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Dietary Fish Oil Alters DNA Methylation of Genes Involved in Polyunsaturated Fatty Acid Biosynthesis in Muscle and Liver of Atlantic Salmon (*Salmo salar*)

Nicola A. Irvine[1] · Bente Ruyter[2] · Tone-Kari Østbye[2] · Anna K. Sonesson[2] · Karen A. Lillycrop[3] · Gerd Berge[4] · Graham C. Burdge[1]

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Abstract Adequate dietary supply of eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) is required to maintain health and growth of Atlantic salmon (*Salmo salar).* However, salmon can also convert α-linolenic acid (18:3n-3) into eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) by sequential desaturation and elongation reactions, which can be modified by 20:5n-3 and 22:6n-3 intake. In mammals, dietary 20:5n-3 + 22:6n-3 intake can modify *Fads2* expression (Δ6 desaturase) *via* altered DNA methylation of its promoter. Decreasing dietary fish oil (FO) has been shown to increase *Δ5fad* expression in salmon liver. However, it is not known whether this is associated with changes in the DNA methylation of genes involved in polyunsaturated fatty acid synthesis. To address this, we investigated whether changing the proportions of dietary FO and vegetable oil altered the DNA methylation of *Δ6fad_b*, *Δ5fad*, *Elovl2,* and *Elovl5_b* promoters in liver and muscle from Atlantic salmon and whether any changes were associated with mRNA expression. Higher dietary FO content increased the proportions of 20:5n-3 and 22:6n-3 and decreased *Δ6fad_b* mRNA expression in liver, but there was no effect on *Δ5fad, Elovl2,* and *Elovl5_b* expression. There were significant differences between liver and skeletal muscle in the methylation of individual CpG loci in all four genes studied. Methylation of individual *Δ6fad_b* CpG loci was negatively related to its expression and to proportions of 20:5n-3 and 22:6n-3 in the liver. These findings suggest variations in dietary FO can induce gene-, CpG locus-, and tissue-related changes in DNA methylation in salmon.

Keywords atlantic salmon · desaturase · DNA methylation · docosahexaenoic acid · eicosapentaenoic acid · elongase · polyunsaturated fatty acid biosynthesis

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Abbreviations

Elovl2	gene encoding elongase-2
Elovl5_b	gene encoding elonagse 5_b
FA	fatty acid
FAME	fatty acid methyl ester
FO	fish oil
MCP	calcium monophosphate
ND	not detected
PUFA	polyunsaturated fatty acids
RT	room temperature
SPC	soy protein concentrate
TSS	transcription start site
Δ5fad	gene encoding Δ5 desaturase
Δ6fad_b	gene encoding Δ6 desaturase_b

Supporting information Additional supporting information may be found online in the Supporting Information section at the end of the article.

✉ Graham C. Burdge
g.c.burdge@soton.ac.uk

[1] Human Development and Health, Faculty of Medicine, University of Southampton, Southampton, UK

[2] Nofima (Norwegian Institute of Food, Fisheries and Aquaculture Research), PO Box 210 1432, Ås, Norway

[3] Centre for Biological Sciences, Faculty of Natural and Environmental Sciences, University of Southampton, Southampton, UK

[4] Nofima (Norwegian Institute of Food, Fisheries and Aquaculture Research), Sjølsengveien 22, 6600 Sunndalsøra, Norway

Introduction

Oily fish, including Atlantic salmon, are the principal source of the n-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) in the human diet (Calder and Yaqoob, 2009). Higher intakes of these fatty acids have been associated with health benefits both in humans by preventing and attenuating a range of inflammatory disorders, including cardiovascular disease (Calder, 2015; Calder and Yaqoob, 2009; Eilander et al., 2007; Rogero and Calder, 2018; Ruxton et al., 2007; Thota et al., 2018; Todorcevic and Hodson, 2015), and in Atlantic salmon by improving robustness under challenging environmental conditions (*Salmo salar) (*Bou et al., 2017a*).*

Consumption of preformed 20:5n-3 and 22:6n-3 is obligatory for carnivorous marine fish, although diadromous species, such as Atlantic salmon, are able to produce 20:5n-3 and 22:6n-3 from 18:3n-3 *via* a pathway (Morais et al., 2012; Ruyter et al., 2016; Tocher, 2010), which is similar to that in rodents (Sprecher et al., 1999) and humans (Burdge, 2004). However, Atlantic salmon have limited capacity for 20:5n-3 and 22:6n-3 synthesis and, therefore, require at least 10 g 20:5n-3 + 22:6n-3 per kg feed depending of life stage to maintain good health and growth (Bou et al., 2017a; Ruyter and Thomassen, 1999). Diets of farmed salmon have traditionally contained high levels of n-3 PUFA from fish oil (FO) and fish meal, but while the production of these ingredients has changed little since the 1970s, the demand for marine ingredients containing n-3 PUFA has been increasing globally (Sprague et al., 2016; Sprague et al., 2017; Ytrestoyl et al., 2015). This has led to the need for replacing a large proportion of the marine ingredients in fish feed with more sustainable plant-based ingredients. While 90% of traditional Norwegian Atlantic salmon diets were composed of marine ingredients in 1990, current diets only contain approximately 30% marine ingredients (Ytrestoyl et al., 2015; Ytrestøyl et al., 2014). Although farmed Atlantic salmon is still considered a major source of n-3 PUFA in the human diet (Henriques et al., 2014; Jensen et al., 2012; Sprague et al., 2016), the reduction in marine ingredients in fish feed has resulted in a substantial decline in the content of 20:5n-3 and 22:6n-3 in farmed salmon fillets (Nichols et al., 2014; Sprague et al., 2016; Ytrestoyl et al., 2015). The absolute content of 20:5n-3 and 22:6n-3 in Scottish and Norwegian farmed Atlantic salmon fillets has decreased from an average of 2.7 g in 2006 to 1.4 g per 100 g fillet and 1.1 g per 100 g fillet in 2015 (Lundebye et al., 2017; Sprague et al., 2016). As further reductions of marine ingredients in the feed not only will reduce the nutritional value of the fish to consumers, but may also influence fish health (Bou et al., 2017) (Bou et al., 2017a), Thus, the limited supply of omega-3 ingredients is a critical factor for further growth in the aquaculture industry (Craze, 2018). It is therefore important to know how to optimise the salmon's innate capacity to produce 20:5n-3 and 22:6n-3 from 18:3n-3. This requires deeper understanding about the mechanisms involved in the regulation of the omega-3 metabolic pathway in Atlantic salmon.

In Atlantic salmon, three genes encode Δ6 desaturase*:* Δ6*fad_a,* Δ6*fadb,* and Δ6*fadc* (Monroig et al., 2010; Monroig et al., 2013; Zheng et al., 2005a), and one gene encodes a Δ5 desaturase: Δ5*fad* (Hastings et al., 2004). Four elongase genes have been cloned and characterized, encoding for elongases with different chain-length specificities: *Elovl5_a* and *Elovl5_b* (Agaba et al., 2005; Morais et al., 2009), *Elovl2* (Morais et al., 2009), and *Elovl4* (Carmona-Antonanzas et al., 2011). Conversion of 18:3n-3 into longer chain n-3 PUFA involves an initial, rate-limiting desaturation by Δ6 desaturase (encoded by *Fads2* in mammals and *Δ6fad_a/b/c* in salmon (Ruyter et al., 2016)) to form 18:4n-3 (Tocher, 2010). Then, 20:4n-3 and 20:5n-3 can be synthesized by sequential carbon chain elongation by elongase 5 (encoded by *Elovl5*) and desaturation at the Δ5 position by Δ5 desaturase (encoded by *Fads1* in mammals and *Δ5fad* in fish (Ruyter et al., 2016)). 20:5n-3 can be converted into 22:5n-3 by elongase 2 or elongase 5, and 24:5n-3 is then formed by further addition of two carbon atoms by elongase 2 (encoded by *Elovl2*). 24:6n-3 is synthesized from 24:5n-3 by Δ6 desaturation and this intermediate can then be translocated to peroxisomes where two carbons are removed by a single cycle of fatty acid β-oxidation (Ruyter et al., 2016; Tocher, 2010).

The presence and activity of the desaturase and elongase enzymes of the pathway determine the capacity for conversion. Several factors influence the activities of these enzymes, including feed composition, life stage, sea temperature, and genetic factors (Berge et al., 2015; Kjær et al., 2008; Zheng et al., 2005). Through this bioconversion, Atlantic salmon has the potential to be a net producer of 22:6n-3, and to a certain degree 20:5n-3 (Bou et al., 2017c; Rosenlund et al., 2016; Sanden et al., 2011; Turchini and Francis, 2009), but only when fed low levels of 20:5n-3 and 22:6n-3 (Rosenlund et al., 2016; Sissener et al., 2016), as the activity of the enzymes in the pathway is inhibited by dietary 20:5n-3 and 22:6n-3 (Betancor et al., 2015; Moya-Falcon et al., 2005; Tocher, 2015; Turchini et al., 2011; Turchini and Francis, 2009). The liver and intestine are considered the major bioconversion sites in Atlantic salmon, but omega-3 bioconversion genes are also expressed in muscle (Codabaccus et al., 2011). There is limited knowledge on its importance of omega-3 bioconversion in determining the FA composition of muscle. The few transcriptome studies of Atlantic salmon skeletal muscle have showed that this tissue expresses FA desaturase and elongase genes (Codabaccus et al., 2011; Zheng et al., 2005a) as well as transcription factors known to regulate lipid metabolism genes (Vegusdal et al., 2004). A recent study showed a low

correlation between liver and muscle n-3 PUFA content (Horn et al., 2019), showing that muscle must be studied separately in order to find out what determines individual variation in levels of n-3 PUFA in Atlantic salmon fillet. The role of epigenetic processes in regulation of the n-3 metabolic pathway in liver and muscle of Atlantic salmon is not known.

Epigenetic processes such as DNA methylation can be modified by environmental factors, including dietary fats, which may lead to altered gene expression and tissue function (Burdge and Lillycrop, 2014). The mRNA expression of *Fads2* has been shown to be regulated by the methylation status of individual CpG loci in its promoter region in rats (Hoile et al., 2012). Moreover, dietary fat can induce changes in the methylation status of specific *FADS2* CpG loci in humans (Hoile et al., 2014) and rodents (Hoile et al., 2012; Kelsall et al., 2012). There is increasing evidence that epigenetic processes are important for the regulation of transcription in teleost fish (Best et al., 2018). These mechanisms have been studied extensively in zebra fish (*Danio rerio*) (Kong et al., 2016); however, some studies have focused on species of importance to food production including salmonids (Best et al., 2018). Diets containing long-chain n-3 PUFA increased the frequency of methylated *Δ6fad* CpG loci in Japanese sea bass (*Lateolabrax japonicas*) (Xu et al., 2014). However, Geay et al. found no significant effect of dietary fish meal on the methylation of individual *Δ6fad* CpG loci in the liver of European sea bass (*Dicentrarchus labrax*) larvae (Geay et al., 2012). Thus, there is uncertainty whether dietary fat can modify DNA methylation of genes involved in PUFA synthesis in fish. This has implications for understanding how dietary fatty acids may modulate growth and health of farmed fish. Moreover, no studies on the effect of dietary fats on DNA methylation of other genes involved in PUFA synthesis have been reported in fish.

To address this, we investigated whether the amount of dietary FO altered the DNA methylation status of individual CpG loci in the 5′-regulatory regions of *Δ6fad_b*, *Δ5fad*, *elovl2,* and *elovl5_b* and whether any such changes were associated with differences in the mRNA expression of these genes in liver from farmed Atlantic salmon. The isoforms of *Δ6fad* and *elovl5* were selected for the epigenetic studies based on their known inhibitory gene expression response to dietary n-3 PUFA (Kjaer et al., 2016).

Materials and Methods

Design of the Fish Feeding Study

The feeding experiment with Atlantic salmon was conducted in compliance with the national regulation for use of experimental animals (FOR-2015-06-18-761) and classified as not requiring a specific license (§2-f, corresponding to Directive 2010/63/EU Article 1, section 5f), since the experimental treatments were not expected to cause any distress or discomfort for the fish. The experimental Atlantic salmon were randomly selected from SalmoBreed AS elite stock (10 brood stock families). The fish were reared under the same conditions (*e.g.* water temperature, light treatment) close to commercial practice in flow-through units at Nofima Sunndalsøra Research Station until smoltification. Then, the fish were vaccinated with Alpha Ject® 6–2 (Pharmaq AS, Oslo, Norway). All fish were fed the same commercial diet produced by Skretting AS (Stavanger, Norway), except during the experiment. Briefly, the experimental fish were weighed individually and 50 fish with similar average start weight of 96 g were distributed equally in six tanks (300 fish in total). All fish were individually tagged (PIT-tags, Passive Integrated Transponder, Biosonic). The tanks were 2 m^2 gray, fiberglass tanks, equipped for daily collection of uneaten feed (Helland et al., 1996), which were supplied with seawater to 60 cm depth. Two experimental diets were fed to fish in triplicate tanks for a period of 79 days until the fish reached average final weights of 480 g (75% FO group: initial weight 96 ± 1 g, final weight 471 ± 5 g; 0% FO group: initial weight 96 ± 1 g, final weight 480 ± 5 g). There were no differences between dietary groups in the initial weight ($p = 0.99$) or final weight ($p = 0.26$). The diets were produced by BioMar AS (Brande, Denmark) and designed to provide different levels of 20:5n-3 and 22:6n-3 by exchanging FO with rapeseed oil. The compositions of the diets, including the fatty acid content, are presented in Table 1. The 75% FO diet contained 22.1% (w/w) 20:5n-3 + 22:6n-3 derived from FO plus fish meal, while the 0% FO diet contained 3.7% (w/w) 22:5n-3 + 22:6n-3 derived from fish meal alone (Table 1).

Measurement of the DNA Methylation Status of Δ6fad, Δ6fad_b, elovl5_b, and elovl2

Sodium bisulphite pyrosequencing was carried out essentially as described (Hoile et al., 2014; Sibbons et al., 2018). Briefly, genomic DNA was isolated as follows: samples were digested overnight at 50 °C in a lysis buffer in the presence of proteinase K (20 μL), and then treated with 6 M NaCl and centrifuged at maximum speed for 10 min. The supernatant was collected and glycogen (3 μL) added, and the DNA was then precipitated in 100% ethanol. The precipitated DNA was washed in 100% ethanol followed by a final wash in 70% ethanol before resuspending the DNA in DNase- and RNase-free distilled water. Following extraction, genomic DNA was treated with sodium metabisulphite using the EZ DNA methylation kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Modified DNA was amplified by PCR

Table 1 Formulation (ingredients in % of total weight) and chemical composition (in % of dry matter) of the diets

	Diet	
	0% FO	75% FO
Fish meal[a]	32.00	32.00
Fish oil[a]	0.00	16.35
Rapeseed oil[b]	21.80	5.45
SPC[c]	23.45	23.45
Horse beans[d]	14.00	14.00
Wheat gluten[d]	5.99	5.99
Amino acids[e]	0.31	0.31
Vit & min mix[f]	0.46	0.46
Pigment[g]	0.04	0.04
MCP[h]	1.89	1.89
Yttrium oxide[i]	0.05	0.05
Chemical composition		
Dry matter (%)	93.1	93.0
% of dry matter		
Lipid	26.9	27.1
Protein	44.3	43.9
Ash	8.0	8.0
Fatty acids (%w/w total fatty acids)		
14:0	0.8	5.7
16:0	6.2	14.1
18:0	2.3	3.1
20:0	0.6	0.4
22:0	0.3	0.2
24:0	0.2	0.2
16:1 n-7	1.0	6.9
17:1 n-7	ND	1.1
18:1 n-11	ND	1.6
18:1 n-9	50.1	19.7
18:1 n-7	2.4	2.9
20:1 n-11	0.3	1.8
20:1 n-9	2.0	1.4
20:1 n-7	0.1	0.3
22:1 n-11	0.6	0.7
24:1 n-9	0.2	0.4
18:2 n-6	19.0	7.1
18:3 n-6	0.1	0.2
18:3 n-4	0.1	0.1
18:3 n-3	8.2	2.8
18:4 n-3	ND	0.1
20:3 n-6	ND	0.1
20:4 n-3	0.1	0.2
20:4 n-6	0.1	0.8
20:5 n-3	2.1	12.9
22:5 n-3	0.2	1.5
22:6 n-3	1.6	9.2

ND, not detected.

[a]Fish meal LT and fish oil, South American.

using KAPA2G Robust Hot Start Taq DNA polymerase (Labtech) (Table S1). Cycling conditions were as follows: 95 °C for 3 min (initial denaturation), then 45 cycles of 95 °C for 15 s (denaturation), followed by an amplicon specific annealing temperature listed in Table S1 for 15 s, 72 °C for 15 s (extension) with a final extension step of 72 °C for 1 min. The amplified products were immobilized on streptavidin–sepharose beads (GE Healthcare UK, Ltd.), washed, denatured, and released into annealing buffer containing the sequencing primers (Biomers, Söflinger, Germany) listed in Table S2. Pyrosequencing was carried out using an SQA kit on a PSQ96MA pyrosequencer (Biotage, Uppsala, Sweden). The percentage methylation at each CpG locus was determined using the PyroQ CpG software (Biotage) (Sibbons et al., 2018). Chromosome locations of individual CpG loci are listed in Table S3. CpG loci were identified using GenBank (NCBI) in the Atlantic salmon (*Salmo salar*) genome current assembly (ICSASG_v2). The transcription start site (TSS) was identified on Genbank for each gene of interest (accession numbers: Δ5fad, GU294485; Δ6fad, GU294486; Elovl5, GU324549; Elovl2, FJ237532) and primers were designed to cover CpGs in the putative promoter regions within 1000 bp upstream of the TSS.

Analysis of Liver Fatty Acid Composition

Analysis of the fatty acid composition of liver was carried out essentially as described (Burdge et al., 2000). All solvents were dried on molecular sieves prior to use. Briefly, tissues (approximately 100 mg) were extracted with chloroform/methanol (2:1, v/v) containing 50 mg/L butylatedhydroxytoluene (Burdge et al., 2000). One milliliter of 1 M NaCl was added, samples vortexed briefly and then centrifuged at 850*g* for 10 min at RT. Total tissue lipids were collected from the lower phase, dried under nitrogen at 40 °C and then dissolved in toluene (500 µL). Methanol containing 2% (v/v) H_2SO_4 (1 mL) was added and the reaction mixture heated at 50 °C for 2 h (Burdge et al., 2000). The reaction mixture was cooled and then neutralized with a solution of 0.5 M $KHCO_3$ and 0.25 M K_2CO_3 (1.0 mL).

[b]Emmelev, Denmark.

[c]SPC, Soy protein concentrate, Agrokorn, Denmark.

[d]Tereos Syral, Belgium.

[e]Lysine, methionine, histidine, Normin, Norway.

[f]Vitamin and mineral mix, according to commercial standards, based on NRC (Hastings et al., 2004), Normin, Norway.

[g]Astaxanthin, Hoffman-LaRoche, Basel, Switzerland.

[h]Calcium monophosphate, Normin, Norway.

[i]VWR, Norway.

Fatty acid methyl esters (FAME) were recovered by hexane extraction. FAME were resolved on a BPX-70 fused silica capillary column (30 m x 0.25 mm x 0.2 μm) using an Agilent 6890 gas chromatograph (Agilent) equipped with flame ionization detection. Fatty acids were identified by their retention times relative to authentic standards. The proportion of each fatty acid was determined by calculation of the area under the signal*time peak using the ChemStation software (Aglilent) expressed relative to the sum of the areas of all fatty acids.

Analysis of mRNA Expression in Liver and Skeletal Muscle by Real Time RTPCR

Total RNA was isolated from liver and skeletal muscle using Tri-reagent (Sigma) (Hoile et al., 2014). Briefly, complimentary DNA was prepared and amplified using realtime RT-PCR, which was performed using QuantiFast SYBR Green PCR kit (Qiagen). RTPCR primers are listed in Supplementary Table S4. All samples were analyzed in duplicate. Cycle parameters were 95 °C for 2 min then 40 cycles of 95 °C for 30 s, followed by specific annealing temperature for each primer (Table S4) and 72 °C for 1 min. Target transcripts were normalized as described (Sibbons et al., 2018) using the geometric mean of three reference genes (*nuor*, *etif3*, and *ef1a*), which were selected for stability across treatments using the GeNorm method, each produced an *M* value of between 0.65 and0.70 (Vandesompele et al., 2002).

Statistical Analysis

The distribution of the data was tested using the Shapiro–Wilk test and by visual inspection. All data were normally distributed. Statistical comparisons between dietary groups for each fatty acid or CpG locus were by Student's unpaired *t*-test. Statistical comparisons between tissues within a dietary group for each fatty acid or CpG locus were by Student's paired *t*-test. Associations between DNA methylation and mRNA expression were tested by calculation of Pearson's correlation coefficient. Statistical significance was assumed for all statistical tests at $p < 0.05$. Retrospective statistical power calculations were carried out for the primary outcomes; the proportion of 22:6n-3 in liver and methylation of individual CpG loci in *Δ5fad* in liver. Twenty-four fish per group provided 87% statistical power for detecting 5% change in the proportion of 22:6n-3 in liver with α 0.05. Twenty-four fish per group provided 90% statistical power for detecting 6% points (pts) change in the methylation of Δ5fad CpG 775 in liver with α 0.05.

Results

The Effect of Diet on Liver Total Fatty Acid Composition

The fatty acid composition of liver was used as a marker of the efficacy of the dietary intervention in changing the supply of fatty acids to tissues. Nineteen fatty acids were identified consistently in liver (Fig. 1). The liver of fish fed the 75% FO diet contained significantly greater proportions of 16:0 (7.2% pts), 16:1n-7 (1.8% pts), 18:0 (1.2% pts), 20:4n-6 (1.6% pts), 20:5n-3 (7.4% pts), 22:5n-3 (2.5% pts) and 22:6n-3 (15.3% pts) compared to fish fed 0% FO (Fig. 1a). This was accompanied by a

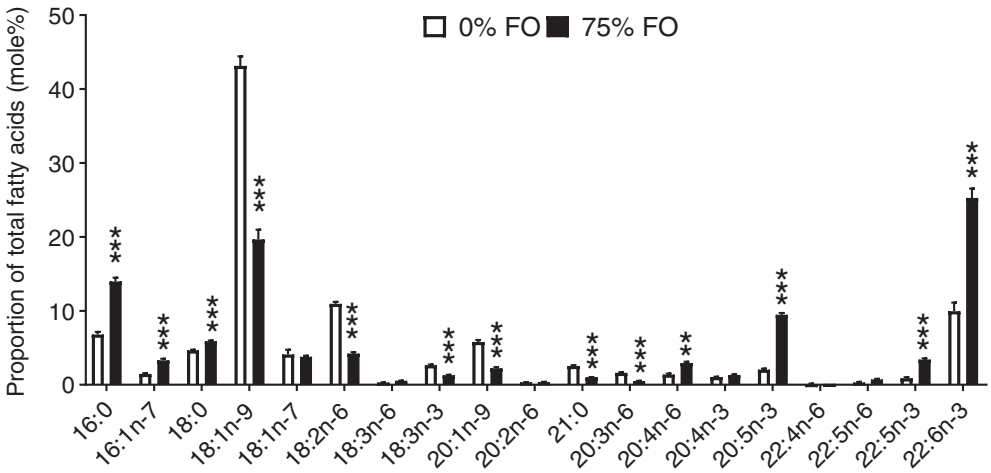

Fig. 1 Fatty acid composition of liver from fish fed diets containing either 0% fish oil (FO) or 75% FO. Values are mean ± SEM of $n = 24$ samples per dietary group. Statistical comparisons between dietary groups were by Student's unpaired *t* test. Means that differed significantly are indicated by $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$

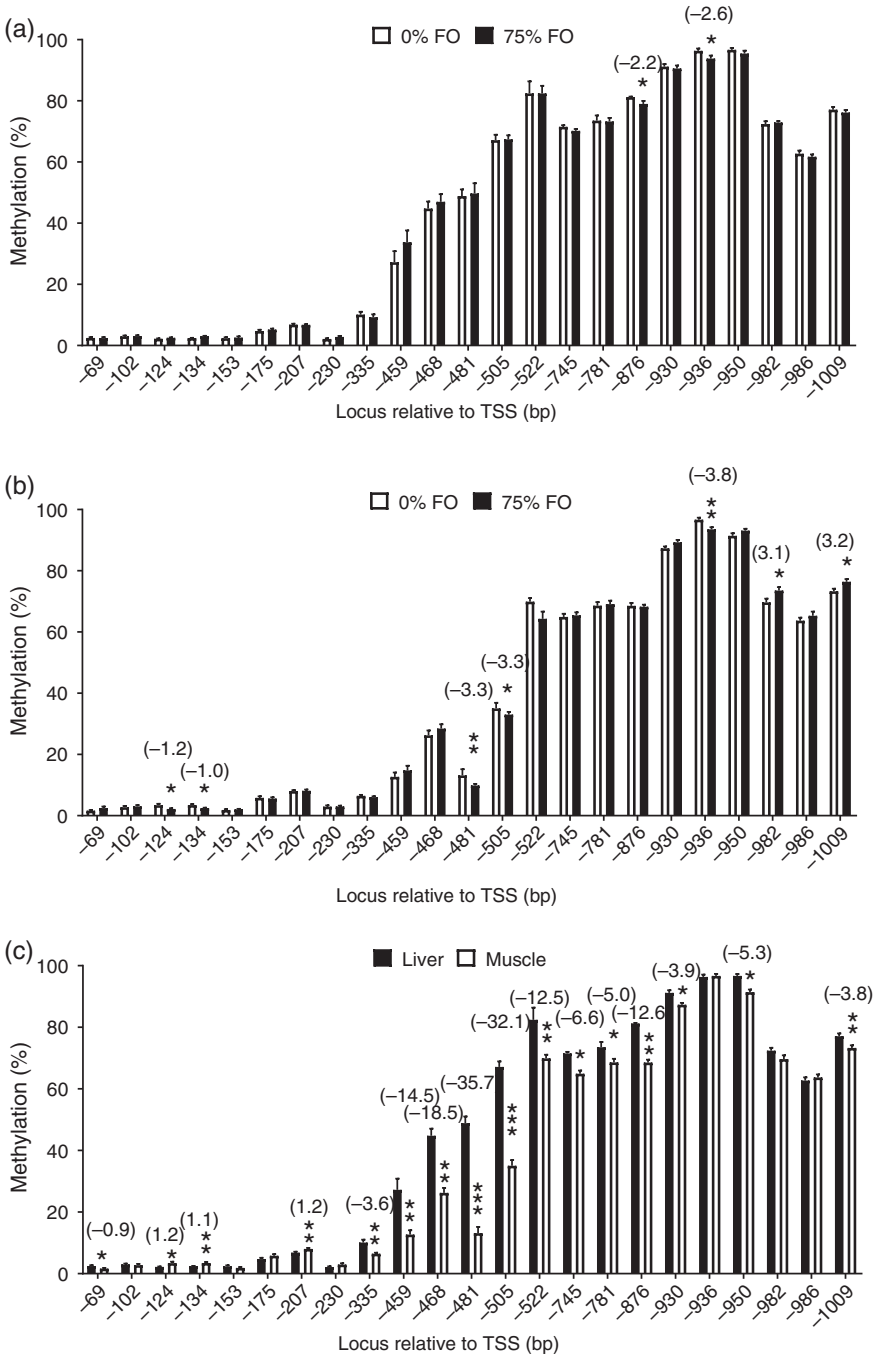

Fig. 2 DNA methylation of individual CpG loci within a 1009 bp region upstream of the Δ*fad_b* transcription start site (TSS) in **(a)** liver and **(b)** skeletal muscle from fish fed diets containing either 0% fish oil (FO) or 75% FO, and **(c)** liver compared to muscle from fish fed a diet containing 0% FO. The x-axis shows locations of CpG dinucleotide pairs relative to the TSS (chromosome locations are shown in Supplementary Table S3). Values are mean ± SEM of $n = 24$ samples per dietary group. Numbers in parenthesis indicate differences between means (% points). Statistical comparisons between dietary groups were by Student's unpaired *t* test. Statistical comparisons between tissues within a dietary group were by Student's paired *t* test. Means that differed significantly between dietary groups or tissues are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$

significant reduction in the proportions of 18:1n-9 (23.5% pts), 18:2n-6 (6.7% pts), 18:3n-3 (1.4% pts), 20:1n-9 (3.6% pts), 21:0 (1.5% pts), and 20:3n-6 (1.1% pts) in fish fed the 75% FO diet compared with fish fed the 0% FO diet (Fig. 1).

The Effect of Diet on the DNA Methylation of Genes Involved in Polyunsaturated Fatty Acid Biosynthesis

Twenty-one CpG loci were measured in a region between the TSS and 1 kb upstream of the *Δ6fad_b* TSS (Fig. 2).

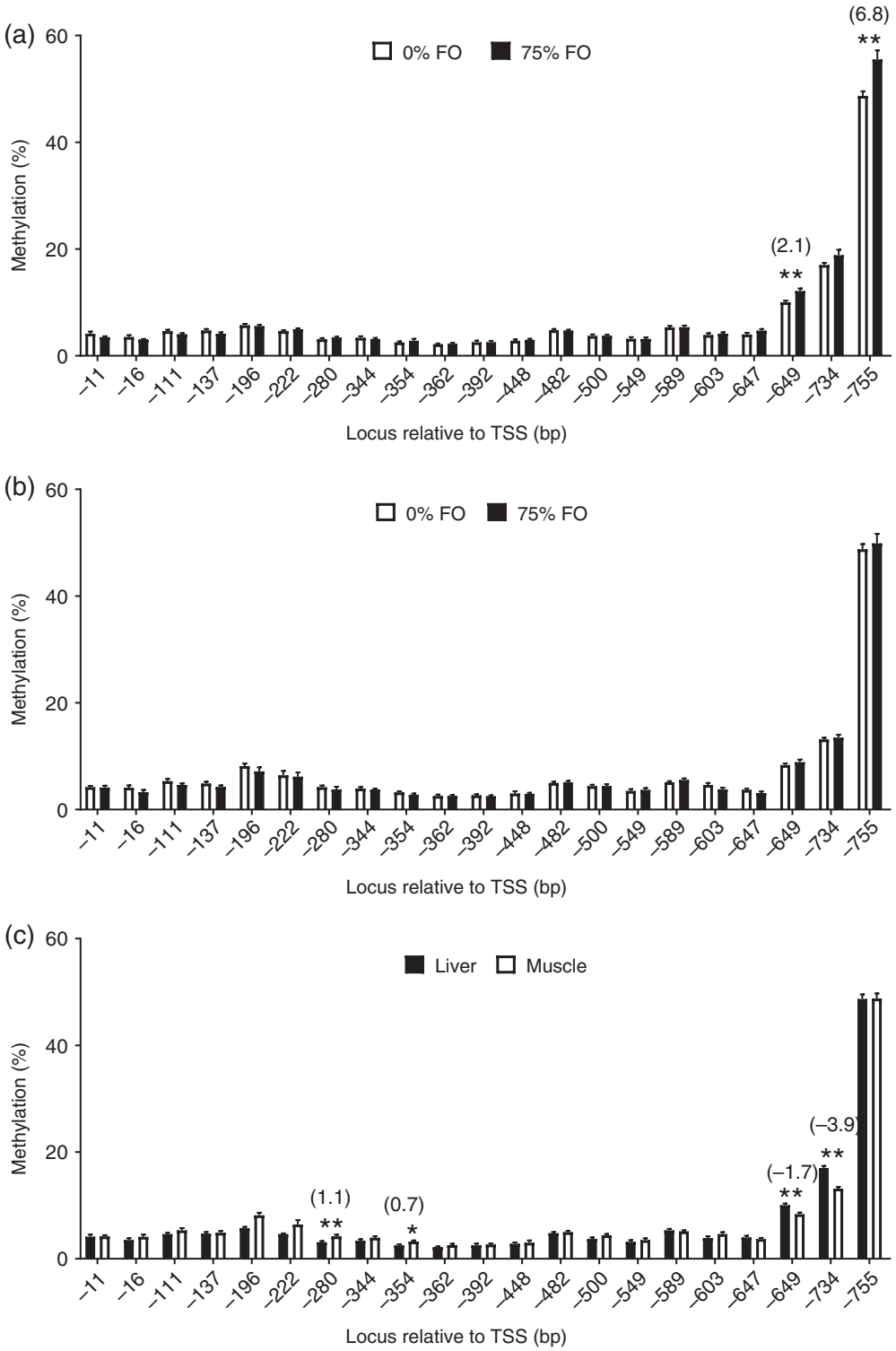

Fig. 3 DNA methylation of individual CpG loci within a 800 bp region upstream of the Δ5*fad* transcription start site (TSS) in **(a)** liver and **(b)** skeletal muscle from fish fed diets containing either 0% fish oil (FO) or 75% FO, and **(c)** liver compared to muscle from fish fed a diet containing 0% FO. The x-axis shows locations of CpG dinucleotide pairs relative to the TSS (chromosome locations are shown in Supplementary Table S3). Values are mean ± SEM of $n = 24$ samples per dietary group. Numbers in parenthesis indicate differences between means (% points). Statistical comparisons between dietary groups were by Student's unpaired *t* test. Statistical comparisons between tissues within a dietary group were by Student's paired *t* test. Means that differed significantly between dietary groups or tissues are indicated by $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$

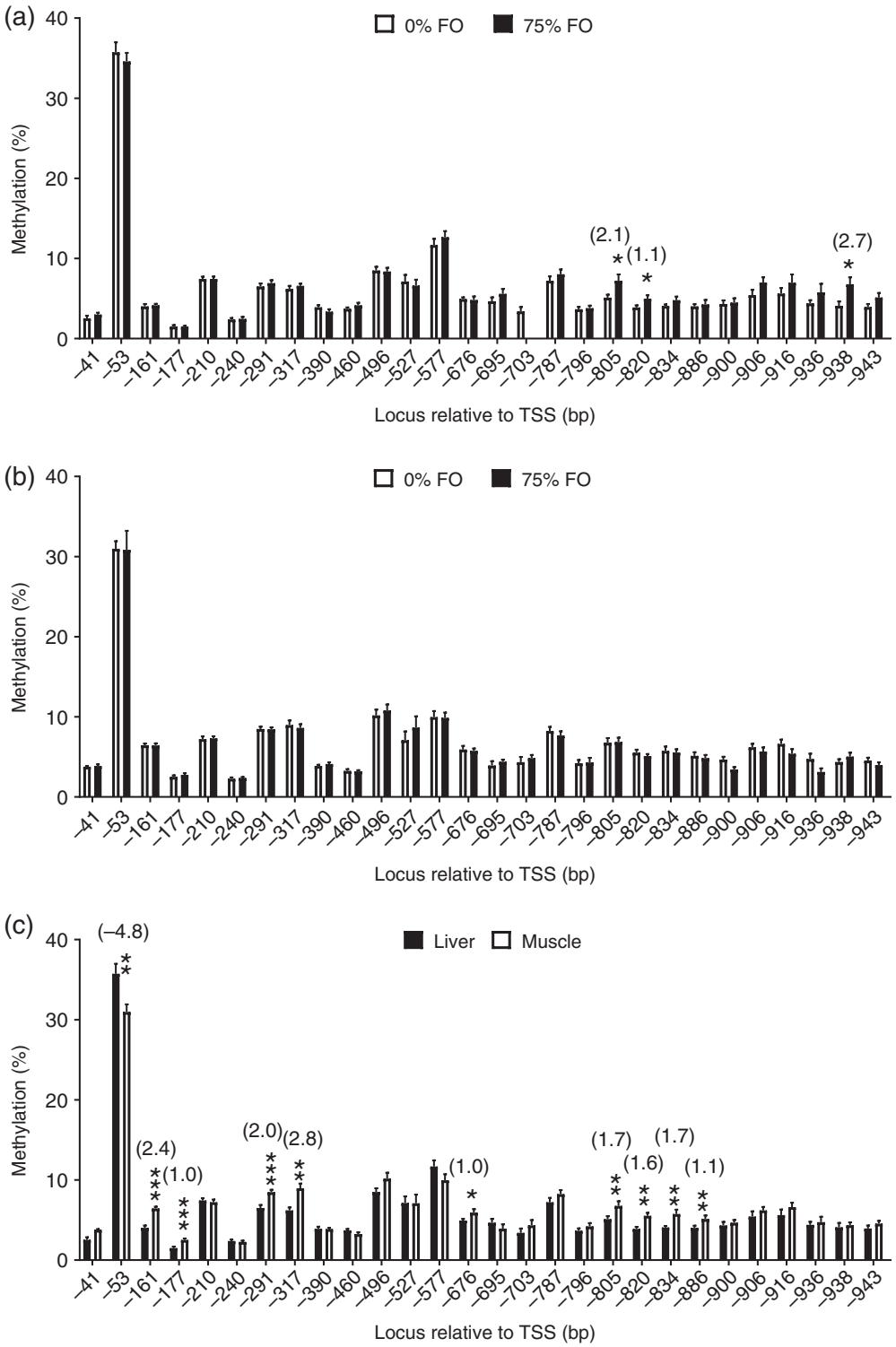

Fig. 4 DNA methylation of individual CpG loci within a 109 bp region upstream of the *elovl5* transcription start site (TSS) in **(a)** liver and **(b)** skeletal muscle from fish fed diets containing either 0% fish oil (FO) or 75% FO, and **(c)** liver compared to muscle from fish fed a diet containing 0% FO. The *x*-axis shows locations of CpG dinucleotide pairs relative to the TSS (chromosome locations are shown in Table S3). Values are mean ± SEM of $n = 24$ samples per dietary group. Numbers in parenthesis indicate differences between means (% points). Statistical comparisons between dietary groups were by Student's unpaired *t* test. Statistical comparisons between tissues within a dietary group were by Student's paired *t* test. Means that differed significantly between dietary groups or tissues are indicated by $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$

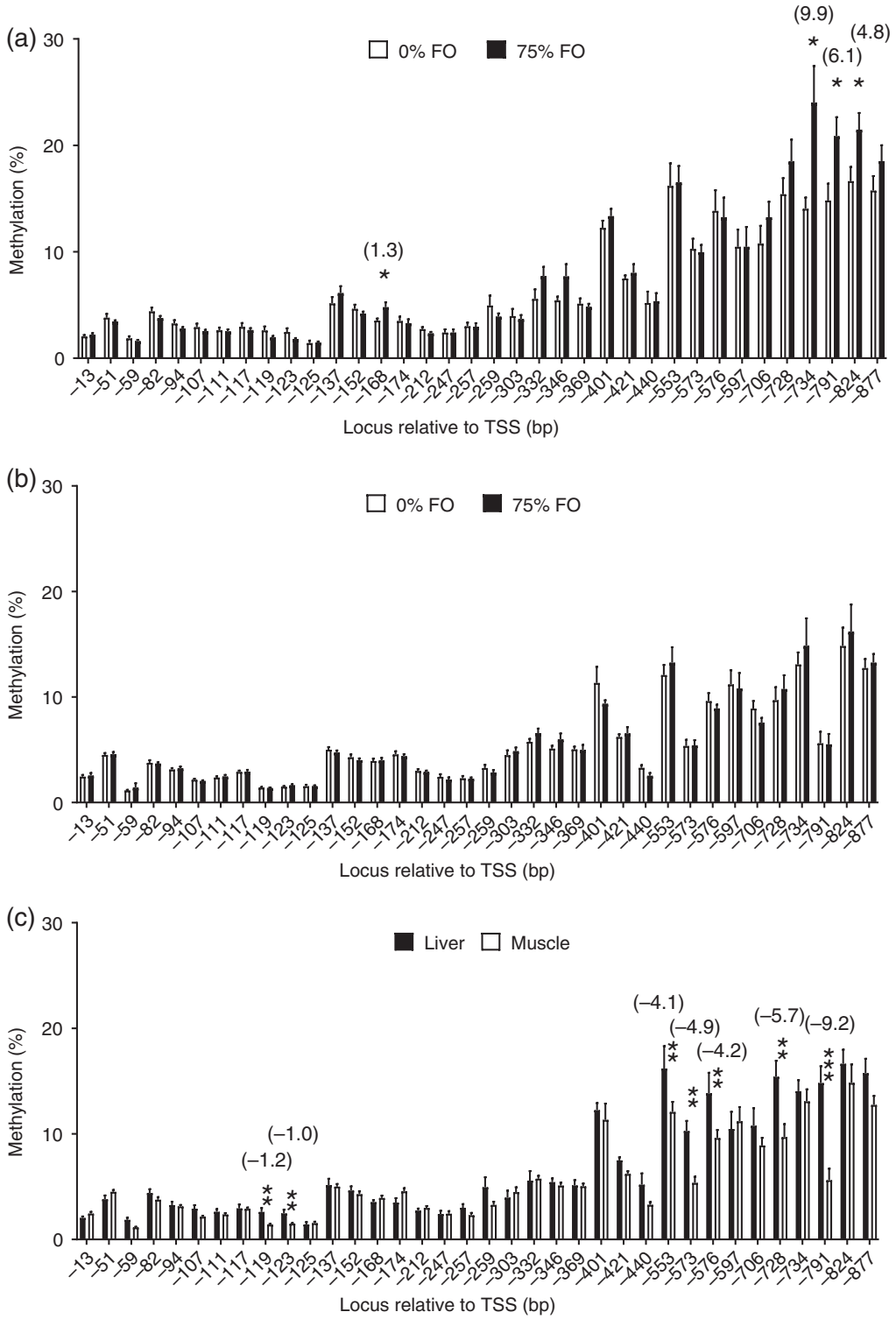

Fig. 5 DNA methylation of individual CpG loci within a 109 bp region upstream of the *elovl2* transcription start site (TSS) in **(a)** liver and **(b)** skeletal muscle from fish fed diets containing either 0% fish oil (FO) or 75% FO, and **(c)** liver compared to muscle from fish fed a diet containing 0% FO. The x-axis shows locations of CpG dinucleotide pairs relative to the TSS (chromosome locations are shown in Table S3). Values are mean ± SEM of $n = 24$ samples per dietary group. Numbers in parenthesis indicate differences between means (% points). Statistical comparisons between dietary groups were by Student's unpaired *t* test. Statistical comparisons between tissues within a dietary group were by Student's paired *t* test. Means that differed significantly between dietary groups or tissues are indicated by $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$

Methylation of CpG −876 and CpG −936 was significantly lower (both <3.0% pts) in liver from fish fed a 75% FO diet compared to fish fed a 0% FO diet (Fig. 2a). The methylation status of seven CpG loci differed significantly (all <4.0% pts) in skeletal muscle from fish fed a 75% FO diet compared to fish fed a 0% FO diet (Fig. 2b). Methylation of two of these loci, CpGs −982 and −1009, was higher in fish fed a 75% FO diet compared to fish fed a 0% FO diet. Only CpG −936 was altered significantly by the amount of dietary FO in both liver and skeletal muscle. Sixteen CpG loci differed in methylation (0.9 to 35.7% pts) between liver and skeletal muscle from fish fed a 0% FO diet (Fig. 2c). The methylation of 13 of these loci was significantly higher in liver compared to skeletal muscle.

Twenty-one CpG loci were measured in a region 1 kb upstream from the Δ5*fad* TSS (Fig. 3). The methylation status of CpG −649 and −755 was significantly greater in the liver of fish fed the 75% FO diet compared to fish fed the 0% FO diet (Fig. 3a). In contrast, the amount of dietary FO did not significantly alter the methylation status of the CpG loci measured in the Δ5*fad* gene in skeletal muscle (Fig. 3b). The methylation status of CpGs −280 and −354 was significantly higher in muscle from fish the 0% FO diet compared to liver, while the level of methylation of CpGs −649 and −734 was significantly lower in muscle than liver from these fish (Fig. 3c).

Twenty-eight CpG loci were measured in a region 1 kb upstream from the *elovl5* TSS (Fig. 4). The methylation status of CpGs −805, −820 and −938 was significantly greater (all less than 3% pts difference) in the liver of fish fed a 75% FO diet compared to fish fed a 0% FO diet (Fig. 4a). There were no significant differences in methylation status of *elovl 5_b* CpGs in skeletal muscle from fish fed a 75% FO diet compared to fish fed a 0% FO diet (Fig. 4b). The methylation status of CpGs −161, −177, −291, −317, −676, −805, −820, −834, −886 and −354 was significantly higher (all difference less than <3.0% pts) in muscle from fish the 0% FO diet compared to liver, while the level of methylation of CpGs −649 and −53 was significantly lower in muscle than liver from these fish (Fig. 4c).

Thirty-six CpG loci were measured in a region 1 kb upstream from the *elovl2* TSS (Fig. 5). The methylation status

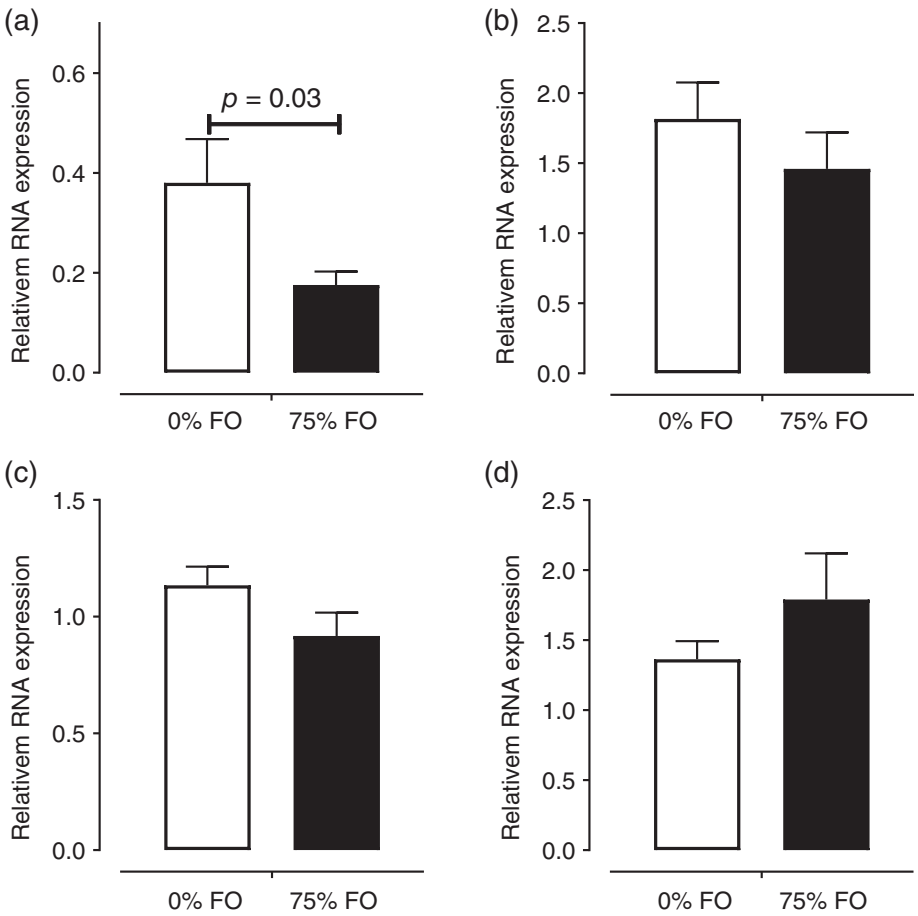

Fig. 6 Relative mRNA expression of **(a)** Δ6*Fad_b*, **(b)** Δ5*Fad*, **(c)** *elovl5* and **(d)** *elovl2* in liver from fish fed diets containing either 0% FO or 75% FO. Values are mean ± SEM of $n = 24$ samples per dietary group. Statistical comparisons between dietary groups were by Student's unpaired *t* test

Table 2 The relationship between the methylation status of individual CpG loci and the proportions of 20:5n-3 and 22:6n-3 or mRNA expression in liver

	Pearson's correlation coefficient (β)	
DM CpG (bp)	20:5n-3 + 22:6n-3 ($n = 48$)	mRNA expression ($n = 48$)
Δ6fad_b		
−124	0.131	−0.1
−134	0.353*	−0.1
−481	0.035	−0.02
−505	−0.130	−0.345*
−745	0.114	−0.339*
−876	−0.391**	−0.13
−936	−0.389*	−0.02
−982	−0.044	0.1
−1009	−0.196	0.2
Δ5fad		
−649	0.161	−0.173
−755	0.230	−0.196
elovl5_b		
−805	−0.211	0.343
−820	−0.088	−0.228
−900	0.154	−0.072
−938	0.288	0.266
elovl2		
−168	0.347	0.09
−734	0.299	0.06
−791	0.123	−0.06
−824	−0.267	−0.148

Values are Pearson's correlation coefficients ($n = 48$) for the relationship between the DNA methylation status of CpG loci that were differentially methylated (DM CpG) between dietary groups and the proportion of 20:5n-3 + 22:6n-3, and mRNA expression of each gene irrespective of the fish oil content of the diet in liver. The locations of individual CpG loci are relative to the transcription start site (bp) (chromosome locations are shown in Table S3. Significant associations are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

of CpGs −168, −734, −791 and −824 was significantly greater in the liver of fish fed a 75% FO diet compared to fish fed a 0% FO diet (Fig. 5a). The amount of dietary FO did not significantly alter the methylation status of the CpG loci measured in the *elovl2* gene in skeletal muscle (Fig. 5b). The methylation status of CpGs −119, −123, −553, −573, −576, −728, and −791 was significantly lower in muscle from fish the 0% FO diet compared to liver, while the level of methylation of CpGs −649 and −53 was significantly lower in muscle than liver from these fish (Fig. 5c).

The Effect of Diet on the mRNA Expression of Genes Involved in Polyunsaturated Fatty Acid Biosynthesis

Feeding a diet containing 75% FO decreased Δ6*fads_b* mRNA expression in liver by 54% ($p = 0.03$), but did not significantly alter the level of Δ6*fads_b* mRNA in skeletal muscle (Fig. 6a). There was no significant effect of diet on the mRNA expression of Δ5*fads*, *elovl5_b* or *elovl2* in liver (Fig. 6b–d). The expression of these genes in skeletal muscle was below the linear range of the assays.

Statistical Associations between DNA Methylation, mRNA Expression and the Proportion of 20:5n-3 Plus 22:6n-3 in Liver

Pearson's correlation coefficients were calculated for CpG loci that showed a significant difference in DNA methylation in liver between fish fed the 0% FO diet and fish fed the 75% FO diet (Table 2). Correlation analysis was carried out without stratification for dietary group. Methylation of Δ6*fad_b* CpG −134 was positively associated with the sum of proportions of 20:5n-3 and 22:6n-3 (Table 2). Δ6*fad_b* CpGs −876 and −939 were negatively associated with 20:5n-3 + 22:6n-3 (Table 2). Δ6*fad_b* CpGs −745 and −505 were negatively associated with the level of the Δ6*fad_b* transcript.

There were no significant associations between Δ5*fads* or *elovl5_b* CpG loci that were differentially methylated between 0% FO diet and fish fed the 75% FO diet and either the mRNA expression of their respective genes or the proportion of 20:5n-3 + 22:6n-3 in liver (Table 2). However, *elovl2* CpG −734 was significantly negatively associated with the proportion of 20:5n-3 + 22:6n-3 (Table 2). Because there were no differentially methylated Δ5*fad*, *elovl5_b or elovl2* CpG loci in muscle and the levels of genes expression were below the limits of detection, associations between DNA methylation and with gene expression or the proportion of 20:5n-3 + 22:6n-3 were not tested.

Discussion

As expected, increasing the FO content of the diet increased the proportions of 20:5n-3, 22:5n-3 and 22:6n-3 in total liver lipids (Bou et al., 2017c). This was accompanied by reduction in the proportion of 18:1n-9 and an increase in 16:0, which may reflect differences in the amounts of these fatty acids between the diets, in agreement with previous results from trials with Atlantic salmon fed increased levels of FO in the diet (Betancor et al., 2014; Bou et al., 2017a; Minghetti et al., 2011).

Increasing the proportion of FO in the diet decreased mRNA expression of Δ6*fad_b* in liver. This is in agreement with previous observations of the effect of varying the FO content of the diet on Δ6*fad_b* mRNA expression (Bou et al., 2017c; Rosenlund et al., 2016; Ruyter et al., 2016; Zheng et al., 2005), which has been shown to involve sterol

regulatory element binding protein and liver–X-receptor networks (Betancor et al., 2014). One possible explanation for the absence of significant change in the mRNA expression of Δ5*fad*, *elovl2* and *elovl5* in liver is that the amount of FO fatty acids that were assimilated by the liver was insufficient to alter the activity of the regulatory networks that control the transcription of these genes in Salmon. This is supported by the observation that in rats the magnitude of change in hepatic DNA methylation of the genes analyzed here is related to the amount of dietary fat (Hoile et al., 2012), although extrapolation from a mammal to a fish needs to be undertaken cautiously. Thus it is feasible that there may be a threshold of fat intake that needs to exceeded to induce changes in DNA methylation. In contrast, increasing the FO content of the diet decreased the expression of Δ6*fad,* Δ5*fad*, and *elovl2*, but not *elovl5* in liver of Atlantic salmon (Bou et al., 2017a; Rosenlund et al., 2016; Zheng et al., 2005), while in meager (*Argyrosomus regius*) an increase in *elovl5* expression was reported (Silva-Brito et al., 2016), which may indicate differences between species in capacity to regulate PUFA synthesis in response to dietary lipid. However, this interpretation may be confounded by differences between studies in the n-3 PUFA content of the diet (Brodtkorb et al., 1997).

Increased dietary intake of n-3 PUFA has been shown to modify DNA methylation of specific CpG loci in the 5′-regulatory regions of *FADS2* and *ELOVL5* in adult humans (Hoile et al., 2014) and of *Fads2* in the adult offspring of rats fed a 20:5n-3 and 22:6n-3 –enriched diet during pregnancy (Hoile et al., 2012). The magnitude, location and direction of such induced epigenetic changes in humans differed between sexes (Hoile et al., 2014), while in rodents the change in DNA methylation was related inversely to the proportion of FO in the maternal diet (Hoile et al., 2012). The present findings show that there were significant differences between liver and muscle in the level of DNA methylation of specific CpG loci in all four genes studied. The methylation status of the majority of differentially methylated loci in Δ6*fad_b* was greater in liver than muscle, while all elovl5 differentially methylated loci showed higher methylation in skeletal muscle than liver. There was no overall pattern of differentially methylated loci *fads1* and *elovl2*. However, the expression of all four genes was significantly higher in liver compared to skeletal muscle. The regulation of DNA methylation and status of individual CpG loci is well known to differ between tissues as a mechanism to tailor the expression of the transcriptome to the function of individual cell types (Godfrey et al., 2015). Thus any suggestion as to why one tissue has a particular DNA methylation pattern compared with another, or why one tissue appears to be more responsive to dietary manipulation than another would just be speculation. However, demonstration of induced epigenetic variation in response to a particular dietary manipulation may provide insights into mechanisms by which dietary changes can alter tissue function. A recent study pointed to a very low correlation regarding 20:5n-3 and 22:6n-3 content between liver and skeletal muscle and suggested that the n-3 PUFA metabolic pathway most probably are of less importance determining the 20:5n-3 and 22:6n-3 content of muscle than that of liver (Horn et al., 2018), also indicating that there are tissue differences related to its regulation.

The methylation status of individual Δ6*fad_b* CpG loci was related negatively to the level of expression of the corresponding mRNA transcript and to the proportions of 20:5n-3 + 22:6n-3 in liver. This is consistent with effect of DNA methylation on the transcription of these genes in mammalian tissues (Hoile et al., 2012; Hoile et al., 2014) which suggests that DNA methylation of specific loci may be involved in the regulation of Δ6*fad_b*, but not of Δ5*fad*, or *elovl2* or *5,* transcription in salmon liver.

Previous studies have shown that feeding adaptation to a vegetarian diet in grass carp (*Ctenopharyngodon idella*) and feeding rainbow trout (*Oncorhynchus mykiss)* diets with different vitamin contents can induce gene-specific changes in DNA methylation (Cai et al., 2018; Liu et al., 2017). Furthermore, feeding n-3 PUFA or FO-enriched diets increased the average methylation of the Δ6*fad* promoter by approximately 3 to 4% points in Japanese seabass compared to fish fed diets enriched in saturated or monounsaturated fatty acid diets (Xu et al., 2014). Increasing the proportion of FO in the diet of salmon induced relatively modest changes in methylation of individual CpG loci in Δ6*Fad_b*, Δ5*Fad*, *Elovl5,* and *Elovl2* in liver, and in Δ6*Fad_b* in muscle. The magnitude of the difference in Δ6*Fad* methylation in liver was similar to that reported in Japanese seabass (Xu et al., 2014). Together these findings suggest that dietary oil can induce in fish tissue-related changes in mRNA expression and in the DNA methylation status of individual CpG loci. One possible further interpretation is that capacity to change specific DNA methylation marks in response to dietary fat intake may have been conserved through evolution since the last common ancestor of mammals and teleost fish as a possible mechanism by which organisms can adapt to variation in food availability. However, since these changes were small, detailed analysis of the role of the differentially methylated loci in the regulation of the respective genes is needed in order to be confident that they represent functionally significant alterations to gene regulation or tissue function.

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Author Contributions N.A.I. and T.-K.Ø. performed all the experiments. N.A.I. and G.C.B. analyzed the data. A.K.S., B.R., K.A.L.,

G.C.B. designed the study and were responsible for its conduct. G.C.B. wrote the first draft of the manuscript with input from the other authors.

Compliance with Ethical Standards The study was conducted in compliance with the national regulation for use of experimental animals (FOR-2015-06-18-761) and classified as not requiring a specific license (§2-f, corresponding to Directive 2010/63/EU Article 1, section 5f).

Conflict of Interest The authors declare that they have no conflict of interest.

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