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Effect of diets low in fish oil and supplemented with chlorogenic acid on fatty acid composition and lipid metabolism in Atlantic salmon (*Salmo salar* L.)

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Abstract

This study aimed to test the use of chlorogenic acid (CGA), a natural antioxidant, which might act improving the retention of eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids. Fish were fed one of three experimental diets. A diet with 160 g kg^{-1} fish oil (FO) and 80 g kg^{-1} vegetable oil (VO) was used as a positive control with high n-3 long-chain polyunsaturated fatty acids (LC)-PUFA, a diet containing 40 g kg^{-1} FO and 200 g kg^{-1} VO was used as a negative control for low n-3 LC-PUFA and 1 g kg^{-1} CGA was added to the negative control diet to test the impact of CGA on n-3 LC-PUFA synthesis. The expression of genes involved in PUFA synthesis was upregulated in the liver of fish fed the low n-3 LC-PUFA diets. This might be due to the activation of sterol regulatory element-binding transcription factor 1 (SREBP1) and liver X receptor. Moreover, malondialdehyde concentration tended to decrease, and accumulation of dietary EPA in the liver in relation to diet was observed in fish fed the low n-3 LC-PUFA diets. However, the concentration of EPA and DHA was higher in fish fed the positive control diet. The inclusion of 1 g kg^{-1} CGA did not exert any additional effect on lipid peroxidation, EPA and DHA concentrations or LC-PUFA metabolism.

KEY WORDS: antioxidants, chlorogenic acid, gene expression, omega 3 fatty acids

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Introduction

In times of a growing world population and rising life expectancy, demand for healthy but affordable food is increasing (FAO, 2014). The consumption of fish as a source of both protein and fatty acids has been constantly rising over the last years. In 2011, it reached a maximum of approximately 19 kg per person worldwide (FAO, 2014). Atlantic salmon (*Salmo salar* L.) is a rich source of the omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). They are important nutrients with reported anti-inflammatory effects and may prevent cardiovascular diseases (Hu *et al.* 2002; Calder 2012; Calder & Yaqoob 2012; Minihane 2013). Fish oil (FO) is the main dietary source of EPA and DHA for farmed salmon. However, it is a finite resource and, because of increasing market demand, is becoming rather unavailable raw material for fish feed which leads to rising prices (Sørensen *et al.* 2011). The possibility of replacing FO partially or completely with vegetable oil (VO) in fish diets has been evaluated in various studies showing that whereas growth, health and feed utilization were not majorly affected, fish fed VO had significantly lower levels of EPA and DHA (Torstensen *et al.* 2000; Bell *et al.* 2001; Regost *et al.* 2003; Menoyo *et al.* 2005). Thus, other nutritional strategies remain to be explored to maximize EPA and DHA contents in Atlantic salmon. A strategy to increase n-3 LC-PUFA content in fish fillet could be fish feed supplementation with bioactive substances to protect n-3 LC-PUFAs from lipid peroxidation or to enhance endogenous n-3 LC-PUFA synthesis.

Interestingly, dietary inclusion of polyphenols may enhance EPA synthesis from C18:3n-3 as could be shown

in rats (Toufektsian *et al.* 2011; de Lorgeril & Salen 2012; Ounnas *et al.* 2014). The polyphenol chlorogenic acid (CGA) is an ester of caffeic acid and quinic acid present in coffee and various other plants, such as apple and tomato (Strack & Gross 1990; Setorki *et al.* 2009; Sato *et al.* 2011). In mice and rats, CGA positively affected oxidative stress levels as it enhanced antioxidant enzyme activity and decreased malondialdehyde (MDA) levels (Wang *et al.* 2012; Chen *et al.* 2013). Furthermore, *in vitro* it showed dose-dependent radical scavenger activity (Xu *et al.* 2012). The antioxidant capacity of CGA may delay the oxidation of lipids and thereby increase the quality of the fillet.

Chlorogenic acid inclusion seems to affect lipid metabolism. As part of a high-fat diet, it increased the expression of the peroxisome proliferator-activated receptor α (PPARα) in rats and mice (Cho *et al.* 2010; Wan *et al.* 2010). PPARα regulates lipid metabolism and is activated under conditions of limited nutrient supply. It not only promotes β-oxidation of lipids but also induces n 3 LC-PUFA syntheses via transcriptional control of fatty acid elongases and desaturases (Rakhshandehroo *et al.* 2007). Mice and hamsters on a CGA-supplemented diet showed lower fat levels in serum and liver (Li *et al.* 2009; Cho *et al.* 2010). In contrast, the addition of 1 g kg^{-1} CGA to a high-fat diet enhanced hepatic lipid levels in mice and decreased fatty acid oxidation (Mubarak *et al.* 2013). Interestingly, the dose used by Mubarak *et al.* was about five times higher than the drug used in previous studies which may explain the contradicting data on CGA and lipid accumulation.

Although direct deposition of EPA and DHA via diet leads to higher n-3 LC-PUFA contents in fish fed FO diets than in fish fed VO diets, the retention of EPA and DHA decreases with rising FO content. This occurs because, when fed at high concentrations, LC-PUFAs are used as energy-yielding substrates (Stubhaug *et al.* 2007; Carmona-Antoñanzas *et al.* 2014). Furthermore, when high FO diets are compared to low FO diets, EPA and DHA synthesis are decreased in the fish (Sørensen *et al.* 2011). Thus, the adequate blend of VO and FO to optimize EPA and DHA concentrations in the fish by dietary intake and *de novo* synthesis needs to be evaluated. As CGA was shown to modulate fatty acid metabolism and may promote fatty acid elongation and desaturation (de Lorgeril & Salen 2012), its combination with dietary VO may be a promising feeding strategy to increase the n-3 LC-PUFA content in salmon fed a low FO diet. As 1 g kg^{-1} CGA as part of a high-fat diet increased lipid levels in mice (Mubarak *et al.* 2013), we chose this concentration for our feeding study in Atlantic salmon.

To increase our knowledge on lipid and LC-PUFA homeostasis in Atlantic salmon, we analysed key lipid regulators such as liver X receptor (LXR), sterol regulatory element-binding transcription factor 1 (SREBP1) or PPARα in fish fed diets with low FO content with and without CGA and high FO content.

To our knowledge, there are no studies evaluating the impact of dietary CGA on Atlantic salmon and fish in general. Due to the positive effects of CGA in mammals, it is of high interest to test its impact in fish.

Materials and methods

Experimental animals

The feeding trial was performed at Skretting Aquaculture Research Center facilities (Lerang Research Station, Jorpeland, Norway) following the Norwegian Animal Research Authority (FDU) guidelines. The experiment was conducted according to Norwegian legislation and approved by The Norwegian animal research authority (NARA/FDU). The scientists performing the samplings were accredited by the Federation of Laboratory Animal Science Associations (FELASA). One hundred and eighty Atlantic salmon postsmolt weighing 137.4 ± 0.9 g were randomly and equally distributed into six tanks and provided with three experimental diets (two tanks per diet). The tanks were supplied with sea water, and the animals were kept at 12.2 ± 0.2 °C.

Experimental diets

Three experimental diets were formulated according to the nutritional requirements of Atlantic salmon (NRC 2011) and produced at Skretting Aquaculture Research Center (Stavanger, Norway). From a common basal composition (Table 1), diets differed in the oil blend and the CGA addition. A diet with 160 g kg^{-1} FO and 80 g kg^{-1} VO (H) was used for high n-3 LC-PUFA fed fish as a positive control, a diet containing 40 g kg^{-1} FO and 200 g kg^{-1} VO (L) was used for low n-3 LC-PUFA fed fish as a negative control, and 1 g kg^{-1} CGA supplied by Stanford Materials Corporation (Irvine, CA, USA) was added to the L diet (LCGA) to test the impact of CGA on n-3 LC-PUFA synthesis in fish fed a high-fat diet low in EPA and DHA. This concentration of CGA is comparable with the dietary concentration of plant bioactives used in other

Table 1 Ingredient and analysed chemical composition of the experimental diets

Item	H	L	LCGA
Ingredients (as fed basis g kg^{-1})			
Wheat	50.0	50.0	49.0
Wheat gluten	170.0	168.1	168.1
Faba beans dehulled	84.4	93.8	93.8
Soy protein concentrate	310.9	310.0	310.0
Fishmeal NA	100.0	100.0	100.0
Palm oil	–	27.1	27.1
Linseed oil	–	11.7	11.7
Rapeseed oil	85.7	163.0	163.0
Fish oil NA	161.4	38.9	38.9
Chlorogenic acid	–	–	1.0
Astaxanthin 10%	0.4	0.4	0.4
Vitamin and mineral mix	37.1	37.1	37.1
Analysed composition (g kg^{-1})			
Moisture	64.0	66.0	63.0
Total fat	272.0	267.0	262.0
Crude protein	458.0	456.0	454.0
Ash	49.0	46.0	54.0
Fatty acids (% of total fatty acids)			
16:0	10.3	10.3	10.2
∑ saturates	17.3	15.3	15.1
18:1n-9	32.6	41.2	40.9
∑ monoenes	43.6	49.1	48.9
18:2n-6	14.5	17.4	18.3
∑ n-6	15.9	18.0	18.8
18:3n-3	5.8	7.6	8.3
20:5n-3	4.3	1.9	1.6
22:6n-3	3.5	1.9	1.4
∑ n:3	15.9	12.4	12.2
n-6:n-3	0.9	1.4	1.5

H, high in fish oil; L, low in fish oil; LCGA, L diet supplemented with 1 g kg^{-1} chlorogenic acid.

feeding trials in fish (D'Souza *et al.* 2005), rats and pigs (De Boer *et al.* 2005; Chougala *et al.* 2012). Ingredients, analysed composition and fatty acid profile are shown in Table 1.

Sample collection

Fish were fed the experimental diets for 16 weeks. Prior to sacrifice, fish were deprived of feed for 12 h, anesthetized with Tricaine Pharmaq (Pharmaq Limited, Pharmaq Limited, UK) and then killed by a blow to the head. Four fish per tank were sacrificed for sampling (eight per diet) to run all the laboratory analysis. Individual samples of liver and fillet were collected, snap-frozen and stored at −80 °C for fatty acid and MDA analysis. For gene expression analysis, approximately 1 g of liver and mucosal scrapings from the pyloric caecum was placed in RNA later (Thermo Fisher Scientific, Madrid, Spain) and stored at −80 °C.

Analysis of the experimental diets

Moisture, total fat, protein and ash in the feeds were analysed using near-infrared reflectance methodology as previously described (Torstensen *et al.* 2008). The fatty acid composition of the experimental diets was determined according to Grahl-Nielsen & Barnung (1985) using gas chromatography and flame ionization detection.

Analysis of tissue fatty acids and MDA

Salmon fillet and liver lipid extraction, and fatty acid methyl esters (FAMEs) were analysed by gas chromatography following the method of Segura & López-Bote (2014). Briefly, lyophilized samples (200 mg) were homogenized in dichloromethane : methanol (8 : 2, by vol.) using a mixer mill (MM400; Retsch technology, Stuttgart, Germany). The final biphasic system was separated by centrifugation. The extraction was repeated three times. Solvent was evaporated under nitrogen stream, and lipids were dried by vacuum desiccation. Total lipid content was determined gravimetrically. FAMEs were prepared from total lipids by transesterification using a mixture of sodium methylate–methanol and methylated in the presence of sulphuric acid. FAMEs were separated using a gas chromatograph (HP 6890 Series GC System; Hewlett Packard Co., Avondale, PA, USA) equipped with flame ionization detector. Separation was performed with a J&W GC Column, HP-Innowax polyethylene glycol (30 m × 0.316 mm × 0.25 μm). Nitrogen was used as a carrier gas.

HPLC analysis of MDA was performed in the liver according to Faizan *et al.* (2014). Briefly, fish liver was homogenized in 1% sulphuric acid, and addition of 0.2 volumes 6 M NaOH was followed by a 30-min incubation at 60 °C. Proteins were then precipitated with perchloric acid, and after centrifugation, the supernatant was incubated with 2,4-dinitrophenylhydrazine and injected into a Jasco HPLC system. Using an isocratic mode with a mobile phase consisting of 0.2% acetic acid (v/v) in water/acetonitrile (42 : 58, v/v), the samples were separated on a Supelco INC water spherisorb ODS2 column (10 cm × 4.6 mm, 3 μm). MDA was analysed at 310 nm.

Gene expression analysis

Total RNA extraction from salmon liver and pyloric caecum tissue samples was realized with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich Corporation, St. Louis, MO, USA) following the manufacturer's instructions. Tissues were disrupted in a mixer mill MM-400

(Retsch). The A_{260} was used to calculate the RNA concentration, and the RNA quality was confirmed with a bioanalyzer (Agilent 2100; Agilent Technologies, Madrid, Spain) showing an average RIN value of 7.1.

Reverse transcription was performed using the SuperScript VILO Master Mix (Invitrogen). qRT-PCR was carried out using the SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA) in a 7300 Real Time PCR System (Applied Biosystems). The relative gene expression of nine target genes and two control reference genes (Table 2) was analysed according to previous publications: the housekeeping genes β-actin and eukaryotic translation elongation factor 1 alpha (EF-1α) were assayed as described by Leaver *et al.* (2008). Genes involved in LC-PUFA metabolism: fatty acid desaturase 6 (Δ6fad) following Leaver *et al.* (2008), fatty acid desaturase 5 (Δ5fad) fad and elongase 2 (Elov2) according to Morais *et al.* (2012) and elongase 5a (Elov5a) according to Minghetti *et al.* (2011). Genes involved in fatty acid transport and oxidation: cluster of differentiation 36 (CD36) was assayed according to Schiller Vestergren. *et al.* (2012), and carnitine palmitoyltransferase 1 (CPT1) and acyl CoA oxidase (ACO) were assayed as described by Leaver *et al.* (2008). Transcription factors involved in lipid metabolism: LXR was assayed as described in Minghetti *et al.* (2011) and SREBP1 and PPARα according to Menoyo *et al.* (2014).

Calculations

The specific growth rate (SGR; %/day) was calculated as 100 × [ln (final BW, g) − ln (initial BW, g)]/days. The fold difference (Δ) between diet and tissue fatty acid values in each experimental group was calculated as (%fatty acid in the liver − %fatty acid in the diet)/(%fatty acid in the diet).

Statistical analysis

Analyses were performed using the mixed-model procedure of SAS (release 9.2; SAS Institute Inc., Cary, NC, USA). The model included the diet as a fixed effect and the tank was considered as a random effect. Differences between experimental diets were analysed by non-orthogonal contrasts, as follows: (i) H versus L, (ii) H versus LCGA and (iii) L versus LCGA. Changes in gene expression resulting from the comparison of the experimental groups L or LCGA relative to the control (H) were determined using a mixed-effects model in which treatment was considered as a fixed effect and the tank as a random effect (Steibel *et al.* 2009). In the model, the residual variance was heterogeneous for the gene effect (Salces-Ortiz *et al.* 2013). For genes displaying efficiencies different from 2 ($E \neq 2$), C_t

Table 2 Genes, accession numbers, forward (Fw) and reverse (Rv) primers used for quantitative PCR and amplification efficiencies in salmon selected tissues

Genes	Accession numbers	Primer sequence Fw	Primer sequence Rv	Amplification Efficiency liver	Amplification Efficiency pyloric caecum	References
Δ6 fad	AY458652	CCCCAGACGTTTGTGTCAG	CCTGGATTGTTGCTTTGGAT	2.01	2.03	Leaver *et al.* (2008)
ACO	DW555420	AAAGCCTTCACCACATGGAC	TAGGACACGATGCCACTCAG	2.06	1.92	Leaver *et al.* (2008)
β-actin	AF012125	ACATCAAGGAGAAGCTGTGC	GACAACGGAACCTCTCGTTA	1.92	1.98	Leaver *et al.* (2008)
EF-1α	AF321836	CTGCCCCTCCAGGACGTTTCAA	CACCGGGCATAGCCGATTCC	1.93	1.93	Leaver *et al.* (2008)
Δ5 fad	AF478472	GTGAATGGGGATCCATAGCA	AAACGAACGGACAACCAGA	1.94	1.85	Morais *et al.* (2012)
Elov2	TC91192	CGGTACAAAATGTGCTGGT	TCTGTTTGCCGATAGCCATT	2.05	1.95	Morais *et al.* (2012)
Elov5a	AY170327	ACAAGACAGGAATCT CTTTCAGATTAA	TCTGGGGTTACTGTGC TATAGTGTAC	1.91	1.94	Minghetti *et al.* (2011)
LXR	FJ470290	GCCGCCGCTATCTGAAATCTG	CAATCCGGCACCAATCTGTAGG	1.97	2.04	Minghetti *et al.* (2011)
CD36	AY606034	GGATGAACTCCCTGCATGTGA	TGAGGCCAAAGTACTCGTCGA	2.09	1.99	Schiller Vestergren *et al.* (2012)
SREBP1	NM_001195818	CACTACTAGCCCCATGTTTTGATTG	CAGCCACTCTCTAAACACACCAA	2.13	2.09	Menoyo *et al.* (2014)
PPARα	AM230809	GCTCCTTGGATGTCCCTGAGT	GCATCTAGAACGGTGGATCCTT	1.95	2.08	Menoyo *et al.* (2014)
CPT1	AM230810	CCTGTACCGTGGAGACCTGT	CAGCACCTCTTTGAGGAAGG	2.04	2.05	Leaver *et al.* (2008)

Δ6 fad, fatty acid desaturase 6; *ACO*, acyl CoA oxidase; *EF-1α*, eukaryotic translation elongation factor 1 alpha; *Δ5 fad*, fatty acid desaturase 5; *Elov2*, elongase 2; *Elov5a*, elongase 5a; *LXR*, liver X receptor); *CD36*, cluster of differentiation 36; *SREBP1*, sterol receptor binding protein 1; *PPARα*, peroxisome proliferator-activated receptor alpha; *CPT1*, carnitine palmitoyltransferase 1.

values were adjusted according to the model described by Steibel *et al*. (2009). The expression of target genes was normalized with the geometric mean of the reference genes β-actin and EF-1α according to Pfaffl *et al*. (2004). The standard error (SE) was used to recalculate the lower and upper 95% confidence intervals for each fold change.

Results

Growth, tissue fatty acid composition and lipid peroxidation

After 12 weeks of feeding, a poor performance was detected for all the fish, showing on average a SGR of 1.18 ± 0.06 and a feed conversion ratio (FCR) of 0.84 ± 0.02. This was because of a natural infection of the salmon with the parasite Ichthyobodo. Fish were then treated with formalin (30-min bath in 1 : 4000 ppm) and fed the experimental diets for another 4 weeks when sampling took place. After the formalin treatment, animal performance was good and there were no differences in final body weight (FBW), SGR or FCR between the three dietary treatments (Table 3).

Saturated and monounsaturated fatty acids in salmon fillet (Table 4) directly reflected that of the diet for fish fed the H and L diets. The concentration of EPA and DHA was significantly higher in fish fed the H diet than in those fed the L or LCGA diets. However, while DHA

Table 3 Effects of experimental diets on Atlantic salmon performance[1]

	Experimental diets				Probability of contrasts		
	H	L	LCGA	SEM	H versus L	H versus LCGA	L versus LCGA
FBW	565	595	577	19.7	0.35	0.68	0.56
SGR	1.51	1.48	1.52	0.09	0.79	0.97	0.76
FCR	0.75	0.75	0.76	0.02	0.94	0.80	0.85

SGR, specific growth rate; LCGA, L diet supplemented with 1 g kg^{-1} chlorogenic acid.
[1] Data correspond to the last month of trial after the formalin treatment.

Table 4 Fatty acid composition (% of total fatty acids) of fillet total lipids in Atlantic salmon fed the experimental diets

	Experimental diets				Probability of contrasts		
Fatty acid	H	L	LCGA	SEM	H versus L	H versus LCGA	L versus LCGA
14:0	3.1	1.5	1.4	0.02	<0.0001	<0.0001	0.002
16:0	12.2	11.9	12.2	0.19	0.27	0.86	0.26
18:0	3.1	3.1	3.2	0.05	0.91	0.38	0.35
∑ saturates[1]	19.0	17.0	17.3	0.25	0.01	0.01	0.50
18:1n-9	37.5	44.6	43.5	0.43	0.001	0.002	0.16
∑ monoenes[2]	48.2	52.3	50.5	0.56	0.01	0.06	0.10
18:2n-6	13.3	14.8	15.7	0.19	0.01	0.003	0.05
20:3n-6	0.5	0.7	0.9	0.02	0.006	0.001	0.03
20:4n-6	0.5	0.3	0.3	0.01	0.001	0.001	0.42
∑ n-6[3]	15.2	16.7	17.6	0.20	0.01	0.003	0.04
18:3n-3	4.7	5.1	5.4	0.11	0.07	0.01	0.13
20:5n-3	2.7	1.6	1.4	0.06	0.0009	0.0006	0.15
22:6n-3	6.1	3.9	3.6	0.11	0.001	0.0007	0.18
∑ n-3[4]	16.9	13.5	13.6	0.23	0.002	0.002	0.69
∑ PUFA	32.5	30.5	31.6	0.39	0.03	0.19	0.14
n-6:n-3	0.9	1.2	1.3	0.01	0.0005	0.0003	0.06
Fold difference (Δ)[5]							
20:5n-3	−0.36	−0.20	−0.16	0.02	0.02	0.01	0.42
22:6n-3	0.71	1.11	1.54	0.05	0.01	0.002	0.01

H, high in fish oil; L, low in fish oil; LCGA, L diet supplemented with 1 g kg^{-1} chlorogenic acid.
[1] Includes 17:0 and 20:0.
[2] Includes 16:1, 17:1, 18:1n-7, 20:1 and 22:1.
[3] Includes 18:3, 20:2, and 22:4.
[4] Includes 18:4, 20:3, 20:4, and 22:5.
[5] Fold difference (Δ) between diet and tissue fatty acid values in each experimental group calculated as follows: Δ = (%fatty acid in the fillet − %fatty acid in the diet)/(%fatty acid in the diet). A positive value means accumulation of a given fatty acid in muscle in relation to diet.

was accumulated in the fillet compared with the diet, EPA showed negative values (Table 4) with a significant lower retention in fish fed the H diet ($P = 0.02$). No major effects of CGA were observed on fillet fatty acid composition.

The effects of the dietary treatments on liver fatty acid composition are shown in Table 5. Despite having similar dietary inputs of C16:0, the concentration of this fatty acid was significantly lower ($P < 0.01$) in the liver of fish fed the L compared with those fed the H diet. MUFA, n-3 and n-6 fatty acid profiles in the liver were closely related to the dietary inputs in fish fed the H and L or LCGA diet. Fish fed the LCGA diet tended to increase ($P = 0.07$) hepatic n-6 fatty acids compared with those fed the L diet with significant accumulation of C20:3n-6 ($P = 0.03$). DHA was accumulated in the liver compared to the diet. However, EPA showed negative values only in fish fed the H diet (Table 5) being thus accumulated in fish fed the L and LCGA diets. There was a tendency ($P \leq 0.08$) to decrease MDA concentrations in the liver of fish fed the L and LCGA diets in contrast to the H diet (Fig. 1).

Lipid metabolism

There was an overall upregulation of genes encoding long-chain PUFA synthesis proteins [Δ5fad ($P = 0.09$), Δ6fad ($P < 0.05$) and Elov2 ($P = 0.10$)], but not for Elov5a, in the liver of fish fed the L and LCGA diets compared with those fed the H diet (Fig. 2a), having no effects in the

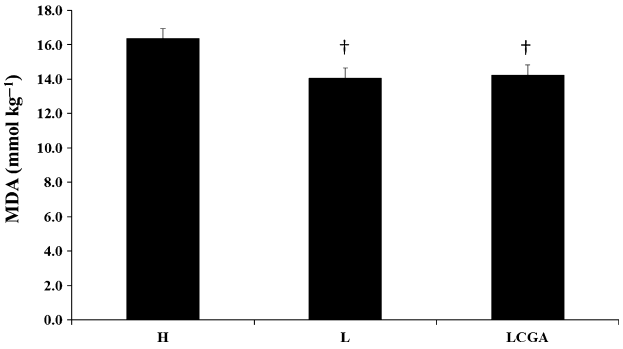

Figure 1 Effect of experimental diets on salmon liver lipid peroxidation measured as malondialdehyde. Data are means ± SE (†$P \leq 0.10$).

Table 5 Fatty acid composition (% of total fatty acids) of liver total lipids in Atlantic salmon fed the experimental diets

	Experimental diets				Probability of contrasts		
Fatty acid	H	L	LCGA	SEM	H versus L	H versus LCGA	L versus LCGA
14:0	1.4	0.9	0.7	0.03	0.001	0.0005	0.01
16:0	13.9	11.4	12.4	0.50	0.003	0.13	0.25
18:0	6.1	5.6	6.1	0.19	0.18	0.91	0.17
∑ saturates[1]	21.8	18.3	19.5	0.57	0.02	0.06	0.24
18:1n-9	24.5	36.2	31.7	1.49	0.01	0.04	0.12
∑ monoenes[2]	33.1	44.1	39.0	1.81	0.02	0.10	0.14
18:2n-6	7.3	10.2	10.0	0.27	0.004	0.006	0.74
20:3n-6	1.3	2.4	3.3	0.16	0.01	0.003	0.03
20:4n-6	2.7	1.8	2.2	0.08	0.15	0.03	0.13
∑ n-6[3]	13.5	14.9	16.0	0.27	0.03	0.008	0.07
18:3n-3	1.7	2.2	2.0	0.10	0.06	0.17	0.40
20:5n-3	4.1	2.7	2.7	0.29	0.04	0.04	0.99
22:6n-3	21.2	13.0	14.9	1.07	0.01	0.02	0.31
∑ n-3[4]	31.1	22.3	25.2	1.37	0.01	0.05	0.24
∑ PUFA	45.1	37.6	41.5	1.42	0.03	0.15	0.16
n-6:n-3	0.4	0.7	0.6	0.03	0.01	0.02	0.53
Fold difference (Δ)[5]							
20:5n-3	−0.05	0.39	0.63	0.11	0. 07	0.02	0.24
22:6n-3	5.0	6.0	9.3	0.5	0.26	0.01	0.02

H, high in fish oil; L, low in fish oil; LCGA, L diet supplemented with 1 g kg^{-1} chlorogenic acid.
[1] Includes 17:0 and 20:0.
[2] Includes 16:1, 17:1, 18:1n-7, 20:1 and 22:1.
[3] Includes 18:3, 20:2, and 22:4.
[4] Includes 18:4, 20:3, 20:4, and 22:5.
[5] Fold difference (Δ) between diet and tissue fatty acid values in each experimental group calculated as follows: Δ = (%fatty acid in the liver − %fatty acid in the diet)/(%fatty acid in the diet). A positive value means accumulation of a given fatty acid in the liver in relation to diet.

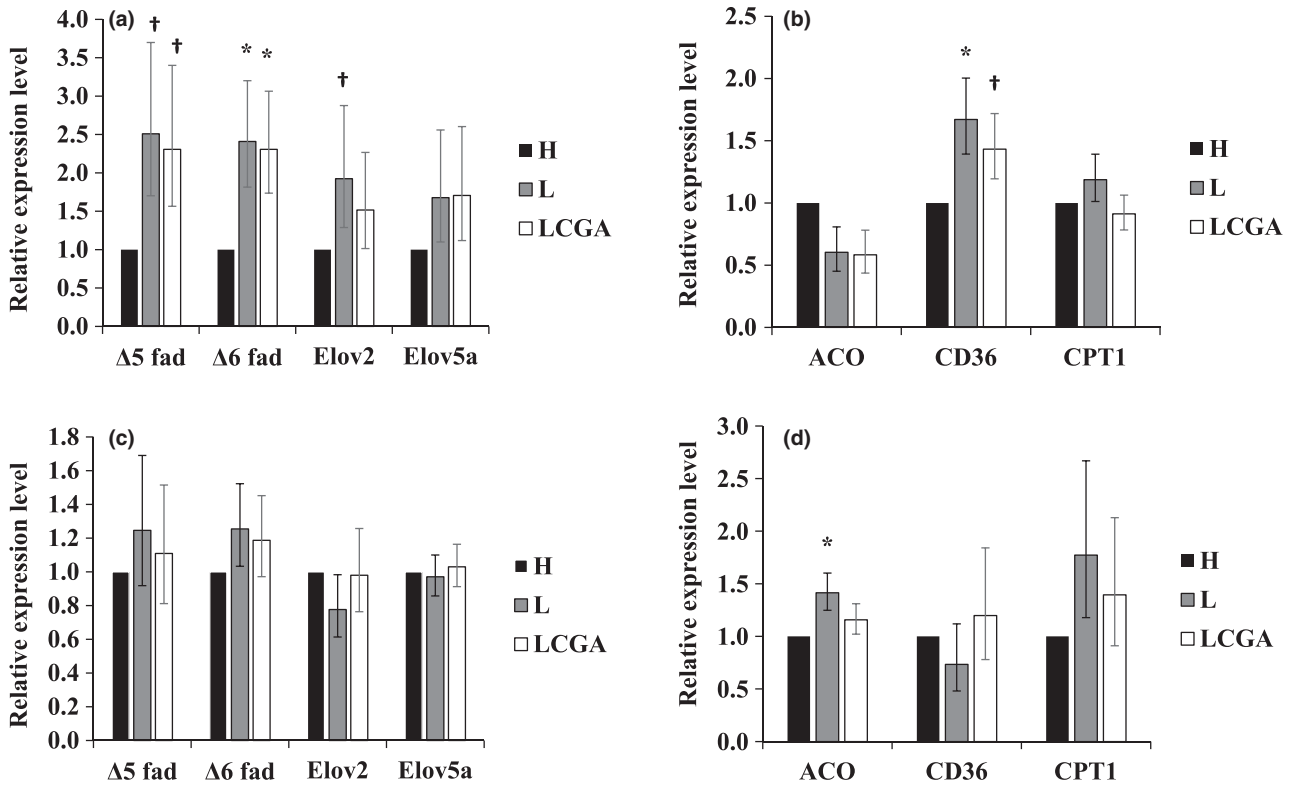

Figure 2 Effects of feeding Atlantic salmon diets high in fish oil (FO) (H), diets low in FO (L) and the L diet supplemented with 1 g kg^{-1} chlorogenic acid (LCGA) on the mRNA levels of fatty acid desaturase 5 (Δ5 fad), fatty acid desaturase 6 (Δ6 fad), elongase 2 (Elov2), elongase 5a (Elov5a), acyl CoA oxidase (ACO), cluster of differentiation 36 (CD36) and carnitine palmitoyltransferase 1 (CPT1) in the liver (a, b) and pyloric caeca (c, d). Relative gene expression values are fold change of L and LCGA diets relative to the control diet (H) set to be 1.0. Bars indicate the 95% confidence interval (Fold change up – Fold change low) (†$P \leq 0.10$; *$P < 0.05$).

pyloric caeca (Fig. 2c). There was a significant ($P < 0.05$) increase of CD36 mRNA in the livers of fish fed the L and LCGA diets (Fig. 2b). Also, there was a tendency ($P = 0.15$) to downregulate ACO gene expression in the liver of salmon fed the L and LCGA diets (Fig. 2b). The expression of ACO was significantly ($P < 0.05$) upregulated in the pyloric caeca of fish fed the L diet compared with those fed the H diet (Fig. 2d). Finally, the expression of SREBP1 was significantly upregulated ($P < 0.05$) in the liver of fish fed the L and LCGA diets (Fig. 3a), following the expression of LXR in a similar pattern ($P = 0.09$). In the pyloric caeca, only the expression of LXR was changed with a significant downregulation ($P < 0.05$) in fish fed the CGA-supplemented diet compared with those fed the H diet (Fig. 3b).

Discussion

Fish oil availability for salmon feed production represents a major challenge for the industry (Sørensen *et al.* 2011). The replacement of this raw material by VO has highlighted the need for greater understanding of salmon lipid metabolism and particularly n-3 LC-PUFA metabolism (Monroig *et al.* 2010; Minghetti *et al.* 2011). Moreover, the way dietary lipids interfere with EPA and DHA direct deposition and *de novo* synthesis is of relevance to optimize their concentrations in the fish tissues. In the present study, Atlantic salmon postsmolts were fed a diet with current commercial FO inclusion levels (160 g kg^{-1}) and a diet with only 40 g kg^{-1} FO providing around 80 and 40 g kg^{-1} of total fatty acids for EPA + DHA, respectively. Consistent with previous studies, EPA and DHA concentrations in the fillet and livers of salmon resembled those of the diet (Torstensen *et al.* 2000; Bell *et al.* 2001; Menoyo *et al.* 2005, 2014; Faizan *et al.* 2013). However, in fish fed the H diet, it was noticeable that while DHA was selectively retained in the liver compared with the diet, EPA was not. We and others have previously reported evidence for selective DHA retention in salmon tissues (Menoyo *et al.* 2003; Stubhaug *et al.* 2007; Codabaccus

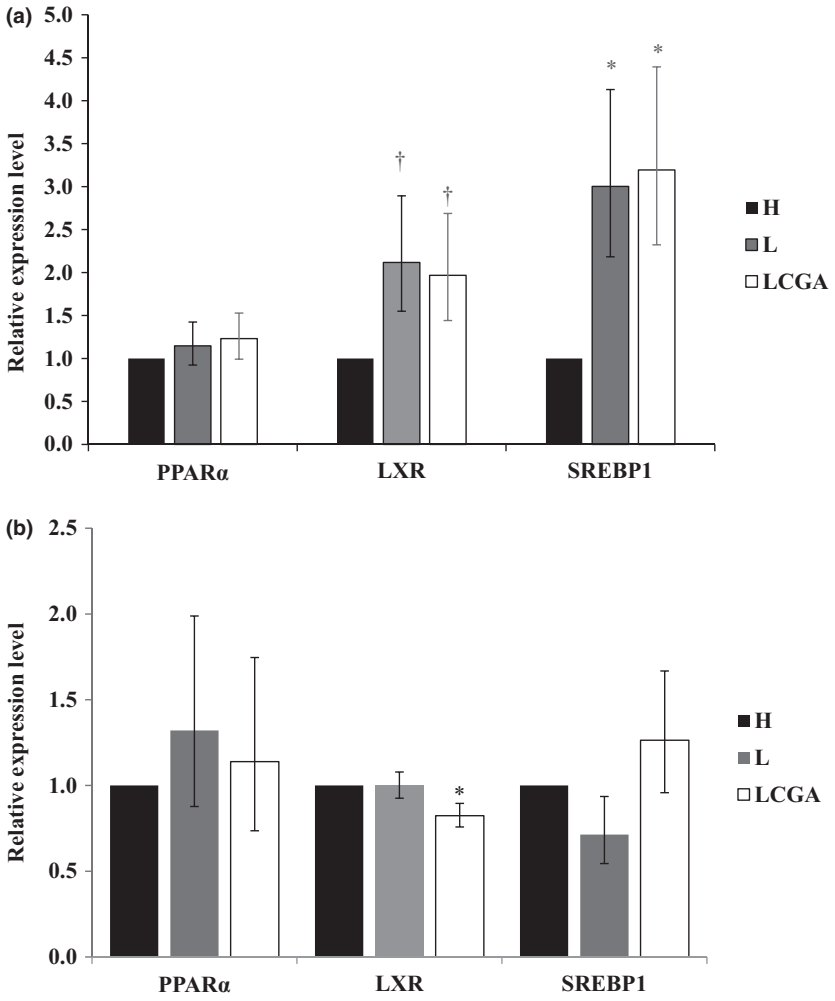

Figure 3 Effects of feeding Atlantic salmon diets high in fish oil (FO) (H), diets low in FO (L) and the L diet supplemented with 1 g kg^{-1} chlorogenic acid (LCGA) on the mRNA levels of peroxisome proliferator-activated receptor alpha (PPARα), liver X receptor (LXR), sterol receptor binding protein 1 (SREBP1) in the liver (a) and pyloric caeca (b). Relative gene expression values are fold change of L and LCGA diets relative to the control diet (H) set to be 1.0. Bars indicate the 95% confidence interval (Fold change up – Fold change low) (†$P \leq 0.10$; *$P < 0.05$).

et al. 2012). In addition, it is known that fish preferably metabolize EPA and save DHA because of its longer metabolic pathway (Madsen *et al*. 1999). Accordingly, fish fed the L and LCGA diets tended to show lower ACO expression and liver MDA concentrations and a net accumulation of EPA in the liver. Therefore, it seems that when EPA and DHA are provided in the diets at low levels (40 g kg^{-1}), both fatty acids are retained in the liver. However, in the fillet, EPA values were lower in the tissue respective to diet with a significant higher loss in fish fed the H diet.

The inclusion of VO at expenses of FO in L and LCGA diets produced an increase in the expression of genes encoding LC-PUFA synthesis proteins (Δ5fad, Δ6fad and Elov2) in the liver. This effect has been reported before (Bell *et al*. 2001; Leaver *et al*. 2008). In fish fed the L and LCGA diets with higher VO concentration, LC-PUFA synthesis might have been enhanced not only due to an increase of substrates (i.e. dietary linolenic or linoleic acid), for the LC-PUFA-synthesizing enzymes, but also because of a decreased dietary input of end products such as ARA and DHA, which were shown to inhibit Δ5fad and Δ6fad activity (Seiliez *et al*. 2001; Thomassen *et al*. 2012). Studies in salmon SHK-1 cells have shown that the inhibitory effect of EPA and DHA on Δ5fad and Δ6fad was mediated by a decrease in SREBP1 (Minghetti *et al*. 2011). It has been hypothesized that LC-PUFAs inhibit SREBP1 expression by accelerating SREBP1 mRNA decay, thereby lowering Δ5fad and Δ6fad expression and consequently linolenic conversion to EPA and DHA (Tang *et al*. 2003). Our results for hepatic SREBP1, Δ5fad and Δ6fad support this hypothesis and previous *in vitro* results (Minghetti *et al*. 2011). In the present study, the replacement of FO with VO also tended to upregulate LXR expression in the liver with no changes on PPARγ expression. Previous studies in Atlantic salmon showed no effects of a VO-FO diet compared with a 100% FO diet on LXR expression in

postsmolts but a significant downregulation in adult individuals (Cruz-Garcia *et al*. 2009). In line with this, increased cholesterol concentration in the culture media increased LXR expression, suggesting that this transcription factor may be a key sensor of hepatic cholesterol concentrations (Minghetti *et al*. 2011). However, in the present study the regulatory effects of LXR are possibly masked by SREBP1 for genes which are regulated by both transcription factors (Minghetti *et al*. 2011). Despite a higher expression of LC-PUFA synthesis related genes with VO, the concentration of EPA and DHA was higher in fish fed the H diet.

While there is an increasing interest in finding omega 3 sources to replace fish oil, less attention has been paid in verifying how the retention of HUFA in fish tissues can be maximized. The use of antioxidant substances can be a way to protect n-3 LC-PUFAs from lipid peroxidation (Dillard *et al*. 1983). Studies in human and animal models have proved CGA as a potent antioxidant with also modulating effects on glucose and lipid metabolism (Xu *et al*. 2012; Meng *et al*. 2013). In the present study, salmon fed the L and LCGA showed similar EPA and DHA concentrations in the studied tissues. Also, liver MDA concentrations were not significantly different between the two diets. Therefore, it seems that feeding Atlantic salmon with 1 g kg^{-1} CGA has not an extra effect on decreasing lipid peroxidation.

Effects of CGA on lipid metabolism also seem to be regulated in a dose-dependent manner. Mice fed a high-fat diet which contained 1 g kg^{-1} CGA showed higher body fat accumulation because of lower β-oxidation whereas supplementing 0.2 g kg^{-1} CGA diminished body fat accumulation compared with non-supplemented control mice (Cho *et al*. 2010; Mubarak *et al*. 2013). Reported lipid lowering effects of CGA were mostly mediated by PPARα enhancing ACO and CPT1 activity (Cho *et al*. 2010). Additionally, CGA was shown to decrease cholesterol via LXR (Huang *et al*. 2014). In the present study, no differences were observed in whole fish fat (data not shown) and PPARα expression levels in pyloric caeca or liver were not affected by dietary means. Moreover, while ACO and CPT1 were significantly upregulated in the pyloric caeca of fish fed the L compared with the H diet, no such response was observed in salmon fed the LCGA diet. However, the expression of LXR was significantly downregulated in the pyloric caeca of fish fed the LCGA diet. As LXR seems to be a master transcription factor sensing cholesterol levels in Atlantic salmon (Minghetti *et al*. 2011), it could be a possible target of CGA, thereby modulating cholesterol metabolism in Atlantic salmon as it has been suggested in rats.

In conclusion, reducing dietary FO from 160 g kg^{-1} to 40 g kg^{-1} in blends with VO fed to salmon upregulates the expression of genes involved in LC-PUFA synthesis and diminishes oxidative stress. However, both effects are not sufficient to maintain EPA and DHA concentrations in fish. The inclusion of 1 g kg^{-1} CGA in salmon does not seem to have an extra protective effect on lipid peroxidation.

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Conflict of interest

None of the authors has any conflict of interests to declare.

References

Bell, J.G., McEvoy, J., Tocher, D.R., McGhee, F., Campbell, P.J. & Sargent, J.R. (2001) Replacement of fish oil with rapeseed oil in diets of Atlantic salmon (*Salmo salar*) affects tissue lipid compositions and hepatocyte fatty acid metabolism. *J. Nutr*., **131**, 1535–1543.

Calder, P.C. (2012) The role of marine omega-3 (n-3) fatty acids in inflammatory processes, atherosclerosis and plaque stability. *Mol. Nutr. Food Res*., **56**, 1073–1080.

Calder, P.C. & Yaqoob, P. (2012) Marine omega-3 fatty acids and coronary heart disease. *Curr. Opin. Cardiol*., **27**, 412–419.

Carmona-Antoñanzas, G., Tocher, D.R., Martinez-Rubio, L. & Leaver, M.J. (2014) Conservation of lipid metabolic gene transcriptional regulatory networks in fish and mammals. *Gene*, **534**, 1–9.

Chen, W.C., Liou, S.S., Tzeng, T.F., Lee, S.L. & Liu, I.M. (2013) Effect of topical application of chlorogenic acid on excision wound healing in rats. *Planta Med*., **79**, 616–621.

Cho, A.S., Jeon, S.M., Kim, M.J., Yeo, J., Seo, K.I., Choi, M.S. & Lee, M.K. (2010) Chlorogenic acid exhibits anti-obesity property and improves lipid metabolism in high-fat diet-induced-obese mice. *Food Chem. Toxicol*., **48**, 937–943.

Chougala, M.B., Bhaskar, J.J., Rajan, M.G.R. & Salimath, P.V. (2012) Effect of curcumin and quercetin on lysosomal enzyme activities in streptozotocin-induced diabetic rats. *Clin. Nutr*., **31**, 749–755.

Codabaccus, B.M., Carter, C.G., Bridle, A.R. & Nichols, P.D. (2012) The "n-3 LC-PUFA sparing effect" of modified dietary n-3 LC-PUFA content and DHA to EPA ratio in Atlantic salmon smolt. *Aquaculture*, **356–357**, 135–140.

Cruz-Garcia, L., Minghetti, M., Navarro, I. & Tocher, D.R. (2009) Molecular cloning, tissue expression and regulation of liver X receptor (LXR) transcription factors of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol. B Biochem. Mol. Biol*., **153**, 81–88.

De Boer, V.C.J., Dihal, A.A., van der Woude, H., Arts, I.C.W., Wolffram, S., Alink, G.M., Rietjens, I.M.C.M., Keijer, J. &

Hollman, P.C.H. (2005) Tissue distribution of quercetin in rats and pigs. *J. Nutr*., **135**, 1718–1725.

Dillard, C.J., Gavino, V.C. & Tappel, A.L. (1983) Relative antioxidant effectiveness of alpha-tocopherol and gamma-tocopherol in iron-loaded rats. *J. Nutr*., **113**, 2266–2273.

D'Souza, N., Skonberg, D.I., Camire, M.E., Guthrie, K.E., Malison, J. & Lima, L. (2005) Influence of dietary genistein levels on tissue genistein deposition and on the physical, chemical, and sensory quality of rainbow trout, *Oncorhynchus mykiss*. *J. Agric. Food Chem*., **53**, 3631–3636.

Faizan, M., Stubhaug, I., Menoyo, D., Esatbeyoglu, T., Wagner, A.E., Struksnæs, G., Koppe, W. & Rimbach, G. (2013) Dietary alpha-tocopherol affects tissue vitamin e and malondialdehyde levels but does not change antioxidant enzymes and fatty acid composition in farmed Atlantic salmon (*Salmo salar* L.). *Int. J. Vitam. Nutr. Res*., **83**, 238–245.

Faizan, M., Esatbeyoglu, T., Bayram, B. & Rimbach, G. (2014) A fast and validated method for the determination of malondialdehyde in fish liver using high-performance liquid chromatography with a photodiode array detector. *J. Food Sci*., **79**, 484–488.

FAO (2014) The State of World Fisheries and Aquaculture. Food and Agriculture Organization of the United Nations, Rome.

Grahl-Nielsen, O. & Barnung, T. (1985) Variations in the fatty acid profile of marine animals caused by environmental and developmental changes. *Mar. Environ. Res*., **17**, 218–221.

Hu, F.B., Bronner, L., Willett, W.C., Stampfer, M.J., Rexrode, K.M., Albert, C.M., Hunter, D. & Manson, J.E. (2002) Fish and omega-3 fatty acid intake and risk of coronary heart disease in women. *JAMA*, **287**, 1815–1821.

Huang, K., Liang, X., Zhong, Y., He, W. & Wang, Z. (2014) 5-Caffeoylquinic acid decreases diet-induced obesity in rats by modulating PPARα and LXRα transcription. *J. Sci. Food Agric*., **95**, 1903–1910.

Leaver, M.J., Villeneuve, L.A., Obach, A., Jensen, L., Bron, J.E., Tocher, D.R. & Taggart, J.B. (2008) Functional genomics reveals increases in cholesterol biosynthetic genes and highly unsaturated fatty acid biosynthesis after dietary substitution of fish oil with vegetable oils in Atlantic salmon (*Salmo salar*). *BMC Genom*., **9**, 299.

Li, S.-Y., Chang, C.-Q., Ma, F.-Y. & Chang-Long, Y. (2009) Modulating effects of chlorogenic acid on lipids and glucose metabolism and expression of hepatic peroxisome proliferator-activated receptor-alpha in golden hamsters fed on high fat diet. *Biomed. Environ. Sci*., **22**, 122–129.

de Lorgeril, M. & Salen, P. (2012) Use of at least one omega-3 fatty acid and of at least one polyphenol for the endogenous synthesis of eicosapentanoic acid and docosahexanoic acid. U.S. Patent No. 13/383,290 (issued Aug. 9, 2012).

Madsen, L., Rustan, A.C., Vaagenes, H., Berge, K., Dyrøy, E. & Berge, R.K. (1999) Eicosapentaenoic and docosahexaenoic acid affect mitochondrial and peroxisomal fatty acid oxidation in relation to substrate preference. *Lipids*, **34**, 951–963.

Meng, S., Cao, J., Feng, Q., Peng, J. & Hu, Y. (2013) Roles of chlorogenic acid on regulating glucose and lipids metabolism: a review. *Evid Based Complement Alternat Med*, **2013**, 1–11.

Menoyo, D., Lopez-Bote, C.J., Bautista, J.M. & Obach, A. (2003) Growth, digestibility and fatty acid utilization in large Atlantic salmon (*Salmo salar*) fed varying levels of n-3 and saturated fatty acids. *Aquaculture*, **225**, 295–307.

Menoyo, D., López-Bote, C.J., Obach, A. & Bautista, J.M. (2005) Effect of dietary fish oil substitution with linseed oil on the performance, tissue fatty acid profile, metabolism, and oxidative stability of Atlantic salmon. *J. Anim. Sci*., **83**, 2853–2862.

Menoyo, D., Sanz-Bayón, C., Hesby Nessa, A. *et al*. (2014) Atlantic salmon (*Salmo salar* L.) as a marine functional source of gamma-tocopherol. *Mar. Drugs*, **12**, 5944–5959.

Minghetti, M., Leaver, M.J. & Tocher, D.R. (2011) Transcriptional control mechanisms of genes of lipid and fatty acid metabolism in the Atlantic salmon (*Salmo salar* L.) established cell line, SHK-1. *Biochim. Biophys. Acta*, **1811**, 194–202.

Minihane, A.M. (2013) Fish oil omega-3 fatty acids and cardiometabolic health, alone or with statins. *Eur. J. Clin. Nutr*., **67**, 536–540.

Monroig, O., Zheng, X., Morais, S., Leaver, M.J., Taggart, J.B. & Tocher, D.R. (2010) Multiple genes for functional 6 fatty acyl desaturases (Fad) in Atlantic salmon (*Salmo salar* L.): gene and cDNA characterization, functional expression, tissue distribution and nutritional regulation. *Biochim. Biophys. Acta*, **1801**, 1072–1081.

Morais, S., Taggart, J.B., Guy, D.R., Bell, J.G. & Tocher, D.R. (2012) Hepatic transcriptome analysis of inter-family variability in flesh n-3 long-chain polyunsaturated fatty acid content in Atlantic salmon. *BMC Genom*., **13**, 410.

Mubarak, A., Hodgson, J.M., Considine, M.J., Croft, K.D. & Matthews, V.B. (2013) Supplementation of a high-fat diet with chlorogenic acid is associated with insulin resistance and hepatic lipid accumulation in mice. *J. Agric. Food Chem*., **61**, 4371–4378.

National Research Council (2011) Nutrient Requirements of Fish and Shrimp. National Academy Press, Washington, DC.

Ounnas, F., Privé, F., Salen, P. *et al*. (2014) Wheat aleurone polyphenols increase plasma eicosapentaenoic acid in rats. *Food Nutr. Res*., **58**, 24604–24609.

Pfaffl, M.W., Tichopad, A., Prgomet, C. & Neuvians, T.P. (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. *Biotechnol. Lett*., **26**, 509–515.

Rakhshandehroo, M., Sanderson, L.M., Matilainen, M., Stienstra, R., Carlberg, C., de Groot, P.J., Müller, M. & Kersten, S. (2007) Comprehensive analysis of PPARα-dependent regulation of hepatic lipid metabolism by expression profiling. *PPAR Res*., **2007**, Article ID 26839.

Regost, C., Arzel, J., Cardinal, M., Rosenlund, G. & Kaushik, S.J. (2003) Total replacement of fish oil by soybean or linseed oil with a return to fish oil in Turbot (*Psetta maxima*). *Aquaculture*, **220**, 737–747.

Salces-Ortiz, J., González, C., Moreno-Sánchez, N., Calvo, J.H., Pérez-Guzmán, M.D. & Serrano, M.M. (2013) Ovine HSP90AA1 expression rate is affected by several SNPs at the promoter under both basal and heat stress conditions. *PLoS ONE*, **8**, e66641.

Sato, Y., Itagaki, S., Kurokawa, T., Ogura, J., Kobayashi, M., Hirano, T., Sugawara, M. & Iseki, K. (2011) In vitro and in vivo antioxidant properties of chlorogenic acid and caffeic acid. *Int. J. Pharm*., **403**, 136–138.

Schiller Vestergren, A., Wagner, L., Pickova, J., Rosenlund, G., Kamal-Eldin, A. & Trattner, S. (2012) Sesamin modulates gene expression without corresponding effects on fatty acids in Atlantic salmon (*Salmo salar* L.). *Lipids*, **47**, 897–911.

Segura, J. & López-Bote, C.J. (2014) A laboratory efficient method for intramuscular fat analysis. *Food Chem*., **145**, 821–825.

Seiliez, I., Panserat, S., Kaushik, S. & Bergot, P. (2001) Cloning, tissue distribution and nutritional regulation of a Delta6-desatur-

ase-like enzyme in rainbow trout. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.*, **130**, 83–93.

Setorki, M., Asgary, S., Eidi, A., Rohani, A.H. & Esmaeil, N. (2009) Effects of apple juice on risk factors of lipid profile, inflammation and coagulation, endothelial markers and atherosclerotic lesions in high cholesterolemic rabbits. *Lipids Health Dis.*, **8**, 39.

Sørensen, M., Berge, G.M., Thomassen, M.S., Ruyter, B., Hatlen, B., Ytrestøyl, T., Aas, T.S. & Åsgård, T.E. (2011) Today's and tomorrow's feed ingredients in Norwegian aquaculture. *Rapport/Report* 52/2011.

Steibel, J.P., Poletto, R., Coussens, P.M. & Guilherme, R.J.M. (2009) A powerful and flexible linear mixed model framework for the analysis of relative quantification RT-PCR data. *Genomics*, **94**, 146–152.

Strack, D. & Gross, W. (1990) Properties and activity changes of chlorogenic acid: glucaric acid caffeoyltransferase from tomato (*Lycopersicon esculentum*). *Plant Physiol.*, **92**, 41–47.

Stubhaug, I., Lie, Ø. & Torstensen, B.E. (2007) Fatty acid productive value and β-oxidation capacity in Atlantic salmon (*Salmo salar* L.) fed on different lipid sources along the whole growth period. *Aquacult. Nutr.*, **13**, 145–155.

Tang, C., Cho, H.P., Nakamura, M.T. & Clarke, S.D. (2003) Regulation of human delta-6 desaturase gene transcription: identification of a functional direct repeat-1 element. *J. Lipid Res.*, **44**, 686–695.

Thomassen, M.S., Rein, D., Berge, G.M., Østbye, T.K. & Ruyter, B. (2012) High dietary EPA does not inhibit Δ5 and Δ6 desaturases in Atlantic salmon (*Salmo salar* L.) fed rapeseed oil diets. *Aquaculture*, **360–361**, 78–85.

Torstensen, B.E., Lie, O. & Frøyland, L. (2000) Lipid metabolism and tissue composition in Atlantic salmon (*Salmo salar* L.) effects of capelin oil, palm oil, and oleic acid-enriched sunflower oil as dietary lipid sources. *Lipids*, **35**, 653–664.

Torstensen, B.E., Espe, M., Sanden, M. *et al.* (2008) Novel production of Atlantic salmon (*Salmo salar*) protein based on combined replacement of fish meal and fish oil with plant meal and vegetable oil blends. *Aquaculture*, **285**, 193–200.

Toufektsian, M.-C., Salen, P., Laporte, F., Tonelli, C. & de Lorgeril, M. (2011) Dietary flavonoids increase plasma very long-chain (n-3) fatty acids in rats. *J. Nutr.*, **141**, 37–41.

Wan, C.W., Wong, C.N., Pin, W.K., Wong, M.H., Kwok, C.Y., Chan, R.Y., Yu, P.H. & Chan, S.W. (2010) Chlorogenic acid exhibits cholesterol lowering and fatty liver attenuating properties by up-regulating the gene expression of PPAR-α in hypercholesterolemic rats induced with a high-cholesterol diet. *Phytother. Res.*, **27**, 545–551.

Wang, J.H., Liu, Y.L., Li, C.L., Yu, J., Li, X., Wang, F. & Li, Y. (2012) Effect of chlorogenic acid extracted from Eucommia Ulmoides Oliv on hyperlipemia of mice induced by high fat diet. *Sci. Technol. Food Ind.*, **15**, 360–362.

Xu, J.G., Hu, Q.P. & Liu, Y. (2012) Antioxidant and DNA-protective activities of chlorogenic acid isomers. *J. Agric. Food Chem.*, **60**, 11625–11630.