTCCACCAAGTTC	156	56 ºC	D: 97 T: 94	NM_001139976.1	New design
mmp13	F: CCAACCCAGACAAGCCAGAT R: GCTCTGAGAGTGGATACGCC	189	56 ºC	D: 95 T: 99	NM_001140524.1	New design
col1a1	F: TGGTGAGCGTGGTGAGTCTG R: TAGCTCCGGTGTTTCCAGCG	188	60 ºC	D: 99 T: 98	FJ195608	New design
opn	F: CTTACTGAGGTGGCCCCTGT R: GCTGTCCGATGTTGGGTCTG	114	60 ºC	D: 100 T: 98	XM_014186048.1	New design
igf1r	F: TGGACATGGAGGTGGAGAAC R: TCAGGGTACTAGAAGGGCCT	168	56 ºC	D: 94 T: 98	XM_014124120.1	New design
Bactin	F: ATCCTGACAGAGCGCGGTTACAGT R: TGCCCATCTCCTGCTCAAAGTCCA	112	60 ºC	D: 99 T: 99	AF012125	McStay *et al*. (2014)
ef1a	F: CACCACCGGCCATCTGATCTACAA R: TCAGCAGCCTCCTTCTCGAACTTC	78	60 ºC	D: 95 T: 99	DQ834870	Wargelius *et al*. (2005)
gapdh	F: TGGTGCAGAACCTCATGGTCCTCA R: ATCCCGGATGATTCCAAAGTCGTC	120	60 ºC	D: 100 T: 97	BT043825	Takle *et al*. (2005)

mpg, matrix gla protein; *bmp2*, bone morphogenetic protein 2; *ostcn*, osteocalcin; *alp*, alkaline phosphatase; *mmp13*, matrix metallopeptidase; *col1a1*, collagen type I alpha 1; *opn*, osteopontin; *igf1r*, insulin-like growth factor 1 receptor; *ef1a*, elongation factor 1 alpha; *Bactin*, β-actin; *gapdh*, glyceraldehyde 3-phosphate dehydrogenase; D, diploids; T, triploids.

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Table 5. Mortality, final weight and somatic indices (mean ± SEM) recorded at the end of the freshwater rearing in juvenile diploid and triploid Atlantic salmon fed low marine diets (FM 15% / FO 8%) with differing micronutrient supplementation level (L1 = 100 %, L2 = 200 % and L3 = 400 % NRC Premix).

	Diploid			Triploid		
Diet	L1	L2	L3	L1	L2	L3
Mortality (%)	4.8 ± 4.3	1.4 ± 0.6	0.6 ± 0.1	1.6 ± 0.5	1.1 ± 0.2	1.1 ± 0.3
Fn. Smolt Wt (g)	94.4 ± 5.8^{b}	113.8 ± 3.8^{a}	94.1 ± 6.4^{b}	75.4 ± 4.8^{c}	75.2 ± 1.8^{c}	77.5 ± 0.6^{c}
SGR_{wt}	0.62 ± 0.01^{b}	0.70 ± 0.03^{a}	0.61 ± 0.03^{b}	0.68 ± 0.02^{b}	0.66 ± 0.03^{b}	0.69 ± 0.02^{b}
TGC	0.88 ± 0.04^{b}	1.02 ± 0.05^{a}	0.86 ± 0.07^{b}	0.82 ± 0.03^{b}	0.80 ± 0.03^{b}	0.83 ± 0.01^{b}
FCR	0.90 ± 0.17^{ab}	0.71 ± 0.07^{b}	0.87 ± 0.02 ^{ab}	0.93 ± 0.09^{ab}	1.02 ± 0.13^{a}	0.92 ± 0.0^{ab}

Superscripts denote significant differences between diet and ploidy (two-way ANOVA).

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Table 6. Relative percentage of deformity within each spinal region of fish recorded as deformed. Bold highlights spinal region within dietary treatment showing predominant area of deformity.

	Diploid			Triploid		
Spinal Region	L1	L2	L3	L1	L2	L3
R1 – Cranial Trunk	13.1	0.0	3.8	9.5	16.0	13.9
R2 – Caudal Trunk	**29.3**	**42.3**	**44.2**	**57.7**	**49.6**	**45.4**
R3 – Tail Region	**37.4**	**38.0**	**25.0**	**21.9**	**21.0**	**30.6**
R4 – Tail Fin	20.2	19.7	26.9	10.9	13.4	10.2
Total (%)	100	100	100	100	100	100

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Table 7. Deformity prevalence (% of population) and concentrations of selected minerals and vitamins (mg.kg^{-1} wet weight) in whole body and liver of diploid and triploid salmon fed low marine diets with differing micronutrient supplementation level.

Ploidy (P)	Diploid			Triploid			*p*-values		
Diet (D)	L1	L2	L3	L1	L2	L3	Ploidy	Diet	P *D
Whole Fish									
Ca*	3782±1018	4788±1182	4634±565	4094±684	3536±378	3850±981	0.28	0.87	0.45
P	3925±369	4616±748	4423±352	4135±324	3918±220	4064±560	0.33	0.73	0.42
Ca:P Ratio	0.96	1.04	1.05	0.99	0.90	0.95	n.a	n.a	n.a
Mg	274±3	292±7	283±16	291±6	277±7	287±33	0.85	0.96	0.39
Zn*	32±1.8	42±11	34±6	39±6	28±0.8	32±1	0.19	0.83	0.43
Se*	0.22±0	0.25±0	0.26±0	0.25±0	0.2±0	0.3±0	0.46	**0.09**	0.60
α-TOH*	28±0^{b}	41±0^{a}	48±2^{a}	28±1 ^{b}	30±3^{b}	40±4 ^{a}	**0.000**	**0.000**	**0.037**
Liver									
Vitamin A_1*	9.5±2.1^{a}	12.0±2.8 ^{a}	21.5±6.4 ^{b}	7.5±2.1 ^{a}	14.0±5.7 ^{a}	19.0±2.8 ^{b}	0.446	**0.012**	0.746
Vitamin A_2	70±12 ^{a}	83±21 ^{a}	135±21 ^{b}	51±5 ^{a}	79±30 ^{a}	115±7 ^{b}	0.089	**0.004**	0.849
Vitamin C*	82±5 ^{a}	108±18 ^{b}	114±2 ^{c}	78±7 ^{a}	102±5 ^{b}	140±10 ^{c}	0.914	**0.000**	0.052
Vitamin K_1	40±9	37±12	31±4	20±8	12±3	17±0	**0.001**	**0.004**	0.238
Vitamin MK_4	31±4	34±10	40±5	14±0	14±3	17±1	**0.000**	0.051	0.292
Deformity (%)	28	24	19	38	33	27			

Data are presented as mean ± SD (n = 2). The supplemented elements are marked with an asterisk.

Significant effect is highlighted in bold (two-way ANOVA) and superscripts indicate differences between groups.

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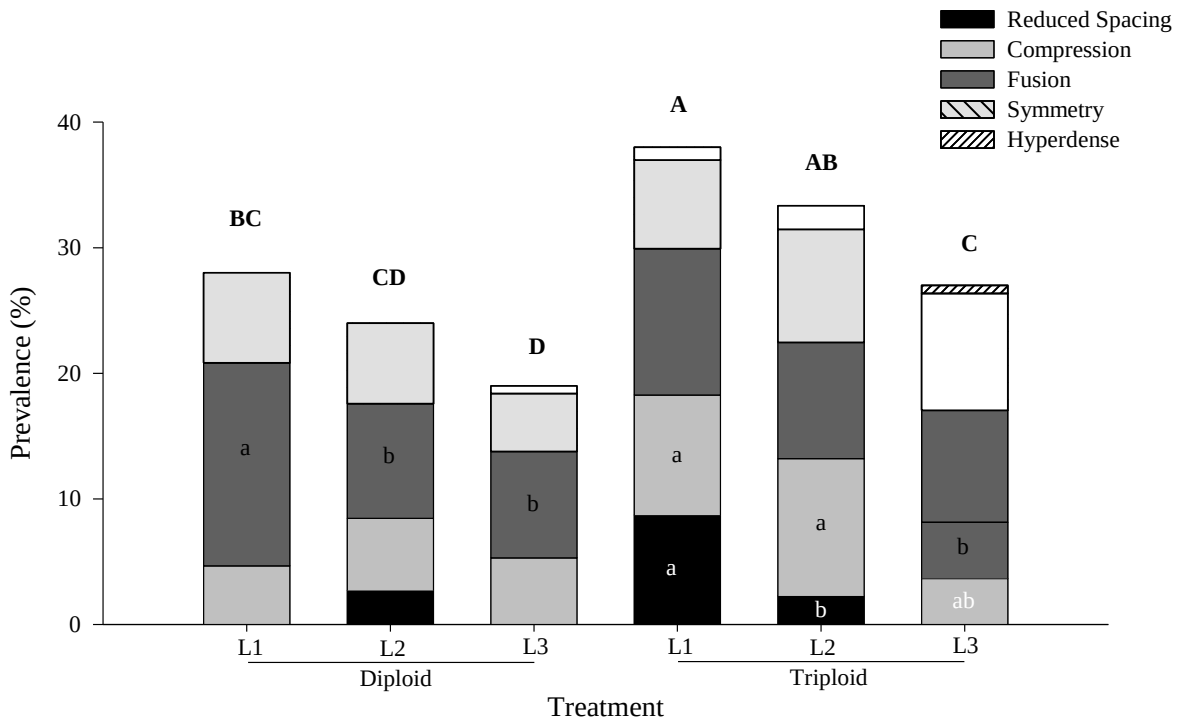

Figure 1

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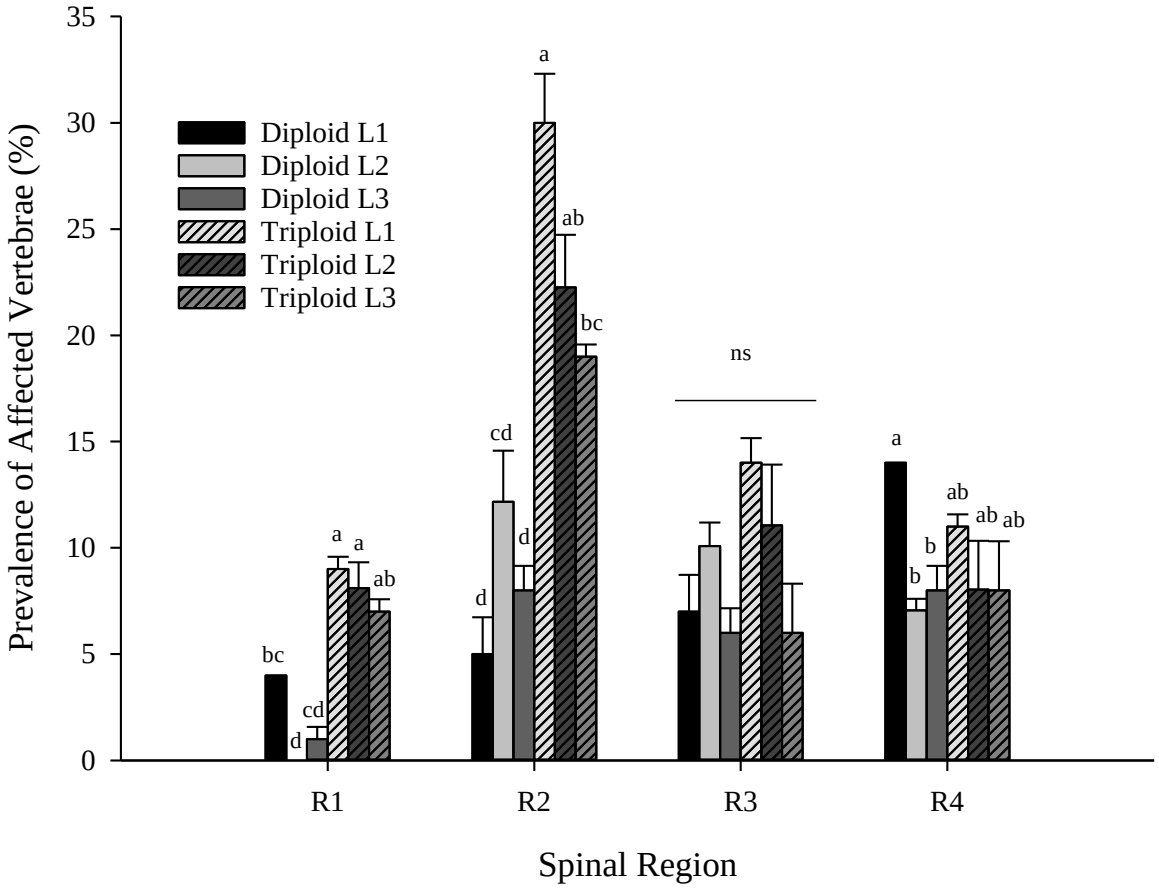

Figure 2

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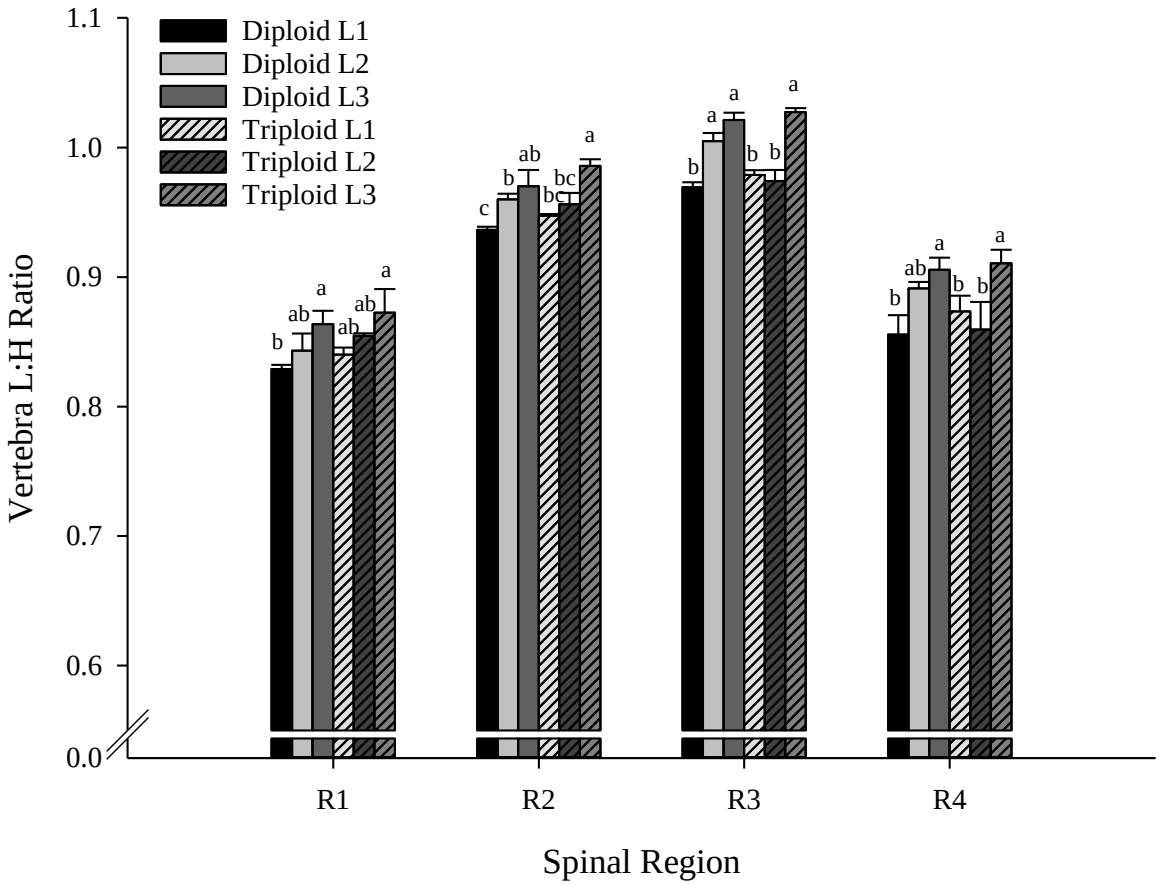

Figure 3

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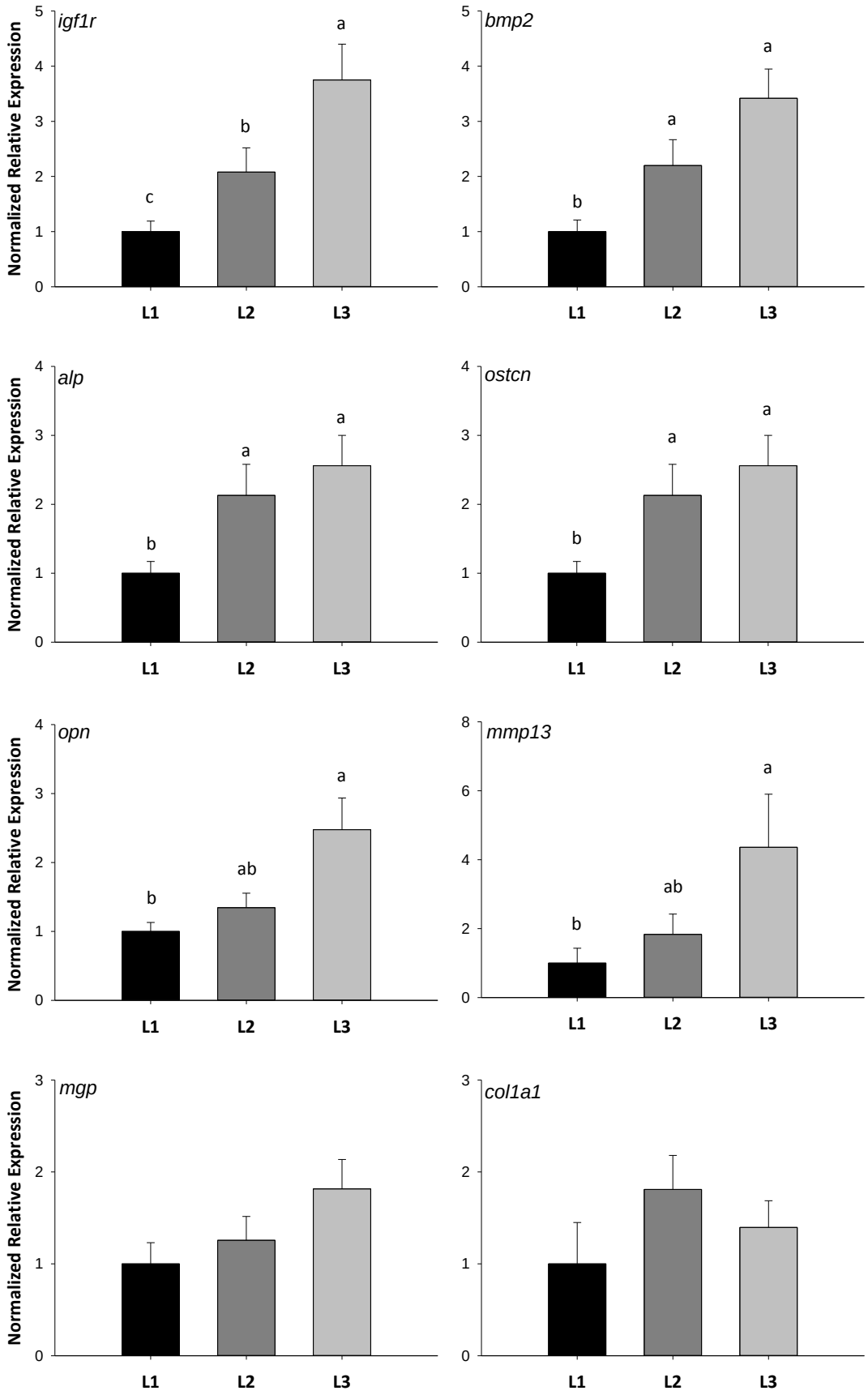

Figure 4

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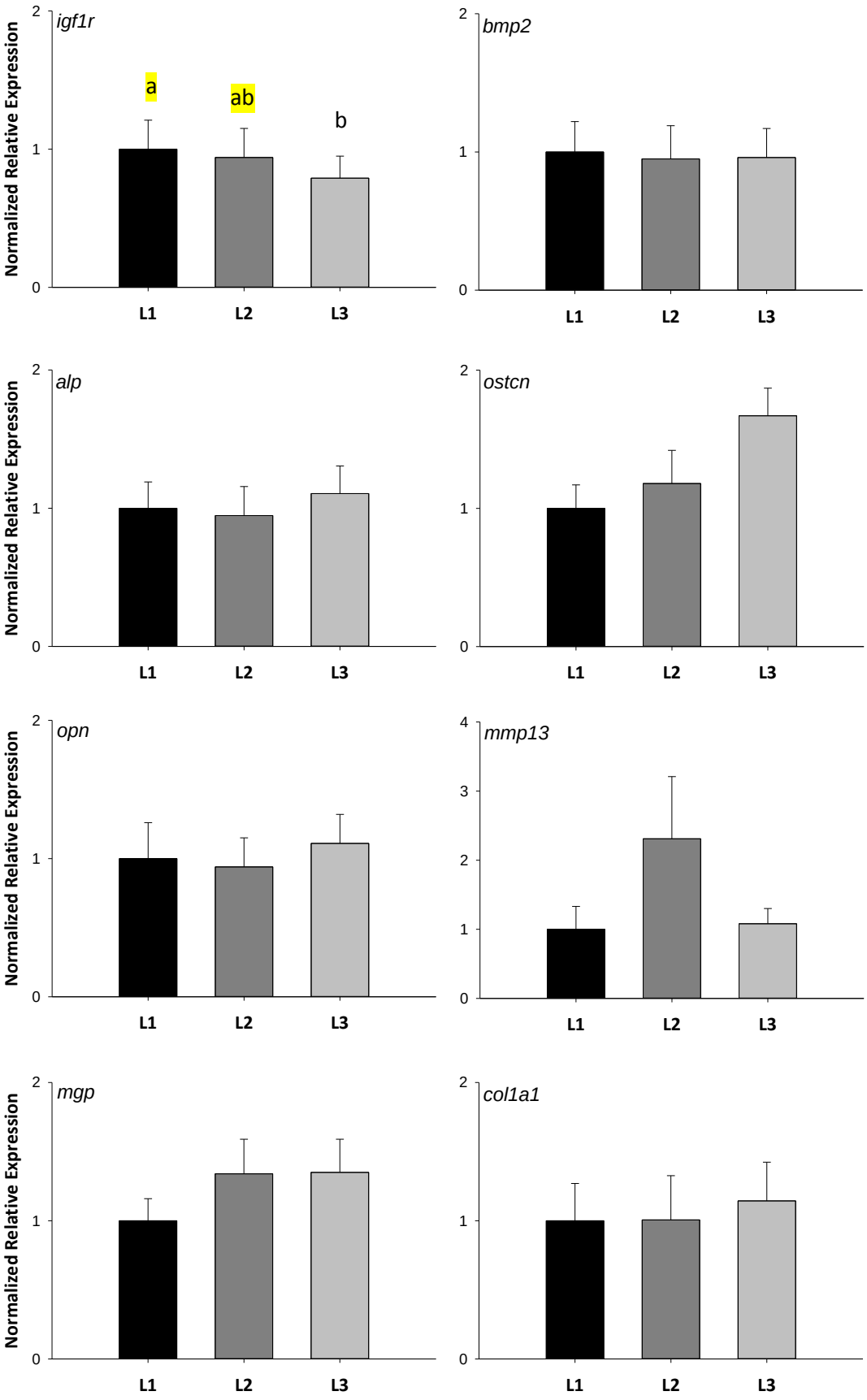

Figure 5

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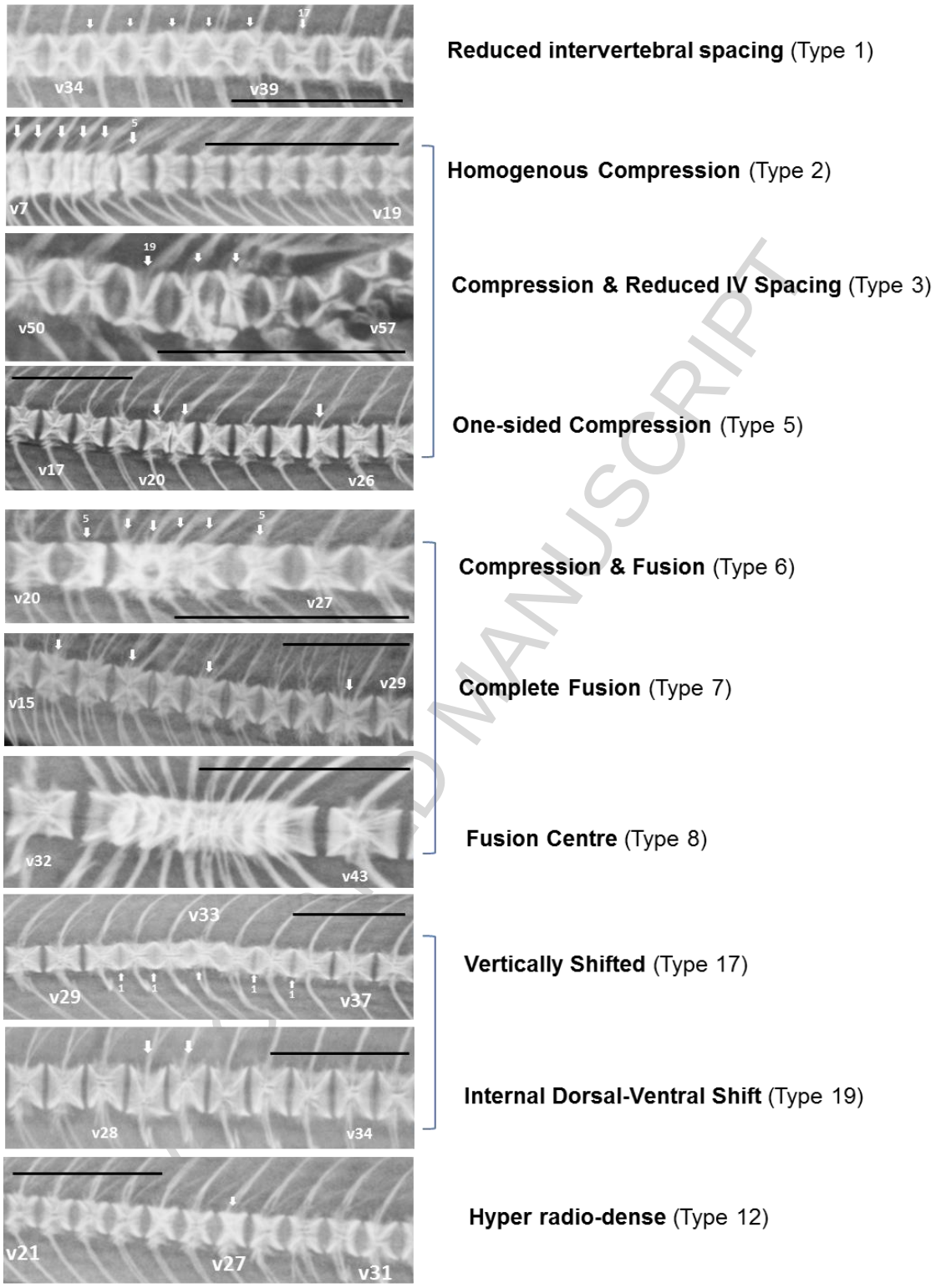
Supplementary file



Supplementary Figure 1. Example radiographs of vertebral body malformations observed in fish fed the experimental diets. Reduced intervertebral spacing (Type 1); Compressions (Type 2 - 5); Fusions (Type 6 - 8); Symmetry Deviations (Type 17 and 19); Radio-dense (Type 12). Black horizontal bars in each radiograph represents 1 cm.

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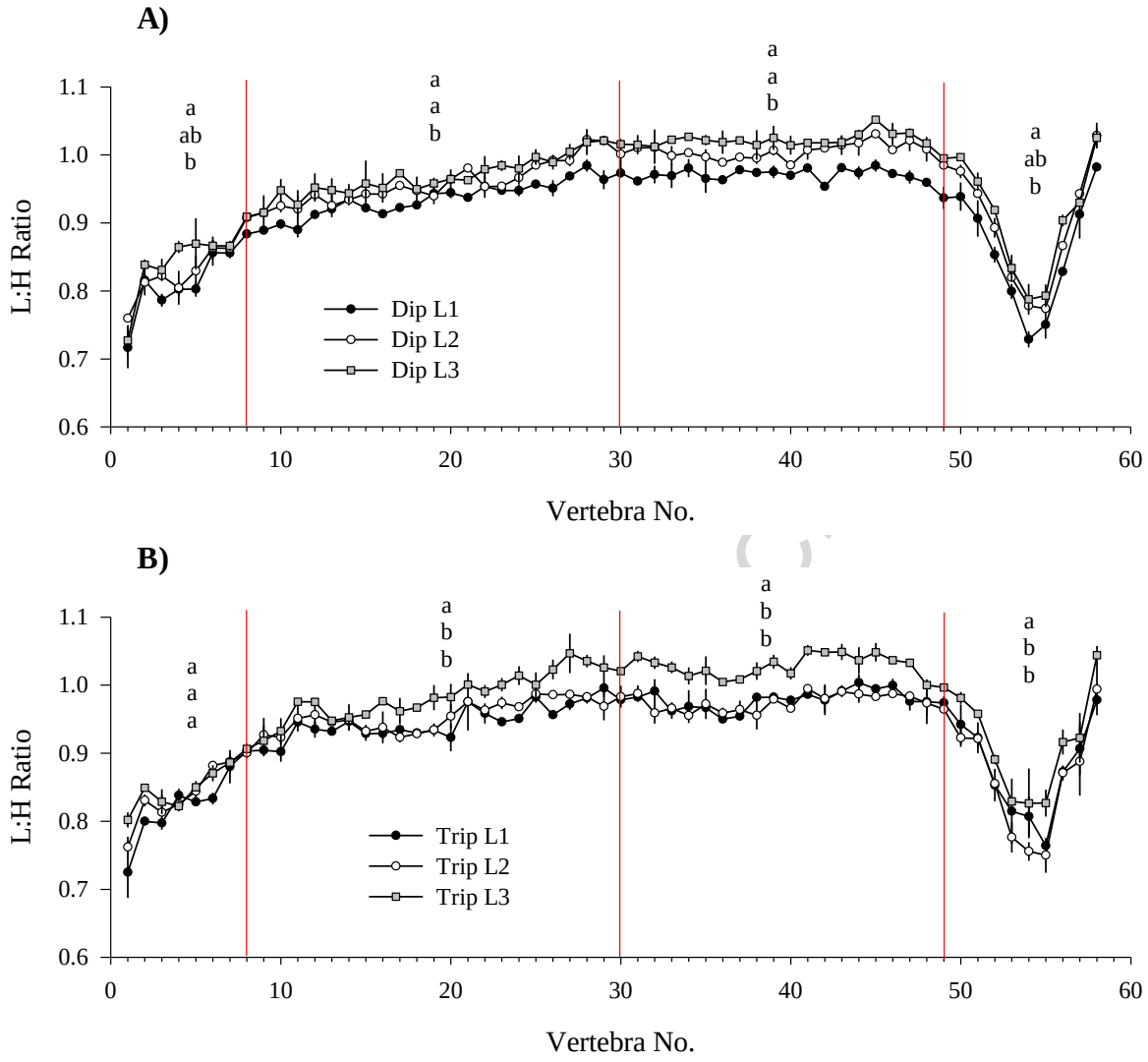

Supplementary Figure 2. Individual vertebral length-height ratio (L:H) along the spinal column of A) juvenile diploid and B) triploid Atlantic salmon at end of freshwater phase having been fed a low marine diet (FM 15 % / FO 8 %) with differing micronutrient supplementation level (L1 = 100 %; L2 = 200 %; or L3 = 400 % NRC Premix). The vertebral column has been divided into four regions as defined by Kacem *et al.*, (1998). Lower case superscripts denote significant differences between diets within ploidy for each region (one-way ANOVA).

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Figure Legends

Fig. 1. Prevalence (Mean ± SEM) of radiologically deformed fish within juvenile diploid and triploid Atlantic salmon at end of freshwater phase having been fed a low marine diet (FM 15 % / FO 8 %) with differing micronutrient supplementation level (L1 = 100 %; L2 = 200 %; or L3 = 400 % NRC Premix). Fill patterns represent the vertebral pathology classification (% of deformed vertebrae) according to Witten *et al.* (2009). Upper case superscripts denote significant differences between diet and ploidy for mean prevalence (two-way ANOVA), while lower case superscripts denote significant differences between diet within ploidy for pathology classification (one-way ANOVA).

Fig. 2. Mean percentage of total deformed vertebra within each spinal region of juvenile diploid and triploid Atlantic salmon at end of freshwater phase having been fed a low marine diet (FM 15 % / FO 8 %) with differing micronutrient supplementation level (L1 = 100 %; L2 = 200 %; or L3 = 400 % NRC Premix). The vertebral column has been divided into four regions as defined by Kacem *et al.* (1998). Lower case superscripts denote significant differences between diet and ploidy for each region (two-way ANOVA).

Fig. 3. Mean vertebral length-height ratio (L:H) within each spinal region of juvenile diploid and triploid Atlantic salmon at end of freshwater phase having been fed a low marine diet (FM 15 % / FO 8 %) with differing micronutrient supplementation level (L1 = 100 %; L2 = 200 %; or L3 = 400 % NRC Premix). The vertebral column has been divided into four regions as defined by Kacem et al., (1998). Lower case superscripts denote significant differences between diet and ploidy for each region (two-way ANOVA).

Fig. 4. Relative expression of genes involved in bone formation and development in vertebrae of diploid Atlantic salmon smolts fed low marine feeds with differing levels

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of micronutrient supplementation. Superscript letters indicate statistically significant differences between treatments.

Fig. 5. Relative expression of genes involved in bone formation and development in vertebrae of triploid Atlantic salmon smolts fed low marine feeds with differing levels of micronutrient supplementation. Superscript letters indicate statistically significant differences between treatments.

Supplementary Figure 1. Example radiographs of vertebral body malformations observed in fish fed the experimental diets. Reduced intervertebral spacing (Type 1); Compressions (Type 2 - 5); Fusions (Type 6 - 8); Symmetry Deviations (Type 17 and 19); Radio-dense (Type 12). Black horizontal bars in each radiograph represents 1 cm.

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Declarations of interest: none.

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Highlights:

- The use of plant ingredients affects the level of micronutrients in salmon feeds.
- Increased micronutrient supplementation reduced the prevalence of spinal deformity.
- Triploid salmon showed higher prevalence of malformation.
- In diploids, the expression of bone genes was affected by micronutrient levels.
- Micronutrients should be supplemented when feeding salmon low marine diets.

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