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A full factorial design to investigate interactions of variable essential amino acids, trace minerals and vitamins on Atlantic salmon smoltification and post transfer performance

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ABSTRACT

To contribute in knowledge for the development of safe, efficient and sustainable functional salmon diets, we ran a feeding trial applying a 2^3 full factorial design to investigate combined effects, on Atlantic salmon smoltification and post transfer performance, of variable supplementation levels of essential amino acid (Lys, Met, Thr and Arg), essential trace mineral (Zn, Fe and Se) and vitamins (E, C and astaxanthin as provitamin A) premixes in low fishmeal diets, using crystalline amino acids, organic trace minerals and synthetic vitamins, respectively. The nutrient levels used in our study were chosen to meet the known requirements of fish reflecting the variation in commercial feeds. Fish performance, nutrient digestibility, skin, and intestinal health were evaluated in Atlantic salmon parr-smolt, the latter by means of qPCR, global transcriptomics, and immunohistochemistry. The results revealed the potential for significant improvement of salmon post smoltification growth by simultaneous dietary level increase of Met, Lys, Thr and Arg (5% higher body weight increase). Significantly negative effect on fish post transfer growth and survival (22.5 % lower body weight growth and 2.6 times higher mortality) was observed in the high dietary vitamin supplementation treatments which was not present in the simultaneous high trace mineral and vitamin supplementation treatments (8% higher body weight increase and 2.8 times lower mortality in the high trace mineral supplementation treatments). In the high trace mineral supplemented dietary treatments was also observed improved FCR (8.5 %) and a further improvement in performance was seen in the treatments with simultaneous high essential amino acid and trace mineral supplementation levels (12.6 % higher body growth increase). Redox-sensitive gene and extracellular matrix components' gene transcription effects and compensatory mechanisms on protein and energy metabolism, immune modulation, skin repair systems and erythropoiesis were observed by transcriptomic and histological analyses in response to the variable dietary essential nutrient levels.

1. Introduction

Responsible growth of the fish farming industry requires simultaneous development of sustainable technologies and knowledge that secure fish performance, health, and welfare in constantly changing and challenging conditions. Yearly farming mortality rates for Atlantic salmon and rainbow trout averaged 13.3 % in 2018 against 14.7 % in 2017 (Fiskeridirektoratet, 2019), and smoltification, sea water transfer, vaccination, infectious disease, treatments, stocking densities and water quality are bottlenecks contributing to stress and limiting fish growth and survival (Hjeltnes et al., 2019). Along with improving farming practices, it is important to balance diets that enable farming of robust fish that can withstand and recover from stress and diseases. Essential nutrient requirements in fish have been defined mainly under optimal conditions, often using diets with high marine content (NRC, 2011). It is likely that requirements change during stressful rearing periods. For

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instance, stress tolerance and reduced skin ulcer healing capacity at smolt transfer are likely to be linked to sub-optimal trace mineral and vitamin nutrition, whereas poor smoltification and transfer performance may be linked to the fish's poor essential amino acid and trace mineral status (Salte et al., 1994; Wahli et al., 2003). For instance, suboptimal histidine (His) levels in the diet are the identified cause behind the development of cataracts in Atlantic salmon smolt following transfer (Remø et al., 2014). The pathology is however more complex involving several other nitrogenous compounds that may be limiting in the diet. For instance cataract has been related with the levels of free anserine in the fish tissues, whose biosynthesis involves either His or methionine (Met), occurring *via* condensation of Nπ-methyl-l-histidine with β-alanine and direct N-methylation of carnosine using S-adenosyl-l-methionine as a methyl group donor (Yamada, 2013).

By 2016, the marine component in diets for Atlantic salmon (*Salmo salar*) in Norway had decreased 3.6 times since 1990, replaced by plant based raw materials. A simultaneous increase, by a factor of four, has been observed in the supplementation of micro-ingredients represented mainly by essential amino acids added in crystalline form, trace minerals, vitamins, phosphorous (P) sources and astaxanthin (Aas et al., 2019). Benchmarking analyses of commercial diets from different suppliers of feeds for the Norwegian salmon market show that there is significant variation in the levels of essential nutrients: Iron (Fe) (108−340 mg/kg; mean: 172 mg/kg), zinc (Zn) (126−232 mg/kg; mean: 170 mg/kg), copper (Cu) (7−14 mg/kg; mean:, 11 mg/kg), manganese (Mn) (21−70 mg/kg; mean: 47 mg/kg), and selenium (Se) (0.3–1.8 mg/kg; mean: 0.8 mg/kg) (Sele et al., 2018). If supplemented more than the naturally present levels in the raw materials, the highest allowed levels for the aforementioned trace minerals in aquafeeds are 750, 200 (180 in salmon), 25, 100 and 0.5 (0.3 and 0.2 added up to 0.5 for inorganic and organic Se, respectively) mg/kg feed for Fe, Zn, Cu, Mn and Se, respectively (Regulation (EC) No 1831, 2003). In addition, different nutrient sources, as those of trace minerals and P, may have different bioavailability (Maage and Sveier, 1998; Standal et al., 1999) and physiological effects (Berntssen et al., 2018a, 2018b). The relative levels of naturally occurring and added vitamins and trace minerals in different forms, and the ratio between essential and non-essential amino acids, will vary in diets changing from marine to plant-based ingredients, as well as between the different sources used.

The dietary requirements of salmon have been established (NRC, 2011) mostly using inorganic compounds in the case of minerals which can become toxic in higher dietary levels (Berntssen et al., 2018a, 2018b), and not covering the degree of marine component replacement by plant ingredients which is practiced today (Aas et al., 2019) nor all farming stages as the challenging smoltification and transfer to the sea periods. Often, in determining the nutrient dietary requirement of trace elements in short trials and optimal rearing conditions, in absence of significant effects on growth and survival, the response parameters used have been the respective element tissue levels (Maage et al., 1991; Maage and Julshamn, 1993; Prabhu et al., 2019). Nutrient requirements in modern fish farming may be increased, as recently reported in the case of niacin and riboflavin by Hemre et al. (2016) showing that the current salmon requirement is more than six and two times higher, respectively, than previously recommended. This is further corroborated by the increasing numbers of functional-health feeds appearing in the salmon feed market offering protection or even recovery from pathological syndromes or parasitic and viral diseases, which are responsible for great losses in salmon farming (Fiskeridirektoratet, 2019), like pancreas disease (PD), amoebic gill disease (AGD), cardiomyopathy syndrome (CMS), heart and skeletal muscle inflammation (HSMI), and winter ulcers. Such functional diets will for instance often contain higher levels of protein or marine ingredients which will contribute with higher dietary levels of the essential nutrients dealt with in the current study, except vitamin C which is lost in the fish meal production processing.

The synergies and interactions between multiple dietary components are highly complex to model. Recent trials are designed to study the effects of micronutrient supplementation in farmed salmon fed low marine diets, by increasing simultaneously the dietary level of a rather large number of dietary substances, including trace minerals and vitamins. Though the source of the observed effects in fish performance and possible interactions in such studies remain unclear (Hemre et al., 2016; Skjærven et al., 2016; Taylor et al., 2019). In the current study, we constructed a feeding trial applying a 2^3 full factorial design to investigate the combined effects on Atlantic salmon smoltification and post transfer performance of variable supplementation of essential amino acids (lysine (Lys), Met, threonine (Thr) and arginine (Arg)), trace minerals (Zn, Fe and Se) and vitamins (vitamin E, C and astaxanthin as a vitamin A precursor) in low fishmeal diets. In salmonids, the synthesis of vitamin A from its carotenoid precursors, such as astaxanthin, is a known and important pathway (Schiedt et al., 1985; Guillou et al., 1989). Following a practical approach, we chose nutrient levels in our design based on the available literature and the respective extreme levels found through our benchmarking study of commercial feeds. Fish performance, nutrient digestibility, skin, and intestinal health were evaluated, the latter by means of qPCR, global transcriptomics, and histology.

2. Materials and methods

2.1. Experimental design

Before designing the experimental feed formulations, we consulted the recent literature and benchmarked a limited number (5) of parr/smolt feeds for salmon for the nutrients of interest in our trial. The choice of the experimental factor levels in the experimental diets was based on the variation in the respective analytical results of commercial salmon diets as well as known levels present in wild fish (Hertrampf and Pascual, 2000). The analyzed levels for the varying nutrients in our study in the commercial and the trial diets are presented in Table 1.

We applied a full 2^3 factorial design (Table 2) with 3 replications at the points of max and min levels of the main factors, to investigate the single and combined factor effects of dietary essential amino acid (EAA), trace mineral and vitamin enrichment levels in low fish meal diets on smoltification and post transfer performance of salmon smolt. The trace minerals used were organic/Bioplex, provided by Alltech Inc. (Dunboyne, Ireland) whereas the added vitamins (Sodium Ascorbyl Phosphate (Stay C), alpha tocopherol, and astaxanthin acting as provitamin A) and amino acids were purchased from Vilomix Norway AS, Norway. All diets contained low fish meal levels and other functional components in equal amounts (Table 3).

The experimental diets were produced at the Feed Technology Centre of Nofima in Bergen, Norway, in the same production series, using a Wenger TX-52 co-rotating twin-screw extruder with 150 kgh-1 capacity. The settings of the extruder were "normal" i.e., the production

Table 1
Atlantic salmon commercial feed benchmarking prior to the trial and current feed trial experimental design.

	Commercial feeds (analyzed)		Trial feeds (analyzed)	
	min	max	low	high
Fe mg/kg	130	260	250−270	330−370
Zn mg/kg	150	210	110−120	250−270
Se mg/kg	0.7	1.4	1−1.1	2.0−2.3
Astaxanthin mg/kg	4.27	8(parr) 32 (smolt)	<2	28−31
VitE alpha-tocopherol mg/kg	147	501	180−184	430−466
VitC mg/kg	187	1160	230−252	1570−1590
Lys % in protein	6.2	6.6	4−5.1	5.1−6.1
Met % in protein	2.5	2.6	1.8−1.9	3.2−3.4
Thr % in protein	3.6	3.8	2.4−2.8	3.4−3.6
Arg % in protein	5.7	6.1	4.2−4.8	5.1−5.4

Table 2
Feeding trial full factorial (2^3) design, where: dietary trace mineral (M) level (varying Se, Fe and Zn), dietary vitamin (V) level (varying VitC, VitE and astaxanthin – provitamin A), and dietary essential amino acid (AA) level (varying Arg, Lys, Met and Thr) represent the variable factors 1,2 and 3, respectively, at two supplementation levels, low (-1 or -) and high (+1 or +).

Treatment	Replicate tanks	Factor 1 Trace minerals (M)	Factor 2 Vitamins (V)	Factor 3 Amino acids (AA)
(-1)M(-1)V(-1) AA	3	–	–	–
(-1)M(-1)V(+1) AA	1	–	–	+
(-1)M(+1)V(-1) AA	1	–	+	–
(-1)M(+1)V (+1)AA	1	–	+	+
(+1)M(-1)V(-1) AA	1	+	–	–
(+1)M(-1)V (+1)AA	1	+	–	+
(+1)M(+1)V (-1)AA	1	+	+	–
(+1)M(+1)V (+1)AA	3	+	+	+

can be up scaled to a feed factory (extruder settings considered: screw configuration (D), die opening (4.5 mm), knife speed (1494−1981 rpm), SME (7.7–9.5 kW), feed rate (150 kg/h) and amount of steam (0 kg/h) and water (0.15−0.41 kg/min) added to the process). The experimental diets, and were relevant fish faeces, were analyzed for proximate composition: Crude protein: (Kjeldahl method N x 6.25; ISO 5983−1997), moisture (ISO 6496−1999), ash (ISO 5984−2002) and lipid (Bligh and Dyer, 1959). Dietary gross energy was determined in a Parr adiabatic bomb calorimeter. For total amino acid profile determination, samples were hydrolyzed in 6 M HCl for 22 h at 110 °C and analyzed by HPLC using a fluorescence technique for detection (Cohen and Michaud, 1993). The astaxanthin content in feeds was performed according to Schierle et al. (2016). Test trace minerals and vitamins in feeds and faeces were analysed by an external laboratory (Eurofins, Hamburg, Germany). The remaining analyses were performed in duplicate. If differences between parallels exceeded standardised values, new duplicate analyses were carried out according to accredited procedures. More in detail (as provided by Eurofins): For the test trace mineral analyses the sample preparation was realised according to §64 LFGB L.00.00–19/1, CON-PV 00001 (2019-03) with microwave digestion. Copper (Cu), iron (Fe), zinc (Zn) and manganese (Mn) were analysed using ICP-OES according to EN ISO 11885 (modified). Selenium (Se) was analysed using ICP-MS according to an analogue method to §64 LFGB L 00.00–19/3. Retinol (vitamin A) was analysed according to EN 12823-1 2014, alpha tocopherol (vitamin E) was analysed according to EN 12822:2014 and DJCPH L-ascorbyl-2-phosphate (stay-C form of vitamin C added in the diets) was analysed by LC-DAD (Table 4).

2.2. Salmon feeding trial

The feeds were given to 12 populations of 99 individual Atlantic salmon parr for a period of 15 weeks, of which 4 weeks were in freshwater and 11 in saltwater. The experiment took place in the land tank facilities of Nofima at Sunndalsøra, Norway. The experimental fish used were non-vaccinated Atlantic salmon parr, with 38 g as mean body weight at start, and mean final body weight at ca. 100 g in all treatments combined. Fish were tagged using passive integrated transponder (PIT) tags (HPT 12, Biomark inc., 2.12 mm diameter and 12.5 mm length), which made it possible to follow individual fish performance, assess fish according to their performance in each tank and potentially exclude much lower performing fish which may have eaten too little to exhibit dietary effects.

The experimental tanks were equipped with continuous light and flow through water systems using UV-treated filtrated water from 40 m depth. The initial stocking density in the tanks was 3.3 kg/m^3 and was roughly tripled at the end of the trial. The fish were fed 120 % of the *ad libitum* feeding levels during the saltwater phase of the trial. Uneaten feed was collected and weighed daily for the estimation of total daily feed intake of the experimental fish populations. During the freshwater phase, the fish were fed at visual satiation, and uneaten feed collection was not practiced. During the freshwater phase, smoltification was induced by photoperiod manipulation.

At start, Atlantic salmon parr were starved for 24 h and the individual weight and length of 100 fish where noted. The range of individual fish weights acceptable for use in the trial was defined by excluding fish belonging to the 10 % highest and lowest size groups. Fish showing deformities or lesions were also excluded. The selected trial fish were pit tagged, individually weighed, and distributed in the experimental tanks in rotation. The fish were first offered feed the following day at gradually increasing amounts to determine the satiation feeding

Table 3
Experimental feed formulation. Ingredient level are provided in % of the total feed raw material mix before extrusion.

Diet Name	(-1)M (-1)V (-1)AA	(-1)M (-1)V (+1)AA	(-1)M (+1) V (-1)AA	(-1)M (+1)V (+1)AA	(+1)M (-1)V (-1)AA	(+1)M (-1)V (+1)AA	(+1)M (+1) V (-1)AA	(+1)M (+1) V (+1)AA
Plant protein mix	48.29	48.29	48.29	48.29	48.29	48.29	48.29	48.29
Horse beans	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Fish meal Norse-LT 94	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Plant oil mix (rapeseed/linseed oil)	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2
Fish oil	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Microalgae meal	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Mineral mix (without – Fe, Se, Zn)	2.88	2.88	2.88	2.88	2.88	2.88	2.88	2.88
Vitamin mix	2.004	2.004	2.004	2.004	2.004	2.004	2.004	2.004
Krill hydrolysate (liquid)	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Soya lecithin	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Microingredients (Choline chloride, Cholestorol, Aquate, inositol, His, Yttrium oxide)	1.54	1.54	1.54	1.54	1.54	1.54	1.54	1.54
Zn Bioplex	0.037	0.037	0.037	0.037	0.140	0.140	0.140	0.140
Selplex	0.015	0.015	0.015	0.015	0.080	0.080	0.080	0.080
Fe Bioplex					0.080	0.080	0.080	0.080
Vitamin C (Stay C)			0.286	0.286			0.286	0.286
Vitamin E			0.062	0.062			0.062	0.062
Astaxanthin (Carop Pink 10 %)			0.030	0.030			0.030	0.030
Lysine	0.320	1.300	0.320	1.300	0.320	1.300	0.320	1.300
Methionine	0.185	1.110	0.185	1.110	0.185	1.110	0.185	1.110
Threonine	0.022	0.610	0.022	0.610	0.0220	0.610	0.022	0.610
Arginine		0.40		0.40		0.40		0.40

Table 4
Experimental diets' nutrient composition (complementary information to that provided in Table 1).

		(-1)M (-1)V (-1)AA	(-1)M (-1)V (+1)AA	(-1)M (+1)V (-1)AA	(-1)M (+1)V (+1)AA	(+1)M (-1)V (-1)AA	(+1)M (-1)V (+1)AA	(+1)M (+1)V (-1)AA	(+1)M (+1)V (+1)AA
Crude protein	%	49.7	50.4	50.7	50.5	49.9	50.6	49.7	50.6
Dry matter	%	92.7	91.6	94.1	91.9	93	91.8	92.8	92.2
Crude lipid (B&D)	%	21.6	20.7	21.7	20.6	21.8	20.8	21.6	20.8
Crude lipid (EU)	%	22.2	21	22	21.2	21.8	21.1	21.7	21
Raw fibers	%	1.8	1.8	1.9	1.8	1.9	2.2	2.2	1.7
Crude energy	Kj/g	22.74	22.32	22.86	22.38	22.53	22.49	22.48	22.17
Asparaginic acid	%	3.1	3	3.3	3.1	3.2	3.1	3.4	3.2
Glutaminic acid	%	9.7	9.8	10.3	9.8	10	9.8	10.4	9.6
Hydroxyproline	%	0.1	0.11	0.11	0.1	0.11	0	0	0.1
Serine	%	2.1	2.1	2.3	2	2	2	2	2
Glycine	%	2	2	2	1.9	1.8	1.8	1.9	1.9
Histidine	%	1.1	1.1	0.96	1	0.95	0.97	1	1
Alanine	%	1.8	1.8	1.9	1.8	2	1.8	1.9	1.7
Proline	%	3.6	3.6	3.7	3.5	3.7	3.4	3.7	3.5
Tyrosine	%	1.6	1.7	1.7	1.5	1.6	1.6	1.6	1.6
Valine	%	2.1	2.1	2.2	2.1	2.1	2.1	2.1	2.1
Isoleucine	%	1.9	1.9	1.9	1.8	1.9	1.8	1.9	1.8
Leucine	%	3.5	3.5	3.7	3.4	3.6	3.5	3.6	3.4
Phenylalanine	%	2.3	2.2	2.3	2.2	2.2	2.1	2.2	2.1
Vitamin A	mg/kg	0.116	0.106	0.111	0.101	0.114	0.110	0.123	0.111

rate levels of each group. Fish were weighed individually before switching from fresh to seawater supply in the experimental tanks. On the last day of the experiment, fish were fed continuously, until individually weighing all fish in all trial tanks. Tissues samples from the 5 largest fish (145 ± 8.1 g) from each tank were taken for calculation of biometric indexes, and further analysis of skin histology and skin and midgut qPCR. Three fish per tank from the treatments without replication (-M-V + AA, -M + V-AA, -M + V + AA, +M-V + AA, +M + V-AA, +M-V-AA), and 2 fish per tank from the treatments with 3 replicates (-M-V-AA, +M + V + AA) were used in the microarray study. Random sampling is common praxis that can yield meaningful results when the sampled population is homogenous and / or n – numbers are relatively large. Due to the high costs of transcriptome analyses only a small number of fish can usually be included and the size variance in the study groups was relatively high. By selecting the largest fish in each tank, we eliminated risk of bias related to undernourishment or other factors affecting feeding and growth and got a picture of the metabolic balance in genomic level in fish fed different diets but achieving similar phenotype (growth). The start, intermediate and final fish number and mean body weights of all fish in the tanks as well as the fish used for the microarray and histology studies, growth rate, FCR and biometrics are provided din Supplementary Table 1.

2.2.1. Growth and feeding performance

Fish growth rate, survival, feed intake rates and feed efficiency (TGC: thermal growth coefficient, FCR: feed conversion ratio, PER: protein efficiency ratio) and fish biometrics (D%: dress out percentage, HSI: hepatosomatic index, CF: condition factor) were measured. Feed intake was expressed as the total feed consumed per tank, mean feed intake per fish, or mean daily feed consumption per fish expressed as % of its body weight. Feed conversion ratio is feed consumed/biomass increase. Feed efficiency is biomass increase/feed consumed. Thermal growth coefficient is TGC = ($w2^{1/3}$- $w1^{1/3}$) x 1000/ $\sum$ (t x feeding days), where $\sum$(t x feeding days) is the sum of water temperatures (^{o}C) for every feeding day in the experiment (Cho, 1992). Protein efficiency ratio is fish weight gain/protein consumption. Condition factor is CF = fish weight (g) x fish fork length^{-3} x 1000. Dress out percentage is D% = gutted fish weight/whole fish weight x 100. Hepatosomatic index (HSI) is the % of liver weight/whole fish weight.

2.2.2. Nutrient apparent digestibility coefficient (ADC)

At the end of the trial all fish from each tank, except for 10, of which 5 fish were used for further biological studies and 5 more kept in store, were stripped and their faeces separated from urine and collected in 1 pre-weighed box per tank. Following sampling of each tank the collected faeces were frozen immediately at −20 °C prior to further freeze drying and analysis. Apparent digestibility coefficient of nutrients and energy in the test diets was calculated from the following formula: ADC = 100 – 100 x Yd x Nf / Nd / Yf where d is diet, f is faeces, Y yttrium content and N nutrient content.

2.2.3. Skin health

Gene expression analysis (qPCR) was performed in skin samples to evaluate how the different feeds affect skin status and health. Selected markers involved in e.g., cell proliferation and differentiation, cell-cell contact, immune response, oxidative stress, apoptosis, mucus production or trace minerals regarding scale development, known to be affected by dietary factors such as trace minerals and vitamins, were analyzed (Table 5). For instance, diets supplemented with organic Zn enhanced the epidermal activity of the metalloproteinases *mmp9* and *13* in experimentally wounded Atlantic salmon (Berge et al., 2019). Skin samples from selected groups was further evaluated using histology and immunohistochemistry (e.g., with specific antibodies for connective tissue, cell-cell contact, stress, or immune markers).

Samples for histological evaluations were taken from fish with end mean body weight of 145 g (n = 5 per group), fixed in formalin (CellstoreTM, CellPath, UK) and stored at 4 °C. Skin samples were further carefully dissected, orientated and placed in tissue embedding cassette (Simport, Quebec, Canada). To decalcify the skin samples, samples were incubated in EDTA (Merck KGaA, Darmstadt, Germany) solution, pH 7 for 4 days. Tissue processing was performed using an automated tissue processor (TP1020, Leica Biosystems, Nussloch GmbH, Germany) where the samples were dehydrated through 100 % alcohol and then a clearent Xylene bath before infiltration in melted 60 °C paraffin (Merck KGaA, Darmstadt, Germany). Paraffin-embedded tissue samples were cut in 5 µm sections using a Microtome (Leica RM 2165), mounted on polysine coated slides (VWR, Avantor, Pennsylvania, USA) and dried overnight at 37 °C. The sections were deparaffinized and rehydrated, and staining was performed using an automated special stainer (Autostainer XL Leica Biosystems, Nussloch GmbH, Germany). Paraffin sections were stained with Hematoxylin/Eosin (HE) and Alcian Blue Periodic Acid Schiff (AB/PAS, pH 2.5, Alcian Blue 8GX, Sigma Aldrich, Darmstadt, Germany), modified Mowry method. Sections were examined by light microscope, Zeiss ZEN Blue software (Zeiss International).

Table 5
Primer pairs and related information for real-time qPCR assays.

Gene		Accession nr.	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
elongation factor 1a	af1a	DQ834870	CACCACCGGCCATCTGATCTACAA	TCAGCAGCCTCCTTCTCGAACTTC
heat shock protein 70	hsp70	BT043589.1	TGACGTGTCCATCCTGACCAT	CTGAAGAGGTCGGAACACATCTC
laminin1	la	FJ195616	CATGTGACATGGACACAGGAA	CGTCCTCAGCCTCATAGGTGTA
elastin1	eln	BF228555	GACCAGGAGGAGCTGCTGCTGGAT	TCTGGGTCGGTGGGTTTGTA
collagen 1a1	col1a1	FJ195608	GGGTCCTGGAAGTCCCTGGAAT	AGAGAGGAGTCATGGGACCCGTT
matrix metalloproteinase 13	mmp13	DW539943	TGATGTCCAAGTCAGCCGCTTC	AAGGAGGCAGGAGGAAGAGG
Decorin	dec	DQ452069	GAACCTGGCTAAGCTGGGTCTAA	GAACAGGCTGATGCCAGAGTACAT
Lysyl oxidase	lox	EF514532	CTGCCTGATCTTGTGCCGGACG	GGAACCTCAGCAGCATCCGGGT
Mucin-lin 2	muc2	JT815394.1	ACCACCCTGAACCATCAGTC	CTCCTTCAACATCGCATCAA
Mucin-like 5ac	muc5	JT819124.1	AGGCGTCCTTGTCCAAATAA	CCTCTGGAAACTGGATGGTC

Skin samples for gene expression analyses were snap frozen in liquid nitrogen and stored at -80 °C. Gene expression analysis was performed using Trizol (Invitrogen) to isolate RNA, cDNA transcribed using SuperScript® VILO cDNA Synthesis Kit (Thermo Fisher Scientific Inc.) and SYBR Green I Master mix (Roche Applied Science, Mannheim, Germany) to quantify relative gene expression levels. Real time qPCR was run on a LightCycler with primers for the genes listed in Table 5. The samples were run on a LightCycler 480 (Roche Diagnostics) under the following conditions: Preincubation at 95 °C for 5 min, amplification with 45 cycles at 95 °C for 15 s and 60 °C for 1 min, melting curve at 95 °C for 5 s and 65 °C for 1 min, cooling at 40 °C for 10 s. All samples were run in parallels. Non-template control and non-RT control were included in the analysis. A melting point analysis was included to confirm specificity of the primers, and the primer efficiencies were evaluate using a standard curve.

2.2.4. Gut transcriptome

Transcriptomic analysis was performed in intestinal tissue from the mid gut of the largest fish in each tank. The DNA oligonucleotide microarray platform developed by Nofima (SIQ-6 microarray: Salmon Immunity and Quality) contains probes for 15 K genes annotated with the bioinformatic pipeline STARS, (Krasnov et al., 2011). Microarrays were fabricated by Agilent Technologies, equipment and reagents unless indicated otherwise were purchased from the same provider. Total RNA was extracted with PureLink RNA Mini Kit (Thermo Fisher Scientific) and RNA quality confirmed (Bioanalyzer). Two-color common reference design was applied where test and reference (equalized mixture of all samples) RNA were labelled with Cy5 Cy3 and competitively hybridized to microarray. Following hybridization and scanning of arrays, spot signal intensities were processed and normalized using STARS.

2.3. Statistics

The biological and analytical data were analyzed for Pearson's correlations using Microsoft Excel for Windows. Biological and analytical data were subjected to General Linear Model Univariate analysis of variance tests using IBM SPSS statistics 24 for detection of between-subjects' effects of main factor and their interaction. When differences among treatments were identified means were ranked using the Duncan post hoc test. Bonferroni adjustment for multiple comparisons was applied for pairwise comparisons of estimated marginal means for the main factors and interaction of 2 main factors at the time. Equality of error variances was tested with Levene's test. Effects were considered at a significance level of $p < 0.05$, and tendencies are discussed at $p<0.1$.

3. Results and discussion

3.1. Smoltification and transfer survival rate

At trial day 1 all fish populations were in fresh water and set at 24 h/day light. On days 26, 33 and 37, one fish per tank was used for salt tolerance test (measure of smoltification success) and on day 38 all fish were weighed and transferred to new tanks supplied with salt water, were they remained until the end of the trial (108 days). There was nearly no mortality in fish (2 dead fish in total) during the first 50 days of the trial (freshwater and transfer phase). During smoltification, in the freshwater phase of the trial and transfer, the average fish body weight in the trial tanks did not change, similarly in all treatments. Nevertheless, this was not the case for individual fish. Approximately a third of the fish in each tank increased in body weight during this period, some individuals up to 50 %, whereas others did not growth or even lost weight (<10 % on average). It was however not possible to predict post transfer performance by the weight change of the fish during smoltification (Fig. 1). A limited number of fish that lost weight during smoltification remained at the same weight during the trial, when other reached highest post transfer performance irrespectively of feeding during the smoltification period (Fig. 1A). Starting 13 days post transfer to salt water, mortality was observed in some dietary treatments, which topped 39 days post transfer, decreased then onwards and stopped 59 days post transfer. Over half of all the fish that died post transfer (29 out of total 55) had increased their weight during smoltification (Fig. 1B) and nearly all are assumed to have been feeding to some degree, justifying our assumption that a significant proportion of the mortality can have been a result of the dietary treatments.

Our further measurements, analyses and presentation of results concern the phase following transfer, after all fish populations started feeding normally.

The most marked dietary effect in the present study was a significantly negative effect of high dietary vitamin levels on total fish growth and survival rate following smoltification and transfer to seawater (Fig. 2 and Table 6). The main contribution to the observed mortality effect is due to 1 treatment (-M + V + AA) whereas all + V treatments yielded lower growth rates as compared to the -V treatments. No definite conclusion can be drawn on weather one or combination of 2 or all 3 vitamin/provitamin components that were used caused the observed effects, and the results are discussed based on this consensus.

The + V diets contained relatively high vitamin C levels and moderately high levels of vitamin E, in addition to the increased astaxanthin levels (as provitamin A) as compared to the -V diets. There is consensus on the fact that vitamin C, being a water-soluble vitamin, is not harmful at high dietary levels and there are speculations and scientific evidence that considerably higher than the known dietary requirement levels of vitamin C may be beneficial for fish, as for instance seen in Atlantic salmon pre-smolt fed up to 4000 mg/kg diet vitamin C (Waagbo et al., 1993). The reported requirement for vitamin C using AMP in Atlantic salmon is in the range of 10−20 mg ascorbic acid equivalents per kg diet (Sandnes et al., 1992). Dietary doses up to 1137 vitamin C in AMP form as the one used in this trial induced no negative or positive effects in farmed juvenile eel (Bae et al., 2012), and in humans a daily intake higher than 33 mg vitamin C per kg body weight (for instance in the case of a 60 kg adult) may cause negative side effects such as nausea and diarrhea (Johnson, 2019). In our trial, with an average daily feed intake rate throughout the experiment of 6 g feed per kg fish, the intake of vitamin C is calculated to have been approx.

Fig. 1. A: Post transfer growth of smoltified Atlantic salmon (TGC; y axis) vs smoltification performance in terms of weight change for one month (x axis, in grams), and B: weight change during smoltification of fish that died post transfer.

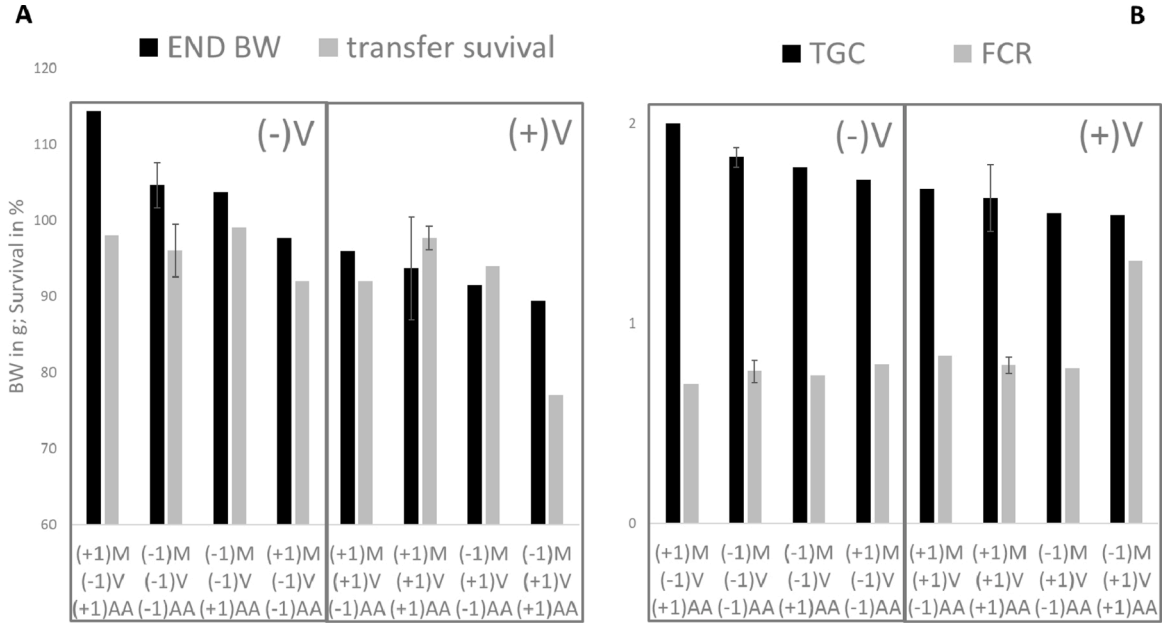

Fig. 2. Fish smoltification-transfer performance: A. End point fish mean body weight in black (BW in g) and transfer survival in grey (%); B. Saltwater phase fish thermal growth coefficient in black (TGC) and feed conversion ratio in grey (FCR) of experimental populations. For -M-V-A and + M + V + A n = 3, for all other treatments n = 1.

Table 6
GLM univariate tests of between-subjects effects results; summary for the effects of main factors and their interactions on salmon performance and biometrics following smoltification and transfer when fed diets with variable levels of trace minerals, vitamins and amino acids, considering all fish in the trial units (all fish per tank considered).

Dependent Variable	Source	df	F	Sig.	Pairwise comparisons
FCR	Corrected Model	7	16.7	0.008	
	Intercept	1	2806	0.000	
	M	1	13.5	0.021	+M < -M
	V	1	33.2	0.004	+V > -V
	AA	1	8.55	0.043	+AA > -AA
	M*V	1	12.57	0.024	+M-V<-M-V<+M + V<-M + V
	M*AA	1	27.56	0.006	+M + AA<-M-AA<+M-AA<-M + AA
	V*AA	1	23.32	0.008	-V + AA<-V-AA<+V-AA<+V + AA
Dead fish numbers (total)	Corrected Model	7	6.64	0.043	
	Intercept	1	36.45	0.004	
	M	1	3.698	ns	
	V	1	10.08	0.034	+V > -V
	AA	1	0.242	ns	
	M*V	1	11.25	0.028	-M-V<+M + V<+M-V<-M + V
	M*AA	1	11.25	0.028	+M + AA<-M-AA<+M-AA<-M + AA
	V*AA	1	7.938	0.048	-V + AA<-V-AA<+V-AA<+V + AA
Post transfer survival %	Corrected Model	7	7.739	0.033	
	Intercept	1	11,637	0.000	
	M	1	3.909	ns	
	V	1	12.39	0.024	+V < -V
	AA	1	0.114	ns	
	M*V	1	11.72	0.027	-M-V>+M-V>+M + V>-M + V
	M*AA	1	13.78	0.021	+M + AA>-M-AA>+M-AA>-M + AA
	V*AA	1	8.653	0.042	-V + AA>-V-AA>+V-AA>+V + AA
Daily feed intake % of body weight	Corrected Model	7	4.112	0.095	
	Intercept	1	2725	0.000	
	M	1	1.136	ns	
	V	1	1.412	ns	
	AA	1	5.194	0.085	+AA>-AA
	M*V	1	1.627	ns	
	M*AA	1	6.116	0.069	-M + AA>+M-AA>+M + AA>-M-AA
	V*AA	1	6.196	0.068	+V + AA>-V-AA>-V + AA>+V-AA
TGC	Corrected Model	7	2.052	ns	
	Intercept	1	1474	0.000	
	M	1	1.028	ns	
	V	1	8.85	0.041	+V < -V
	AA	1	0.079	ns	
	M*V	1	0.037	ns	
	M*AA	1	1.081	ns	
	V*AA	1	0.846	ns	
BW increase	Corrected Model	7	2.64	ns	
	Intercept	1	1228	0.000	
	M	1	1.028	ns	
	V	1	11.52	0.027	+V < -V
	AA	1	0.596	ns	
	M*V	1	0.081	ns	
	M*AA	1	1.407	ns	
	V*AA	1	1.55	ns	
END BW	Corrected Model	7	3.126	ns	
	Intercept	1	3436	0.000	
	M	1	0.838	ns	
	V	1	13.64	0.021	+V < -V
	AA	1	0.702	ns	
	M*V	1	0.14	ns	
	M*AA	1	1.69	ns	
	V*AA	1	2.213	ns	
END CF (all fish)	Corrected Model	7	15.716	0.000	
	Intercept	1	124,405	0.000	
	M	1	0.968	ns	
	V	1	3.262	0.071	-V>+V
	AA	1	84.82	0.000	+AA>-AA
	M*V	1	0.000	ns	
	M*AA	1	0.581	ns	
	V*AA	1	6.461	0.011	-V + AA>+V + AA>+V-AA>-V-AA
END CF (sample fish)	Corrected Model	7	4.105	0.001	
	Intercept	1	18,562	0.000	
	M	1	0.014	ns	
	V	1	0.304	ns	
	AA	1	22.658	0.000	+AA>-AA
	M*V	1	0.965	ns	
	M*AA	1	1.471	ns	
	V*AA	1	0.011	ns	
END D% (sample fish)	Corrected Model	7	0.883	ns	
	Intercept	1	179,028	0.000	
	M	1	0.254	ns	
	V	1	0.569	ns	
	AA	1	1.401	ns	
	M*V	1	0.976	ns	
	M*AA	1	0.686	ns	
	V*AA	1	0.161	ns	
END HSI (sample fish)	Corrected Model	7	4.241	0.001	
	Intercept	1	3823.4	0.000	
	M	1	1.441	ns	
	V	1	0.152	ns	
	AA	1	17.188	0.000	+AA<-AA
	M*V	1	0.059	ns	
	M*AA	1	7.069	0.011	+M + AA<-M + AA<-M-AA<+M-AA
	V*AA	1	2.590	ns	

1 mg/kg and 9 mg/kg fish body weight in the -V and + V treatments, respectively. Nevertheless, during smoltification, osmoregulation is challenged and so may be the capacity of fish to excrete superfluous vitamin C amounts, which could have caused that instead of exerting antioxidant effect, excess vitamin C promoted oxidative stress, compromising thus, fish performance, either alone or in combination with the relatively increased dietary astaxanthin and vitamin E levels.

In fish, vitamin E deficiency has been studied in several species (NRC, 2011) inducing symptoms such as reduced immune responses in rainbow trout (Blazer and Wolke, 1984). Increased dietary vitamin E levels were reported to improve immune responses of Atlantic salmon (Hardie et al., 1990) but not that of rainbow trout (Kiron et al., 2004). Studies in fish with moderately high dietary vitamin E levels are scarce. Reduced blood erythrocytes in juvenile brook trout blood (Poston and Livingston, 1969) and increased lipid peroxidation in sweet smelt (Kaewsrithong et al., 2001) were reported at very high dietary vitamin levels (5000 and 10,000 mg/kg, respectively), which are more than ten times higher than the levels we used in our study.

Though vitamin E is vital for protection of cells from oxidative stress (Verhagen et al., 2006), high intake levels of vitamin E (e.g., in the form of supplements) may have negative health effects. Thus, the Food and Nutrition Board (FNB) at the Institute of Medicine and Food and Nutrition Board (2000) has set the Tolerable Upper Limit levels (Uls) for

human adults to 1500 IU/day for supplements made from the natural form of vitamin E and 1100 IU/day for supplements made from synthetic vitamin E (1 mg of alpha-tocopherol is equivalent to 1.49 IU of the natural form or 2.22 IU of the synthetic form). The current fish trial diets were analyzed to contain 150-200 mg/kg (-V treatments) or 500−600 mg/kg (+V treatments) vitamin E. At 0.6 % mean daily feed intake per body weight in our trial, fish had an average daily vitamin E intake of 1 (when fed the 200 mg/kg vitamin E diet) or 4 mg kg body weight (when fed the 600 mg/kg vitamin E diet), corresponding to safe intake levels in humans.

Our trial data show significant interactions among the main dietary test factors on fish survival including interaction i) between dietary trace mineral and vitamin levels, ii) between trace mineral and amino acid levels, and iii) vitamin and amino acid levels (Table 6). Mortality rates were reduced in the groups with simultaneous high dietary supplementation of both organic trace minerals and vitamins. Similarly, mortality rates were significantly lower in the dietary treatments with balanced (low/low or high/high) dietary trace mineral and amino acid levels, lowest when both nutrient premixes were supplemented at high as compared to low levels. Last, fish mortality was reduced moderately (ca. 4%) in the high amino acid supplementation treatments, but only at low dietary vitamin levels.

In salmon, Se deficiency is found to induce several negative effects, lethargy, loss of appetite, growth retardation, reduced muscle tone, and mortality which can be partly alleviated by adequate vitamin E supplementation (Poston et al., 1976; Poston and Combs, 1979). Tocopheroxyl radicals (Vitamin E-O*) are recycled by a network of antioxidants (hydrogen donors) including ascorbate (Vitamin C) and thiols, including glutathione (in Traber, 2014; Packer et al., 1979). In our study it was mainly the increased trace mineral supplementation, likely leading to increased production of enzymes with antioxidant functions, as for instance selenopeptides (Sele et al., 2018) and selenoproteins, that may have contributed to alleviating the putative detrimental effects of high vitamin levels and potential presence of accumulated reactive oxygen species (ROS) through suboptimal vitamin E recycling and antioxidant function.

With respect to the observed improvement in salmon smolt survival by increased levels of dietary essential amino acid and trace mineral supplementation, it is expected that fish fed balanced diets with better nutritional status will be more robust in overcoming the osmotic stress and increased energy needs during smoltification and transfer from fresh to saltwater. During this period feed intake can be reduced or cease for variable periods of time, as also seen in our study in the case of two thirds of the fish populations. The tissues involved in osmoregulation of euryhaline fish species fuel their increased energy requirement during osmotic acclimation by metabolites as amino acids, lactate, and glucose (Sangiao-Alvarellos et al., 2003, 2005; Soengas et al., 2008). It has been demonstrated in rainbow trout that dietary supplementation of the essential amino acids Lys, Met and Leu in free form is effective in raising their plasma concentration above levels found in fish fed similar amounts bound in proteins (Tantikitti and March, 1995). It may be that besides growth, fish survival following transfer can improve by the presence of higher available free amino acid levels in the diet, allowing the fish to maintain normal physiological functions (in Matthews, 2014: Cahill and Aoki, 1980).

During the freshwater phase of the trial, i.e., before transfer, no significant effects or interactions of the main factors were observed in terms of survival rate, as seen also in Espe et al. (2019).

3.2. Feed intake and feed conversion efficiency

There were no significant correlations between feed intake and the test variables and their interactions. Nevertheless, there was a tendency for higher daily feed intake rate at + AA as compared to -AA as well as interaction between AA and M and AA and V treatments. Higher feed intake at + AA did not lead to significantly higher growth but had a negative effect on FCR (increasing FCR). Daily feed intake rate ranged between 0.85 and 1% BW/day except for the -M + V + AA treatment where DFI was 1.2. In this treatment there were the fewest fish in the tank following the highest mortality rate among all treatments and included the fish with the lowest BW. Smaller fish grown at the lowest fish density are expected to grow at higher rates than larger fish. This last treatment excluded, feed intake rate correlated near linearly with final fish weight ($R^2 = 0.756$, $P < 0.05$).

In terms of FCR, all trial fish per tank included, the analysis show significant effects of all main factors and their interactions (Table 6). Those included increased FCR at + V or + AA and improved (decreased) FCR at + M. FCR improved with simultaneously high or low supplementation of organic trace minerals and essential amino acids, demonstrating that the dietary balance of those essential nutrients is important for best nutrient utilization efficiency of the fed diets. Moreover, FCR was lower at high vitamin and trace mineral supplementation levels, when compared to the diets where vitamins but not the essential trace minerals were supplemented at higher levels. FCR was largely affected by the observed growth inhibition and increased fish mortality in the high vitamin groups, thus, the mechanisms described in sections 3.1 and 3.3 are considered relevant here too.

3.3. Growth and fish biometrics

Final body weight, transfer survival, TGC and FCR of fish in the different dietary treatments are shown in Fig. 2. In terms of growth performance of the whole tank populations, a significantly negative effect of high vitamin supplementation level was observed. Trace mineral and amino acid supplementation level had no significant effect on growth, and no significant interactions between the main factors were observed (Table 6). Espe et al. (2019), in a, experimental set up with Atlantic salmon fed first in fresh and then in saltwater, saw increased salmon performance through feeding a plant based diet fortified with Met (9.2 g/kg), vitamin B12 (0.18 mg/kg), folic acid (4.8 mg/kg) and vitamin B6 (8.45 mg/kg), and growth reduction when the respective premix was included in excess (Met: 11.7 g/kg; Vitamin B12: 0.20 mg/kg; B6: 11 mg/kg and folic acid: 6.3 mg/kg). In our study Met levels in the high AA treatment were higher than the respective excess treatment in Espe et al. (2019) and did not affect the growth in a negative way, leading us to the conclusion that it is more likely that it is the excess vitamins in the diet that caused the negative effects in fish performance in both studies.

Lee and Dabrowski (2004) found that high supplementation levels of dietary vitamin C (250 mg/kg) alone or combined with vitamin E (160 mg/kg) improved growth rates and survival in yellow perch. The assigned low levels (-V) of the Vitamin C and E in the diets of our study were similar to the high levels used by Lee and Dabrowski (2004) and showed better growth as compared to even higher dietary supplementation levels. Vitamin C contributes to regeneration of vitamin E by tocopheroxyl radicals (Packer et al., 1979) restoring the impaired vitamin E status. In the presence of high levels of vitamin C, high (e.g., more than 150 mg/kg vitamin E) supplementation of vitamin E may be superfluous (Hamre et al., 1997), or even detrimental as may have been the case in our study. Very high levels of dietary vitamin E supplementation levels (1000 ppm against 50−100 ppm) had neither beneficial nor detrimental effects in growth, feed efficiency or survival of channel catfish, but induced reduced protease, lipase, and alkaline phosphatase in hepatopancreas and the gut at similar levels as the treatments with no vitamin E supplementation (He et al., 2017). In our study we saw significant upregulation of several protease coding genes as for instance coding for trypsin, carboxypeptidase precursors A1; A2 and B, Chymotrypsin-like protease CTRL-1 precursor, Elastase-1, carboxylic ester hydrolase, Proproteinase E precursor a.o. (results not shown) in the midgut of the five biggest fish in the tanks of the + V treatments, apparently exhibiting compensatory mechanisms to the negative effects induced by excess vitamin supplementation levels. High

doses of vitamin E antagonize vitamin K, reducing the vitamin K-dependent coagulation factors II, VII, IX and X (Corrigan, 1992). According to the FAO, published toxicity effects in salmonids due to hypervitaminosis with vitamin A and D, include growth reduction, increased mortality, and fin erosion at 2.2–2.7 million IU vitamin A/kg diet (Poston et al., 1966;1971), growth reduction, lethargy, and dark coloration by high levels vitamin D (Halver, 1980). Vitamin D supplementation in our study was similar in all diets, whereas the variable levels of dietary supplemented astaxanthin, a precursor of vitamin A, would result in far lower levels of vitamin A as compared to the levels that induced toxicity in the aforementioned studies.

Some dietary factor effects in our study were more prominent, or only existed when considering smaller population size of largest fish per tank. The smaller the fish, the least prominent is expected to be the actual dietary effect which gets diluted by fish that eat less or not at all, due to for instance established hierarchies common in Atlantic salmon populations. Considering body growth rate in groups of the fish populations in each treatment by size ranking, we found a positive effect of + AA on body growth in the 5% largest fish. High dietary Met intake (1000−2000 mg/100 g diet) by rainbow trout fingerlings reversed or ameliorated some of the toxic effects of excess vitamin A (2000–4000 IU/100 g diet) (Eckhert and Kemmerer, 1974), but this did not occur in our study against the negative effects of the used vitamin premix. Among the largest 10 % of the fish in the populations, the higher vitamin supplementation levels show significantly negative effects, however a significantly positive effect of higher amino acid supplementation level was still present. No effect of trace mineral supplementation level or interactions were observed on fish growth of the largest fish groups. By increasing the population size considered, at the largest 25 % of the fish in the populations, increased vitamin supplementation still resulted in a negative effect, whereas there was only a tendency for a positive effect of the higher amino acid supplementation level that was no more present when all fish populations were considered.

Only few and small significant treatment effects were found in the biometric indexes measured. Dietary AA level significantly affected the condition factor of the fish, with + AA yielding fish with higher CF, similarly for sample and all fish considered, which agrees well with the observed higher feed intake and FCR, not followed by higher body growth in the + AA groups. Nevertheless, the opposite effect was observed in the relative liver size of the fish (HSI). Fish CF considering all the fish in the tanks was, as expected, negatively affected by high dietary vitamin levels, as in these treatments fish body growth was significantly lower. Significant interaction was observed between dietary vitamin and AA level on the CF of fish when all individuals were considered, whereas no such effect was detected for the sample fish. Last, significant interaction was observed between dietary trace mineral and AA level on the HSI of sample fish, as also observed in the case of the relative feed intake levels of the fish (Table 6).

3.4. ADC of dietary energy and nutrients

No effect of the main factors was seen on ADC of protein, lipid, or energy (Supplementary Table 2). There were nevertheless observed significant interactions between vitamin and trace mineral levels in the ADC of both lipids ($P = 0.031$), and energy ($P = 0.017$). ADC_{lipid} was reduced at -M + V (90.5 %) as compared to + M + V (92.6 %). The opposite was observed at low vitamin levels where ADC_{lipid} in -M-V (92.9 %) was higher as compared to + M–V (91.7 %). Similar effects as for ADC_{lipid} were observed in ADC_{energy} which was reduced at -M + V (83.8 %) as compared to + M + V (85.4 %). The opposite was again observed at low vitamin levels where ADC_{energy} in -M-V (85.7 %) was higher as compared to + M–V (84.0 %). This result agrees with the performance data where fish fed the -M + V diets had the lowest total growth (TGC) and final body weight among all treatments. Moreover, as in growth performance, ADC_{energy} of the -M-AA (85.7 %) and + M + AA (84.8 %) treatments were higher as compared to the -M + AA (83.8 %) and + M-AA (84.6 %).

Olsen et al. (2015) suggested that in salmon vitamin E supplementation prevents uptake and accumulation of fatty acids, which agrees partly with our observation of increased FCR with higher vitamin supplementation in the diets as well as the observed effect in ADC of lipid and energy which were reduced in the treatments with high vitamin and low trace mineral levels.

Simultaneous high supplementation of the essential amino acids Thr, Lys, Met and Arg in crystalline form significantly improved the digestibility of total dietary Thr (ADC_{Thr}-1AA = 87.7 %; ADC_{Thr}+1AA = 91.2 %), Lys (ADC_{Lys}-1AA = 92.9 %; ADC_{Lys}+1AA = 94.6 %), Met (ADC_{Met}-1AA = 94.8 %; ADC_{Met}+1AA = 96.8 %) and Arg (ADC_{Arg}-1AA = 95.5 %; ADC_{Arg}+1AA = 96.2 %) (Supplementary Tables 2–3). This effect may be related to the potentially higher availability of the supplemented crystalline amino acids. As mentioned previously, higher dietary levels of available essential amino acids could be utilised even more efficiently both as an energy source *via* gluconeogenesis and in protein synthesis to maintain vital physiological functions in 'fasting' or fish feeding at reduced rates during smoltification and immediately following transfer to saltwater. There was a significant interaction of dietary vitamins and amino acid supplementation levels. Higher vitamin supplementation had a significantly negative effect on Thr ADC, only at low AA supplementation rates (Corrected model: $P < 0.05$; Vitamins x Amino: acids $P < 0.05$). The ADC of dietary Gly and His were higher at high dietary supplementation of Thr, Lys, Arg and Met, and simultaneous high dietary vitamin supplementation levels. This may highlight the need for generation of compounds involved in antioxidant functions and tissue regeneration and healing processes (His) and connective tissue (Gly), induced by high vitamin levels which had negative effects on both fish growth and survival, which can be facilitated by higher dietary supplementation of essential amino acids. Both corrected models had a p value between 0.1 and 0.05 and showed indications for effects from both variable trace mineral supplementation and the interaction of trace mineral and vitamin supplementation, highlighting the need for nutrient balance in diets when formulating feed for the different life stages of fish.

3.5. Skin and intestinal health – qPCR

Transcriptional changes in skin from the variable dietary trace mineral, vitamin, and amino acid levels, were found (Table 7 and Supplementary Table 4). High trace mineral as well as high vitamin premix supplementation levels upregulated *col1a1* transcription in both skin and intestine ($P < 0.01$) (Supplementary Tables 4 and 5, respectively). In addition, only in the intestine but not in the skin, higher levels of supplementary essential amino acids led to downregulation of this gene, whereas there were also seen interaction effects between essential amino acid and trace mineral supplementation. No significant treatment effects were detected in the skin for the other genes studied.

In the intestine, we saw, in addition, significant transcriptional effect of vitamin levels on *muc5mc* (+V > -V; $P < 0.05$) and interaction effects between essential amino acid and trace mineral supplementation were observed. Unlike *col1a1* and *muc5mc*, transcription of intestinal *hsp70* was downregulated by higher vitamin supplementation levels. A tendency for higher transcription of *hsp70* in the −1 M treatments and interactions between vitamin and amino acid supplementation levels were also observed. Last, there were observed tendencies for increased transcription of *mmp13* at higher organic trace mineral supplementation levels and for interactions between vitamin and amino acid supplementation levels.

3.6. Morphological evaluation of skin

Few histological differences were found between the groups (Table 8 and Supplementary Table 6). However, an effect of trace mineral

Table 7
GLM univariate tests of between-subjects effects results; summary for the effects of main factors and their interactions on salmon skin and intestine tissue relative expression of barrier tissue structural and health indicating genes following smoltification and transfer when fed diets with variable levels of trace minerals, vitamins and amino acids, considering all fish in the trial units and (presented are results where significant differences were found).

	Source	SS	df	F	Sig.	Pairwise comparisons
Skin Col1a1	Corrected Model	59.13	9	6.668	0.000	
	Intercept	68.32	1	69.349	0.000	
	Trace minerals	8.593	1	8.721	0.005	+M > -M
	Vitamins	13.25	1	13.447	0.001	+V > -V
	Amino acids	0.449	1	0.456	ns	
	Trace minerals*vitamins	0.316	1	0.321	ns	
	Trace minerals*amino acids	2.607	1	2.646	ns	
	Vitamins*amino acids	1.183	1	1.200	ns	
	Error	47.29	48			
Intestine Col1a1	Corrected Model	115.7	8	12.831	0.000	
	Intercept	152.8	1	135.561	0.000	
	Trace minerals	9.336	1	8.284	0.006	+M > -M
	Vitamins	11.37	1	10.092	0.003	+V > -V
	Amino acids	7.553	1	6.703	0.013	-AA > +AA
	Trace minerals*vitamins	0.613	1	0.544	ns	
	Trace minerals*amino acids	5.946	1	5.276	0.026	+M-AA>+M + AA>-M-AA>-M + AA
	Vitamins*amino acids	1.590	1	1.411	ns	
	Error	56.34	50			
Intestine muc5ac	Corrected Model	84,153	8	1.656	0.133	
	Intercept	57,328	1	9.025	0.004	
	Trace minerals	1735	1	0.273	ns	
	Vitamins	30,625	1	4.821	0.033	+V > -V
	Amino acids	3550	1	0.559	ns	
	Trace minerals*vitamins	1476	1	0.232	ns	
	Trace minerals*amino acids	23,015	1	3.623	0.063	-M-AA>+M + AA>+M-AA>-M + AA
	Vitamins*amino acids	4597	1	0.724	ns	
	Error	317,599	50			
Intestine hsp70	Corrected Model	22.80	8	3.034	0.007	
	Intercept	0.546	1	0.581	ns	
	Trace minerals	2.778	1	2.958	0.092	-M > +M
	Vitamins	7.357	1	7.834	0.007	-V > +V
	Amino acids	0.328	1	0.349	ns	
	Trace minerals*vitamins	0.781	1	0.831	ns	
	Trace minerals*amino acids	0.311	1	0.331	ns	
	Vitamins*amino acids	3.728	1	3.969	0.052	-V-AA>-V + AA>+V + AA>+V-AA
	Error	47.00	50			
Intestine mmp13	Corrected Model	4.25	8	1.627	0.141	
	Intercept	22.68	1	69.433	0.000	
	Trace minerals	1.294	1	3.963	0.052	+M > -M
	Vitamins	0.666	1	2.039	ns	
	Amino acids	0.279	1	0.855	ns	
	Trace minerals*vitamins	0.015	1	0.047	ns	
	Trace minerals*amino acids	0.027	1	0.082	ns	
	Vitamins*amino acids	0.977	1	2.992	0.090	+V-AA>-V + AA>+V + AA>-V-AA
	Error	16.33	50			

supplementation level on the thickness of the dermis was observed, with fish in the -M treatment having increased dermis thickness. Collagen 1a1 is the major component of the dermis, and differences in thickness were reflected in transcriptional changes seen for this gene in response to altered trace mineral supplementation. An increased number of mucous cells at the surface of the epidermis was found in the -AA as compared to + AA treatment. Fish fed + M + V + A had higher ratio of purple mucous cells compared to the -M-V-A fed fish. Dietary components have previously shown to impact the skin structures differently (Ytteborg et al., 2018) and mucous cells in the outer part of the epidermis to indicate higher stress levels (Sveen et al., 2018). Examples of skin tissue histology samples in fish of the present trial are shown in Fig. 3.

Though fish tissues where not analyzed for the test dietary components in the present study, recently published studies show that for instance, Atlantic salmon readily accumulates Se in its tissues when fed increasing dietary levels of organic Se, far more efficiently as compared to inorganic selenium (Prabhu et al., 2020). The gut transcriptomic data of the current study showed significant upregulation of the zinc transporter gene *zip4* in the -M ($p < 0.001$), +AA ($p < 0.01$) and M/AA interaction $p < 0.001$) in a similar manner as observed with the performance results indicating increased trace mineral need at -M supplementation levels. In a preliminary study we observed that increased dietary levels of organic Se and Zn lead to higher tissue levels (including fillet, gills, skin, and liver) of the respective trace minerals in Atlantic salmon smolts without compromising the deposition of Cu, Mn and Fe in the respective tissues (Kousoulaki, 2017), as it has been reported in Nile tilapia in the case of dietary inorganic trace minerals (Huang et al., 2015). As in our trial we used organic trace minerals and available and stable forms of vitamins and amino acids we are confident that the observed effects in the analyzed tissues (in this case skin) were a result of the dietary treatments.

3.7. Gut metabolism and health

The transcription of 15 000 genes was assessed in midgut samples of fish from all experimental treatments. Several functional groups of genes were significantly affected by the main variables in the study. Interaction effects were also observed.

3.7.1. Cytoskeleton

The microarray platform contains 241 probes to genes for cytoskeleton components and 58 were significantly up or downregulated the dietary factors of the study. Those with a higher response are listed in Table 9 and Supplementary Table 7. Several actin, desmin, calponin,

Table 8
GLM univariate tests of between-subjects effects results; summary for the effects of main factors and their interactions on salmon skin histological measurements in fish following smoltification and transfer when fed diets with variable levels of trace minerals, vitamins and amino acids, considering all fish in the trial units and (presented are results where significant differences or indications were found). Purple and blue mucus cells were counted only in the + M + V + A and -M-V-A treatments.

Dependent Variable	Source	Type III SS	df	F	Sig.	Pairwise comparisons
Mean dermis thickness μm	Corrected Model	2141.768a	7	1.669	0.152	
	Intercept	446175.637	1	2433.185	0.000	
	Trace minerals	650.910	1	3.550	0.069	-M>+M
	Vitamins	419.140	1	2.286	ns	
	Amino Acids	280.000	1	1.527	ns	
	Trace minerals * Vitamins	598.163	1	3.262	0.080	-M-V>-M + V>+M + V>+M-V
	Trace minerals * Amino acids	4.989	1	0.027	ns	
	Vitamins * Amino Acids	145.764	1	0.795	ns	
Mucus cells (ratio outer/total %)	Corrected Model	5221.150a	7	1.730	0.137	
	Intercept	160898.823	1	373.229	0.000	
	Trace minerals	299.811	1	0.695	ns	
	Vitamins	0.021	1	0.000	ns	
	Amino Acids	4102.691	1	9.517	0.004	-AA>+AA
	Trace minerals * Vitamins	430.507	1	0.999	ns	
	Trace minerals * Amino Acids	139.062	1	0.323	ns	
	Vitamins * Amino Acids	182.850	1	0.424	ns	
Mucus cells Ratio purple/blue	Between Groups	0.320	1	10.409	0.010	+M + V + A>-M-V-A
	Within Groups	0.277	9			
	Total	0.597	10			

annexin, LIM domain protein, plasticin, filamin, nestin and other cell cytoskeleton protein coding genes were downregulated by high dietary trace mineral and vitamin and low dietary amino acid levels (except a molecule interacting with CasL 2b gene that showed opposite effect). Several interactions between the three main factors were observed showing similar trends among the different genes. Common interactions were downregulation of genes coding cytoskeleton proteins at + M + V but not at + M–V, downregulation at + M-AA and + V-A but not at + AA.

3.7.2. Cell redox

Of 81 genes related to the redox status, 19 genes showed tendency or significant differential expression (Table 10 and Supplementary Table 8). In the high vitamin supplementation treatments, downregulation of redox related genes was observed, in several cases combined with high AA supplementation, whereas the treatments with combined high vitamin and organic trace mineral supplementation were associated with upregulation of several genes encoding thioredoxine, selenoprotein and glutathione peroxidase. All but two regulated genes were also significantly affected by interactions between the main factors (V, M or AA supplementation levels). Most regulated genes were significantly affected by the treatments with variable levels of AA. In the + AA treatments downregulation of e.g., a thioredoxin-interacting protein, a selenoprotein (M) and a glutathione peroxidase coding gene was observed. This occurred particularly in treatments with both high AA and V supplementation, as for instance the upregulation of selenoprotein (Pa), two different glutathione related proteins coding genes a.o.

Reduced expression of redox-sensitive genes seen at the high dietary vitamin supplementation treatments, can increase risk for oxidative stress, which in our study was associated with smoltification and seawater transfer. In the present study, increased supplementation of vitamin E, C and astaxanthin was associated with increased mortality. It is not possible to separate the effects of individual vitamins in our trial; thus, we can only speculate on a plausible scenario. We found no evidence for possible adverse effects of high vitamin C levels in Atlantic salmon, only the contrary. On the other hand, excessive intake of vitamin E has been found to be harmful, in other model animal studies, due to a decrease in the activity of antioxidant enzymes in rat erythrocytes (Eder et al., 2002) or to the induction of toxicity when male albino rats are administered in high doses (2000 mg / kg body weight, vitamin E) daily for 30 days) (EL-Hak et al., 2019).

The effects observed in the + V treatments on genes of redox metabolism (e.g. thioredoxin-interacting protein) were reversed in the + V + M treatments, agreeing with our hypothesis that increased levels of antioxidant molecules such as selenomethionine can mitigate the putative prooxidative effect of excess dietary vitamins.

3.7.3. Iron and heme metabolism

Of 53 genes on the platform, 14 genes responded to the main dietary factors of the study and interactions between them were also observed (Table 11 and Supplementary Table 9). The need for erythropoiesis which in turn regulates plasma iron turnover and the regulation of iron uptake from the gastrointestinal tract (Weintraub et al., 1965). Effects on iron transporter transferrin and depot proteins ferritins could be expected as absorption and storage of Fe is facilitated by ascorbic acid (vitamin C) and amino acids (Conrad and Umbreit, 1993). On the other hand, persistently higher levels of trace mineral supplementation and levels of available Fe can reduce the efficiency of Fe absorption. Accordingly, most of the responsive genes in the intestine of salmon in our study were downregulated in the treatments with combined high trace mineral and amino acid supplementation levels but remained upregulated in the treatments with low trace mineral supplementation levels as well as high vitamin supplementation levels. The stimulated genes are involved in biosynthesis, transport, and degradation of heme. An interaction effect was seen between M and V for a series of genes responsible for iron storage and biosynthesis of heme which followed the logic of bioavailable iron requirement of fish. Therefore, the iron and heme homeostasis related genes were upregulated at -M-V (need for more Fe), downregulated at -M + V (growth impairment in + V and thus lower need for Fe and/or increased Fe uptake by high dietary vit C levels), decreased at + M–V (adequate Fe supplementation) and again increased at + M + V (at + M growth was impaired to a lower degree by + V maintaining thus higher Fe requirement).

3.7.4. Growth factors and related proteins

The expression of 147 genes encoding growth factors and related proteins was analysed and 27 genes were affected showing tendency (3) or significant (24) up or downregulation (Table 12 and Supplementary Table 10). Like genes coding proteins of cytoskeleton and extracellular matrix (ECM), the growth factors and related genes were downregulated in the + M treatments and in general also in the + V treatments and were upregulated in the + AA treatments. A common interaction was observed: the inhibitory effect of + V was neutralized in the + AA treatments. A suite of genes was downregulated in the high vitamin and

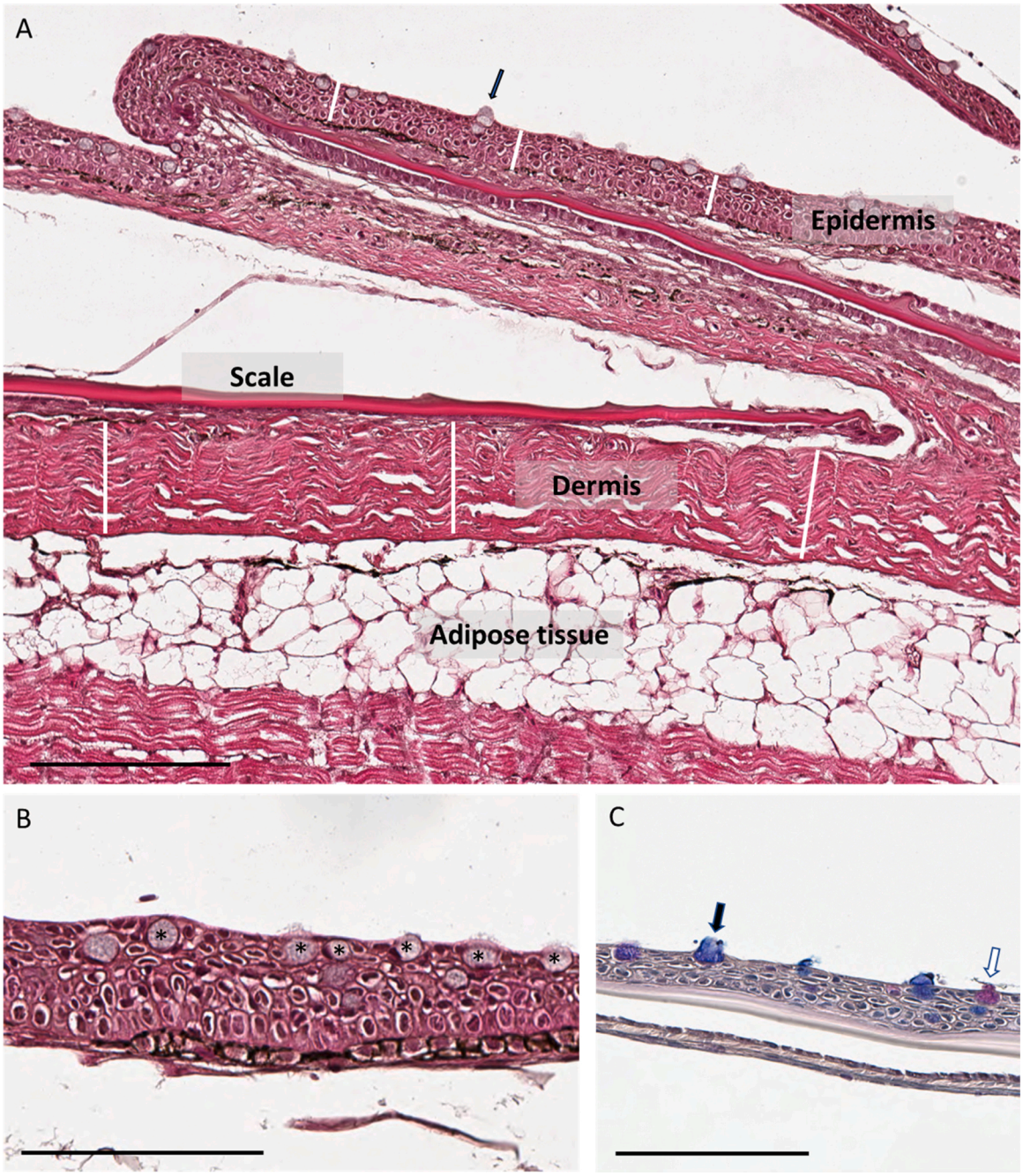

Fig. 3. Histology of skin included measuring the different layers in skin A) epidermal and dermal thickness (white lines) and counting mucous cells (arrow) that included B) outer mucous cells (star) and inner mucous cells, and C) differentiation of blue (black arrow) and purple (white arrow) cells, as visualized through AB/PAS staining. Scale bar = 100 μm.

trace mineral supplementation treatments and upregulated in the high amino acid supplementation treatments. This was true among other for follistatin-related protein 1 precursor (FSTL1 with a role in nervous system and skeletal development in mammals (Sylva et al., 2011; Yang et al., 2009)), insulin-like growth factor (IGF) binding protein, several growth factor-receptor bound proteins and transforming growth factor induced proteins, three precursors of sparcs, which regulate cell growth through interactions with the extracellular matrix and cytokines (Termine et al., 1981; Gaudet et al., 2011) and two mimecan precursors.

3.7.5. Extracellular matrix (ECM) and secreted proteins

The mechanical properties of the connective tissue and ECM in barrier tissues such as skin and intestinal epithelium, is determined by the abundance and modifications of collagens, other extracellular proteins and glycans. Out of 83 genes encoding collagens and modifying enzymes, 27 were affected by the dietary factors of the study (Tables 13, 14 and Supplementary Tables 11,12). Vitamin C is required for hydroxylation of proline residues in procollagen stabilizing the triple helical structure (reviewed in Peterkofsky, 1998). In our study, increased vitamin premix supplementation led to higher expression of COL1a1 both in the skin and the intestine (relevant observation in histology in the + V groups). Nevertheless, microarray analysis showed increased expression of collagen genes in the low (-1) vitamin treatments (in particular the −1 M-1V-1AA) and downregulation of multiple genes in the high (+1) vitamin supplementation treatment +1M+1V-1AA (e.g. collagen type XI alpha1 short isoform, collagen type X alpha 1 precursor,

Table 9
Significant effects ($p \leq 0.05$) and tendencies ($p \leq 0.1$) of dietary factors (low -1, and high +1, levels of Trace minerals: M, Vitamins: V, amino acids: AA) on the RGE (Relative gene expression) of genes related to **cytoskeleton** (n = 3-6 individual fish per treatment). The direction of expression differences is indicated in columns Trace minerals (M), Vitamins (V) and Amino acids (AA) by ↓ for downregulation or ↑ for upregulation by high dietary nutrient supplementation level. Symbols indicate: # = $p \leq 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ for the statistical significance level of main effects and interactions, and: – no significant effect or interaction.

Gene	M	V	AA	M*V	M*AA	V*AA
Phosphatase and actin regulator 3	** M↓	–	# AA↓	# [1]	** [2]	–
PDZ and LIM domain protein 3	** M↓	# V↓	# AA↑	# [3]	–	# [4]
LIM and SH3 domain protein 1	** M↓	–	# AA↑	# [5]	–	–
Alpha actin	** M↓	–	# AA↑	* [5]	–	–
Annexin A6	*** M↓	# V↓	** AA↑	* [5]	–	* [6]
Desmin	*** M↓	# V↓	# AA↑	** [5]	–	** [7]
Filamin-A	* M↓	–	* AA↑	* [5]	–	* [11]
Filamin-A	** M↓	# V↓	–	# [5]	–	** [11]
Nestin - Ident 27	** M↓	–	–	* [5]	–	# [11]
Plasticin	*** M↓	–	*** AA↑	* [5]	** [8]	** [9]
Desmin	*** M↓	* V↓	# AA↑	* [5]	# [8]	** [10]
Desmin	*** M↓	* V↓	–	** [5]	# [8]	** [7]
Actin	** M↓	–	–	# [5]	# [8]	–
Proline-serine-threonine phosphatase-interacting protein 2	* M↓	–	–	–	** [8]	–
Microfibrillar-associated protein 2 precursor	** M↓	** V↓	# AA↑	–	# [8]	* [14]
Calponin-1	*** M↓	# V↓	–	# [5]	* [13]	* [10]
Actin	* M↓	–	** AA↑	** [12]	# [13]	–
Annexin A6	** M↓	* V↓	** AA↑	* [5]	* [15]	* [9]
Calponin-3	* M↓	* V↓	# AA↑	–	–	* [6]
Smoothelin-like	** M↓	* V↓	–	–	–	** [7]
Molecule interacting with CasL 2b	** M↑	–	# AA↓	–	–	–

[1] Highest downregulation by -M-V followed by + M + V, highest RGE at + M–V followed by -M + V.
[2] Downregulation at -M + AA and + M-AA, highest upregulation at -M-A followed by + M + AA.
[3] Downregulation at + M more at + V, upregulation at -M irrespective of V level.
[4] Downregulation at + V-A, upregulation at + V + AA, no different RE at -V irrespective of AA.
[5] Downregulation at + M + V, upregulation at -M.
[6] Downregulation at + V-A, upregulation at + V + AA and -V.
[7] Downregulation at + V-A, upregulation at + V + AA and -V-AA.
[8] Downregulation at + M-AA, upregulation at -M.
[9] Downregulation at + V-A, upregulation at + AA.
[10] Downregulation at + V-A, upregulation at -V-AA.
[11] Downregulation at + V-A, upregulation at + V + AA.
[12] Downregulation at + M + V, upregulation at -M + V.
[13] Downregulation at + M-AA, upregulation at + AA.
[14] Downregulation at + M + V, upregulation at -M-V.
[15] Downregulation at + M-AA, upregulation at -M + AA.

Table 10
Significant effects ($p \leq 0.05$) and tendencies ($p \leq 0.1$) of dietary factors (low -1, and high +1, levels of Trace minerals: M, Vitamins: V, amino acids: AA) on the RGE (Relative gene expression) of genes related to cell **redox metabolism** (n = 3-6 individual fish per treatment). The direction of expression differences is indicated in columns Trace minerals (M), Vitamins (V) and Amino acids (AA) by ↓ for downregulation or ↑ for upregulation by high dietary nutrient supplementation level. Symbols indicate: # = $p \leq 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ for the statistical significance level of main effects and interactions, and: – no significant effect or interaction.

Gene	M	V	AA	M*V	M*AA	V*AA
Thioredoxin-interacting protein	–	–	* AA↓	* [1]	–	–
Selenoprotein M [SePM]	–	–	* AA↓	** [2]	–	# [5]
cytoglobin	–	–	# AA↓	–	–	* [4]
DJ-1	–	–	* AA↓	–	* [10]	–
Catalase	–	–	# AA↓	–	* [7]	–
Glutathione peroxidase	** M↑	–	# AA↓	–	–	–
Selenoprotein Pa [zSelPa]	–	–	* AA↓	* [2]	* [6]	–
checkpoint kinase 2	–	–	# AA↑	–	* [6]	–
Glutathione S-transferase P	–	–	# AA↑	# [12]	** [7]	–
Extracellular superoxide dismutase precursor	# M↓	–	* AA↑	–		–
Glutathione peroxidase 7 precursor	–	* V↓	* AA↑	–	* [8]	–
Glutathione S-transferase P	–	* V↑	–	–	* [7]	–
Interactor protein for cytohesin exchange factors 1	–	# V↓	–	–	–	* [9]
Thioredoxin domain-containing protein 12	* M↓	# V↓	–	–	# [3]	# [4]
Peroxiredoxin-6	* M↓	# V↓	–	–		# [9]
glutathione peroxidase 4b	# M↑	–	–	–	* [6]	–
Thioredoxin-dependent peroxide reductase, mitochondrial precursor	–	–	–	* [2]	–	–
Thioredoxin domain-containing protein 9	–	–	–	* [2]	–	–
Arrestin domain-containing protein 2	–	–	–	–	* [3]	–
glutathione S-transferase kappa 1-like	–	–	–	** [11]	–	–

[1] Downregulation + V-M, neutralised moderate upregulation + V + M.
[2] Downregulation at + V-M and -V + M, upregulation at -V-M and + V + M.
[3] Downregulation at + M-AA and -M + AA, upregulation at -M-AA and + M + AA.
[4] Downregulation at + V + AA and -V-AA, upregulation at + V-AA and -V + AA.
[5] Downregulation at + V + AA, upregulation at + V-AA.
[6] Downregulation at -M-AA, upregulation at -M + AA and no effect at + M.
[7] Downregulation at -M-AA and + M + AA, upregulation at + M-AA and -M + AA.
[8] Downregulation at + M-AA, upregulation at + M + AA.
[9] Downregulation at + V-AA, moderate upregulation else.
[10] Downregulation at + M + AA, moderate upregulation else.
[11] Downregulation + V-M and -V + M, upregulation -V-M and less at + V + M.
[12] Downregulation at + V-M and -V + M, upregulation at -V-M and + V + M.

collagen a3(I); a1(I) and type I collagen alpha 2 chain, collagen type XI, protein similar to vertebrate collagen, COL6A3, collagen I alpha 2 chain, col6a2 protein, collagen 2a1 and 1a, collagen V alpha 3 and procollagen-lysine,2-oxoglutarate 5-dioxygenase 3 precursor). Several genes encoding other ECM protein were downregulated in the + M

Table 11
Significant effects ($p \leq 0.05$) and tendencies ($p \leq 0.1$) of dietary factors (low -1, and high +1, levels of Trace minerals: M, Vitamins: V, amino acids: AA) on the RGE (Relative gene expression) of genes related to iron **heme metabolism** (n = 3-6 individual fish per treatment). The direction of expression differences is indicated in columns Trace minerals (M), Vitamins (V) and Amino acids (AA) by ↓ for downregulated or ↑ for upregulated by high dietary nutrient supplementation level. Symbols indicate: # = $p \leq 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ for the statistical significance level of main effects and interactions, and: – no significant effect or interaction.

Gene	M	V	AA	M*V	M*AA	V*AA
Heme-binding protein 2	# M↓	# V↓	–	–	# [1]	** [2]
Uroporphyrinogen-III synthase	* M↓	–	–	–	* [5]	** [4]
HIRA-interacting protein 5	# M↓	–	# AA↓	–	# [7]	–
Heme oxygenase	* M↓	# V↑	–	–	–	–
5-aminolevulinate synthase. nonspecific. mitochondrial precursor	–	** V↑	* AA↓	–	–	# [6]
Transferrin	–	* V↑	–	–	# [7]	–
Ferritin. middle subunit	–	–	# AA↓	# [3]	–	–
Ferritin-H subunit	–	–	* AA↓	–	–	–
5-aminolevulinate synthase. erythroid-specific. mitochondrial precursor	–	–	–	* [3]	–	–
Delta-aminolevulinic acid dehydratase	–	–	–	* [3]	–	** [4]
Ferritin middle subunit	–	–	–	* [3]	* [5]	–
FRIM SALSA RecName: Full = Ferritin. middle subunit; Short = Ferritin M	–	–	–	** [3]	–	* [4]
Ferritin. middle subunit	–	–	–	# [3]	** [5]	# [4]

[1] +AA balance gene RGE which is lowest at + M-AA and highest at -M-AA.
[2] +AA balance gene RGE which is lowest at + V-AA and highest at -V-AA.
[3] At -M increased RGE at -V, decreased at + V, at high trace minerals opposite effect.
[4] At -V increased RGE at + AA, decreased at -AA, at high vitamins opposite effect.
[5] At -M increased RGE at + AA, decreased at -AA, at high trace minerals opposite effect.
[6] Upregulation of RGE at + V-AA, low else.
[7] At -M increased RGE at + AA, decreased else.

and + V and upregulated in the + AA treatments.

Interactions between dietary trace mineral and vitamin level supplementation were also observed. Typically, the upregulation effects in the + AA treatments in the expression of this group of genes was absent in the -V but present in the + V treatments. Nevertheless, single genes were upregulated at + AA at both -V and + V. The lack of clear effects on collagen production in the transcription level in our trial is in line with the trial results of Li et al. (2007) who saw that healthy juvenile Atlantic salmon reared under controlled growth conditions require 79 mg kg diet vitamin C inclusion in order to synthesize adequate soluble muscle collagen, and no further increase in collagen production was promoted by higher dietary vitamin C levels up to 424 mg/kg diet.

3.7.6. *Plasma proteins*

The microarray used in this study contained probes to 90 genes for secreted proteins and 8 genes were differentially expressed (Table 15 and Supplementary Table 13). Those, including 3 apolipoprotein A and B coding genes, were upregulated in the + M and downregulated in the + AA treatments mostly irrespective of V, and in some cases the downregulation in + AA was stronger than the upregulation in + M. Several interactions of the main variables were observed, mainly upregulation of genes in the group in the + V-AA treatments and

Table 12
Significant effects ($p \leq 0.05$) and tendencies ($p \leq 0.1$) of dietary factors (low -1, and high +1, levels of Trace minerals: M, Vitamins: V, amino acids: AA) on the RGE (Relative gene expression) of genes related to tissue **growth factors** (n = 3-6 individual fish per treatment). The direction of expression differences is indicated in columns Trace minerals (M), Vitamins (V) and Amino acids (AA) by ↓ for downregulated or ↑ for upregulated by high dietary nutrient supplementation level. Symbols indicate: # = $p \leq 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ for the statistical significance level of main effects and interactions, and: – no significant effect or interaction.

Gene	M	V	AA	M*V	M*AA	V*AA
Transforming growth factor beta-1-induced transcript 1 protein	** M↓		* AA↑	# [2]	–	* [1]
Follistatin-related protein 1 precursor	* M↓	# V↓	* AA↑	–	–	* [1]
IGF binding protein 6, IBP6	* M↓	* V↓	* AA↑	–	–	** [1]
Transforming growth factor-beta-induced protein ig-h3 precursor	** M↓	# V↓	** AA↑	–	–	* [1]
SPARC precursor	# M↓	* V↓	# AA↑	–	–	–
SPARC precursor	# M↓	* V↓	* AA↑	–	–	# [1]
Mimecan precursor	# M↓	** V↓	–	–	–	# [1]
Insulin-like growth factor-binding protein 7 precursor	* M↓	* V↓	–	–	–	–
Growth factor receptor-bound protein 2	# M↓	** V↓	–	–	# [3]	–
latent transforming growth factor binding protein	* M↓	* V↓	–	–	** [6]	** [1]
Transcription factor Smad4	# M↓	* V↑	–	–	–	–
WNT1 inducible signaling pathway protein 1b	–	* V↓	* AA↑	–	–	–
Serine protease HTRA1	–	** V↓	** AA↑	–	–	* [1]
cysteine rich angiogenic inducer	–	* V↓	* AA↑	–	–	# [1]
SPARC precursor	–	* V↓	# AA↑	–	–	# [1]
Bone morphogenetic protein 4	–	* V↓	* AA↑	–	–	–
Growth factor receptor-bound protein 10	–	–	** AA↑	–	–	# [1]
Rhomboid-like protease 4	–	–	* AA↑	–	# [4]	* [1]
Relaxin-3	–	–	** AA↓	–	–	–
Serine protease HTRA1	–	–	# AA↓	–	–	–
Cation-independent mannose 6-phosphate receptor	–	–	# AA↓	* [5]	–	# [1]
transforming growth factor, beta receptor III, TGBR3	–	# V↓	–	# [5]	–	** [1]
IGF binding protein 5	–	*** V↓	–	–	–	–
Mimecan precursor	–	** V↓	–	–	–	–
Heparin-binding EGF-like growth factor precursor	–	** V↑	–	–	–	* [7]
Bone morphogenetic protein 2 precursor	–	–	–	–	–	** [1]
Granulins precursor	–	–	–	–	* [8]	–

[1] Cancellation of + V downregulation at + AA.
[2] Downregulation at + V + M and upregulation at -M, no effect at -V + M.
[3] Downregulation at + M-AA, upregulation at -M-AA else no effect.
[4] Downregulation at + M-A, else little effect.
[5] Downregulation at + V + M and upregulation at + V-M or -V + M.
[6] Downregulation at -M + AA and + M-AA and upregulation at + M + AA or -M-AA.
[7] Upregulation at + V-AA else little effect.
[8] Upregulation at -M + AA and + M-AA and downregulation at + M + AA or -M-AA.

Table 13
Significant effects ($p \leq 0.05$) and tendencies ($p \leq 0.1$) of dietary factors (low -1, and high +1, levels of Trace minerals: M, Vitamins: V, amino acids: AA) on the RGE (Relative gene expression) of genes related to **EMC** (n = 3-6 individual fish per treatment). The direction of expression differences is indicated in columns Trace minerals (M), Vitamins (V) and Amino acids (AA) by ↓ for downregulated or ↑ for upregulated by high dietary nutrient supplementation level. Symbols indicate: # = $p \leq 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ for the statistical significance level of main effects and interactions, and: – no significant effect or interaction.

	M	V	AA	M*V	M*AA	V*AA
Chondroitin sulfate proteoglycan 5	*** M↓	# V↓	–	* [7]	# [9]	* [5]
Transgelin	*** M↓	* V↓	** AA↑	* [7]	–	* [5]
Periostin precursor	** M↓	* V↓	* AA↑	–	–	–
Lumican	* M↓	* V↓	* AA↑	–	–	** [8]
Fibronectin type III domain-containing protein	# M↓	** V↓	* AA↑	–	–	–
Protein-lysine 6-oxidase	# M↓	* V↓	# AA↑	–	–	* [4]
Leprel2 protein	# M↓	* V↓	# AA↑	–	–	–
Decorin precursor	# M↓	* V↓	** AA↑	–	–	# [6]
Galectin-3	# M↓	* V↓	# AA↓	–	–	** [5]
Matrix Gla protein precursor	# M↓	* V↓	–	–	–	* [4]
A disintegrin and metalloproteinase with thrombosp	# M↓	* V↓	–	–	–	–
Matrix Gla protein precursor	# M↓	* V↓	–	–	–	* [5]
Laminin subunit beta-1; Laminin. beta 1. isoform C	** M↓	–	* AA↑	* [1]	–	# [2]
Leucine-rich repeat transmembrane protein	** M↓	–	–	–	* [9]	–
Exostosin-1c	** M↓	–	–	–	–	–
Microfibrillar-associated protein 2 precursor	* M↓	–	# AA↓	# [1]	–	–
Procollagen C-endopeptidase enhancer 1 precursor	* M↓	–	* AA↑	–	–	–
Hyaluronidase-2 precursor	# M↓	–	# AA↑	–	–	** [2]
Fibulin-7	# M↓	–	* AA↑	–	–	# [2]
osteocalcin	* M↑	** V↓	# AA↓	* [14]	* [15]	* [4]
NHL repeat-containing protein 3 precursor	* M↑	** V↑	–	–	–	–
Laminin alpha 3b	# M↑	** V↑	–	–	–	# [3]
Lumican	–	* V↓	–	–	–	# [4]
Prolyl 4-hydroxylase subunit alpha-2 precursor	–	* V↓	# AA↑	–	–	# [4]
Extracellular matrix protein 1 precursor	–	# V↓	–	–	–	* [2]
GalNAc alpha 2.6-sialyltransferase	–	# V↓	–	–	–	** [13]
Fibronectin	–	–	* AA↑	–	–	–
Alkaline phosphatase	–	–	–	–	* [11]	–
Protein-lysine 6-oxidase	–	–	* AA↑	* [10]	–	* [2]

[12] At -V no effect of M, at high V reduced RGE by -M and increased at + M.
[1] At low V no effect of M, at high V reduced RGE by + M and increased at -M.
[2] At low V no effect of AA, at high V reduced RGE by -AA and increased at + AA.
[3] Higher RGE at + V, more at low than high AA.
[4] Higher RGE at -V, lowest at + V -AA.
[5] At high AA, no effect of V, at low AA decreased RGE by + V and increased at -V.
[6] Upregulation by + AA, lowest RGE at -AA and + V.
[7] Reduced RGE by + M even more pronounced at + V.
[8] At -V no effect of AA, at + V reduced RGE by -AA only and similar to -V by + V + AA.
[9] Reduced RGE by + M even more pronounced at -AA.
[10] Reduced RGE by + M + V increased at + M–V, opposite effect at -M.
[11] Reduced RGE by + AA at -M and increased at + M.
[13] At -AA upregulation at -V and downregulation at + V, opposite effect but smaller at + AA.
[14] Increased RGE at + M–V, decreased at + V and low at -M-V.
[15] Increased RGE at + M-AA, decreased at + AA and low at -M-AA.

downregulation in the -M + V treatments. Apolipoproteins are involved in the transport and uptake of lipids and cholesterol, which is crucial for nutrient utilisation and growth. Three apolipoprotein coding genes were upregulated at + M, which agrees with the observed increase in ADC of lipids and energy at + M + V. The opposite was observed in the + M-V treatment. In the + M treatments there was also seen increased expression of the AMBP protein coding gene, a protein that interacts with numerous plasma proteins (Berggård et al., 1997). Valdenegro-Vega et al. (2014) noted a significant downregulation of AMPB protein coding gene in Atlantic salmon gill cells infected by AGD. Increased relative gene expression (RGE) of Alpha-1-antiproteinase-like protein coding gene, protecting proteins against proteases released from inflammatory cells' (Mackiewicz, 1993), which was in turn downregulated in the high dietary vitamin supplementation treatments.

3.7.7. *Mucus proteins*

Of 26 genes encoding mucus proteins and related genes, 12 were regulated in relation to the dietary factors (Table 16 and Supplementary Table 14). Upregulation of two giant mucus protein (GMP) and two spondin coding genes at + M, of mucin 5B2, a spondin and a pancreatic secretory granule membrane major glycoprotein (GP2) coding gene at + V, and of mucin 5AC and a spondin coding gene by + AA was observed. Several interactions of the main factors were also seen. Mucus, composed of highly glycosylated proteins suspended in a solution of electrolytes, is secreted by goblet cells in the intestinal epithelium and serves multiple functions, as for instance protection against shear stress and chemical damage (Gendler and Spicer, 1995). In our study, in the increased dietary vitamin (C, astaxanthin and E), organic trace mineral and essential amino acid supplementation treatment there was upregulation of genes involved in mucus homeostasis in the mid intestine of salmon. In our study we supplemented astaxanthin (provitamin A) to enhance the production of vitamin A without risking adverse effects of too high dietary doses of this vitamin (Grisdale-Helland et al., 1991). In channel catfish variable vitamin E supplementation, between 50 and 1000 mg/kg, did not cause differences in the thickness of the mucus membrane (He et al., 2017).

4. Conclusions

The main findings of this study are related to the negative effects on Atlantic salmon post-smoltification and transfer growth and survival from relatively high dietary vitamin C and E supplementation levels. In the groups that overall exhibited reduced growth at the time of sampling we saw significant upregulation of several protease coding genes in the midgut of the biggest fish in the tanks of the high vitamin treatments, which exhibited apparently compensatory mechanisms to the negative effects induced by high vitamin supplementation levels and/or compensatory growth.

Several observations in the current study indicate that balance between essential trace minerals and essential amino acids in the diet is important for fish digestion and health and welfare physiological status.

Increased trace mineral supplementation in organic form (the only

Table 14
Significant effects ($p \leq 0.05$) and tendencies ($p \leq 0.1$) of dietary factors (low -1, and high +1, levels of Trace minerals: M, Vitamins: V, amino acids: AA) on the RGE (Relative gene expression) of genes related to **collagen homeostasis** (n = 3-6 individual fish per treatment). The direction of expression differences is indicated in columns Trace minerals (M), Vitamins (V) and Amino acids (AA) by ↓ for downregulated or ↑ for upregulated by high dietary nutrient supplementation level. Symbols indicate: # = $p \leq 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ for the statistical significance level of main effects and interactions, and: – no significant effect or interaction.

Gene	M	V	AA	M*V	M*AA	V*AA
Collagen alpha-1(VI) chain	# M↓	* V↓	–	–	–	** [2]
type I Collagen alpha 2 chain	# M↓	** V↓	–	–	–	* [2]
Type I Collagen Alpha 2 chain	# M↓	** V↓	–	–	–	* [2]
Collagen 1a	# M↓	** V↓	–	–	–	* [2]
type I Collagen alpha 2 chain	# M↓	** V↓	–	–	–	* [2]
Collagen a3(I)	# M↓	* V↓	–	–	–	# [2]
Collagen alpha-3(VI) chain	# M↓	* V↓	–	–	–	* [2]
ProCollagen-lysine.2-oxoglutarate 5-dioxygenase 3 precursor	# M↓	* V↓	* AA↑	–	–	* [2]
Adiponectin	** M↓	** V↓	** AA↑	–	–	** [2]
Dipeptidase 1	** M↓	–	# AA↑	–	–	–
Collagen type XI alpha1 short isoform	* M↓	–	* AA↑	–	–	* [2]
Collagen type XI	** M↓	–	–	# [3]	–	* [2]
Collagen a3(I)	–	** V↓	–	–	–	* [2]
Collagen a3(I)	–	* V↓	–	–	–	* [2]
Collagen Type XI Alpha2	–	# V↓	–	–	# [10]	–
Novel protein similar to vertebrate Collagen type VI alpha 3 (COL6A3) - Ident 41	–	* V↓	–	–	–	# [2]
Col6a2 protein - Ident 96	–	# V↓	–	–	–	# [2]
Collagen a3(I)	–	* V↓	–	–	–	* [2]
Col6a2 protein - Ident 96	–	* V↓	–	–	–	* [2]
type I Collagen alpha 2 chain	–	* V↓	# AA↑	–	–	* [2]
Col14a1 protein	–	* V↓	# AA↑	* [9]	–	* [2]
SSR alpha subunit	–	–	# AA↑	** [4]	–	–
ProCollagen-lysine.2-oxoglutarate 5-dioxygenase 3 precursor	–	–	# AA↑	–	* [7]	–
ProCollagen-lysine.2-oxoglutarate 5-dioxygenase 3 precursor	–	–	** AA↑	–	–	–
Collagen a3(I)	* M↑	** V↑	–	* [8]	–	–
Collagen type XI	–	–	–	* [8]	–	–
Collagen Type XI Alpha2	–	–	–	* [8]	–	–
Collagen type XI	–	–	–	–	** [5]	–
Alkaline phosphatase	–	–	–	–	* [5]	–
Collagen Type XI Alpha2	–	–	–	–	* [6]	–
Collagen triple helix repeat-containing protein 1 precursor	–	–	–	–	–	* [1]

[1] Downregulation at -V-AA, upregulation by + V-AA>-V + AA.
[2] Downregulation at + V-AA, upregulation at -V-AA, +AA cancels the + V effect or induces upregulation.
[3] Downregulation at + V + M, upregulation at + V + M & -V-M.
[4] Downregulation at + V-M, upregulation at -/+V-M, no effect -V + M.
[6] Downregulation at -M-AA, upregulation at -M + AA and + M-AA.
[7] Downregulation at + M-AA, upregulation at + M + AA, else little effect.
[9] Downregulation at + V + M, upregulation at -V + M.
[5] Downregulation at -M + AA & +M-AA, upregulation at + M + AA (in some cases also -M-A).
[10] Downregulation at + M + AA & -M-AA, upregulation at -M + AA.
[8] Downregulation at -V-M, else no/little effect.

Table 15
Significant effects ($p \leq 0.05$) and tendencies ($p \leq 0.1$) of dietary factors (low -1, and high +1, levels of Trace minerals: M, Vitamins: V, amino acids: AA) on the RGE (Relative gene expression) of genes related to **tissue secretory functions** (n = 3-6 individual fish per treatment). The direction of expression differences is indicated in columns Trace minerals (M), Vitamins (V) and Amino acids (AA) by ↓ for downregulated or ↑ for upregulated by high dietary nutrient supplementation level. Symbols indicate: # = $p \leq 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ for the statistical significance level of main effects and interactions, and: – no significant effect or interaction.

Gene	M	V	AA	M*V	M*AA	V*AA
Apolipoprotein A-I-2	* M↑	–	# AA↑	# [1]	–	* [2]
Apolipoprotein A-I-1 precursor	* M↑	–	*** AA↓	# [1]	** [3]	** [2]
Alpha-1-antiproteinase-like protein	*** M↑	–	*** AA↓	** [1]	* [6]	** [2]
Apolipoprotein B-100	** M↑	–	–	–	–	–
AMBP protein precursor	* M↑	** V↑	–	–	–	–
Von Willebrand factor [vWF]	* M↓	* V↑	–	–	–	–
Haptoglobin	–	** V↓	** AA↓	–	–	–
Heparinase	–	–	# AA↓	** [4]	* [5]	–

[1] Downregulation at + V and -M, upregulation at + V + M, no effect at -V.
[2] Downregulation at + V and + AA, upregulation at + V-AA, no effect at -V.
[3] Downregulation at -M + AA, lower upregulation at -AA irrespective of M status.
[4] Lower upregulation ag -V-M and + V + M and downregulation else.
[5] Downregulation at + M + AA minor upregulation + M-AA, else little effect.
[6] Upregulation ag + M-AA, lower downregulation at -M + AA, else little effect.

form used in this study) alleviated the negative physiological effects observed in the high vitamin premix supplemented treatments. Accordingly, the expression of genes regulating the redox status was reduced at high dietary vitamin levels which was possibly associated with lower post transfer performance. However, combined high dietary organic trace mineral and vitamin levels increased transcription of several redox related genes such as thioredoxine, selenoprotein and glutathione peroxidase.

During and following smoltification feed intake is commonly reduced dramatically which in turn is known to deplete fish's glycogen stores and body proteins. Increased dietary essential amino acid supplementation in combination with increased trace mineral supplementation led to improved post transfer survival of salmon smolt, with indication of higher feed intake but deterioration of FCR. ADC of protein, lipid or energy was not affected by the main dietary factors, but significant interactions among them were present. Moreover, the ADC of threonine, lysine, methionine, and arginine was significantly higher in the treatments where they were supplemented at higher levels, possibly due to the higher availability of the supplemented crystalline amino acids which fish could use as energy source *via* gluconeogenesis and in protein synthesis to maintain vital physiological functions in previously 'fasting' fish during smoltification and immediately following transfer to saltwater. In the low performing high vitamin treatments, increased Met, Lys, Thr and Arg supplementation also led to higher Gly and His ADC, possibly allowing fish to increase synthesis of collagen and compounds with antioxidant and tissue regeneration function, strengthening

Table 16
Significant effects ($p \leq 0.05$) and tendencies ($p \leq 0.1$) of dietary factors (low -1, and high +1, levels of Trace minerals: M, Vitamins: V, amino acids: AA) on the RGE (Relative gene expression) of genes related to **mucus production and homeostasis** (n = 3-6 individual fish per treatment). The direction of expression differences is indicated in columns Trace minerals (M), Vitamins (V) and Amino acids (AA) by ↓ for downregulated or ↑ for upregulated by high dietary nutrient supplementation level. Symbols indicate: # = $p \leq 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ for the statistical significance level of main effects and interactions, and: – no significant effect or interaction.

Gene	M	V	AA	M*V	M*AA	V*AA
SCO-spondin 1	* M↑	# V↑	–	–	–	# [1]
SCO-spondin 2	* M↑	–	# AA↓	* [20]	–	# [21]
GMP Giant Mucus Protein 3	* M↑	–	–	–	–	# [14]
GMP Giant Mucus Protein 4	* M↑	–	–	–	–	–
Mucin 5AC	* M↓	–	** AA↑	** [15]	# [16]	** [17]
Mucin 5B2	–	* V↑	–	* [11]	** [12]	** [13]
Pancreatic Secretory Granule Membrane Major Glycoprotein GP2	–	# V↑	–	** [3]	–	# [4]
GMP Giant Mucus Protein 1	–	–	–	–	** [2]	–
GMP Giant Mucus Protein 2	–	–	–	# [7]	–	** [8]
Large Mucosal Protein	–	–	–	–	* [9]	* [10]
Secreted Protein Acidic Rich in Cysteine (*Oncorhynchus mykiss*)	–	–	–	# [5]	–	** [6]
Sushi Domain Containing Protein 2	–	–	–	** [18]	# [19]	–

[1] At -V, +AA reduce RGE but at +1 V increases.
[2] At -M, +AA increase RGE but at +1 M reduces.
[3] At -M, +V increase RGE but at +1 M reduces.
[4] At -V, +AA reduce RGE but at +1 V increases.
[5] At -M, +V reduce RGE but at +1 M increases.
[6] At -V, +AA increase RGE but at +1 V reduces.
[7] At -M, +V increase RGE but at +1 M reduces.
[8] At -V, +AA reduce RGE but at +1 V increases.
[9] At -M, +AA reduce RGE but at +1 M increases.
[10] At -V, +AA increase RGE but at +1 V reduces.
[11] At -M, +V increase RGE but at +1 M reduces.
[12] At -M, +AA reduce RGE but at +1 M increases.
[13] At -AA, -V reduce RGE but at +1AA increases, no effect at + AA.
[14] At -AA, -V reduce RGE but at +1AA increases, no effect at + AA.
[15] +V reduce RGE only at +1 M.
[16] +M reduce RGE only at -AA.
[17] +V reduce RGE only at -1AA.
[18] At -M-AA and + M + AA low whereas + M + AA higher RGE.
[19] At -M, +V reduce RGE but at +1 M increases.
[20] +V increase RGE in -AA and reduce RGE at +1AA.

the animals' barrier tissues. This suggestion was supported by our gene expression data. Dietary trace mineral, essential amino acid and vitamin levels also affected the genes for mucus component increasing the transcription rate at higher dietary levels.

Higher trace mineral supplementation resulted in significantly lower FCR. FCR improved also with simultaneously high or low supplementation of organic trace minerals and essential amino acids, demonstrating the importance of dietary balance between essential nutrients for optimal dietary amino acid and energy utilization efficiency. Compensatory mechanisms were observed in the transcription of genes for cytoskeleton components with downregulation at high trace mineral and vitamin and low essential amino acid dietary levels. Similar effects were also observed in the regulation of genes encoding growth factors and related proteins as well as extracellular matrix components, such as collagens. The regulation of apolipoprotein coding genes was also affected by dietary essential nutrients, with potential implications in fish's energy metabolism. Last, the regulation of heme homeostasis genes was affected by dietary trace mineral and vitamin levels indicating increased need at low trace mineral supplementation or higher fish growth.

Author contributions

Planning and preparing experiment: KK, JS, RM, EY and AK. Participated in carrying out experiment and analyses: KK, EY, AK, MEP, VH and MC. Writing and editing: KK, EY, AK, JS and RM.

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Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

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References

Aas, T.S., Ytrestøyl, T., Åsgård, T., 2019. Utilization of feed resources in the production of Atlantic salmon (*Salmo salar*) in Norway: an update for 2016. Aquac. Rep. 15 (November), 100216 https://doi.org/10.1016/j.aqrep.2019.100216, 2019.

Bae, J.-Y., Park, G.-H., Yoo, K.-Y., Lee, J.-Y., Kim, D.-J., Bai, S.C., 2012. Re-evaluation of the optimum dietary vitamin C requirement in Juvenile Eel, *Anguilla japonica* by using L-ascorbyl-2-monophosphate. Asian-Australas. J. Anim. Sci. 25, 98–103. https://doi.org/10.5713/ajas.2011.11201.

Berge, G.M., Ytteborg, E., Østbye, T.-K., Sundh, H., Rud, I., Sveen, L., Bæverfjord, G., Karlsen, C., Krasnov, A., Øgaard, J., Sundell, K., Pedersen, M., Halvorsen, B., Ruyter, B., 2019. The Importance of Nutrition for Skin, Gut and Gill Health in Salmon. Nofima Report 17,2019 (*In Norwegian*; Norwegian title: Ernæringens betydning for skinn-, tarm- og gjellehelse hos laks).

Berggård, T., Thelin, N., Falkenberg, C., Enghild, J.J., Akerström, B., 1997. Prothrombin, albumin and immunoglobulin A form covalent complexes with alpha1-microglobulin in human plasma. Eur. J. Biochem. 245, 676–683.

Berntssen, M.H., Amlund, H., Sele, V., Ørnsrud, R., 2018a. Project Report. "Evaluation of the Maximum Limits for Selenium in Atlantic Salmon Feeds. FHF" (Project no. 900871). Report from the Norwegian Institute of Marine Research p.186. (*in Norwegian*).

Berntssen, M.H.G., Betancor, M., Caballero, M.J., Hillestad, M., Rasinger, J., Hamre, K., Sele, V., Amlund, H., Ørnsrud, R., 2018b. Safe limits of selenomethionine and selenite supplementation to plant-based Atlantic salmon feeds. Aquaculture 495, 617–630.

Blazer, V.S., Wolke, R.E., 1984. The effects of α-tocopherol on the immune response and non-specific resistance factors of rainbow trout (*Salmo gairdneri* Richardson). Aquaculture 37, 1–9.

Cahill Jr, G.F., Aoki, T.T., 1980. Partial and total starvation. In: Kinney, J.M. (Ed.), Assessment of Energy Metabolism in Health and Disease. Report of the First Ross Conference on Medical Research. Ross Laboratories, Columbus, OH, pp. 129–134.

Cho, C.Y., 1992. Feeding systems for rainbow trout and other salmonids with reference to current estimates of energy and protein requirements. Aquaculture 100, 107–123.

Cohen, S.A., Michaud, K.E., 1993. Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high-performance liquid chromatography. Anal. Biochem. 211, 279–287.

Conrad, M.E., Umbreit, J.N., 1993. A concise review: iron absorption - the mucin-mobilferrin-integrin pathway. A competitive pathway for metal absorption. Am. J. Hematol. 42, 67.

Corrigan, J.J.Jr., 1992. Effects of vitamin E in warfarin-induced vit K. deficiency. Ann. N. Y. Acad. Sci. 82, 361.
Eckhert, C.D., Kemmerer, A.R., 1974. Effect of vitamin A and methionine on growth and amino acid composition of body protein in rainbow trout fingerlings. J. Nutr. 104, 865–870.
Eder, K., Flader, D., Hirche, F., Brandsch, C., 2002. Excess dietary vitamin e lowers the activities of antioxidative enzymes in erythrocytes of rats fed salmon oil. J. Nutr. 132, 3400–3404.
EL-Hak, H.N.G., ELaraby, E.E., Hassan, A.K., Abbas, O.A., 2019. Study of the toxic effect and safety of vitamin E supplement in male albino rats after 30 days of repeated treatment. Heliyon 5, e02645.
Espe, M., Vikesa, V., Thomsen, T.H., Adam, A.C., Saito, T., Skjaerven, K.H., 2019. Atlantic salmon fed a nutrient package of surplus methionine, vitamin B12, folic acid and vitamin B6 improved growth and reduced the relative liver size, but when in excess growth reduced. Aquacult. Nutr. https://doi.org/10.1111/anu.13010. Early Access.
Fiskeridirektoratet, 2019. Rapport: Statistikk for akvakultur 2018 – foreløpige tall (in Norwegian).
Gaudet, P., Livstone, M.S., Lewis, S.E., Thomas, P.D., 2011. Phylogenetic-based propagation of functional annotations within the gene ontology consortium. Brief. Bioinformatics 12, 449–462.
Gendler, S.J., Spicer, A.P., 1995. Epithelial mucin genes. Annu. Rev. Physiol. 57, 607–634.
Grisdale-Helland, B., Helland, S.J., Asgard, T., 1991. Problems associated with the present use of menadione sodium bisulphite in diets for Atlantic salmon. Aquaculture 92, 351–358.
Guillou, A., Choubert, G., Storebakken, T., de la Noile, J., Kaushik, S.J., 1989. Bioconversion pathway of astaxanthin into retinol 2 in mature rainbow trout (*Salmo gairdneri*). Comp. Biochem. Physiol. 94B, 481–485.
Halver, J.E., 1980. The vitamins. Fish Feed Technology. FAO, Rome. ADCP/REP/80/11 65-103.
Hamre, K., Waagbø, R., Berge, R.K., Lie, O., 1997. Vitamin C and E interact in juvenile Atlantic salmon. Free Radic. Biol. Med. 22, 137–149.
Hardie, L.J., Fletcher, T.C., Secombes, C.J., 1990. The effect of vitamin E on the immune response of the Atlantic salmon (*Salmo salar* L.). Aquaculture 87 (1), 1–13.
He, M., Wang, K., Liang, X., Fang, J., Geng, Y., Chen, Z., Pu, H., Hu, Y., Li, X., Liu, L., 2017. Effects of dietary vitamin E on growth performance as well as intestinal structure and function of channel catfish (*Ictalurus punctatus*, Rafinesque 1818). Exp. Ther. Med. 14 (6), 5703–5710. https://doi.org/10.3892/etm.2017.5295.
Hemre, G.-I., Lock, E.-J., Olsvik, P.A., Hamre, K., Espe, M., Torstensen, B.E., Silva, J., Hansen, A.-C., Waagbø, R., Johansen, J.S., Sanden, M., Sissener, N.H., 2016. Atlantic salmon (*Salmo salar*) require increased dietary levels of B-vitamins when fed diets with high inclusion of plant-based ingredients. PeerJ 4, e2493. https://doi.org/10.7717/peerj.2493.
Hertrampf, J.W., Pascual, F.P., 2000. Handbook on Ingredients for Aquaculture Feeds. Pp. 573..
Hjeltnes, B., Bang Jensen, B., Bornø, G., Haukaas, A., Walde, C.S., 2019. Fiskehelserapporten 2018. Veterinærinstituttet 2019 pp.132.
Huang, F., Jiang, M., Wen, H., Wu, F., Liu, W., Tian, J., Yang, C., 2015. Dietary zinc requirement of adult Nile tilapia (*Oreochromis niloticus*) fed semi-purified diets, and effects on tissue trace mineral composition and antioxidant responses. Aquaculture 439, 53–59.
Institute of Medicine, Food and Nutrition Board, 2000. Dietary Reference Intakes: Vitamin C, Vitamin E, Selenium, and Carotenoids. National Academy Press, Washington, DC.
Johnson, L.E., 2019. Vitamin C Toxicity (Ascorbic Acid Toxicity). MSD Manual. https://www.msdmanuals.com/professional/nutritional-disorders/vitamin-deficiency-dependency-and-toxicity/vitamin-c-toxicity.
Kaewsrithong, J., Ohshima, T., Ushio, H., Nagasaka, R., Maita, M., Sawada, M., 2001. Effects of an excess dose of dietary α-tocopherol on hydroperoxide accumulation and erythrocyte osmotic fragility of sweet smelt *Plecoglossus altivelis* (Temminck et Schlegel). Aquac. Res. 32, 191–198.
Kiron, V., Puangkaew, J., Ishizaka, K., Satoh, S., Watanabe, T., 2004. Antioxidant status and nonspecific immune responses in rainbow trout (*Oncorhynchus mykiss*) fed two levels of vitamin E along with three lipid sources. Aquaculture 234, 361–379.
Kousoulaki, K., 2017. A Study of Trace Minerals in Farmed Atlantic Salmon. February 2017 - International Aquafeed Magazine, pp. 32–35. https://issuu.com/international_aquafeed/docs/iaf1702_w1/34.
Krasnov, A., Timmerhaus, G., Afanasyev, S., Jørgensen, S.M., 2011. Development and assessment of oligonucleotide microarrays for Atlantic salmon (*Salmo salar* L.). Comp. Biochem. Physiol. D 6, 31–38.
Lee, K.-J., Dabrowski, K., 2004. Long-term effects and interactions of dietary vitamins C and E on growth and reproduction of yellow perch, *Perca flavescens*. Aquaculture 230, 377–389.
Li, X., Bickerdike, R., Nickell, D., Campbell, P., Dingwall, A., Johnston, I.A., 2007. Investigations on the effects of growth rate and dietary vitamin C on skeletal muscle collagen and hydroxylysyl pyridinoline cross-link concentration in farmed Atlantic salmon (*Salmo salar*). J. Agric. Food Chem. 55, 510–515. https://doi.org/10.1021/jf062251i. PMID: 17227086.
Maage, A., Julshamn, K., 1993. Assessment of zinc status in juvenile Atlantic salmon (*Salmo salar*) by measurement of whole body and tissue levels of zinc. Aquaculture 117, 179–191.
Maage, A., Sveier, H., 1998. Addition of dietary iron (III) oxide does not increase iron status of growing Atlantic salmon. Aquac. Int. 6 (38), 249–252, 1998.
Maage, A., Lorentzen, M., Bjornevik, M., Julshamn, K., 1991. Zinc requirement of Atlantic salmon (*Salmo salar*) fry during start feeding. Fish Nutrition in Practice. Biarritz (France), June 24-27, 1991. Ed. INRA Paris 1993 (Les Colloques, n°61).
Mackiewicz, K.A., 1993. The Acute Phase Response: an Overview. Acute-phase Glycoproteins: Molecular Biology, Biochemistry and Clinical Applications. CRC Press, pp. 3–19.
Matthews, D.E., 2014. Proteins and amino acids. In: Ross, A.C., Caballero, B., Cousins, R. J., Tucker, K.L., T.R, Ziegler (Eds.), Modern Nutrition in Health and Disease, 11th ed., pp. 3–35.
NRC, 2011. Nutrient Requirements of Fish and Shrimp. National Academies Press, Washington D. C., USA. https://doi.org/10.17226/13039.
Olsen, P.A., Berntssen, M.H.G., Søfteland, L., 2015. Modifying effects of vitamin E on chlorpyrifos toxicity in Atlantic Salmon. PLoS One 10 (3). https://doi.org/10.1371/journal.pone.0119250 e0119250.
Packer, J.E., Slater, T.F., Wilson, R.L., 1979. Direct observation of a free radical interaction between vitamin E and vitamin C. Nature 278, 737–738.
Peterkofsky, B., 1998. Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy. Am. J. Clin. Nutr. 54, 1135S–1140S.
Poston, H.A., 1971. Effect of feeding excess vitamin A on the carbohydrate and lipid metabolism and growth of brook trout. Fish. Res. Bull. 34, 22–26. State of New York Conserv. Dept. Albany.
Poston, H.A., Combs, G.F., 1979. Interrelationships between requirements for dietary selenium, vitamin E, and L-ascorbic acid by Atlantic salmon (*Salmo salar*) fed a semipurified diet. Fish Health News 8 (4), VI–VII.
Poston, H.A., Livingston, D.L., 1969. Effects of Massive Doses of Dietary Vitamin E on Fingerling Brook Trout. Fisheries Research Bulletin 33. State of New York Conservation Department, Albany, pp. 6–12.
Poston, H.A., Livingston, D.L., Pyle, E.A., Phillips, A.M.Jr., 1966. The toxicity of high levels of vitamin A in the diet of brook trout. Fish. Res. Bull. 29, 20–24. State of New York Conserv. Dept., Albany.
Poston, H.A., Combs, G.F., Leibovitz, L., 1976. Vitamin E and selenium interrelations in the diet of Atlantic salmon (*Salmo salar*): gross, histological and biochemical signs. J. Nutr. 106, 892–904.
Prabhu, A.J., Holen, E., Espe, M., Silva, M.S., Holme, M.-H., Hamre, K., Lock, E.-J., Waagbø, R., 2020. Dietary selenium required to achieve body homeostasis and attenuate pro-inflammatory responses in Atlantic salmon post-smolt exceeds the present EU legal limit. Aquaculture 526, 735413, 2020.
Regulation (EC), 2003. No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition, pp. 29.
Remø, S., Hevrøy, E., Olsvik, P., Fontanillas, R., Breck, O., Waagbø, R., 2014. Dietary histidine requirement to reduce the risk and severity of cataracts is higher than the requirement for growth in Atlantic salmon smolts, independently of the dietary lipid source. Br. J. Nutr. 111, 1759–1772. https://doi.org/10.1017/S0007114513004418.
Salte, R., Rørvik, K.A., Reed, E., Norberg, K., 1994. Winter ulcers of the skin in Atlantic salmon, *Salmo salar* L - pathogenesis and possible aetiology. J. Fish Dis. 17, 661–665.
Sandnes, K., Torrissen, O., Waagbø, R., 1992. The minimum dietary requirement of vitamin C in Atlantic salmon (*Salmo salar*) fry using Ca ascorbate-2-monophosphate as dietary source. Fish Physiol. Biochem. 10, 315–319. https://doi.org/10.1007/BF00004480.
Sangiao-Alvarellos, S., Laiz-Carrión, R., Guzmán, J.M., Martín del Río, M.P., Míguez, J. M., Mancera, J.M., Soengas, J.L., 2003. Acclimation of *S. aurata* to various salinities alters energy metabolism of osmoregulatory and nonosmoregulatory organs. Am. J. Physiol. Regul. Integr. Comp. Physiol. 285, R897–R907.
Sangiao-Alvarellos, S., Arjona, F.J., Martín del Río, M.P., Míguez, J.M., Mancera, J.M., Soengas, J.L., 2005. Time course of osmoregulatory and metabolic changes during osmotic acclimation in *Sparus auratus*. J. Exp. Biol. 208, 4291–4304.
Schiedt, K., Leuenberger, F.J., Vecchi, M., Glinz, E., 1985. Absorbtion, retention and metabolic transformations of carotenoids in rainbow trout, salmon and chicken. Pure Appl. Chem. 57, 685–692.
Schierle, J., Frey, C., Riegert, V., 2016. Determination of Carophyll Pink Astaxanthin in Dilutions, Premixes and Fish Feeds, Version 1.9.
Sele, V., Sanden, M., Berntssen, M.H.G., Lunestad, B.T., Espe, M., Lie, K.K., Amlund, H., Lundebye, A.-K., Hemre, G.-I., Waagbø, R., Ørnsrun, R., 2018. Program for Fish Feed Monitoring – Yearly Report for Samples Collected in 2017. A Norwegian Institute of Marine Research Report. ISSN 1893-4536. (in Norwegian).
Skjærven, K.H., Jakt, L.M., Dahl, L.M., Espe, M., Aanes, H., Hamre, K., Fernandes, J.M.O., 2016. Parental vitamin deficiency affects the embryonic gene expression of immune-, lipid transport- and apolipoprotein genes. Sci. Rep. 6, 34535. https://doi.org/10.1038/srep34535.
Soengas, J.L., Sangiao-Alvarellos, S., Laiz-Carrión, R., Mancera, J.M., 2008. Energy metabolism and osmotic acclimation in teleost fish. In: Baldisserotto, B., Mancera, J. M., Kapoor, B.G. (Eds.), Fish Osmoregulation. Science Publishers, Inc., Enfield, IBH Publishing. Co. Pvt. Ltd, New Delhi, pp. 278–307.
Standal, H., Dehli, A., Rorvik, K.A., Roem, A., 1999. Iron status and dietary levels of iron affect the bioavailability of haem and non-haem iron in Atlantic salmon, *Salmo salar*. Aquac. Nutr. 5, 193–198.
Sveen, L.R., Timmerhaus, G., Krasnov, A., Takle, H., Stefansson, S.O., Handeland, S.O., Ytteborg, E., 2018. High fish density delays wound healing in Atlantic salmon (*Salmo salar*). Sci. Rep. 8 (1), 16907.
Sylva, M., Li, V.S., Buffing, A.A., van Es, J.H., van den Born, M., van der Velden, S., Gunst, Q., Koolstra, J.H., Moorman, A.F., Clevers, H., van den Hoff, M.J., 2011. The BMP antagonist follistatin-like 1 is required for skeletal and lung organogenesis. PLoS One 6 (8). https://doi.org/10.1371/journal.pone.0022616 e22616.

Tantikitti, C., March, B.E., 1995. Dynamics of plasma free amino acids in rainbow trout (Oncorhynchus mykiss) under variety of dietary conditions. Fish Physiol. Biochem. 14, 179–194.

Taylor, J.F., Vera, L.M., De Santis, C., Lock, E.-J., Espe, M., Skjærven, K.H., Leeming, D., del Pozo, J., Mota-Velasco, J., Migaud, H., Hamre, K., Tocher, D.R., 2019. The effect of micronutrient supplementation on growth and hepatic metabolism in diploid and triploid Atlantic salmon (*Salmo salar*) parr fed a low marine ingredient diet. Compar. Biochem. Physiol. Part B 227, 106–121.

Termine, J.D., Kleinman, H.K., Whitson, S.W., Conn, K.M., McGarvey, M.L., Martin, G.R., 1981. Osteonectin, a bone-specific protein linking trace mineral to collagen. Cell 26, 99–105.

Traber, M.G., 2014. Vitamin E. In: Ross, A.C., Caballero, B., Cousins, R.J., Tucker, K.L., T. R, Ziegler (Eds.), Modern Nutrition in Health and Disease, 11th ed., pp. 293–304.

Valdenegro-Vega, V.A., Crosbie, P., Bridle, P., Leef, M., Wilson, R., Nowak, B.F., 2014. Differentially expressed proteins in gill and skin mucus of Atlantic salmon (Salmo salar) affected by amoebic gill disease. Fish Shellfish Immunol. 40, 69–77.

Verhagen, H., Buijsse, B., Jansen, E., Bueno-de-Mesquita, B., 2006. The state of antioxidant affairs. Nutr. Today 41, 244–250.

Wahli, T., Verlhac, V., Girling, P., Gabaudan, J., Aebischer, C., 2003. Influence of dietary vitamin C on the wound healing process in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 225, 371–386. https://doi.org/10.1016/S0044-8486(03)00302-8 (2003).

Weintraub, L.R., Conrad, M.E., Crosby, W.H., 1965. Regulation of the intestinal absorption of iron by the rate of erythropoiesis. Br. J. Hematol. 2, 432.

Yamada, S., 2013. Biosynthesis and degradation of bioactive imidazole-related dipeptides. Marine Enzymes for Biocatalysis Sources, Biocatalytic Characteristics and Bioprocesses of Marine Enzymes Woodhead Publishing Series in Biomedicine, pp. 295–331.

Yang, Y., Liu, J., Mao, H., Hu, Y.A., Yan, Y., Zhao, C., 2009. The expression pattern of Follistatin-like 1 in mouse central nervous system development. Gene Expr. Patterns 9, 532–540.

Ytteborg, E., Sveen, L.R., Østby, T.K., Ruyter, B., Høst, V., Berge, G.M., 2018. In Norwegian: Kan vi sjonglere med sink og omega-3 i fôret for å styrke lakseskinnet? Conference Mariculture, Oslo, Norway, April 18th – 20th.