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Dietary micronutrient composition affects fillet texture and muscle cell size in Atlantic salmon (*Salmo salar*)

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Abstract

During the past 20 years, plant ingredients have taken over as the main constituents in feed for Atlantic salmon. This has changed the nutrient composition of the feeds, the bioavailability of nutrients and perhaps nutrient metabolism. Plant-based diets also contain more anti-nutrients. The EU-funded project ARRAINA re-evaluated recommendations for micronutrient supplementation to Atlantic salmon feeds, and the present study compared a diet supplemented with micronutrients according to NRC (2011) (control diet, 100% NP (nutrient package)) with a diet supplemented according to the new recommendations (New NP). Tissue concentrations of pyridoxine, pantothenic acid, niacin, vitamin C, Zn and Se were significantly higher; and Cu was lower in Atlantic salmon fed the diet with the New NP compared to the control fish. The New NP also gave a near significant effect on growth, decreased muscle firmness and increased muscle cell size, and it affected metabolism of nitrogen-containing metabolites in the muscle. While we cannot be certain which micronutrient(s) gave these effects, the B vitamins are probable candidates, since they are mediators of intermediary metabolism and have been shown to influence some of the affected metabolites.

KEYWORDS

Atlantic salmon, growth, micronutrient requirements, muscle cellularity, muscle quality, plant-based diets

1 | INTRODUCTION

In the past, intensive farming of Atlantic salmon, as of other fish species, depended on use of fishmeal and fish oil as the main feed ingredients. With increasing aquaculture production and stagnating wild fisheries (FAO, 2011), there has been a need for alternative protein and lipid sources for farming of fish. During the past 20 years, plant ingredients have taken over as main constituents and now feeds for Atlantic salmon may contain more than 70% plant ingredients (Ytrestoyl, Aas, & Asgard, 2015).

Fishmeal and fish oil are extremely nutritious and match well with the requirements of carnivorous fish (NRC, 2011; Ytrestoyl et al., 2015). In Atlantic salmon, the majority of research on effects of using plant ingredients, and of dietary modifications needed, has focused on optimal ingredient replacement levels (Torstensen et al., 2008), amino acid (Espe, Lemme, Petri, & El-Mowafi, 2006,

2007), n-3 fatty acid requirements (Torstensen et al., 2008) bioavailability and utilization of minerals, and effects of anti-nutrients (Krogdahl, Hemre, & Mommsen, 2005). The aim of the EU-funded project ARRAINA was to provide updated recommendations for micronutrient supplementation in diets with high levels of plant ingredients for several fish species. These studies provided practical recommendations for vitamin and trace mineral supplementation for Atlantic salmon based on two full regression studies (Hamre et al., 2016; Hemre et al., 2016; Prabhu et al., 2019).

The regression studies included vitamins, trace minerals, selected amino acids and cholesterol as a nutrient package (NP), added in graded levels (0%–400%) to a basic diet, where 100% NP was generally the requirements given by NRC (2011) for salmonids, mostly based on experiments with rainbow trout juveniles, in many cases fed purified diets. It is therefore necessary to establish the requirements for Atlantic salmon at relevant conditions for modern fish farming. Water-soluble vitamins act as co-factors of enzymes in intermediary metabolism and maximum activities of these enzymes demand that they are bound to their coenzymes. The requirements of water-soluble vitamins were therefore estimated as the dietary level where tissues became saturated with the vitamin in question as shown for pyridoxine (Albrektsen, Waagbø, & Sandnes, 1993; Hemre et al., 2016). Tissue levels of lipid-soluble vitamins generally follow a linear relationship with dietary levels, and the recommendation for supplementation of vitamin E was therefore based on previous knowledge (Hamre, 2011; Hamre et al., 2016).

Trace mineral requirement studies in Atlantic salmon are very limited with published reports lacking for selenium and iodine (NRC, 2011). The estimates available are more often based on data obtained with purified diets and hence do not take into consideration the interaction effects encountered when fish are fed with practical feeds. For instance, NRC (2011) recommendations are on an available basis, and it is difficult to provide a value for availability of trace minerals with practical ingredients. Hence, it is required to have an estimate of total trace mineral levels in diets that can fulfil the requirement. Moreover, the nutrient package design also helps to take into consideration the different antagonistic interactions while making the recommendation for dietary levels required.

Fillet quality was one of the response measurements in the present study. Dietary protein level affected both number and average area of white muscle fibres in a standardized transverse section of blackspot seabream (*Pagellus bogaraveo*) muscle (Silva et al., 2009). A diet rich in soy protein for rainbow trout gave smaller white muscle fibres than a diet containing more fishmeal (Alami-Durante, Wrutniak-Cabello, Kaushik, & Medale, 2010), and a high-fat diet fed early in development of rainbow trout decreased white muscle diameter, both on short and long term (Alami-Durante et al., 2014). Feeding copepods (high nutritional value), instead of rotifers to cod larvae, gave a large increase in larval growth and increased the number of white and red fibres in the muscle of the resulting cod juvenile (T. A. Vo, T. F. Galloway, T. Bardal, K. Hamre, P. Velmurugu, T. van der Meeren, & E. Kjørsvik, unpublished data). Moreover, dietary supplementation of glutamate to Atlantic salmon, increased fillet firmness measured post rigour (Larsson et al., 2014). To our knowledge, there are no studies on effects of micronutrients on muscle quality. Many factors other than nutrients also affect muscle growth and may thereby influence cellularity and texture, including temperature (Campos, Valente, Conceicao, Engrola, & Fernandes, 2013; Campos, Valente, Conceicao, Engrola, Sousa, et al., 2013; Johnston, Manthri, Alderson, et al., 2003) and photoperiod (Johnston, Manthri, Smart, et al., 2003). Muscle growth also seems to be regulated by the circadian clock in zebrafish (Amaral & Johnston, 2012).

The above-mentioned studies showed that nutrition may alter the mode of muscle growth; however, muscle growth in fish has been only partly elucidated (Fuentes, Valdes, Molina, & Bjornsson, 2013) and the mechanisms behind nutritional effects are poorly understood. Fasting and refeeding of *Paralichthys adspersus* led to muscle atrophy followed by hypertrophy, respectively. Blocking of the target of rapamycine (TOR) prevented growth and hypertrophy

TABLE 1 Formulation (g/kg) and calculated macronutrient composition (g/kg) and energy (MJ/kg) of the experimental diets

	100% NP	New NP
Ingredients		
Fishmeal LT	100	100
Fishmeal SA	100	100
Krill meal	42	42
Soy protein concentrate	149.6	149.6
Pea protein concentrate	102	102
Wheat gluten	100	100
Corn gluten	50	50
Wheat meal	101.3	101.3
Fish oil	100	100
Rapeseed oil	100	100
Monocalcium phosphate	33.6	33.6
Lucantin Pink (astaxanthin)	0.5	0.5
Yttrium oxide	0.5	0.5
Amino acid mix	14.6	14.6
Premix 100% NP	6	0
Premix New NP	0	6
Total	1,000	1,000
Gross composition (as fed)		
Crude protein	463	463
Crude lipid	22.7	22.7
Fibre	10.1	10.1
Ash	68.2	68.2
Gross energy	22.8	22.8
Total P	15.0	15.0
Ca	16.6	16.6
Na	1.5	1.5

during the refeeding period (Fuentes et al., 2015). The TOR signalling pathway is activated by the insulin-like growth factors (IGFs), controls protein synthesis, and is also regulated by amino acids and intracellular energy status (Loewith & Hall, 2011), cited by Fuentes et al. (2015)). Blocking the MAPK (mito-activated protein kinase) pathway, which may control myogenic cell proliferation in fish, also partially impaired muscle hypertrophy and growth (Fuentes et al., 2015).

The NRC (2011) states that recommended dietary levels provided in the document assume 100% availability and that under practical conditions, higher levels are likely to be needed to optimize fish and feed performance. The purpose of the present study was to measure effects of feeding Atlantic salmon postsmolts plant-based diets with increased micronutrient supplementation compared to NRC, 2011. The control diet (100% NP) contained micronutrients approximately according to the requirements given by NRC (2011), and an experimental diet (New NP) contained micronutrients according to the new recommendations for Atlantic salmon from the EU-funded project ARRAINA (http://www.arraina.eu/). Dietary amino acids were not varied. When analysed, a few of the nutrients were different from planned levels, but effects such as growth and tissue uptake of micronutrients were still largely as predicted by results in ARRAINA. We also found changes in fillet quality measured as texture and muscle cell size distribution.

2 | MATERIALS AND METHODS

2.1 | Experimental diets and fish rearing

Two feeds were formulated and produced for the present trial, using a common basal formulation (formulation details and theoretical macronutrient composition can be seen in Table 1). Diet 100% NP was a control diet containing close to 100% of the micronutrient requirements for Atlantic salmon, as stated in the NRC (2011). Diet New NP was an experimental diet aiming to contain the improved nutrient package micronutrient mix developed during the EU-FP7 project, ARRAINA (Hamre et al., 2016; Hemre et al., 2016; Prabhu et al., 2019). The analysed dietary concentrations of vitamins and minerals, and their target values in the New NP diet, are given in Table 2. Of the vitamins, cobalamin and thiamine were added below planned levels, while many of the minerals were above anticipated requirements.

Each experimental diet was tested in quadruplicate over a period of 100 days. This growth trial was conducted between 17 June and 3 October 2016 in the experimental facilities of Gildeskål Forskningsstasjon (GIFAS), starting with a total of 880 Atlantic salmon, which were randomly distributed between eight 125,000 L cages (mean initial body weight of 197.1 ± 1.8 g). Fish were fed twice daily (including weekends) throughout the trial, with a slight overfeeding and collection of waste feed, to ensure ad libitum feeding and calculate accurate feed intake. Fish were subjected to natural temperature (monthly average temperatures ranging from 13.4 to 4.7°C), salinity and photoperiod conditions (67°N).

TABLE 2 Analysed concentrations in the two diets and New NP targets of vitamins and minerals (mg/kg)

	100% NP	New NP	New NP target
Pyridoxine, B_6	5	8.7	12
Cobalamin[a]	0.063	0.071	0.45
Folate	0.99	1.8	2
Pantothenic acid	13	42	50
Riboflavin	8.5	11	11
Biotin	0.41	0.39	0.38
Thiamine[a]	2.4	1.9	10
Niacin	79	88	75
Vitamin C	57	201	200
Vitamin E	133	172	178
Selenium	1.2	1.5	1
Copper	13	11	9
Manganese	85	89	40
Iron	660	550	250
Zinc	110	230	180
Calcium	15,000	17,000	4,920
Magnesium	3,000	3,300	1920
Phosphorus	17,000	18,000	15,000

[a]Lower concentrations than planned.

Twenty fish per cage were sacrificed by killing with a sharp blow to the head without previous starvation. Fish were sacrificed before the first morning meal, and faeces stripped for removing any remains of feed and faeces before further processing. Wet weight (precision 1 g) and fork length of each fish were then measured. Ten fish per cage were then pooled and minced together, and two representative subsamples (100 g each) vacuum packed, frozen and stored at −20°C, for proximate composition analysis. The other 10 fish per cage were used to analyse tissue mineral/vitamin status, fillet texture and cellularity. Ten fish per cage were shipped in ice to Nord University for further analysis of fillet quality. For tissue mineral/vitamin status, samples of gill, liver and muscle (the dorsal muscle behind the pelvic fin) were pooled per cage, frozen and stored at −80°C, until analysis.

2.2 | Chemical analyses

Moisture was measured by drying at 103°C for 24 hr, and lipid after extraction with ethyl acetate and isopropanol for fish tissues (Lie, Waagbø, & Sandnes, 1988) and acid extraction for fish feed (European Commission, 1998). Nitrogen was measured with a nitrogen analyser (Vario Macro Cube, CN, Elementar Analysensysteme GmbH) according to AOAC official methods of analysis (Sweeney & Rexroad, 1987), and protein calculated as N x 6.25. Total amino acids in diets were determined after hydrolysis in 6N HCl at 22°C using the UPLC

(ultra-performance liquid chromatography) method as described by Andersen et al. (2013). Free amino acids and nitrogen metabolites in muscle were analysed using the Biochrome with postcolumn derivatization with ninhydrin as described by Espe, Lemme, Petri, and El-Mowafi (2006). The B vitamins biotin, niacin, folate, pantothenic acid and cobalamin were all determined by microbiological methods (Feldsine, Abeyta, & Andrews, 2002; Mæland, Rønnestad, Fyhn, Berg, & Waagbø, 2000). Some of the B vitamins were determined by HPLC, including thiamine (CEN, 2003), vitamin B6 (CEN, 2005) and riboflavin (Brønstad, Bjerkås, & Waagbø, 2002). Vitamin C and E were measured by HPLC according to Mæland and Waagbø (1998) and Hamre, Kolås, and Sandnes (2010), respectively. Microminerals were analysed as in Julshamn, Lundebye, Heggstad, Berntssen, and Boe (2004) and macrominerals according to Liaset, Julshamn, and Espe (2003).

2.3 | Fillet quality

Ten fish per cage held on ice, were sent from GIFAS to Nord University, Bodø, where it arrived the next day, for fillet quality measurements. In all the fish, the tail was cut off immediately behind the dorsal fin according to Norwegian Quality Cut (NQC—Norwegian standard procedure—NS 9401, 1994). From five fish per cage, three muscle bocks (5 × 5 × 5 mm^3) were cut from the front of the tail piece (Figure 1), covered with Shandon Cryomatrix (Thermo Fisher Scientific) and flash-frozen in 2-methylbutane cooled to the freezing point (−159°C) by immersion in liquid nitrogen. The blocks were kept at −80°C until cell size analysis. The number of analysed fish was less than sampled; 100% NP n = 8 and New NP, n = 12. The remaining fish were held on melting ice until the end of rigour (3 days).

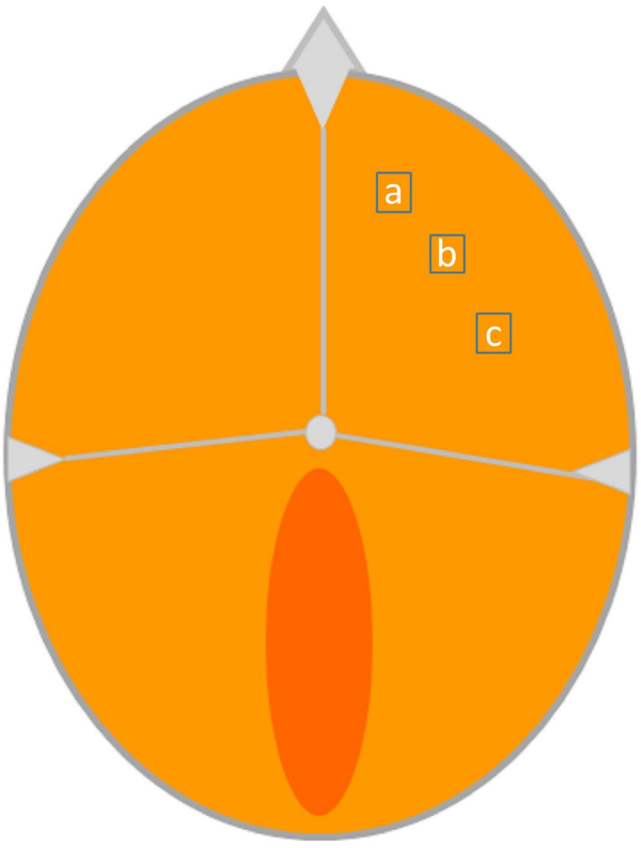

FIGURE 1 Muscle blocks (5 × 5 × 5 mm^3) taken for sectioning of Atlantic salmon for muscle for cell size analyses. The transverse cut was made just behind the dorsal fin

Texture analyses were performed using a TA-XT2 PLUS texture analyser with Texture Expert Exceed 2.52 software (Stable Micro System). A probe (10 mm diameter) was pressed to 60% of fillet thickness at 1 mm/s in the left fillet on three standardized places under the dorsal fin. Maximal force (in Newton, N) and total work (N/cm^2) were registered.

Cell sizes were measured by muscle histology analyses according to Johnston (1999). The muscle blocks were sectioned transverse of the muscle fibres in sections of 7 µm (CryoStar NX50 Thermo Fischer Scientific) mounted on glass slides and stained by haematoxylin (Harris haematoxylin). The sections were analysed using a light microscope with a digital camera (Axioskop 2 mot plus, Carl Zeiss) and image analysis software (Axio Vision Rel. 4.2, Carl Zeiss). The circumference of a minimum of 450 cells per fish was measured, and the cell area and diameter were calculated.

2.4 | Calculations and statistics

Zootechnical performance parameters (feed conversion ratio, weight gain and biomass gain) were calculated using the following formulae:

$$\mathrm{FCR} = \frac{\mathrm{FI}}{W_f - W_i}$$

$$\mathrm{WG}\,(\%) = 100 \times \frac{W_f - W_i}{W_i}$$

$$\text{Biomass gain}\,(\%) = 100 \times \frac{\text{Biomass}_f - \text{Biomass}_i}{\text{Biomass}_i}$$

where FI is the total feed intake for a cage (in grams), W_i is the initial biomass weight for a cage (in grams), and W_f is the final biomass weight for a cage (in grams).

The software Statistica (ver 13, Dell Inc.) was used for statistical analyses. Data were analysed using Student's t tests or Mann–Whitney U tests when variances were non-homogenous. The relationships between treatment groups, carcass weight and texture were described by correlations categorized by groups. Differences and effects were considered significant at $p < .05$. For the frequency distributions of cell sizes, the number of cells within each size class was counted for each fish and the per cent of cells in the given size class out of total cell count in this fish was calculated. Then, the average percentage of cells in each size class was used in a series of t tests. Also, the average cell size per fish was calculated and a t test was run on fish fed the two different diets.

TABLE 3 Growth, mortality and feed conversion in Atlantic salmon fed diets with different levels of micronutrients (mean ± *SD*, *n* = 4, *significant difference, *t* test, $p < .05$)

	100% NP	New NP
Final weight (g)	937 ± 41	968 ± 27
Final biomass (kg)	81 ± 3	86 ± 3*
Weight gain (g)	739 ± 41	771 ± 26
Weight gain (%)	373 ± 21	392 ± 11
Biomass gain (kg)	59 ± 3	65 ± 3*
Biomass gain (%)	272 ± 13	299 ± 12*
Mortality no	23 ± 4	21 ± 1
Mortality biomass (kg)	7.9 ± 3.1	6.0 ± 0.9
Feed intake (kg)	56 ± 3	59 ± 3
FCR	0.95 ± 0.07	0.92 ± 0.02

Note: Initially 110 fish per cage, mean individual weight 198 ± 2g

3 | RESULTS

Over the course of the growth trial, fish in all cages (regardless of dietary treatment) displayed good growth performance (FCR < 0.95 and WG > 3.7–3.9 fold for all tanks). No significant effects were observed in terms of individual weight gain, mortality or feed conversion, but biomass gain, the combined effect of average weight gain and mortality, was higher in fish fed the New NP diet (Table 3, $p = .03$). There was a difference in muscle dry matter at the final sampling, with a slightly higher level in fish fed the New NP diet ($p = .048$). Otherwise, there were no differences in dry matter, protein or lipid in muscle or whole body, and protein and lipid in liver, between the two groups of fish (Table 4).

The concentrations of the B vitamins pyridoxine (muscle), pantothenic acid (gills) and niacin (whole body), and that of vitamin C (whole body), were higher in fish fed the diet with New NP than in those fed the control diet (Table 5, $p = 4*10^{-6}$, .002, .05, and .01, respectively). The concentration of riboflavin in muscle was higher in the control group than in fish fed the diet with New NP (Table 5, $p = .02$). There were no differences in cobalamin, thiamine, biotin (muscle), cobalamin or folate (liver), alpha- or gamma-tocopherol (whole body) between the two groups (Table 5). Fish fed the New NP diet had higher levels of Zn and Se in whole body compared to the control fish ($p = .002$, .001, respectively) while Cu was higher in the control fish ($p = .02$). Otherwise, there were no differences in mineral concentrations between the two groups of fish (Table 6; Ca, Na, K, Mg, P, Mn and Fe analysed).

Among the 40 fish per treatment that were sampled for texture analyses, fish fed the diet with New NP weighed slightly more ($p = .048$) and had a tendency for higher carcass weight ($p = .11$) than the control fish, while length and condition factor were similar (Table 7). Maximum force required to press a probe to 60% of fillet thickness was higher in control fish than in fish fed the New NP diet ($p = .0005$, Table 7). Thus, fish fed the New NP diet had softer fillets than the control fish and Figure 2 shows that fillet firmness was affected by dietary treatment ($r = .30$, $p < .05$), but not by carcass weight ($r = .004$, n.s.).

The length and carcass weight of fish used in cell size analyses were not significantly different; however, there was a tendency of higher carcass weight in fish fed the New NP ($p = .055$, Table 8). Muscle cells in fish fed diet New NP were generally larger than in the control fish, as indicated by the measured cell circumference ($p = .01$) and the calculated cell area and diameter ($p = .02$, Table 8). Figure 3 shows that there were fewer small cells and more of the larger cells in the muscle of fish fed diet New NP.

There were differences between the two fish groups in some free amino acids and other nitrogen metabolites (Table 9). The concentrations of free glutamine ($p = .02$), free lysine ($p = .008$) and beta-alanine ($p = .02$) were higher, while the concentration of cystathionine was lower ($p = .004$) in fish fed the diet with New NP compared to control fish.

TABLE 4 Dry matter, lipid and protein (g/kg WW) in tissues and whole body of Atlantic salmon fed diets with different levels of micronutrients (mean ± *SD*, *n* = 4, *significant difference, *t* test, $p < .05$)

	100% NP	New NP
Muscle		
Dry matter	250 ± 0	260 ± 10*
Lipid	37 ± 1	39 ± 2
Protein	210 ± 10	210 ± 10
Liver		
Lipid	46 ± 1	47 ± 2
Protein	200 ± 30	180 ± 10
Whole body		
Dry matter	330 ± 0	330 ± 0
Lipid	124 ± 3	127 ± 1
Protein	180 ± 10	190 ± 10

4 | DISCUSSION

Fish fed the diet with New NP had softer fillets with larger cells than the control fish. This relationship between cell size and texture has been found before (Kiessling, Ruohonen, & Bjørnevik, 2006). One could question if the difference in size of the fish sampled for texture analyses could have given the difference in texture, but Figure 2 shows that there was no significant effect of fish size on texture so the difference was due to the treatments. Torgersen et al. (2014) characterized salmon from a slaughter line according to muscle firmness and studied matched muscle sections by histology. The soft fillets had detached cells with mitochondrial dysfunction, large glycogen aggregates and enlarged intercellular area. This appears to be a pathological situation, while the change in muscle quality in the present study seems to be a result of dynamic changes in muscle cell growth. Since the muscle cells were larger in the New NP group and the fish showed a tendency of higher carcass weight, one can speculate that these fish had higher muscle hypertrophic growth

TABLE 5 Concentrations of vitamins in fish tissues (mg/kg WW, mean ± *SD*, *n* = 4, *significant differences, *t* test, $p < .05$, when non-homogenous variances, Mann–Whitney *U* test)

	100% NP	New NP
Muscle		
Cobalamin	0.020 ± 0.002	0.020 ± 0.001
Pyridoxine	4.9 ± 0.2	7.8 ± 0.3*
Riboflavin	0.62 ± 0.02*	0.59 ± 0.01
Thiamine	0.8 ± 0.3	0.8 ± 0.2
Biotin	0.043 ± 0.008	0.032 ± 0.005
Liver		
Cobalamin	0.35 ± 0.02	0.37 ± 0.01
Folate	6.3 ± 1.1	7.1 ± 0.4
Gill		
Pantothenic acid	7 ± 1	12 ± 2*
Whole body		
Niacin	48 ± 3	57 ± 7*
Vitamin C	13 ± 4	29 ± 4*
alpha-TOH	30 ± 4	34 ± 3
gamma-TOH	13 ± 1	13 ± 0

TABLE 6 Whole-body concentrations of minerals in Atlantic salmon fed diets with different concentrations of micronutrients (mg/kg WW, mean ± *SD*, *n* = 4, *significant differences, *t* test, $p < .05$)

	100% NP	New NP
Ca	2,850 ± 412	2,925 ± 377
Na	918 ± 72	950 ± 64
K	3,925 ± 320	4,075 ± 287
Mg	313 ± 21	320 ± 22
P	4,125 ± 403	4,225 ± 171
Mn	1.08 ± 0.22	0.89 ± 0.12
Fe	10.5 ± 0.6	10.1 ± 0.7
Cu	2.68 ± 0.38*	2.03 ± 0.17
Zn	23 ± 2	28 ± 1*
Se	0.23 ± 0.01	0.27 ± 0.01*

due to stimulation of protein synthesis by the more balanced diet with the New NP.

Johnston, Manthri, Alderson, et al. (2003) showed that Atlantic salmon postsmolt undergo strong muscle hyperplasia while growing from 60 to 1,000 g body weight under descending photoperiod in the autumn. At the same time, there was a large decrease in fibre density, indicating growth in average cell size, e.g. hypertrophy. The number of muscle fibres in the standardized transverse section stabilized in November and increased only slightly until August the following year in fish held at ambient photoperiod (Johnston, Manthri, Smart, et al., 2003). Texture has also been shown to vary with season and to be negatively correlated to specific growth rate (Mørkøre & Rørvik, 2001).

TABLE 7 Length, weight, condition factor, weight of carcass without viscera and texture of the fish used for texture analyses (mean ± *SD*, *n* = 40, *significant differences, *t* test, $p < .05$. Firmness is maximal force used when a probe is pressed to 60% of fillet thickness, and total work is the integrated area under the curve of force vs. time)

	100% NP	New NP
Length (cm)	41.8 ± 1.7	42.7 ± 1.8
Weight (g)	916 ± 119	986 ± 131*
Condition	1.25 ± 0.06	1.26 ± 0.06
Carcass weight (g)	795 ± 124	847 ± 99
Firmness/Max force (*N*)	5.77 ± 0.77*	5.18 ± 0.58
Total work (N/cm^2)	16 ± 3	16 ± 3

Fish in the present study were in a similar size range and had similar growth as in autumn in the study of Johnston, Manthri, Alderson, et al. (2003), and due to the high growth rate, firmness should be relatively low (Mørkøre & Rørvik, 2001). Similarly, the softer fillet in the present study also correlated with a slight growth stimulation in the New NP group, compared to the control.

Tissue concentrations of pyridoxine, pantothenic acid, niacin and vitamin C were significantly higher in fish fed the diet with the New NP compared to the control fish. This would be expected as the New NP diet had elevated levels of these vitamins, and the previously estimated requirements, based on tissue saturations, were higher than those given by NRC (2011) (Hamre et al., 2016; Hemre et al., 2016). With respect to trace minerals, higher levels of Zn and Se in the New NP diet were beneficial in improving the whole-body status of Atlantic salmon, while Mn, Cu and Fe levels in New NP did not have an impact.

It is, of course, difficult to say which of the nutrients with different tissue concentrations in fish fed the diet with New NP actually affected fillet quality, but we measured free amino acids and some other nitrogen-containing metabolites and searched the literature for pathways that could have been affected. We found that the muscle concentrations of glutamine, lysine, cystathionine and beta-alanine differed between fish from the two treatments, even though the dietary concentrations of amino acids were similar (data not shown). There was also a slightly higher dry matter in the muscle of fish fed the diet with the New NP, than in the control.

Glutamine is the most abundant free amino acid in the body of mammals, with high concentrations in plasma and skeletal muscle (Cruzat, Pantaleao, Donato, De Bittencourt, & Tirapegui, 2014; Cruzat & Tirapegui, 2009; Tapiero, Mathe, Couvreur, & Tew, 2002). It is a non-essential amino acid, synthesized by binding of an amino group to glutamic acid in ε-position, but may become essential under stress conditions such as sepsis and prolonged exercise (Cruzat et al., 2014; Cruzat & Tirapegui, 2009). Glutamine has multiple functions, such as being a precursor in nucleotide, glucose and amino sugar biosynthesis, glutathione homoeostasis, protein synthesis and a source of oxidative energy (Tapiero

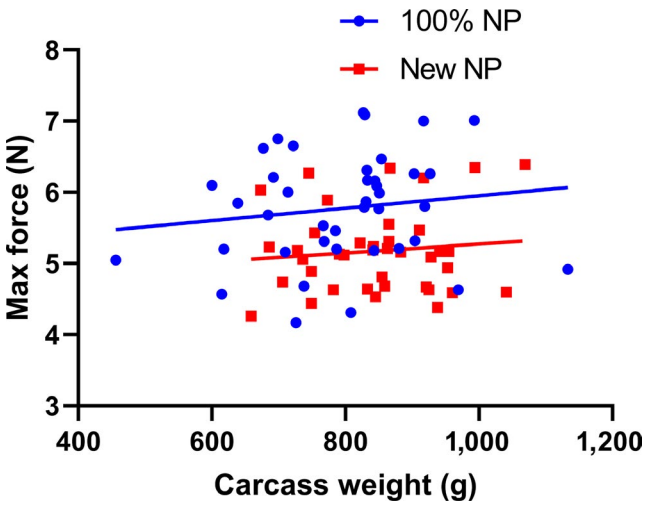

FIGURE 2 The relationships between carcass weight and fillet texture (Max force needed to press a probe to 60% of fillet thickness) in Atlantic salmon fed the two diets with different levels of micronutrients

TABLE 8 Length, carcass weight and measures of average cell sizes in fish used for cell size analyses (mean ± *SEM*, 100% NP, *n* = 8, New NP, *n* = 12, circumference in minimum 450 cells per fish were measured, and area and diameter calculated, *significant differences, *t* test, $p < .05$)

	100% NP	New NP
Length (cm)	41 ± 1	42 ± 1
Carcass weight (g)	745 ± 41	846 ± 30
Cell circumference (μm)	250 ± 10	280 ± 10*
Cell area (μm^2)	5,190 ± 470	6,520 ± 270*
Cell diameter (μm)	71 ± 3	80 ± 2*

et al., 2002). It serves as a non-toxic transport form of ammonium in plasma and may donate its ε-amino group for biosynthesis or for acid-base regulation. One of the pathways for glutamic acid synthesis goes through transamination of α-ketoglutarate and requires pyridoxine.

Lysine is an essential amino acid, which also carries an ε-amino group and is most abundant in muscle tissue. Upon determining the requirement for lysine in Atlantic salmon, no differences in growth occurred, but protein deposition and accretion was significantly affected showing that lysine preferentially is deposited in muscle (Espe, Lemme, Petri, & El-Mowafi, 2007). Lysine is also part of other nitrogen-containing metabolites, such as carnitine, which is a cofactor in fatty acid metabolism. The concentration of free lysine was almost twice as high in muscle of fish fed the diet with New NP compared to the control. Plant-based diets for carnivorous fish are often supplemented with lysine to meet the requirements and, in the present study, both diets contained assumed requirement levels (Espe et al., 2007). It is difficult to pinpoint which micronutrients led to the difference in muscle lysine levels, and to get more knowledge, a study focusing muscle signalling through the mTOR cascade needs to be conducted in combination with the availability of B vitamins.

Cystathionine is part of the transsulfuration pathway from homocysteine to taurine or glutathione, and production and degradation of cystathionine are catalysed by the pyridoxine-dependent enzymes cystathionine beta-synthase (CBS) and cystathionine gamma-lyase, respectively. Alternatively, homocysteine can be re-methylated to produce methionine by the enzyme methionine synthethase, a pathway that requires cobalamin as a cofactor and folate as methyl donor (Espe, Hevroy, Liaset, Lemme, & El-Mowafi, 2008). It has been shown that the plasma and urine concentrations of cystathionine increase in humans with critical confirmed deficiencies in cobalamin, folate or pyridoxine (Stabler, Lindenbaum, Savage, & Allen, 1993). The lower cystathionine concentration in fish fed the control diet is therefore an additional indication of deficiency in one or more of these vitamins, most probably pyridoxine, being the cofactor for CBS.

In beta-alanine, the amino group is bound to the (terminal) beta-position in the carbon chain. It is not used in protein synthesis, but is part of the dipeptides carnosine and anserine, and of pantothenic acid. Carnosine (β-alanyl-L-histidine) is an important intramuscular buffer, constituting 10%–20% of total buffering capacity in muscle cells. Supplementation with beta-alanine can decrease fatigue and increase performance in athletes (Derave et al., 2007;

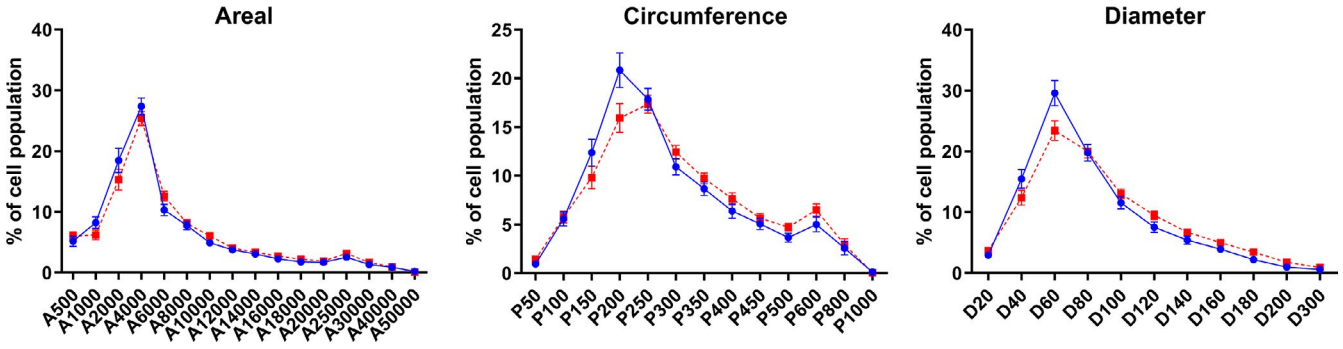

FIGURE 3 Proportion of the cell population distributed in different size classes of area, circumference and diameter (A500, cells with areas less than 500 μm^2; A1000, cells with area from 500 to 100 μm^2 and so on. Circumference and diameter are in μm. Mean ± *SEM*, *n* = 8 (100% NP, blue) or 12 (New NP, red), minimum 450 cells measured per fish)

TABLE 9 Levels of free amino acids and other nitrogen-containing metabolites (μmol/g WW) in muscle of Atlantic salmon fed diets with different composition of micronutrients (mean ± *SD*, *n* = 4, *significant differences, *t* test, $p < .05$)

	100% NP	New NP
Sum free AA	11.9 ± 0.9	12.0 ± 0.8
L-Glutamine	0.33 ± 0.04	0.46 ± 0.08 *
L-Lysine	0.26 ± 0.05	0.42 ± 0.06*
Other *N*-metabolites		
Cystathionine 2	0.10 ± 0.03*	0.02 ± 0.02
Beta-Alanine	0.28 ± 0.04	0.38 ± 0.06*

Hill et al., 2007; Mannion, Jakeman, Dunnett, Harris, & Willan, 1992). There was no difference in muscle carnosine or anserine between the two groups (data not shown), and how beta-alanine was affected by the dietary differences in the present study requires further studies.

It is unclear how the higher muscle dry matter in the muscle of fish fed the diet with New NP would have affected the fillet texture. To our knowledge, there is no literature on this topic, but intuitively, it should lead to a harder fillet, counteracting the main effect in the present study.

The lack of a significant positive response of new NP on average fish growth, unlike that observed in ARRAINA (Hemre et al., 2016), has several explanations. (a) The analysed levels of some of the vitamins (cobalamin, folate, thiamine) deviated from formulated levels, possibly caused by loss during feed processing (Barrows, Gaylord, Sealey, Porter, & Smith, 2008). (b) The level of certain trace minerals (e.g. Fe, Se, Mn) in the control diet (on total basis) was high to compensate for the effects of anti-nutrients and calcium phosphate in plant ingredients in reducing micro-mineral availability to salmonids (Cheng & Hardy, 2002; Prabhu et al., 2014). (c) The growth effect found in ARRAINA (Hemre et al., 2016; Taylor et al., 2019) was caused by graded levels of methionine, histidine and taurine, which were maintained at similar levels in the two diets in the present study, although their levels were formulated to be above requirements.

5 | SUMMARY AND CONCLUSIONS

Tissue concentrations of pyridoxine, pantothenic acid, niacin, vitamin C, Zn and Se were significantly higher in Atlantic salmon fed the diet with the New NP compared to the control fish in the present study. The different micronutrient supplementation in the two diets gave a near significant effect on growth, decreased muscle firmness and increased muscle cell size. It also affected metabolism of nitrogen-containing metabolites in the muscle, which could have changed protein turnover and thereby muscle cell size. The B vitamins are the most probable candidates to have caused these effects, since B vitamins are mediators of intermediary metabolism. In summary, pyridoxine, pantothenic acid, niacin, vitamin C, Zn and Se are the most likely candidates to have given a tendency of growth stimulation and altered fillet quality in fish in the present study.

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