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Dietary plant proteins and vegetable oil blends increase adiposity and plasma lipids in Atlantic salmon (*Salmo salar* L.)

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Abstract

In order to study whether lipid metabolism may be affected by maximum replacement of dietary fish oil and fish meal with vegetable oils (VO) and plant proteins (PP), Atlantic salmon (*Salmo salar* L.) smolts were fed a control diet containing fish oil and fish meal or one of three plant-based diets through the seawater production phase for 12 months. Diets were formulated to meet all known nutrient requirements. The whole-body lipid storage pattern was measured after 12 months, as well as post-absorptive plasma, VLDL and liver TAG. To further understand the effects on lipid metabolism, expression of genes encoding for proteins involved in VLDL assembly (apoB100), fatty acid uptake (FATP1, cd36, LPL and FABP3, FABP10 and FABP11) were measured in liver and visceral adipose tissue. Maximum dietary VO and PP increased visceral lipid stores, liver TAG, and plasma VLDL and TAG concentrations. Increased plasma TAG correlated with an increased expression of apoB100, indicating increased VLDL assembly in the liver of fish fed the high-plant protein- and VO-based diet. Atlantic salmon fed intermediate replacement levels of VO or PP did not have increased body fat or visceral mass. Overall, the present results demonstrate an interaction between dietary lipids and protein on lipid metabolism, increasing overall adiposity and TAG in the body when fish meal and fish oil are replaced concomitantly at maximised levels of VO and PP.

Key words: **Vegetable oil: Plant protein: Fish meal: Fish oil: Lipid metabolism: TAG: Adiposity: Visceral fat: Atlantic salmon**

Farmed Atlantic salmon (*Salmo salar* L.) have traditionally been fed diets containing major amounts of fish oil and fish meal. The steady increase in aquaculture production volume of 8–10 % per year[1] has resulted in an increased use of alternative proteins and oils in aqua feeds. Vegetable oils are recognised as suitable alternatives to fish oils[2,3]. Vegetable oils are, however, devoid of marine *n*-3 PUFA (EPA (20 : 5*n*-3), docosapentaenoic acid (22 : 5*n*-3) and DHA (22 : 6*n*-3)) while the levels of linoleic acid (18 : 2*n*-6) and monoene fatty acids are usually high, resulting in low dietary *n*-3:*n*-6 ratios. Likewise, sustainable alternatives to fish meal are included among plant protein sources, such as vegetable meals, with protein contents of 20 to 50 %[4]. However, the indispensable amino acid profile of plant proteins differs from that of fish meal. Sensible blending of different protein sources are adopted to complete the indispensable amino acid composition, while low levels of selected crystalline amino acids may also be added to fulfil amino acid requirements.

Studies on metabolic effects when replacing marine ingredients have concentrated either on fish oil[3–5] or fish meal replacement[6–8] independently. Typically, replacing fish oil with vegetable oils and thus decreasing marine *n*-3 PUFA and the *n*-3:*n*-6 ratio as well as increasing oleic acid (18 : 1*n*-9) has been reported to increase hepatic lipid stores in Atlantic salmon[9], particularly at low water temperatures[10]. Plasma cholesterol levels have been reported to decrease, whereas plasma TAG was unaffected by fish oil replacement[9]. Dietary fatty acid composition may also regulate the expression of genes encoding for lipid metabolism-related genes[5–11].

Some of the most frequently used vegetable oils contain high levels of 18 : 1*n*-9 which has been shown in different cell-culture systems[12,13] and rats[14] to affect liver lipid and lipoprotein metabolism. EPA and DHA are reported to affect liver TAG metabolism and β-oxidation capacity[15–18], with especially EPA having a plasma lipid-lowering effect in rats[19,20]. Furthermore, *n*-3 PUFA is suggested to inhibit the secretion of TAG-rich VLDL particles by inhibiting the rate-limiting enzyme diacylglycerol acyltransferase (*EC* 2.3.1.20)[15,16] and by inhibiting the assembly of VLDL particles in the liver[21–23]. Several studies

Abbreviations: 40PP70VO, 40 % plant protein and 70 % vegetable oil blend; 80PP35VO, 80 % plant protein and 35 % vegetable oil blend; 80PP70VO, 80 % plant protein and 70 % vegetable oil blend; FABP, fatty acid binding protein; FATP, fatty acid transport protein; FMFO, 100 % fish meal and 100 % fish oil; LPL, lipoprotein lipase.

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in human subjects have shown that dietary EPA and DHA decrease plasma TAG[24,25] and protect against CHD[26,27].

A large body of literature presents studies on alternative protein sources to fish meal in feed to salmonids, both by using blends of plant proteins with or without amino acid supplementation, as well as total replacement of fish meal[6,28–32]. A high inclusion of dietary plant protein mix has been reported to increase lipid retention in rainbow trout[33] whereas the opposite effects was reported in Atlantic salmon fed diets without any fish meal[29]. The reduced lipid deposition disappeared when only 5 % fish meal was added to the diets[30]. Furthermore, suboptimal dietary levels of methionine in high-plant protein diets have been reported to increase liver TAG concentration, fatty acid synthase activity and the 18 : 1:18 : 0 ratio in Atlantic salmon[34], resembling the signs observed in early stages of non-alcoholic fatty liver disease in rodent models. Suboptimal lysine in high-plant protein diets increased the whole-body lipid concentration[30] and affected the partitioning of growth in on-growing Atlantic salmon post-smolt[35].

Replacement of both fish oil and fish meal in diets fed to Atlantic salmon has been reported to result in decreased growth, which could only partially be explained by reduced feed intake during the first 3 months[36]. The aim of the present study, which was part of a larger project[36], was to further elucidate the metabolic effects of the combined and maximised replacement of fish meal and fish oil with vegetable feed ingredients with special emphasis on lipid metabolism.

Materials and methods

Feeding trial

The experiments complied with the guidelines of the Norwegian Regulation on Animal Experimentation and European Community Directive 86/609/EEC, and the protocol was approved by competent individuals at the laboratory unit at the Institute of Marine Research (Bergen, Norway) and the National Animal Research Authority.

The feeding trial was carried out at the Institute of Marine Research, Matre (Matredal, Norway; 60°52′N, 05°35′E) during the period from 22 June 2006 to 15 June 2007. The Atlantic salmon were obtained from AkvaGen A/S (Tingvoll, Norway). In June 2006, approximately 6000 smolt with a mean weight of 355 (SD 92) g were distributed equally into twelve 10 m^3 indoor fibreglass tanks containing 7 m^3 seawater, with a continuous flow-through (about 52 litres/min) of seawater (salinity 34·9 g/l) from a deepwater inlet (80 m deep, Matrefjorden). Temperature was kept constant at 8·9 ± 0·1°C, with continuous recording and an automatic record system. O_2 was also automatically recorded in the outlet water and was never decreased below 80 % saturation. The fish were acclimatised to the experimental conditions for 2·5 weeks before being fed the experimental diets on 22 June 2006. Fish in three randomised tanks were fed four different extruded diets: (1) a diet with maximum inclusion of fish meal (FM) and fish oil (FMFO); (2) a diet with an estimated safe maximum replacement of both fish meal and fish oil with plant meal (80 % plant protein) and vegetable oil (70 % vegetable oil) (80PP70VO); (3) a diet with half the maximum replacement with plant meal and maximum replacement with vegetable oil (40PP70VO); and (4) a diet with maximum replacement with plant meal and half the maximum replacement with vegetable oil (80PP35VO). Diets were produced by Skretting ARC (Stavanger, Norway). The four experimental diets were fed throughout the entire seawater production phase, with all diets changing in pellet size and lipid content after a 3-month feeding period (Table 1). Diet with pellet size 4 mm was fed to the fish from 20 June 2006 to 20 September 2006, and from 20 September 2006 to 12 June 2007 the fish were fed the 6 mm diets. Capelin oil (Fish Oil Nordic, Nordsildmel, Norway) was used as the dietary fish oil, and was the main source for very-long-chain highly unsaturated *n*-3 PUFA. A mixture of rapeseed oil, palm oil and linseed oil (55:30:15, by vol.) was used as the replacement for fish oil (Table 1). The mixture was selected to obtain a lipid profile of SFA, MUFA and *n*-3 PUFA as close as possible to capelin oil[2].

As replacement for fish meal, a mixture of maize gluten, wheat gluten, soya concentrate and krill meal was used (Table 1). A small component (< 50 g/kg) of krill meal (Aker Seafoods ASA, Oslo, Norway) was added to the experimental diets, with the aim to improve palatability and thus voluntary feed intake[37,38]. All diets were formulated to meet nutrient requirements of fish according to the National Research Council[39] recommendations. After 0, 3, 5, 8 and 12 months fish were weighed, and the amount of feed given was adjusted according to their biomass. Fish were reared under a natural light regimen until October 2006 when a 10 h light–14 h dark regimen was maintained throughout the winter until March 2007, when the fish returned to the natural light regimen. From June 2006 until February 2007, the feed consumption per tank was recorded daily. Fish were fed in excess twice per d (at 07.00 and 14.00 hours) with automatic feeders for 30 min, followed by feed collection 30 min after each feeding. Measuring of individual weights of at least 30 % of the biomass per tank and sampling of fish for whole-body analysis (six pooled fish per tank) were performed in June 2006 (initial stage), September 2006 (T = 3 months), November 2006 (T = 5 months), February 2007 (T = 8 months) and June 2007 (T = 12 months). Measuring individual weights of all fish at each sampling point was avoided to minimise handling stress and ensure optimal growth conditions. Growth, nutrient retention and digestibility are reported elsewhere[36].

Sampling

Samples of each experimental diet and the oil and meal ingredients used in the feeds for each feed production batch were stored at −20°C. From each tank, eleven fish were anaesthetised with benzocaine (7 g/l) and killed by a blow to the head. Of these, three fish from each tank were sampled for whole-body proximate composition, plasma lipids and lipoprotein separations, and these were not fed 2 d before sampling. Blood was collected from the caudal vein using

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Table 1. Feed composition (g/kg) and proximate composition (fat, protein, starch, ash and DM (g 100 g/g wet weight) and energy as kJ/g) of two pellet sizes of the four experimental diets

Pellet size…	4 mm				6 mm			
Diet…	FMFO	80PP35VO	40PP70VO	80PP70VO	FMFO	80PP35VO	40PP70VO	80PP70VO
Ingredients*								
Wheat	127	110	104	111	157	123	127	124
Wheat gluten	–	150	96	150	–	150	90	150
Maize gluten	–	150	150	150	–	150	150	150
Soyabean meal extracted	–	130	50	130	–	110	14	110
Krill meal	–	50	25	50	–	50	25	50
LT South American	620	120	300	120	560	120	300	120
Linseed oil	–	17	30	30	–	18	36	36
Palm oil	–	33	60	60	–	32	61	61
Rapeseed oil	–	61	110	110	–	56	110	110
Fish oil (Nordic capelin oil)	250	160	65	70	280	188	84	86
L-Lysine	–	13	6	13	–	–	–	–
Histidine-HCl	–	1	–	1	–	–	–	–
DL-Methionine	–	1	–	1	–	–	–	–
Vitamins and minerals†	3	4	4	4	3	3	3	3
Proximate composition (g/kg)								
Fat (±5%)‡	286	281	286	281	343	318	339	328
Protein (±3%)	431	442	433	442	422	424	412	426
Ash (±3%)	108	58·2	72·4	58·4	67·2	54·4	65	54·5
Starch	85·7	97·4	97·4	101	91·2	88·4	80·7	86·6
DM (±3%)	928	939	932	943	923	925	924	932
Energy (kJ/g)	238	249	245	248	251	254	254	253

FMFO, 100% fish meal and 100% fish oil; 80PP35VO, 80% plant protein and 35% vegetable oil blend; 40PP70VO, 40% plant protein and 70% vegetable oil blend; 80PP70VO, 80% plant protein and 70% vegetable oil blend; LT, low temperature .

* Wheat: Statkorn, Oslo, Norway; wheat gluten: Cerestar Scandinavia AS, Copenhagen, Denmark; maize gluten: Cargill, Minneapolis, MN, USA; soyabean meal extracted: Denofa, Fredrikstad, Norway; krill meal: Aker Seafoods ASA, Oslo, Norway; LT South American: Consortio, Arequipa, Peru; linseed oil: Elbe Fetthandel GmbH, Geesthacht, Germany; palm oil: Denofa; rapeseed oil: Emmelev AS, Otterup, Denmark; fish oil: Nordsildmel, Fyllingsdalen, Norway.

† Vitamin and mineral supplementation is estimated to meet the requirements according to National Research Council[39] recommendations.

‡ The method uncertainty is based on internal reproducibility, analyses of reference material and between-laboratory test of quality-assured methods.

EDTA vacutainers for plasma samples for further lipoprotein separation and heparin for the plasma samples for further analyses of nutrients. At 6 h after the last feeding, samples were collected from five fish per tank and pooled for tissue lipid and amino acid analyses and the determination of plasma amino acids. Visceral fat index was based on the visceral weight of eight fish from each tank (totally twenty-four fish from each dietary treatment). For RNA isolation and gene expression analyses, liver and visceral adipose tissue from three fish from each tank (in total nine fish per dietary treatment and collected 6 h after last feeding) were sampled individually and flash-frozen on liquid N_2. All samples were stored at −80°C until analysed.

Dietary amino acids and nitrogen metabolites in liver

Dietary amino acids were analysed after hydrolysis in 6 M-HCl as described previously for the growth and accretion data of the present trial[36]. N metabolites in the liver at the end of the feeding experiment were extracted and analysed as described[29].

Lipid extraction and fatty acid analysis

The lipid content of diets, whole fish and flesh was determined gravimetrically as the sum of free and bound fat. Free or loosely bound fat was extracted with petroleum ether and dried at 103 ± 1°C. The samples were thereafter hydrolysed with HCl in a Tecator Soxtec Hydrolysing unit (Foss Tecator AB, Höganös, Sweden) to release the bound fat, which was extracted with petroleum ether and dried at 103 ± 1°C. Total lipid was extracted from diets, liver and flesh by homogenisation in chloroform–methanol (2:1, v/v) with 19 : 0 methyl ester as the internal standard. Fatty acid methyl esters were prepared from total lipid by boron trifluoride following saponification, as described previously[40,41]. Thermo Finnegan Trace 2000 GC equipped with a fused silica capillary column was used (CP-sil 88; 50 m × 0·32 mm internal diameter; Chrompak Ltd, Middelburg, The Netherlands) with temperature programming of 60°C for 1 min, 160°C for 28 min, 190°C for 17 min, and finally 220°C for 10 min with all intervening temperature ramps being at 25°C per min. Individual methyl esters were identified by comparison with known standards and on the basis of published values[42]. Data were collected and processed using Totalchrom software (version 6.2; PerkinElmer, Waltham, MA, USA).

Lipid classes of salmon liver and diets were determined essentially as described by Jordal *et al.*[9] based on Bell *et al.*[43]. Briefly, lipids were extracted from the liver and diets by homogenising in chloroform–methanol (2:1, v/v) with 0·01% butylated hydroxytoluene (BHT). The samples were filtrated, evaporated after the addition of isopropanol and dissolved in chloroform–0·01% BHT before separation on high-performance TLC plates. Total lipid (10 μg) was applied to a 10 × 20 cm high-performance TLC plate that had been pre-run in hexane–diethyl ether (1:1, v/v) and activated at 110°C for 30 min. The plates were developed

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at 5·5 cm in methyl acetate–isopropanol–chloroform–methanol–0·25 % (w/v) aqueous KCl (25:25:25:10:9, by vol.) to separate phospholipid classes with neutral lipids running at the solvent front[44]. After drying, the plates were developed fully in hexane–diethyl ether–acetic acid (80:20:2, by vol.) to separate neutral lipids and cholesterol. Lipid classes were visualised by charring at 160°C for 15 min after spraying with 3 % copper acetate (w/v) in 8 % (v/v) phosphoric acid and identified by comparison with commercially available standards. Lipid classes were quantified by scanning densitometry using a CAMAG TLC Scanner 3 and calculated using an integrator (WinCATS-Planar Chromatography, version 1.2.0; CAMAG, Berlin, Germany). Quantitative determination (mg lipid class/g tissue) of lipid classes was performed by establishing standard equations for each lipid class within a linear area, in addition to including a standard mixture of all the lipid classes at each high-performance TLC plate for corrections between plate variations.

Plasma and lipoprotein lipids were analysed using a clinical bioanalyser (Maxmat PL analyser; MaxMat S.A., Montpellier, France) according to standardised procedures, reagents and controls. Plasma lipoproteins (VLDL, LDL and HDL) in plasma were obtained by sequential centrifugal flotation[45,46] as described by Lie *et al.*[47] using a Beckman Optima™XL-100K Ultracentrifuge equipped with a SW41Ti rotor. The centrifugation was done at 197 600 $\boldsymbol{g}_{av}$ and 4°C. The density intervals were obtained by the addition of solid KBr[48], and the run times for the separation of lipoproteins were: VLDL, density (d) < 1·015 g/ml for 20 h; LDL, 1·015 g/ml < d < 1·085 g/ml for 20 h; and HDL, 1·085 g/ml < d < 1·21 g/ml for 44 h. The lipoprotein fractions were stored at −80°C until further analysis.

For gene expression analysis total RNA was isolated from 100–500 μg of tissue sample by the standard TRIzol extraction method (Invitrogen Ltd, Paisley, Renfrewshire, UK) and recovered in 100 μl molecular diethylpyrocarbonate (DEPC)-treated water. In order to remove any possible genomic DNA contamination, the total RNA samples were pretreated using DNA-*free*™ DNase treatment and removal reagents kit (Ambion Inc., Austin, TX, USA) following the manufacturer's protocol.

A two-step real-time RT-PCR protocol was developed to measure the mRNA levels of the target genes in Atlantic salmon liver and visceral adipose tissue. The RT reactions were run in triplicates on ninety-six-well reaction plates with the GeneAmp PCR 9700 instrument (Applied Biosystems, Foster City, CA, USA) using TaqMan Reverse Transcription Reagent containing Multiscribe RT (50 U/μl) (N808-0234; Applied Biosystems). For efficiency calculations, twofold serial dilutions of total RNA were made. A dilution curve was recorded using four serial dilutions (250–31 ng), with each concentration being run in triplicate. The samples were analysed by quantitative RT-PCR in separate sample wells and the resulting cycle thresholds (C_t) recorded. Total RNA input was 125 ng in each reaction for all genes. No template control (ntc) and RT-control (a duplicate RNA sample analysis where only the RT enzyme is left out) reactions were run for quality assessment. RT-controls were not performed for each sample, but were run for each assay or gene, with the same sample as used to make the dilution curves on the ninety-six-well plates. Reverse transcription was performed at 48°C for 60 min by using oligo dT primers (2·5 μM) in 50 μl total volume. The final concentrations of the other reagents in the RT reaction were: $MgCl_2$ (5·5 mM), dNTP (500 μM of each), 10X TaqMan RT buffer (1X), RNase inhibitor (0·4 U/μl) and Multiscribe RT (1·67 U/μl). cDNA (2·0 μl) from each RT reaction for all genes was transferred to a new ninety-six-well reaction plate, and the real-time PCR run in 20 μl reactions on the LightCycler® 480 Real-Time PCR System (Roche Applied Sciences, Basel, Switzerland). The final concentration of the primers was 500 nM. Real-time PCR was performed using TaqMan universal PCR master mix (LightCycler 480 SYBR Green master mix kit; Roche Applied Sciences) containing FastStart DNA polymerase, and gene-specific primers. PCR was achieved with a 5 min activation and denaturising step at 95°C, followed by forty cycles of a 15 s denaturing step at 95°C, a 60 s annealing step and a 30 s synthesis step at 72°C. The annealing temperature and sequences for the primer pairs are presented in Table 2.

Data analyses and statistics

Q-Gene was used for the normalisation and calculation of relative expression data[49]. Q-Gene takes into account the PCR efficacy, calculated based on dilution curves. The gene expression levels were normalised towards a reference gene. For all dietary treatments, three different reference genes

Table 2. Primers for quantitative PCR assays*

Gene	GenBank accession number	Forward primer	Reverse primer	Annealing temperature (°C)
FATP1	CA373015/AF023258	TGGGAGCTTGTGGGTTCAA	ACTTTCATGAGGCGGATTGG	58
CD36	AY606034	TTTCCTGCTGCGCACCTT	GGTGCGGGTCATGAAGATTT	54
LPL	gi:14582900	GCCCGACCTTTGAGTTTGC	ACGTCCACAAAGAGAGCATCGT	60
FABP3	AY509548	CACCGCTGACGACAGGAAA	TGCACGTGAACCATCTTACCA	60
FABP11	DR695475	CCGCCGACGACAGAAAAA	TTTTGCACAAGGTTGCCATTT	60
FABP10	BG935057	TTGCCCATTTTGGTGAAGGA	TGAGATGGCCCTGAGGAACT	60
apoB100	gi:854619	TTGCAGAGACCTTTAAGTTCATTCA	TGTGCAGTGGTTGCCTTGAC	60
ARP (reference gene)	AY255630	GAAAATCATCCAATTGCTGGATG	CTTCCCACGCAAGGACAGA	60
β-Actin (reference gene)	BG933897	CCAAAGCCAACAGGGAGAAG	AGGGACAACACTGCCTGGAT	60
$EF1A_B$ (reference gene)	BG933853	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACTG	60

FATP1, fatty acid transport protein 1; LPL, lipoprotein lipase; FABP, fatty acid binding protein; ARP, acidic ribosomal phosphoprotein; $EF1A_B$, elongation factor $1A_\beta$.
* All sequences are presented as 5′ to 3′.

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were measured; acidic ribosomal protein (ARP), β-actin and elongation factor 1-αβ isoform ($EF1A_B$) were the reference genes that were analysed based on previous Atlantic salmon reference gene validation[50]. The reference gene stability was tested by *geNorm*[51], and all reference genes were found to have stability within acceptable limits. β-Actin, however, exhibited low PCR efficacy in liver and was hence disqualified as a reference gene. The most stable of these three reference genes ($EF1A_B$) was used for normalisation with the Q-Gene software (http://www.qgene.org).

All statistical analyses were performed using the program Statistica (version 9.0; Statsoft Inc., Tulsa, OK, USA). Significant differences among dietary treatments were assessed by a one-way ANOVA[52]. Where the null hypothesis (H_0, no difference between treatments or within treatment at different time intervals) was rejected, significant differences were tested using Tukey's honestly significant difference test ($P<0{\cdot}05$; Sokal & Rohlf[53]). A Kolmogorov–Smirnov test was used to assess the normality of distribution of each treatment[52]. All data were normally distributed. Dependent variables were checked for homogeneity of variance by the Levene test and transformed whenever necessary[52].

Results

After 3 months of feeding, the pellet size was adjusted according to fish size, from 4 mm to 6 mm pellets. Lipid level increased as pellet size increased, resulting in a slightly higher lipid:protein ratio, while the ratio of plant ingredients relative to marine ingredients was kept constant. Dietary amino acids differed due to the substitution of plant protein for fish meal and reflected the amino acid composition of the different protein ingredients in the various experimental diets. The indispensable amino acids:dispensable amino acids ratio was lower in the replacement groups than in the FMFO group[36]. The higher the inclusion level of plant protein, the lower the amount of taurine in these diets and the lower the concentration of non-amino acid-N. However, only minor

Table 3. Fatty acid composition (area % wet weight), total fatty acids (mg/g) and amino acids (g/16 g nitrogen) of the four experimental diets at two different pellet sizes

Pellet size…	4 mm				6 mm			
Diet…	FMFO	80PP35VO	40PP70VO	80PP70VO	FMFO	80PP35VO	40PP70VO	80PP70VO
SFA								
14 : 0	4·5	2·8	1·8	1·7	6·4	5·0	2·6	2·8
16 : 0	14·0	15·1	16·7	16·5	15·2	15·0	16·3	16·1
18 : 0	2·0	2·1	2·6	2·5	2·5	2·4	2·8	2·6
Sum SFA	21·7	21·3	22·1	21·8	25·4	23·5	22·8	22·8
Monoenes								
16 : 1*n*-7	5·3	3·1	1·8	1·8	4·7	4·1	1·9	2·1
18 : 1*n*-7	3·2	2·8	2·5	2·5	2·0	2·2	2·4	2·3
18 : 1*n*-9	11·0	24·5	33·6	34·9	9·5	17·6	30·0	28·9
20 : 1*n*-9	9·7	6·6	3·3	3·5	6·7	5·3	3·0	3·3
20 : 1*n*-11	1·6	1·1	0·5	0·5	0·5	0·4	0·2	0·2
22 : 1*n*-9	1·9	1·3	0·7	0·8	0·9	1·1	1·3	1·3
22 : 1*n*-11	11·8	7·8	3·3	3·6	10·4	7·7	3·5	4·1
24 : 1*n*-9	1·1	0·6	0·3	0·4	1·1	0·8	0·5	0·5
Sum monoenes	47·0	48·7	46·3	48·4	36·7	39·8	43·3	43·3
n-6								
18 : 2*n*-6	1·6	8·9	13·7	13·4	2·3	7·5	12·8	12·7
20 : 2*n*-6	0·3	0·2	0·0	0·0	0·3	0·2	0·1	0·1
20 : 3*n*-6	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
20 : 4*n*-6	0·6	0·3	0·2	0·1	0·9	0·5	0·3	0·3
Sum *n*-6	2·5	9·3	13·9	13·5	3·4	8·2	13·3	13·1
n-3								
18 : 3*n*-3	0·8	5·7	9·1	9·7	1·4	5·3	9·4	9·3
18 : 4*n*-3	2·2	1·3	0·7	0·6	3·5	2·6	1·2	1·3
20 : 3*n*-3	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
20 : 4*n*-3	0·6	0·4	0·2	0·2	0·7	0·5	0·2	0·3
20 : 5*n*-3	8·6	4·8	2·9	2·5	9·8	7·5	3·5	3·9
22 : 5*n*-3	1·0	0·5	0·3	0·3	1·2	0·8	0·4	0·4
22 : 6*n*-3	11·4	6·1	3·8	3·0	13·0	8·5	5·0	4·7
Sum *n*-3	25·2	18·8	17·1	16·3	30·5	26·0	19·9	20·3
n-3:*n*-6	9·9	2·0	1·2	1·2	8·9	3·2	1·5	1·6
Total fatty acids (mg/g)	228	214	235	218	289	288	305	295
Amino acids*								
Lysine	7·2	5·9	6·2	6·0	6·8	5·3	5·7	5·4
Methionine	2·9	2·2	2·3	2·4	2·6	2·6	3·2	2·5
Taurine	0·9	0·2	0·4	0·2	0·9	0·2	0·5	0·3
Non-amino acid-N	12·1	5·5	5·3	6·2	12·1	4·2	3·2	4·3

FMFO, 100 % fish meal and 100 % fish oil; FMFO, 100 % fish meal and 100 % fish oil; 80PP35VO, 80 % plant protein and 35 % vegetable oil blend; 40PP70VO, 40 % plant protein and 70 % vegetable oil blend; 80PP70VO, 80 % plant protein and 70 % vegetable oil blend.

* Lysine, methionine and taurine (g/16 g N) contents of the experimental diets are listed. The rest of the dietary amino acid compositions are given in Torstensen *et al.*[36].

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Table 4. Dietary phospholipid and sterol composition (mg/g) of the four experimental diets

Diet…	FMFO	80PP35VO	40PP70VO	80PP70VO
SM	0·9	nd	nd	nd
PC	15·4	6·6	7·9	7·3
PI	0·8	nd	nd	nd
PS	nd	nd	nd	nd
PE	2·4	nd	0·1	nd
STEROLS	10·6	10·5	11·0	11·3

FMFO, 100 % fish meal and 100 % fish oil; 80PP35VO, 80 % plant protein and 35 % vegetable oil blend; 40PP70VO, 40 % plant protein and 70 % vegetable oil blend; 80PP70VO, 80 % plant protein and 70 % vegetable oil blend; SM, sphingomyelin; nd, not detected; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; STEROLS, cholesterol and phytosterol.

differences in indispensable amino acids were observed between the 4 and 6 mm feeds[36]. The vegetable oil blend was formulated to mimic fish oil in total SFA, MUFA and PUFA content but with no highly unsaturated *n*-3 PUFA, and this was largely achieved. Replacement of fish oil with the vegetable oil blend resulted in increased percentages of 18 : 3*n*-3, 18 : 2*n*-6 and 18 : 1*n*-9, with concomitant decreased proportions of highly unsaturated *n*-3 PUFA and long-chain monoenoic fatty acids such as 20 : 1 and 22 : 1. These differences were quantitatively greater in the diets with the higher level of fish oil replacement, in diets 40PP70VO and 80PP70VO. In contrast to the replacement of protein sources, substitution of fish oil with the vegetable oil blend had similar effects in the 6 and 4 mm diets. Total levels of monoenoic fatty acids, however, were similar in the 4 mm diets and elevated in the 6 mm plant diets, mainly due to 18 : 1 *n*-9 (Table 3). The ash content of diets was highest in the FMFO diet and decreased with increasing plant protein inclusion (Table 1). Similarly, non-starch, ash, protein or fat DM increased when fish meal was replaced by plant protein and krill meal (Table 1).

Dietary lipid class composition was significantly affected by the raw materials used (Table 4). All phospholipids decreased with decreasing fish meal and fish oil inclusion whereas NEFA concentration increased. Total amount of sterols (cholesterol + phytosterols) in all experimental diets remained the same. The phytosterol composition of the diets is reported elsewhere (BE Torstensen, M Espe and Ø Lie, unpublished results). When quantifying lipid classes by high-performance TLC, the amount of a lipid class is dependent on the number of double bonds in the lipid class. Assuming that the difference in dietary fatty acids (Table 3) is representative also for the phospholipids, approximately 20 % of the reduction in phosphatidylcholine may be due to a reduced number of double bonds.

Whole-body lipid stores – adiposity

High inclusion levels of plant proteins combined with the vegetable oil blend (80PP70VO) resulted in significantly increased whole-body lipids compared with high inclusion of plant proteins together with low vegetable oil (80PP35VO) (Fig. 1(A)). This was not reflected in total fillet lipid level (Fig. 1(B)). Indeed, the visceral somatic index was significantly higher in fish fed 80PP70VO compared with the other dietary groups (Fig. 1(C)). It is well known that fillet and whole-fish lipid level can be positively correlated with fish weight. Fish fed 80PP70VO and 80PP35VO had significantly lower final weight[36] and may therefore have decreased relative fillet and whole-fish lipid level due to this. Therefore all lipid data were presented per kg fish to eliminate this confounding factor.

Liver lipid stores and fatty acid composition

Liver lipid stores (TAG) increased in fish fed the plant ingredient diets differently through the 12 months, with TAG being

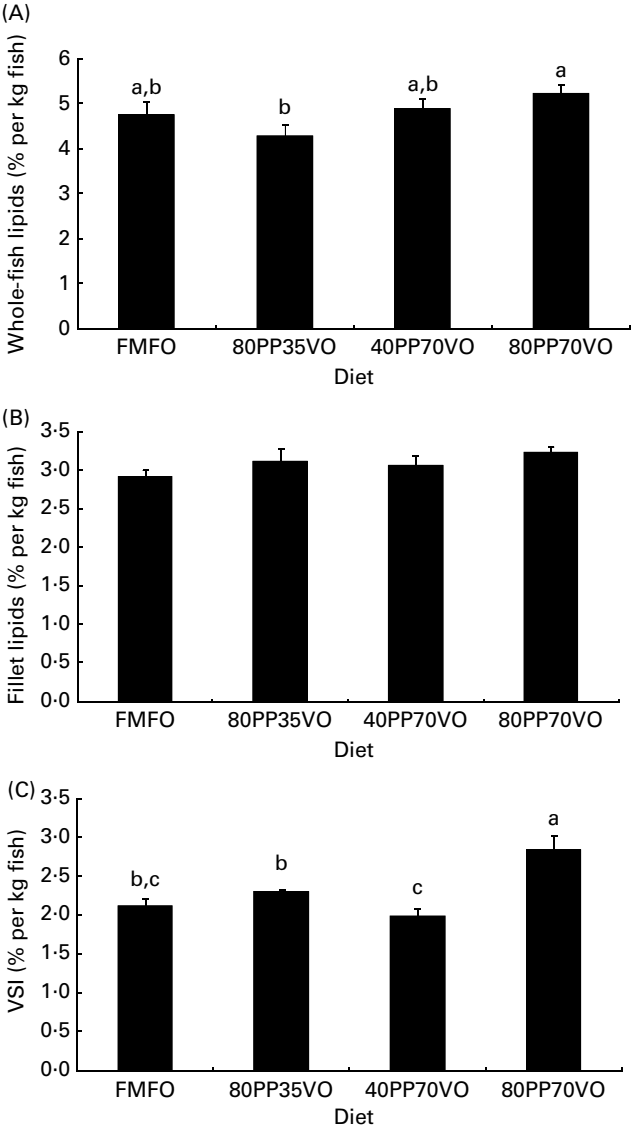

Fig. 1. Whole-fish lipid level (A), fillet lipid level (B) and visceral somatic index (VSI) (C), all corrected for differences in final fish weight, from Atlantic salmon (*Salmo salar* L.) fed either 100 % fish meal and 100 % fish oil (FMFO), 80 % plant proteins and 35 % vegetable oil blend (80PP35VO), 40 % plant proteins and 70 % vegetable oil blend (40PP70VO) or 80 % plant proteins and 70 % vegetable oil blend (80PP70VO) for 12 months. Values are means (*n* 3), with standard deviations represented by vertical bars. [a,b,c] Mean values with unlike letters were significantly different ($P<0·05$; one-way ANOVA).

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more than twofold in the 80PP70VO group compared with the FMFO control after 12 months of feeding (Fig. 2). It is important to note that TAG accumulation was affected differently during the seawater period (Fig. 2). After 3 months, all groups of fish fed diets containing plant protein and vegetable oils showed significantly increased TAG concentration compared with the FMFO-fed fish. After 8 months, however, no significant differences were observed but there were large standard deviations in the 80PP70VO group. After 12 months, the combined high-plant protein and high-vegetable oil group (80PP70VO) showed significant TAG accumulation compared with the FMFO and 80PP35VO groups. Hepatic TAG level of the 40PP70VO-fed fish, however, was in between the other dietary treatments and was not statistically different. Considering that differences in liver fatty acid composition (Table 5) influence the quantification of TAG, TAG in the two 70VO groups would be underestimated by approximately 14 % (due to 14 % fewer double bonds in the livers of 70VO compared with fish oil-fed fish). In this case, the consequence would be even larger differences in TAG level between fish oil- and 35 % vegetable oil (35VO)-fed fish compared with the two groups of 70VO-fed fish.

Liver fatty acid composition was highly influenced by the dietary fatty acid composition after 3, 8 (data not shown) and 12 months of feeding (Table 5). Overall 18 : 1*n*-9, 18 : 2*n*-6 and 18 : 3*n*-3 increased. They were at similar levels in the two 70 % vegetable oil groups whereas the marine *n*-3 fatty acids and 20 : 4*n*-6 were significantly decreased when vegetable oils replaced fish oil (Table 5). In the FMFO diets, 20 : 4*n*-6 was higher (Table 3), whereas the levels of 18 : 2*n*-6 were 6-fold higher in the 70 % vegetable oil diets compared with the fish oil diet and 6-fold higher in the salmon livers. The concentration of 18 : 3*n*-3, however, was almost 9-fold higher in the vegetable oil diets compared with fish oil and 6-fold higher in the salmon livers (Table 5). The 18 : 1*n*-9 content was approximately 3-fold higher in the 70 % vegetable oil diets, whereas 2-fold and significantly higher in the livers of salmon fed the 70VO diets compared with FMFO controls (Tables 3 and 5).

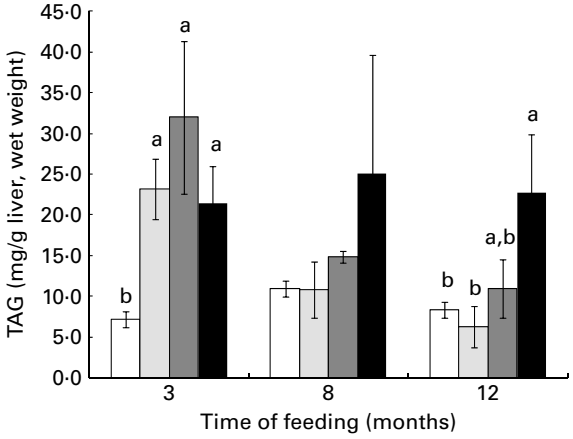

Fig. 2. Liver lipid stores (TAG) from Atlantic salmon (*Salmo salar* L.) fed either 100 % fish meal and 100 % fish oil (□), 80 % plant proteins and 35 % vegetable oil blend (□), 40 % plant proteins and 70 % vegetable oil blend (■) or 80 % plant proteins and 70 % vegetable oil blend (■) for 12 months. Values are means (*n* 3), with standard deviations represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P<0·05$; one-way ANOVA).

Liver nitrogen metabolites

Liver taurine concentration was significantly lower in fish fed the two diets with high plant protein inclusion (diets 80PP35VO and 80PP70VO) at the final sampling after 12 months of feeding. The non-protein-bound glycine, serine, methionine and lysine were not different in fish fed any of the plant diets (Table 5). Liver cystathionine concentration was significantly lower in fish fed the high-plant protein, low-vegetable oil diet (80PP35VO) as compared with the fish fed the total replacement diet (80PP70VO). No significant differences in liver phosphatidylethanolamine concentration were detected in any of the dietary groups.

Plasma and VLDL TAG

Plasma and VLDL TAG significantly increased when both fish meal and fish oil were replaced with maximum levels of vegetable oil (70 %) and 80 % plant protein mix (Fig. 3), being significantly higher after 3 and 12 months of feeding. The two intermediate replacement groups (80PP35VO and 40PP70VO) varied more in plasma and VLDL TAG concentration and with higher levels after 8 months compared with 12 months of feeding. There were no significant differences between 80PP35VO and 40PP70VO and FMFO or 80PP70VO at any of the three sampling periods (Fig. 3). The sampling point after 8 months of feeding exhibited less difference between the groups and no statistical significant differences were observed (Fig. 3).

Expression of genes encoding lipid transport and uptake

The increased plasma TAG in 80PP70VO compared with FMFO was opposite to the expression of the visceral adipose tissue fatty acid binding protein (FABP11), which was down-regulated in visceral adipose tissue of 80PP70VO-fed fish (Fig. 4(A)). The expression of FABP11 was approximately 10-fold higher compared with lipoprotein lipase (LPL) and fatty acid transport protein (FATP) in visceral adipose tissue (Fig. 4(B)). Furthermore, the expression of cd36 was only at trace levels compared with the other lipid uptake and transport proteins measured in visceral adipose tissue (Fig. 4(B)). No changes in the expression of fatty acid uptake genes (LPL, FATP1 and cd36; Fig. 4(B)) were present, which may indicate no difference in the uptake of fatty acids in visceral adipose tissue.

In the liver, the expression of genes related to fatty acid uptake (cd36) and intracellular transport (FABP10) was not significantly affected when fish meal and fish oil were replaced with 80 % plant protein and 70 % vegetable oil (Fig. 5(A) and (B)); however, there seemed to be a trend of increased expression of cd36 and FABP10 in 80PP70VO-fed salmon. The level of expression of FATP1 and cd36 was comparable in liver, whereas FABP10 was expressed 100-fold

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Table 5. Liver fatty acid composition (area %) and nitrogen metabolites (μmol/100 g wet weight) from Atlantic salmon (*Salmo salar* L.) after being fed the four experimental diets for 12 months

(Mean values and standard deviations for three fish per diet)

Diet…	Liver, 12 months							
	FMFO		80PP35VO		40PP70VO		80PP70VO	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
SFA								
14 : 0	2·2[a]	0·2	1·6[b]	0·1	1·1[c]	0·1	1·1[c]	0·1
16 : 0	12·0	0·5	13·1	0·4	11·6	1·3	12·5	0·4
18 : 0	5·6	0·1	5·6	0·3	5·9	0·4	6·4	0·5
20 : 0	0·1	0·1	0·1	0·1	0·2	0·0	0·2	0·1
Sum SFA	22·0	0·3	21·6	0·7	19·7	1·8	20·7	0·8
Monoenes								
16 : 1*n*-7	2·1[a]	0·3	1·6[b]	0·2	1·3[b]	0·3	1·3[b]	0·1
18 : 1*n*-7	2·5[a]	0·1	2·2[b]	0·1	2·2[b]	0·2	2·0[b]	0·1
18 : 1*n*-9	10·5[c]	0·8	16·6[b]	1·1	22·3[a]	6·1	22·7[a]	0·5
18 : 1*n*-11	2·0[a]	0·2	1·4[b]	0·1	0·8[c]	0·1	0·8[c]	0·1
20 : 1*n*-9	4·2	0·2	3·7	0·5	4·0	0·6	3·7	0·2
22 : 1*n*-9	0·3	0·1	0·3	0·0	0·3	0·1	0·4	0·1
22 : 1*n*-11	2·1[a]	0·3	1·4[b]	0·2	0·8[c]	0·1	0·7[c]	0·1
24 : 1*n*-9	0·3	0·1	0·3	0·1	0·3	0·1	0·2	0·1
Sum monoenes	25·5	2·0	28·4	2·0	32·7	7·4	32·6	0·6
n-6								
18 : 2*n*-6	1·3[c]	0·1	5·1[b]	0·1	6·9[a]	0·7	6·7[a]	0·3
20 : 2*n*-6	0·5[c]	0·1	1·4[b]	0·1	2·0[a]	0·1	2·0[a]	0·1
20 : 3*n*-6	0·2[d]	0·0	0·4[c]	0·0	0·6[b]	0·1	0·8[a]	0·1
20 : 4*n*-6	3·5[a]	0·3	2·5[b]	0·2	2·4[b]	0·6	1·9[b]	0·1
Sum *n*-6	5·5[a]	0·3	9·4[b]	0·2	12·0[c]	0·4	11·4[c]	0·5
n-3								
18 : 3*n*-3	0·6[c]	0·0	2·1[b]	0·1	3·3[a]	0·5	2·8[a]	0·2
18 : 4*n*-3	0·4[a]	0·1	0·2[b]	0·1	0·2[b]	0·0	0·2[b]	0·0
20 : 3*n*-3	0·2[c]	0·0	0·6[b]	0·1	1·0[a]	0·1	0·9[a]	0·1
20 : 4*n*-3	1·8[a]	0·1	1·5[b]	0·0	1·4[b]	0·1	1·3[b]	0·1
20 : 5*n*-3	9·6[a]	0·3	7·9[b]	0·2	6·2[c]	1·2	6·9[c]	0·5
22 : 5*n*-3	4·2[a]	0·1	3·2[b]	0·3	2·3[c]	0·6	2·3[c]	0·2
22 : 6*n*-3	25·8[a]	1·4	22·6[a,b]	0·8	19·6[b]	4·2	19·5[b]	0·2
Sum *n*-3	42·5[a]	1·5	38·3[a,b]	1·1	34·0[b]	5·5	33·9[b]	0·3
n-3:*n*-6	7·6[a]	0·2	4·1[b]	0·1	2·9[c]	0·5	3·0[c]	0·1
Remaining fatty acids	4·4	0·1	2·3	0·1	1·7	0·3	1·3	0·1
N metabolites								
Lysine	164[a]	14	137[a]	21	130[a]	7	137[a]	14
Methionine	58[a]	7	58[a]	7	54[a]	6	57[a]	4
Glycine	373[a]	27	386[a]	13	360[a]	13	332[a]	13
Serine	247[a]	29	228[a]	10	200[a]	19	219[a]	19
Cystathionine	15[a,b]	3	7[b]	2	12[a,b]	2	21[a]	2
Phosphatidylethanolamine	35[a]	1	35[a]	3	38[a]	3	33[a]	1
Taurine	1383[a]	24	927[b]	64	1327[a]	16	943[b]	96

FMFO, 100 % fish meal and 100 % fish oil; 80PP35VO, 80 % plant protein and 35 % vegetable oil blend; 40PP70VO, 40 % plant protein and 70 % vegetable oil blend; 80PP70VO, 80 % plant protein and 70 % vegetable oil blend.

[a,b,c,d] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$; one-way ANOVA).

higher. The expression of the gene encoding for apoB100, being a part of the VLDL particle, was significantly up-regulated in the 80PP70VO-fed fish compared with the FMFO-fed fish, while the intermediate groups (80PP35VO and 40PP70VO) were in between (Fig. 5(C)). The expression of LPL in the liver was not affected by dietary plant raw materials (data not shown).

Discussion

Replacing fish meal and fish oil with high levels of a plant protein mixture and vegetable oil blend resulted in increased overall adiposity in Atlantic salmon post-smolt including increased visceral adipose tissue, liver lipids and plasma TAG contents after 12 months of feeding. Intermediate replacement levels (40PP70VO and 80PP35VO) did not have the same effects, indicating that Atlantic salmon metabolism was affected in such a way that the fish developed increased adiposity through interactions between high levels of plant protein and vegetable oil inclusion. To our knowledge, no previous studies have been performed with combined high replacement of fish meal and fish oil in Atlantic salmon focusing on fish lipid metabolism and deposition following a long-term feeding experiment. Thus, the present study has demonstrated for the first time the interaction between plant proteins and vegetable oils in the lipid metabolism of fish. In mammalian models, reports show that either protein or lipid source affects lipid deposition and metabolism[54–58],

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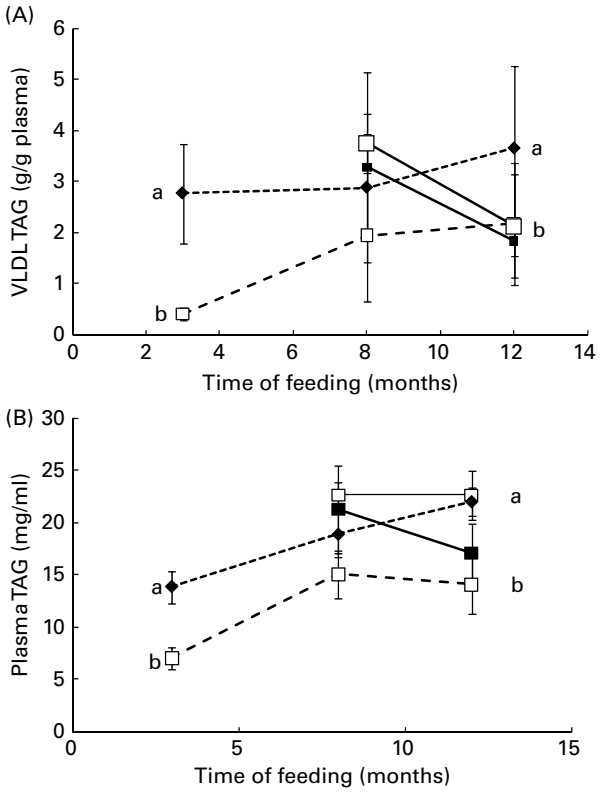

Fig. 3. Plasma TAG (mg/ml) (A) and VLDL TAG (mg TAG/g plasma) (B) after 3 months from Atlantic salmon (*Salmo salar* L.) fed either 100 % fish oil (FMFO; --□--) or 80 % plant proteins and 70 % vegetable oil blend (80PP70VO; --◆--), and after 8 and 12 months from Atlantic salmon fed either FMFO, 80 % plant proteins and 35 % vegetable oil blend (80PP35VO; –□–) or 40 % plant proteins and 70 % vegetable oil blend (40PP70VO; –■–) or 80PP70VO. Values are means (n 3), with standard deviations represented by vertical bars. Statistical differences within each time point were tested by one-way ANOVA. [a,b]Mean values with unlike letters were significantly different ($P<0·05$). No letters were assigned for groups not being significantly different from the other groups (i.e. 80PP35VO and 40PP70VO).

but even in these models the combined effect was very seldom studied[59].

Atlantic salmon fed high levels of both plant protein and vegetable oil seem to store significantly more lipids in visceral adipose tissue. In rodents, fish oil is known to decrease adiposity through reducing the adipocyte cell size and thereby improving insulin sensitivity and decreasing the release of NEFA[60]. Atlantic salmon adipocytes decrease their lipid accumulation when stimulated with marine n-3 fatty acids compared with 18 : 1n-9 *in vitro* [61] and this negatively correlated with FATP1 expression, indicating a role of FATP1 in adipocyte lipid accumulation[62]. The suppression of TAG accumulation by n-3 PUFA has also been reported in studies with 3T3-L1 pre-adipocytes[63], and in mammalian studies where n-3 PUFA limits the hypertrophy of fat depots compared with high-fat diets containing SFA[64]. Hence, in several rodent models n-3 PUFA decreased adipocyte differentiation[65] and reduced fat accumulation[66–69]. However, although differences in dietary n-3 PUFA levels may be an important factor in the development of visceral adipose lipid accumulation also in Atlantic salmon, dietary fatty acids alone cannot explain the lipid accumulation in the 80PP70VO group since 40PP70VO had the same dietary fatty acid composition and fish oil level but not the same visceral lipid accumulation. The present increased visceral lipid levels in 80PP70VO-fed salmon indicate that although known requirement levels were met in the current diets, combined decreased levels of fish oil together with low fish meal levels induce metabolic changes resembling lysine deficiency[30] and/or n-3 PUFA deficiency at dietary levels considered adequate based on current knowledge[39]. Thus, adequate dietary levels of one critical nutrient (for example, methionine and lysine or taurine or EPA and DHA) to avoid the development of fatty liver and or reduce visceral adiposity may be underestimated when other critical nutrients (for example, methionine or EPA and DHA) are available. This synergistic effect when high levels of plant proteins and vegetable oils replace fish meal and fish oil clearly shows the importance for nutrient requirement studies taking nutrient interaction effects into account when marine ingredients are replaced.

Visceral fat levels increased, whereas the expression of the gene encoding for FABP11 decreased in visceral adipose tissue when salmon were fed 80PP70VO. Down-regulation of FABP11 has also previously been reported in visceral adipose tissue and myosepta of Atlantic salmon fed a 100 % vegetable oil blend[11]. In mammals it is well established that the adipocyte FABP (AFABP/aP2) forms a physical complex with hormone-sensitive lipase that affects basal and

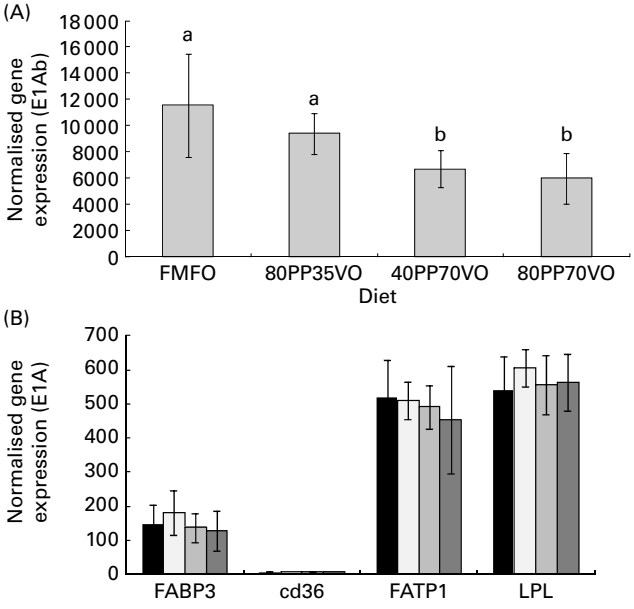

Fig. 4. Normalised gene expression levels (normalised against the reference gene elongation factor 1α_B (EF1A$_B$)) of fatty acid binding protein 11 (A) and of fatty acid binding protein 3 (FABP3), cd36, fatty acid transport protein 1 (FATP1) and lipoprotein lipase (LPL) (B) in visceral adipose tissue from Atlantic salmon (*Salmo salar* L.) fed either 100 % fish meal and 100 % fish oil (FMFO; ■), 80 % plant proteins and 35 % vegetable oil blend (80PP35VO; □), 40 % plant proteins and 70 % vegetable oil blend (40PP70VO; ▨) or 80 % plant proteins and 70 % vegetable oil blend (80PP70VO; ▩) for 12 months. Values are means, with 95 % CI represented by vertical bars. [a,b]Mean values with unlike letters were significantly different ($P<0·05$; one-way ANOVA).

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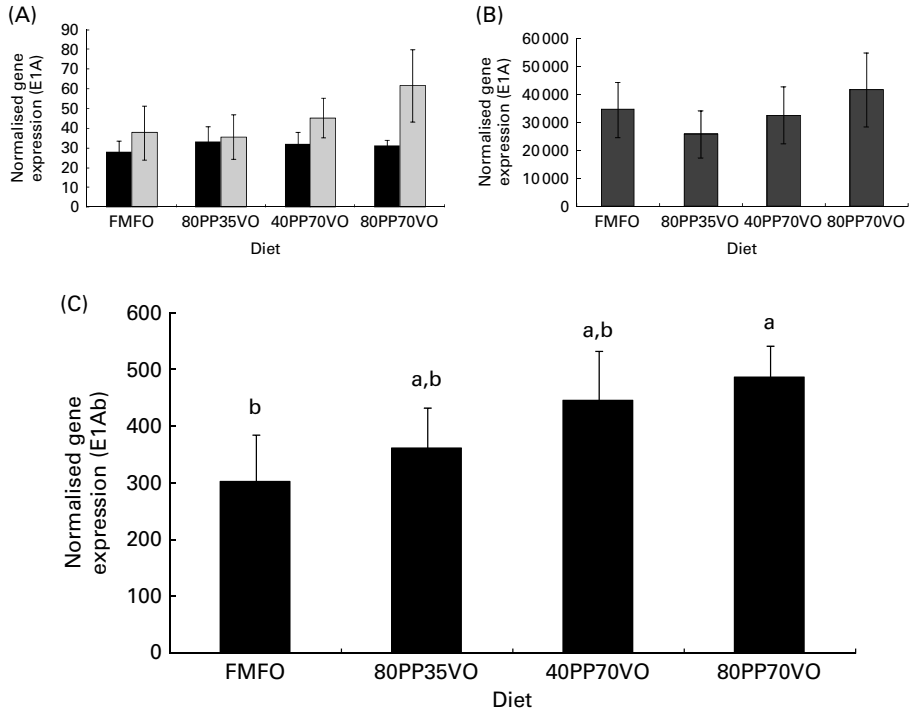

Fig. 5. Normalised gene expression levels (normalised against the reference gene elongation factor $1\alpha_B$ ($EF1A_B$)) of fatty acid transport protein 1 (■) and cd36 (□) (A), fatty acid binding protein 10 (B) and apoB100 (C) in the liver from Atlantic salmon (*Salmo salar* L.) fed either 100 % fish meal and 100 % fish oil (FMFO), 80 % plant proteins and 35 % vegetable oil blend (80PP35VO), 40 % plant proteins and 70 % vegetable oil blend (40PP70VO) or 80 % plant proteins and 70 % vegetable oil blend (80PP70VO) for 12 months. Values are means, with 95 % CI represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P<0{\cdot}05$; one-way ANOVA).

hormone-stimulated adipocyte fatty acid efflux[70,71] and specifically that knock-down of AFABP in mice resulted in decreased adipocyte fatty acid efflux[71]. Although not yet studied in Atlantic salmon, the adipocyte-specific FABP in Atlantic salmon (FABP11)[11,72] may have a similar function of regulating the adipocyte efflux of NEFA. The down-regulation of FABP11 expression, but not of the genes encoding for fatty acid uptake (FATP1, LPL, cd36), indicate that the mechanism of increased lipid level in the visceral adipose tissue of 80PP70VO-fed salmon was mainly decreased efflux of lipids from the adipose tissue rather than increased uptake.

Atlantic salmon fed high levels of both plant protein and vegetable oil had significantly increased plasma, VLDL and liver TAG levels. In contrast, completely replacing fish oil with vegetable oils in diets with high dietary fish meal inclusions was reported to have no effect on plasma and lipoprotein TAG concentration in Atlantic salmon, but significantly increased liver TAG[9] and especially so at low water temperatures[10]. This was confirmed in the 40PP70VO group of the present study, but only after 3 months of feeding. Importantly, the liver TAG stores developed during the 12 months' experiment, with all fish meal and fish oil replacement groups having significantly higher hepatic TAG levels after 3 months, whereas after 8 months no significant differences appeared due to high variation in the 80PP70VO group. After 12 months the liver TAG concentration from fish fed 80PP70VO was almost twofold higher than the other dietary groups, being statistically different from 80PP35VO- and FMFO-fed fish. Ruyter *et al.*[10] demonstrated the importance of water temperature for liver TAG storage when fish oil was replaced by soyabean oil. However, no increase in liver lipids was detected at 12°C but was present at 5°C. The present experiment was performed at a constant temperature at 9°C, which may explain the initial effects in the 80PP35VO and 40PP70VO groups that stabilised after 8 and 12 months of feeding. In the 80PP70VO-fed salmon, however, hepatic TAG accumulation was not reversed and was continuously increased also after 12 months of feeding at 9°C, indicating a synergistic effect of plant proteins and vegetable oil resulting in a metabolic imbalance which was independent of temperature.

Also, limitations in the indispensable amino acids lysine and methionine affect the lipid stores in post-smolt Atlantic salmon[30,34,35]. Suboptimal dietary levels of methionine were reported to increase liver TAG concentration, fatty acid synthase activity and the 18 : 1:18 : 0 fatty acid ratio in Atlantic salmon, but had no impact on plasma TAG[34]. Lysine limitation in post-smolt Atlantic salmon did not affect plasma or liver TAG or the fatty acid composition. Carcass lipid, however, increased upon lysine limitation[30], whereas neither liver nor muscle fat increased[35], indicating increased viscera adipose stores. Dietary levels of indispensible amino acids, including lysine and methionine, in the present study were similar in all diets and all were above defined requirements for on-growing Atlantic salmon[36]. Obviously, the arbitrary voluntary feed intake will affect the availability of nutrients for general metabolism. As reported by Torstensen *et al.*[36], Atlantic salmon fed 80PP70VO had decreased voluntary feed

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intake and thus growth during the first 3 months of the experiment, resulting in significantly lower final body weight (about 17 % lower than FMFO-fed fish). However, the voluntary feed intake was equal in all treatment groups thereafter until the final sampling, indicating that it was not lower intake of indispensable amino acids that could explain the increased liver TAG at the end of the experiment. We have found that liver concentrations of neither free methionine[34] nor lysine[35] were affected by limitations in these amino acids. However, postprandial plasma concentration after 5 h was significantly affected by both lysine and methionine limitations[34,35]. In the present study plasma free lysine was about half in the fish meal and fish oil replacement groups as compared with the FMFO-fed fish, but no differences between any of the replacement diets were present (FMFO, 410 μmol/l; 80PP35VO, 40PP70VO and 80PP70VO, range 210–240 μmol/l). On the other hand, only the fish fed the 80PP35VO diet had significantly less plasma free methionine (140 μmol/l) as compared with 80PP70VO- (250 μmol/l) and FMFO-fed fish (190 μmol/l). Therefore it is unlikely that deprivation of indispensable amino acids explains the fat accumulation in the liver of fish fed 80PP70VO. However, taurine concentration was lower in both diets containing the low level of fish meal inclusion (80PP). Although Atlantic salmon has the capacity to synthesise taurine through trans-sulfuration[73], liver taurine concentration was still significantly lower in fish fed these diets after 12 months of feeding. Cystathionine concentration in liver, the first metabolite following the rate-limiting enzyme in trans-sulfuration[74], was also affected; however, it was only significantly decreased in fish fed 80PP35VO. This may indicate that trans-sulfuration was affected in fish fed the diets containing high fish meal replacement. Dietary and liver taurine was significantly decreased when fish meal was replaced by plant proteins. Taurine is conjugated to bile acids in fish and improves the export of bile from the liver, and as such is linked to both the digestibility and clearance of cholesterol[75–77]. Deficiency of taurine has been reported to decrease the total bile acid excretion in faeces[78]. Increasing the bile acid concentration by dietary cholic acid in mice has been reported to decrease liver and plasma TAG concentrations[79]. The intake of protein sources high in taurine has been linked to reduced visceral lipid accumulation in rodent models[55]. However, taurine alone through reduced bile acid cannot explain the increased fat in 80PP70VO-fed fish, since 80PP35VO-fed salmon had significantly less whole-fish and visceral fat levels but the same dietary and liver taurine levels. Although methionine limitation reduced plasma and faecal bile acid concentrations in post-smolt Atlantic salmon, the reduction did not reach statistical difference[80]. Also, enrichment with crystalline DL-methionine did not increase plasma bile acids significantly in post-smolt Atlantic salmon[34]. In juvenile Atlantic salmon, on the other hand, addition of taurine reduced the whole-body lipid:protein ratio[81]. Since the two dietary groups where fish meal was replaced by 80PP had the same dietary amino acid levels but not the same response regarding whole-body and visceral fat levels, it is not likely that differences in bile acid concentration alone explain the increased visceral mass in the present study. Overall, these results indicate that at low EPA and DHA, combined with possible suboptimal levels of amino acids, the requirements of lysine and methionine increase compared with plant protein diets in which fish oil constitutes the lipid source[34]. However, since no difference in the liver 18 : 1:18 : 0 ratio was observed, firm conclusions cannot be drawn, and further studies are required to elucidate the potential role of dietary amino acids on increased liver TAG in fish meal and fish oil replacement diets.

The three replacement diets contained about half the amount of phospholipids as compared with the FMFO control diet. The lower phosphatidylcholine may interact with protein metabolism because during low dietary choline or phosphatidylcholine intake the animals are able to synthesise phosphatidylcholine from phosphatidylethanolamine through three successive methylation reaction in the liver[82,83]. In the present study liver phosphatidylethanolamine was similar in all groups, indicating similar phosphoethanolamine synthetase (pemt) activity and hence endogenous synthesis of phosphatidylcholine. However, in the present study neither phosphoethanolamine synthetase activity nor *S*-adenosylmethionine or choline status was analysed. On the other hand, we have previously reported that methionine availability affected liver *S*-adenosylmethionine[73] and reduced liver pemt activity (M Espe, EM Hevrøy and B Liaset, unpublished results). Furthermore, phospholipid deficiency and particularly choline deficiency are well-known causes of hepatic lipid accumulation and decreased plasma VLDL levels in rodents[84], especially when methionine is also low[85–87]. The phospholipid requirement has not been established for adult fish, but is considered to be essential for optimal growth and development at juvenile stages[88]. Thus, the increased TAG accumulation in 80PP70VO-fed salmon may be due to an interaction also with dietary phospholipids. However, a classical choline deficiency decreases plasma VLDL and hence plasma TAG, which was the opposite of the effect on plasma and VLDL TAG in 80PP70VO-fed fish. Hence, decreased dietary phospholipids may play a role together with other amino acids and fatty acids in the overall increase in liver TAG and VLDL TAG.

Increases in plasma and VLDL TAG levels were found in both groups of Atlantic salmon fed diets where 80 % of the fish meal protein was replaced by the plant protein mix; these increases were observed most profoundly and consistently in the 80PP70VO group. Several studies in human subjects have shown that dietary EPA and DHA decrease plasma TAG[24,25] and protect against CHD[26–27]. More recently it was demonstrated that high dietary EPA and DHA decreased hepatic TAG secretion in Atlantic salmon compared with a standard fish oil diet and with a rapeseed oil-based diet[89]. In mammals fish oil has been demonstrated to interfere with assembly of the initial VLDL precursor particles in hepatocytes[21,90,91], all supporting that increased plasma TAG in the 80PP70VO-fed salmon may be a result of increased secretion of TAG-rich VLDL particles from the liver in the post-absorptive phase regulated by dietary fatty acid composition. The assembly process of hepatic VLDL in mammals is recognised to be initiated in the endoplasmic

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reticulum as soon as apoB100 is translated and translocated into the lumenal side where the elongating apoB100 polypeptide chain recruits various lipids co-translationally (for a review, see Sundaram & Yao[92]). Interestingly, the gene encoding for apoB100 was increasingly expressed when the inclusion of vegetable oil and plant proteins increased. Furthermore, although not statistically significant, there was a trend towards increased fatty acid uptake and intracellular transport of fatty acids through the increased RNA expression of cd36 and FABP10 in the livers of 80PP70VO-fed Atlantic salmon. In summary, this indicates a more active hepatic fatty acid turnover, accumulation and transport of neutral lipids in high-plant protein- and vegetable oil-fed fish compared with the traditional fish oil- and fish meal-fed fish as well as the lower replacement level diets. Previous studies replacing fish oil with vegetable oil but not replacing dietary fish meal have reported no changes in plasma or VLDL TAG[9], confirming the findings of Kjær *et al.*[89] of no differences in hepatic TAG secretion when comparing fish oil and rapeseed oil-fed fish. In post-smolt Atlantic salmon, limitation in either lysine or methionine had no significant impact on plasma TAG[34,35]. In European seabass fed plant protein-based diets, plasma TAG was reduced as compared with fish meal-fed fish[6]. Again, significantly and consistently increased plasma and VLDL TAG levels in the combined high-plant protein- and vegetable-oil-fed fish demonstrate an interaction between dietary lipids and proteins with lipid metabolic consequences.

Intestinal lipid droplets have been reported to appear in vegetable oil-fed fish[10,93–95], which may be due to a slower transport of lipids from the intestinal cells to the circulation. Hence, the increased plasma TAG observed in 80PP70VO-fed salmon may be due to a slower lipid uptake detected 48 h after the last meal. Furthermore, the apparent digestibility of 16 : 0 was 16 % lower in 80PP70VO-fed fish compared with the other three groups[36], possibly slightly decreasing the lipid uptake. Plasma TAG was also analysed 6 h after the last meal, revealing no significant differences (data not shown). This, together with the discrepancy in effect between the two high vegetable oil groups, indicates that increased plasma TAG was not solely due to a possible increased intestinal lipid accumulation in vegetable oil-fed fish.

In mammals, the metabolic syndrome is related to increased central (visceral) adiposity, increased plasma TAG, decreased HDL-cholesterol, hypertension, glucose intolerance and type 2 diabetes, which together are well-documented risk factors for CVD[96]. In conclusion, Atlantic salmon fed 80PP70VO demonstrated increased visceral adiposity, increased plasma and VLDL TAG as well as increased hepatic TAG stores and decreased plasma HDL-cholesterol levels (BE Torstensen, M Espe and Ø Lie, unpublished results). Compared with the metabolic syndrome in mammals, these changes in lipid metabolism in 80PP70VO-fed Atlantic salmon together indicate a metabolic imbalance that may affect fish health and especially cardiovascular health. These issues will be investigated in further studies.

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There are no conflicts of interest to report.

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