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Atlantic salmon (*Salmo salar*) muscle structure integrity and lysosomal cathepsins B and L influenced by dietary n-6 and n-3 fatty acids

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ABSTRACT

This study had two main objectives: first, to evaluate the impact of different types and levels of dietary n-6 and n-3 fatty acids (FAs) on Atlantic salmon muscle structure integrity; second, to highlight a possible role of lysosomes and lysosomal degrading enzymes, cathepsins, in fish muscle structure integrity, in relation to dietary fatty acids. Four groups of Atlantic salmon (90 g starting weight) in fresh water tanks were fed one of four diets containing 23% crude lipids, with 100% of the added oils as either fish oil (FO), rapeseed oil (RO), eicosapentaenoic acid (EPA) enriched-oil or docosahexaenoic acid (DHA) enriched-oil. The RO diet was characterised by low levels of EPA + DHA (10% of total FAs), whereas the EPA and DHA diets were characterised by very high levels of EPA + DHA (>50% of total FAs). Fatty acid composition of the muscle crude lysosomal fraction (CLF) generally reflected the diets. Salmon fed the RO diet presented a muscle CLF FA composition close to the one of the FO group, showing moderate PUFA levels, and comparable cathepsin B and cathepsin L activities, relative gene expression of cathepsin B and cathepsin L in the muscle and rate of myofibre–myofibre detachments post-mortem. Salmon fed the EPA and DHA-enriched-oil diets presented a fairly similar muscle CLF FA composition, but different from the FO and RO groups. In the EPA and DHA groups, the percentage of PUFAs in the muscle CLF, the rate of myofibre–myofibre detachments and the relative gene expression of cathepsin B were higher than in the FO and RO groups. Cathepsin B and cathepsin L total activities in the muscle were however lower in the EPA and DHA groups 0 h post-mortem. Dietary lipids influenced the level of lysosomal degrading enzyme activity cathepsin B and cathepsin L as well as the relative gene expression of cathepsin B. Feeding Atlantic salmon with rapeseed oil and extreme levels of EPA + DHA highlighted the impact of fatty acid composition of the diet on salmon muscle integrity and the complexity of the process involving muscle lysosomes and cathepsins in relation to these dietary fatty acids.

1. Introduction

Fish oils are characterised by high levels of long-chain n-3 polyunsaturated fatty acids (PUFAs) (20 and 22 carbon atoms) and low levels of n-6 fatty acids (FAs), whereas plant oils often contain shorter FAs (18 carbon atoms and less) and high levels of n-6 FAs. Atlantic salmon have low ability to convert linolenic acid (18:3 n-3) into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Bell, Tocher, Henderson, Dick, & Crampton, 2003) and thus, these FAs need to be provided in the diet. Fish oils have traditionally been the main dietary lipid source used for Atlantic salmon farming. However, the farming industry is today growing, while natural resources of dietary fish oils are declining. New alternatives to fish oils are therefore needed and have already been largely studied in order to encounter this decline. At present, vegetable oils seem to constitute the most suitable lipid sources to partially or totally replace fish oil in salmon feed (Bell et al., 2001, 2002; Olsen et al., 2006; Rosenlund, Obach, Sandberg, Standal, & Tveit, 2001; Torstensen, Froyland, Ornsrud, & Lie, 2004; Thomassen & Røsjø, 1989; Torstensen et al., 2005). In most studies, changes in muscle fatty acid composition and nutritional quality have been in focus. To what extent substantial changes in dietary fatty acid composition may influence other quality parameters is however not well known. To our knowledge, the present study is the first one highlighting a possible role of muscle lysosomes and lysosomal enzymes, cathepsins, in fish flesh quality/degradation in relation to dietary fatty acids. Thomassen and Røsjø (1989) found significant effects on odour, taste, colour shade and colour

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intensity in Atlantic salmon given a feed containing the highest amount of vegetable oil (with up to 68% replacement of fish oil by soybean or rapeseed oil) and Izquierdo et al. (2005) showed that at least 60% replacement of fish oil by soybean oil in seabream diet (diet with 60% PUFAs among which 50% were n-3 FAs (% of total FAs)) given for 7 months, resulted in a softer texture of the flesh.

Several studies suggested that gradual degradation of the extracellular matrix as well as breakages in the muscle cell cytoskeleton and connective tissue (myofibre–myofibre and myofibre–myocommata detachments) were one of the causes of post-mortem fillet softening and could determine flesh texture (Ando, Toyohara, Shimizu, & Sakaguchi, 1991; Bahuaud et al., 2008; Ofstad, Olsen, Taylor, & Hannesson, 2006; Taylor, Fjæra, & Skjervold, 2002). Köhler (1991) showed that the lysosomal system reacted to injury in the liver of flounder, with a two step response: first an adaptive and protective response with an increase in number and size of lysosomes; and second a step where the lysosomal membrane stability decreases to release the degrading enzymes. Lysosomal enzymes have thus been associated with post-mortem fish muscle tenderisation (Ando et al., 2001; Bahuaud et al., 2008; Chéret, Delbarre-Ladrat, de Lamballerie-Anton, & Verrez-Bagnis, 2007; Jessen, 2008; Yamashita & Konagaya, 1991). Jessen (2008) suggested that cathepsin B and cathepsin D were related to flesh texture and participated in the softening of rainbow trout muscle. In several other fish species, cathepsins B, D and L seem to be the most involved enzymes in post-mortem muscle degradation (Ando et al., 2001; Jiang, Wang, & Chen, 1992; Ladrat, Verrez-Bagnis, Noel, & Fleurence, 2003), with a particularly high participation of cathepsin L in salmon (Yamashita & Konagaya, 1990; Yamashita & Konagaya, 1991). In a recent study (Bahuaud et al., 2008), our results indicated a correlation between lysosomal breakages, release of cathepsins and muscle degradation rates.

The aim of the present study was to evaluate the effects of different levels and sources of dietary n-6 and n-3 FAs on Atlantic salmon muscle structure integrity and to reveal a possible role of muscle lysosomes and lysosomal degrading enzymes, cathepsins, on flesh degradation processes in relation to dietary fatty acid composition.

2. Materials and methods

2.1. Fish and diets

Four groups of Atlantic Salmon (*Salmo salar*) with an initial average weight of 90 g were distributed in 3 cylinder-conical (0.85 m diameter) fresh water tanks per group (temperature of the water 10.1 °C) at Nofima Marin Research Station (previously AKVAFORSK), Sunndalsøra, Norway. Four different extruded diets (Table 1) were prepared with 50% (of total weight) crude proteins and 23% (of total weight) crude lipids, with 100% of the added oils as fish oil (FO), rapeseed oil (RO), eicosapentaenoic acid (EPA)-enriched-oil or docosahexaenoic acid (DHA)-enriched-oil.

Each salmon group was fed one of the four diets for 21 weeks until reaching an average weight of 344 g (±69.8). Three fish per group taken randomly from the different tanks were anaesthetized in metacain (MS-222) provided by Norsk Medisinaldepot (Norway), killed with a blow to the head, gill cut and stored in a refrigerated room (4 °C) for 96 h until further analyses.

2.2. Subcellular fractionation of the muscle

2.2.1. Preparation of the muscle crude lysosomal fraction (CLF)

Subcellular fractionation was made immediately post-mortem on fresh muscle samples taken from each salmon in all dietary groups (FO, RO, EPA and DHA), cranial of the back fin. We used a lysosomal isolation kit (LYSIS01, SIGMA) following the procedure given by the manufacturer. Briefly, 5 g of muscle were taken from the upper layer of the front part of each salmon, in the dorsal section, above the mid-line of the fillet. The muscle samples were first homogenised in 20 ml of Extraction Buffer 1 × (Extraction Buffer 5 × (Product Code E 1156, SIGMA) diluted 5 times in ultra-pure water) mixed with a Protease Inhibitor Cocktail (Product Code P 8340, SIGMA) at a final concentration of 1% (v/v). The homogenate was fractionated by a first centrifugation at 1000*g* for 10 min at 4 °C. While the obtained supernatant was transferred into a new tube, the pellet was homogenised a second time in 2 volumes of Extraction Buffer 1× and centrifuged again at 1000*g* for 10 min at 4 °C. The obtained supernatant was then mixed with the previous supernatant and centrifuged at 20,000 *g* for 20 min at 4 °C. The obtained pellet (Crude Lysosomal Fraction (CLF)) was resuspended in 4 ml of Extraction Buffer 1x and kept frozen at −20 and −80 °C until further analyses. According to the manufacturer of the kit, the muscle CLF primarily consists of an up-concentrated fraction of lysosomes, but will not be completely purified, and contains a small degree of contamination with mitochondria, peroxisomes and endoplasmic reticulum.

Table 1
Formulation and chemical composition of the diets.

Formulation (% of total weight)	FO	RO	EPA	DHA
Fish meal, LT	67.9	67.9	67.9	67.9
Fish oil	13.5			
Rapeseed oil		13.5		
EPA[a]			13.5	
DHA[b]				13.5
Wheat	17.1	17.1	17.1	17.1
Vitamin premix[c]	1.0	1.0	1.0	1.0
Mineral premix[c]	0.4	0.4	0.4	0.4
Yttrium oxide[d]	0.01	0.01	0.01	0.01
Carophyll Pink[e] (8%)	0.064	0.064	0.064	0.064
Chemical composition				
Dry matter (%)	92.3	92.2	93.0	92.2
% of dry matter				
Crude proteins	55.4	54.6	55.1	54.4
Crude fat	22.5	23.0	22.7	23.3
Ash	9.5	9.9	9.5	9.7
Gross energy (MJ/kg)	23.8	23.9	23.6	23.8

150 ppm BHT were added to the oils used in the diets. LT = Low Temperature.
[a] Incromega EPA 500TG SR, Croda Chemicals Europe Ltd., Goole, England.
[b] Incromega DHA 500TG SR, Croda Chemicals Europe Ltd., Goole, England.
[c] Mundheim, Aksnes, and Hope (2004).
[d] Inert marker, Y_2O_3, Sigma.
[e] Hoffman-LaRoche, Basel, Switzerland.

2.2.2. Preparation of the muscle homogenates

Some muscle samples were further taken from each salmon in all dietary groups (FO, RO, EPA and DHA), cranial of the back fin 0 h post-mortem, and immediately frozen in liquid nitrogen before being stored at −80 °C until further analyses. Three hundred milligrams of frozen muscle were homogenised in 1 ml extraction buffer (100 mM Na-acetate in 0.2% Triton X-100, pH 5.5) in Precellys tubes, in a Precellys 24 homogeniser (Bertin Technologies, France) using 2 cycles of 20 s at 5500 rpm, separated by a 10 s break. The obtained homogenates were centrifuged at 16,000 *g* for 30 min and the supernatants were used to measure cathepsin B + L, cathepsin B and cathepsin L activities.

2.3. Lipid class and fatty acid compositions of the muscle crude lysosomal fraction (CLF)

2.3.1. Lipid class composition of the muscle CLF

Lipids from the muscle CLF samples were extracted by adding chloroform/methanol (2:1, v/v) and 19:0 methylester was added

as an internal standard. After extraction of lipids, the samples were filtered and an aliquot was removed for determining lipid class composition. Lipid class composition of the muscle CLF was quantified using high-performance thin-layer chromatography (HPTLC) as described by Bell, Dick, Vicar, Sargent, and Thompson (1993) and Jordal, Lie, and Torstensen (2007). Ten μg total lipid were applied by using an automatic sample applicator (ATS4, CAMAG, Muttenz, Switzerland) onto a 10 × 20 cm HPTLC 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 at 5.5 cm in methyl acetate: isopropanol: chloroform: methanol: 0.25% (w/v) aqueous KCl (25:25:25:10:9 by volume) to separate phospholipid classes with neutral lipids running at the solvent front (Vitello & Zanetta, 1978) using an automatic developing chamber (AMD2, CAMAG, Muttenz, Switzerland). After drying, the plates were developed fully in hexane: diethyl ether: acetic acid (80:20:2, v/v/v) to separate neutral lipids and cholesterol. Lipid classes were visualised by charring at 160 °C for 15 min after dipping the plate in a glass tank with 3% copper acetate (w/v) in 8% (v/v) phosphoric acid. The lipid classes were identified by comparison with commercially available standards and quantified by scanning densiometry using a CAMAG TLC Scanner 3 and calculated using an integrator (WinCATS-Planar Chromatography, Version 1.3.3). Further, 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 mix of all the lipid classes at each HPTLC plate for corrections of between plate variations.

2.3.2. Fatty acid composition of total fatty acids of the muscle CLF

Fatty acid composition of the muscle CLF samples was analysed. After determination of lipid class composition as described above, the remaining samples were saponified and methylated using 12% BF_3 in methanol. Fatty acid composition of total lipids was analysed using methods described by Lie and Lambertsen (1991) where the methyl esters were separated using a Trace gas chromatograph 2000 (Fison) ("cold on column" injection, 60 °C for 1 min, 160 °C for 28 min, 190 °C for 17 min, 220 °C for 10 min), equipped with a 50 m CP-sil 88 (Chromopack) fused silica capillary column (id: 0.32 mm). The fatty acids were identified by retention time using standard mixtures of methylesters (Nu-Chek, Elyian, USA). All samples were integrated using the Totalchrom software (ver. 6.2, Perkin Elmer) connected to the GLC.

2.4. Microscopic observations of the muscle

Muscle blocks (2 × 2 × 3 mm) were taken from each salmon in all dietary groups (FO, RO, EPA and DHA), from the upper layer of the front part of each salmon, in the dorsal section of the fillet, above the mid-line, and fixed in 2.5% glutaraldehyde in cacodylate buffer, pH 7.3. Sampling was performed 0 h, 12 h, 24 h and 96 h post-mortem. The fixed samples were then transported in refrigerated boxes to the laboratory and kept there at cool temperature (4 °C) until they were embedded for light microscopy analyses.

Histological samples were prepared as described by Bahuaud et al. (2008). Briefly, after being rinsed in cacodylate buffer for 2 × 15 min, the muscle blocks were dehydrated in different gradients of ethanol (70% and 96% for 2 × 20 min, 100% for 1 × 20 min) and embedded in a cold plastic resin based on hydroxyethylmethacrylate (Technovit 7100, Heraeus Kulzer). The direction of the myofibres in the embedded samples was oriented so as to obtain both cross- (for all time points) and longitudinal- (for the 96 h samples) sections. Light microscopy observations at 10× magnification were made on thin sections (3 μm), stained using the method of Ofstad et al. (2006) slightly modified, in 0.05 g/100 ml toluidine blue dissolved in 0.1 M aqueous sodium acetate buffer.

In the cross-section samples, muscle degradation was evaluated according to the method of Taylor et al. (2002) slightly modified. Briefly, we counted the percentage of degraded endomysium between myofibres (myofibre–myofibre detachments), as well as detachments between myofibres and connective tissue (myofibre–myocommata detachments). Two samples per fish and per time point were analysed. For each sample, detachments were counted based on 250 junctions between myofibres, and 50 junctions between myofibres and myocommata.

In the longitudinal-oriented 96 h samples, muscle quality was determined by counting myofibre breakages and contracted myofibres. Two samples per fish were analysed. For each sample, percentages of myofibre breakages and contracted myofibres were evaluated, based on a total of 50 myofibres per sample.

2.5. Cathepsin B + L, cathepsin B and cathepsin L activities in the muscle

Cathepsin B + L activity was measured fluorimetrically according to the method of Kirschke, Wood, Roisen, and Bird (1983) on the 0 h sample muscle CLF, muscle CLF supernatants, and muscle homogenates. The procedure used N-CBZ-L-phenylalanyl-L-arginine-7-amido-4-methylcoumarin (Z-Phe-Arg-Nmec) as a substrate. We determined the release of the fluorogenic reagent 7-amido-4-methylcoumarin by fluorescence measurement at excitation 360 nm and emission wavelengths 460 nm. The assays were run twice in triplicates.

Separate cathepsin B and cathepsin L activities were determined on the 0 h sample muscle homogenates only. Cathepsin B activity was determined the same way as cathepsin B + L, but using N-CBZ-L-arginyl-L-arginine-7-amido-4-methylcoumarin (Z-Arg-Arg-Nmec) as a substrate. Cathepsin L activity was determined by subtracting the results from cathepsin B activity from the cathepsin B + L activity results. In all cases, the assays were run twice in triplicates.

2.6. Cathepsin B and cathepsin L relative gene expression

The epaxial muscle in the back fin of Atlantic salmon was dissected immediately post-mortem (0 h samples) and skin and red muscle removed. The tissue samples were kept at −80 °C until analysis. Total RNA was isolated using TRIzol® Reagent (Invitrogen, USA) according to the manufacturer instructions. Total RNA was DNase I and was treated with a Turbo DNA-free kit (Ambion, USA). Purification of RNA was performed by precipitation using a 0.1 volume of 3 M Sodium Acetate and 3 volumes of 99% ethanol. After 30 min incubation at room temperature, centrifugation for 10 min at 4 °C and 10,000 *g*, the pellet was dried and then resuspended in 40 μL RNase free water.

Concentration and quality of RNA was measured with a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, USA). cDNA was synthesised using oligod(T) primers from 0.5 μg RNA in a total volume of 50 μL using Taqman® reverse transcriptase reagents (Applied Biosystems, USA) according to the manufacturer protocol. BLAST search and Vector NTI Advance 10 (Invitrogen, USA) was used to identify and analyse salmon cathepsin L and cathepsin B sequences. Vector NTI Advance 10 was used to design real time PCR primers which were purchased from Invitrogen (Invitrogen, USA).

Real time PCR was performed in a LightCycler 480 Instrument (Roche Applied Science, Germany) with gene-specific primers for cathepsin B and cathepsin L. Both RNA polymerase 2 and eukaryotic translation initiation factor 3 were evaluated as reference genes, and the latter was used due to more stable gene expression. Table 2 gives the real time PCR primer sequences for relative expression of cathepsin B, cathepsin L and eukaryotic translation

Table 2
Real time PCR primer sequences for relative gene expression of cathepsin B and cathepsin L in the muscle.

Primer	Genbank no.		5′–3′
Cathepsin B	DR696159	Ssa cathepsin B F1	AGGGGGGAACTCCTTACTGGCT
		Ssa cathepsin B R1	CGATGCCACAGTGGTCCTTACCT
Cathepsin L	CB502996	Ssa cathepsin L F1	GTATAGTGAAATGTGTGACC
		Ssa cathepsin L R2	AACCAGAGCAATAATTCAAG
Eukaryotic translation initiation factor 3	DW542195	Ssa etif3 F1	CAGGATGTTGTTGCTGGATGGG
		Ssa etif3 R1	ACCCAACTGGGCAGGTCAAGA

initiation factor 3. PCR master mix consisted of 1 μL forward and reverse primer (final concentration of 0.5 μM), 4 μL 1:10 dilution of cDNA and 5 μL LightCycler 480 SYBR Green I Master (Roche Applied Science, Germany). All samples were analysed in triplicates with non-template control for each gene. The reaction condition was 95 °C for 5 min, 45 cycles of 95 °C/15 s and 60 °C for 1 min. Melting curve analysis (95 °C for 5 s and 65 °C for 1 min, 97 °C) was run to confirm the presence of a single PCR product. Primer efficiency was calculated for each primer pair.

The relative gene expression level was calculated according to the ΔΔCt method and adjusted for differences in primer efficiency (Plaffl, 2001). The fish oil (FO) group was used as a control, and the other dietary groups were compared to this.

2.7. Statistical analyses

The relation between the result data (Y) and dietary groups (D) was assumed to be linear during the experiment and parallel for the four dietary groups FO, RO, EPA and DHA. For the cathepsin B + L, cathepsin B and cathepsin L activities, cathepsin B and cathepsin L gene expression, contracted myofibres and lipid class/fatty acid composition, the variable "diet" was tested and the model that explained the data was therefore:

$$Y_{ij} = \mu + bD_{ij} + e_{ij}$$

where Y_{ij} represents cathepsin B + L, cathepsin B or cathepsin L activities (mU/g muscle), contracted myofibres (%) or lipid class/fatty acid composition (% of total FAs) for individual j in group i; D_{ij} represents the diet given to individual fish j in dietary group i ($n = 3$); μ is the overall mean; b the unknown parameter to be estimated and e_{ij} the random errors.

For myofibre–myofibre and myofibre–myocommata detachments, the variable "time" was also added, and the model that explained the data was therefore:

$$Y_{ij} = \mu + b_1 D_i + b_2 T_j + b_3 I_{ij} + e_{ij}$$

where Y_{ij} represents myofibre–myofibre or myofibre–myocommata detachments for individual j in group i; D_i represents the diet given in dietary group i ($n = 3$); T represents the Time post-mortem for individual j and I_{ij} represents the Interaction Diet*Time for individual j in dietary group i; μ is the overall mean; b_1, b_2 and b_3 the unknown parameters to be estimated and e_{ij} are the random errors.

Mean results per dietary group were analysed by the General Linear Model (GLM). We used SAS (Statistical Analysis System) release 8.02 (SAS Institute Inc., Cary, NC, USA) as statistical software, with "diet" and "time" as explanatory variables. Significant differences among means were ranked by Least Squares Means at $p < 0.05$.

3. Results

3.1. Fish and diets

Fatty acid composition of the four diets is described in Table 3. The FO diet contained more than twice the amount of saturated fatty acids (SFAs) as the three other diets. The FO and RO diets were characterised by the highest percentage of monounsaturated fatty acids (MUFAs), whereas the EPA and DHA diets contained almost twice their percentage of PUFAs, reaching more than 60% of the total fatty acids, with 50% consisting of EPA + DHA. The EPA and DHA diets contained almost three times the percentage of EPA + DHA than the FO diet, and five times that of the RO diet. The RO diet presented the lowest n-3/n-6 ratio and percentage of EPA + DHA.

No significant differences in weight ($p = 0.683$) and length ($p = 0.269$) were observed between the dietary groups at the day of sampling.

3.2. Lipid class and total fatty acid compositions of the muscle crude lysosomal fraction (CLF)

Table 4 gives the percentages of lipid class and total fatty acid compositions of the muscle CLF of salmon fed from the four diets (FO, RO, EPA and DHA).

Table 3
Fatty acid composition of the diets (% of total FAs).

Fatty acids	Diets			
	FO	RO	EPA	DHA
14:0	6.88	1.99	2.09	2.22
16:0	12.87	7.58	4.91	5.36
18:0	1.46	1.53	0.62	1.50
20:0	3.66	0.44	4.57	0.39
22:0	0.07	0.30	0.07	nd
24:0	0.03	0.08	0.12	0.47
Total saturated[a]	25.85	12.19	12.68	10.27
16:1n-7	4.26	1.23	1.27	1.37
18:1n-7	1.67	2.77	0.88	1.14
18:1n-9	7.84	40.22	3.73	5.26
20:1n-9	9.96	4.27	3.65	5.18
22:1n-11	15.76	0.19	5.28	6.59
Total monounsaturated[b]	43.07	49.87	18.37	23.83
18:2n-6	2.70	14.09	2.20	1.61
18:3n-3	1.71	7.27	1.02	0.91
18:3n-6	0.11	nd	0.31	0.52
20:3n-6	0.40	0.19	2.35	1.34
20:4n-3	1.04	0.45	0.65	0.67
20:4n-6	nd	0.06	0.12	0.32
20:5n-3 (EPA)	7.12	4.95	42.87	8.68
22:5n-3	0.76	0.29	1.58	3.27
22:5n-6	0.39	2.56	1.29	0.53
22:6n-3 (DHA)	12.18	4.96	12.36	41.73
Total polyunsaturated[c]	28.14	36.14	66.63	63.23
Total n-3	23.34	18.79	59.38	56.13
Total n-6	4.50	17.05	6.80	6.61
n-3/n-6	5.19	1.10	8.73	8.49
EPA + DHA	19.30	9.91	55.23	50.41

nd = not detected.
[a] Includes 15:0, 17:0 and 19:0.
[b] Includes 14:1n-5, 16:1n-9, 17:1n-7, 18:1n-11, 20:1n-7, 22:1n-7, 22:1n-9 and 24:1n-9.
[c] Includes 16:2n-3, 16:2n-6, 16:3n-4, 18:3n-4, 18:4n-3, 20:2n-6, 20:3n-3, 21:5n-3 and 22:4n-6.

Table 4
Fatty acid and lipid class compositions (% of total FAs) in the crude lysosomal fraction (CLF).

Fatty acids	Diets			
	FO	RO	EPA	DHA
14:0	2.1 ± 0.1[a]	1.2 ± 0.0[b]	1.2 ± 0.0[b]	1.4 ± 0.2[b]
16:0	18.0 ± 0.3[a]	16.0 ± 0.5[c]	15.5 ± 0.4[bc]	14.7 ± 0.2[b]
18:0	4.3 ± 0.2	4.2 ± 0.4	4.4 ± 0.3	4.2 ± 0.2
Total Saturated	24.7 ± 0.6[a]	21.5 ± 0.7[b]	21.4 ± 0.7[b]	20.6 ± 0.0[b]
16:1n-7	2.2 ± 0.8	2.1 ± 0.7	1.8 ± 0.4	1.5 ± 0.1
18:1n-7	1.5 ± 0.0[b]	2.3 ± 0.2[a]	1.2 ± 0.1[b]	1.4 ± 0.2[b]
18:1n-9	5.1 ± 0.1[b]	13.9 ± 1.1[a]	4.1 ± 0.4[b]	7.2 ± 2.9[b]
18:1n-11	0.8 ± 0.0[a]	0.3 ± 0.0[c]	0.4 ± 0.0[bc]	0.4 ± 0.0[b]
20:1n-9	1.8 ± 0.1[a]	1.2 ± 0.1[bc]	0.9 ± 0.1[c]	1.6 ± 0.3[b]
22:1n-9	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.2 ± 0.0
22:1n-11	1.4 ± 0.2[a]	0.7 ± 0.1[bc]	0.7 ± 0.2[c]	1.2 ± 0.2[b]
Total Monounsaturated	13.0 ± 0.8[b]	20.7 ± 1.2[a]	9.3 ± 0.6[b]	14.2 ± 3.7[b]
18:2n-6	1.1 ± 0.0[b]	4.8 ± 0.7[a]	0.9 ± 0.0[b]	1.6 ± 0.9[b]
20:4n-6	1.2 ± 0.0[b]	1.6 ± 0.1[a]	1.7 ± 0.1[a]	1.6 ± 0.1[a]
Sum n-6	2.6 ± 0.1[b]	7.8 ± 1.0[a]	2.9 ± 0.2[b]	3.5 ± 0.8[b]
18:3n-3	0.7 ± 0.0[b]	2.8 ± 0.4[a]	0.4 ± 0.0[b]	0.6 ± 0.4[b]
18:4n-3	0.6 ± 0.0[a]	0.4 ± 0.1[b]	0.7 ± 0.0[a]	0.3 ± 0.1[b]
20:4n-3	1.2 ± 0.1[a]	0.9 ± 0.1[a]	1.3 ± 0.1[b]	0.5 ± 0.0[c]
20:5n-3 (EPA)	6.2 ± 0.3[b]	5.0 ± 0.6[b]	17.6 ± 0.9[a]	4.7 ± 0.5[b]
22:5n-3	1.7 ± 0.2[a]	1.2 ± 0.2[b]	3.5 ± 0.2[c]	1.4 ± 0.1[a]
22:6n-3 (DHA)	45.1 ± 1.1[a]	37.8 ± 2.0[b]	41.0 ± 0.2[b]	51.8 ± 4.5[a]
Sum n-3	55.3 ± 1.7[a]	48.0 ± 1.3 [b]	64.4 ± 0.8[c]	59.2 ± 4.6[a]
Total Polyunsaturated	57.9 ± 1.8[bc]	55.8 ± 1.2[b]	67.3 ± 0.8[a]	62.7 ± 3.8[ac]
n-3/n-6	21.7 ± 0.5[a]	6.2 ± 1.0[b]	22.2 ± 1.4[a]	17.7 ± 5.4[a]
Lipid class composition (% of total lipids)				
SM	6.2 ± 1.6[ab]	4.7 ± 0.7[a]	8.5 ± 1.2[b]	7.3 ± 1.1[ab]
PC	34.6 ± 0.7	34.1 ± 2.6	36.5 ± 1.5	33.5 ± 3.0
PS	2.3 ± 0.3[ab]	1.8 ± 0.3[b]	2.5 ± 0.2[a]	2.5 ± 0.3[ab]
PI	6.9 ± 0.7	6.1 ± 1.0	6.6 ± 0.6	5.7 ± 0.4
CL	2.6 ± 0.2	2.4 ± 0.4	n.d. ±	n.d. ±
PE	15.3 ± 0.4[ab]	15.5 ± 0.8[ab]	16.5 ± 0.9[a]	13.2 ± 1.3[b]
Sum PL	67.9 ± 2.5	64.6 ± 3.5	70.6 ± 3.9	62.2 ± 6.0
DAG	2.0 ± 0.2	2.3 ± 0.2	2.0 ± 0.3	2.1 ± 0.2
CHOL	10.2 ± 2.4	10.3 ± 2.3	9.3 ± 1.3	8.3 ± 1.2
FFA	3.8 ± 0.4	3.8 ± 0.5	3.8 ± 0.7	3.6 ± 0.4
TAG	16.1 ± 2.1	19.0 ± 1.9	14.3 ± 3.9	23.8 ± 6.8
Sum NL	32.1 ± 2.5	35.4 ± 3.5	29.4 ± 3.9	37.8 ± 6.0

Different letters denote significant differences between the dietary groups.
CL = cardiolipin; CHOL = cholesterol; DAG = diacylglycerol; FFA = free fatty acid; NL = neutral lipid; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PL = polar lipid; PS = phosphatidylserine; SM = sphingomyelin; TAG = triacylglycerol.

No significant differences were noticed in the percentage of muscle CLF total phospholipids or neutral lipids between the four dietary groups. Significant differences were however observed for some individual phospholipids. No cardiolipin (CL) was detected in the muscle CLF of salmon fed the EPA or DHA diet, whereas 2.6% and 2.4% of CL were found in the FO and RO groups, respectively.

In general, the lipid composition of the muscle CLF reflected the composition of the diets in all groups. The FO diet was characterised by the highest percentage of SFAs, also leading to the highest percentage in the muscle CLF of salmon belonging to this group. The RO diet was characterised by the highest percentages of MUFAs and n-6 FAs as well as the lowest ratio n-3/n-6. The same pattern was observed in the muscle CLF of salmon belonging to this group. The EPA and DHA diets were characterised by the highest percentages of PUFAs, especially n-3 FAs, EPA+DHA, also leading to the highest percentages of these FAs in the muscle CLF of salmon belonging to these groups.

Replacing fish oil (FO) by rapeseed oil (RO) in salmon diet reduced the percentage of SFAs and increased the percentage of MUFAs in the muscle CLF, especially oleic acid (18:1 n-9). The percentages of linoleic (18:2 n-6) and linolenic (18:3 n-3) acids were also increased as well as the total n-6 FAs, but the percentage of n-3 FAs and the ratio n-3/n-6 decreased. The percentage of EPA in the muscle CLF of the RO group was however not significantly different from the FO group, and the percentage of DHA was only 6% lower. The percentage of EPA stayed stable, whereas the percentage of DHA was almost ten times higher in the muscle CLF of salmon than in the original RO diet. In general, the differences observed in the muscle CLF between the FO and RO groups were not as high as found between the FO and RO diets.

Replacing dietary FO by an EPA- or DHA-enriched-oil also decreased the percentage of SFAs but increased the percentage of PUFAs in the muscle CLF of salmon belonging to these groups, especially the n-3 FAs. These effects were more pronounced with the EPA than with the DHA diet.

The percentage of DHA was almost four times higher in the DHA diet than in the FO diet. The percentage of DHA in the muscle CLF of the DHA group was however not significantly different from the FO group. Besides, the percentage of EPA in the EPA diet was six times higher than in the FO diet, but the percentage of EPA in the muscle CLF of the EPA group was only three times higher than in the FO group.

3.3. Microscopy of the muscle

3.3.1. Myofibre–myofibre detachments

Pictures from light microscopy observations of salmon muscle are presented in Figs. 1–4 at different time-points post-mortem. Fig. 5 shows the percentage of myofibre–myofibre detachments between 0 h and 96 h post-mortem. In general, the EPA diet caused significantly more myofibre–myofibre detachments than the FO diet ($p = 0.022$) and to some extent, more than the RO diet ($p = 0.073$).

In general, myofibre–myofibre detachments increased with time ($p < 0.0001$) in the four dietary groups until 24 h post-mortem. However, no differences in rates of myofibre–myofibre detachments were noticed between 24 h and 96 h post-mortem.

Immediately post-mortem, more than twice the percentage of myofibre–myofibre detachments was found in the EPA and DHA groups than in the FO group, even if these results were not significantly different, whereas the RO group presented more similar results to the FO group. Twelve hours post-mortem, the EPA and DHA groups still differed from the others, even if these differences were not statistically significant: the EPA group presented 5% and the DHA group 10% more myofibre–myofibre detachments than the FO group. Twenty-four hours post-mortem, the DHA group revealed almost the same rate of myofibre–myofibre detachments as the FO and RO groups, whereas the EPA group still distinguished itself with more than 5% of myofibre–myofibre detachments than in the three other dietary groups, even if this difference was not statistically significant. Ninety-six hours post-mortem, it seemed that all groups had reached the same rate of myofibre–myofibre detachments.

3.3.2. Myofibre–myocommata detachments, myofibre breakages and contracted myofibres

Microscopic observations of myofibre–myocommata detachments did not show any clear differences between dietary groups and no specific trend was observed through time post-mortem (results not shown).

Microscopic observations of longitudinal sections 96 h post-mortem, did not allow detecting any myofibre breakages in any of the dietary groups.

Microscopic observations of longitudinal-oriented muscle samples 96 h post-mortem revealing contracted myofibres in the four dietary groups are presented in Fig. 6. Fig. 7 shows the percentage

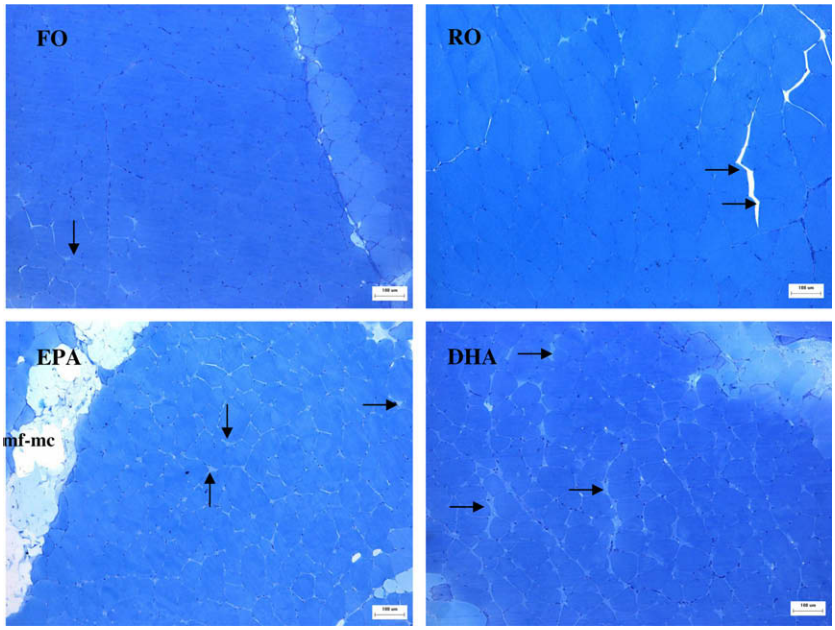

Fig. 1. Microscopic observations (cross sections) of muscle of Atlantic salmon fed the four different diets (FO, RO, EPA and DHA), 0 h post-mortem. Magnification 10×; scale bar = 100 μm; arrows: myofibre–myofibre detachments; mf-mc: myofibre–myocommata detachments.

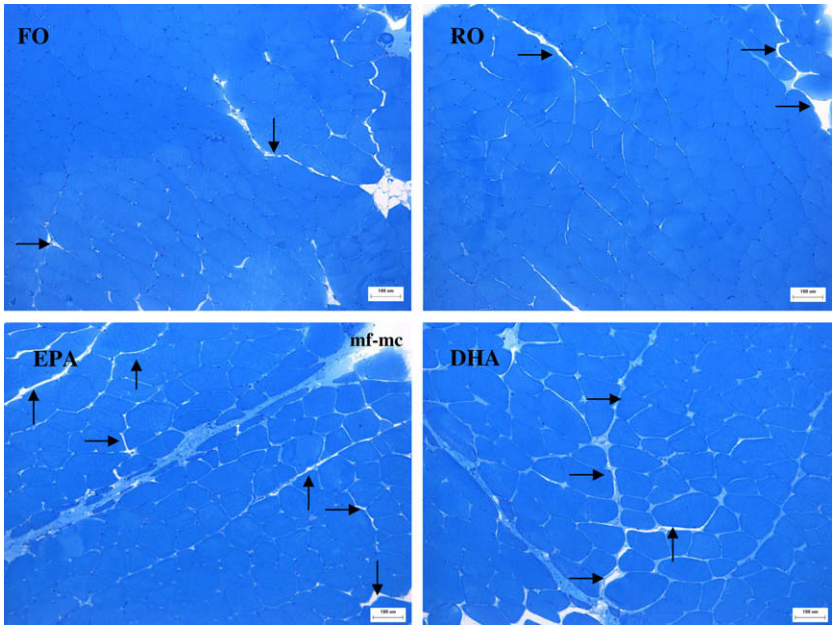

Fig. 2. Microscopic observations (cross sections) of muscle of Atlantic salmon fed the four different diets (FO, RO, EPA and DHA), 12 h post-mortem. Magnification 10×; scale bar = 100 μm; arrows: myofibre–myofibre detachments; mf-mc: myofibre–myocommata detachments.

of contracted myofibres in salmon fed the four diets, 96 h post-mortem. The DHA group presented significantly more contracted myofibres than the three other groups. Feeding salmon with the DHA diet resulted in twice as much contracted myofibres as in the FO group ($p = 0.058$).

3.4. Cathepsin B + L, cathepsin B and cathepsin L activities

Results from 0 h post-mortem cathepsin B + L activities in the frozen muscle CLF and muscle CLF supernatant of salmon fed the four diets are presented in Fig. 8. Results from cathepsin B + L, cathepsin B and cathepsin L activities in the 0 h post-mortem muscle homogenates are shown in Fig. 9. For the four dietary groups, cathepsin L activity was found dominant when compared to cathepsin B activity, as we observed almost four times more cathepsin L than cathepsin B activity in the muscle homogenate.

In the muscle CLF, cathepsin B + L activity was almost similar in salmon fed the FO and RO diets on one hand, and the EPA and DHA diets on the other hand. The cathepsin B + L activity was lower in the muscle CLF of salmon fed the EPA and DHA diets than salmon fed the FO and RO diets, even if these differences were not significant. When comparing the four dietary groups together, salmon fed the EPA diet presented the lowest cathepsin B + L activity, whereas the FO group showed the highest activity.

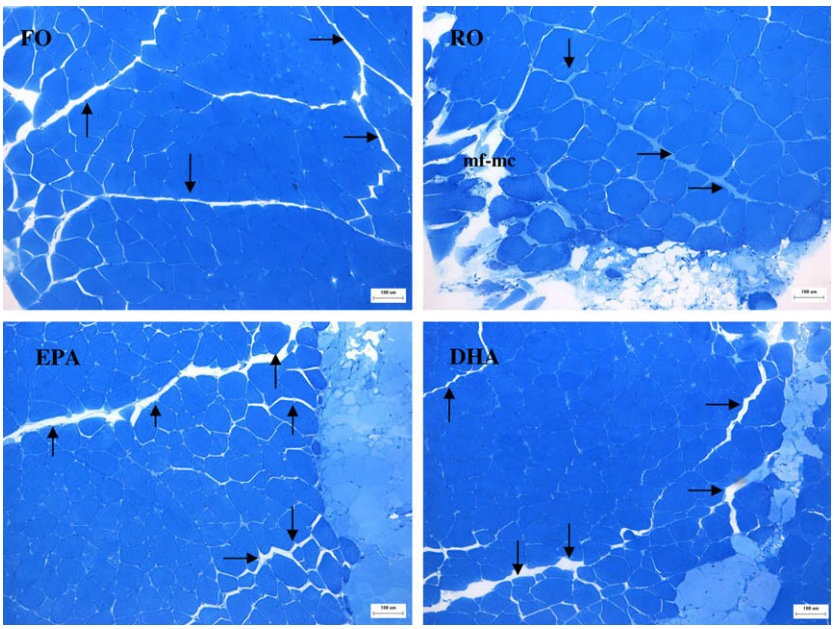

Fig. 3. Microscopic observations (cross sections) of muscle of Atlantic salmon fed the four different diets (FO, RO, EPA and DHA), 24 h post-mortem. Magnification 10×; scale bar = 100 μm; arrows: myofibre–myofibre detachments; mf-mc: myofibre–myocommata detachments.

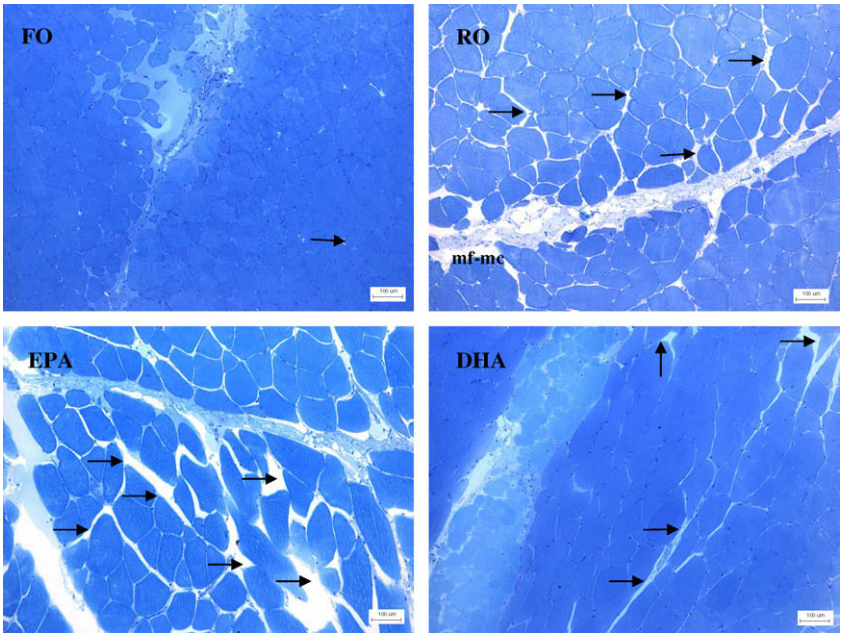

Fig. 4. Microscopic observations (cross sections) of muscle of Atlantic salmon fed the four different diets (FO, RO, EPA and DHA), 96 h post-mortem. Magnification 10×; scale bar = 100 μm; arrows: myofibre–myofibre detachments; mf-mc: myofibre–myocommata detachments.

In the muscle CLF supernatant, cathepsin B + L activity was significantly lower in the RO group than in the FO group ($p = 0.050$), but similar in salmon fed the EPA and DHA diets. Cathepsin B + L activity was significantly lower in the EPA and DHA groups than in the FO group ($p_{(EPA-FO)} = 0.036$ and $p_{(DHA-FO)} = 0.021$).

In the muscle homogenate, cathepsin B+L activity was similar to salmon fed the FO and RO diets, as were the separate cathepsin B and cathepsin L activities. Cathepsin B + L activity was also the same in salmon fed the EPA and DHA diets, as were separate cathepsin B and cathepsin L activities. Cathepsin B + L activity in the muscle homogenate of salmon fed the EPA and DHA diets was significantly lower than in the FO group ($p_{(FO-EPA)} = 0.002$ and $p_{(FO-DHA)} = 0.001$) and in the RO group ($p_{(RO-EPA)} = 0.0002$ and $p_{(RO-DHA)} < 0.0001$). The same pattern was observed for separate cathepsin B ($p_{(FO-EPA)} = 0.0002$; $p_{(FO-DHA)} = 0.0002$ and $p_{(RO-EPA)} < 0.0001$; $p_{(RO-DHA)} < 0.0001$) and cathepsin L activities ($p_{(FO-EPA)} = 0.004$; $p_{(FO-DHA)} = 0.002$ and $p_{(RO-EPA)} = 0.0005$; $p_{(RO-DHA)} = 0.0002$).

3.5. Cathepsin B and cathepsin L relative gene expressions

Relative gene expressions of cathepsin B and cathepsin L are presented in Fig. 10. Results showed that only the expression of cathepsin B was influenced by the diet ($p = 0.016$), whereas the

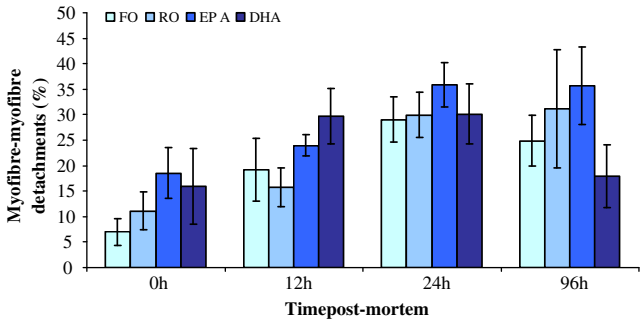

Fig. 5. Myofibre–myofibre detachments (%) during storage time (0 h to 96 h post-mortem) in Atlantic salmon fed the four different diets (FO, RO, EPA and DHA). Each histobar represents the mean value ± SE of one dietary group at one time point (n = 3).

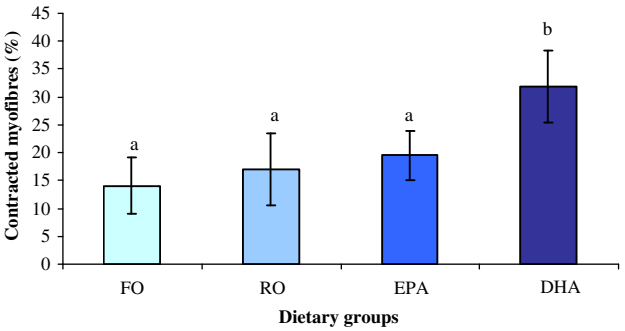

Fig. 7. Contracted myofibres (%) in Atlantic salmon fed the four different diets (FO, RO, EPA and DHA), 96 h post-mortem. Each histobar represents the mean value ± SE of one dietary group (n = 3). Different letters denote significant differences with the other dietary groups.

expression of cathepsin L was not significantly different between dietary groups.

Relative gene expression of cathepsin B was higher in the EPA and DHA groups than in the FO and RO groups but significant differences were found only between the FO and DHA groups (p = 0.027), between the RO and EPA groups (p = 0.045), and the RO and DHA groups (p = 0.003).

4. Discussion

By feeding Atlantic salmon with the RO diet, we aimed at evaluating the impact of this diet on muscle structure stability. Feeding salmon with the EPA and DHA diets, considered as extreme diets potentially representing a stress for the fish, allowed us to highlight the potential implication of lysosomes and lysosomal enzymes, cathepsins, in salmon muscle degradation, in relation to dietary fatty acids. The FO diet served as a control for comparison of the effects with the other diets.

Determination of lipid class composition in the muscle CLF showed no cardiolipin in salmon fed the EPA or DHA diet, whereas 2.6% and 2.4% were found in the FO and RO groups respectively. Cardiolipin is an important component of the inner mitochondrial membrane. The absence of cardiolipin in the muscle CLF of salmon fed the EPA or DHA diet may indicate an increased incidence of lipid peroxidation and oxidative stress, and disrupted mitochondrial membranes (Kirkland, Adibhatla, Hatcher, & Franklin, 2002; Petrosillo, Ruggiero, Pistolese, & Paradies, 2001). In the same study using the same salmon as in the present study, Kjær, Todorcevic, Torstensen, Vegusdal, and Ruyter (2008) and Todorcevic et al. (2008) observed evidence of excessive oxidative stress with less major phospholipids in mitochondrial membranes, higher membrane damages and a lack of mitochondrial beta-oxidation capacity in liver and adipose tissue of salmon fed the EPA or DHA diet. Kurz, Terman, Gustafsson, and Brunk (2008) suggested a close relationship between lysosomes and mitochondria, where oxidative stress due to production of hydrogen peroxide by mitochondria will impair lysosomes; eventually releasing lysosomal enzymes that will again damage the mitochondria and induce more oxidative stress. Consequently, damages observed in mitochondria may also be found at the lysosomal level; suggesting that the EPA and DHA

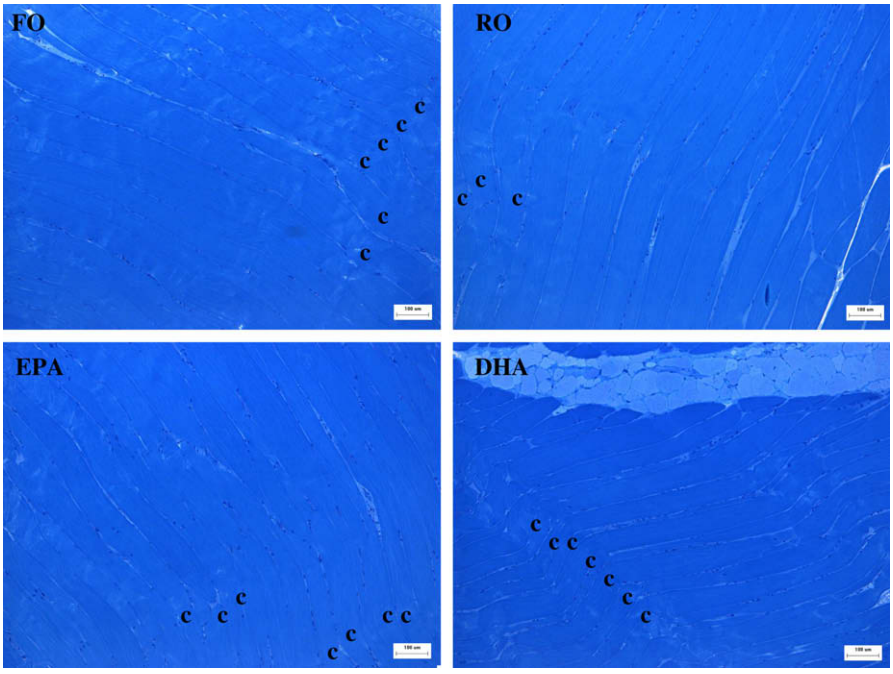

Fig. 6. Microscopic observations (longitudinal sections) of muscle of Atlantic salmon fed the four different diets (FO, RO, EPA and DHA), 96 h post-mortem. Magnification 10×; scale bar = 100 μm; c: contracted myofibres.

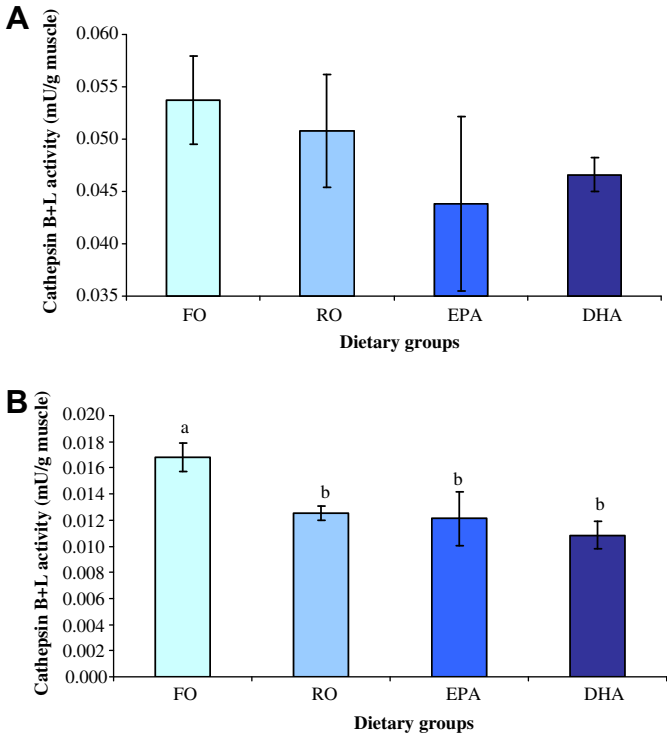

Fig. 8. (A) Cathepsin B + L activity (mU/g muscle) in the muscle CLF; (B) cathepsin B + L in the muscle CLF supernatant (mU/g muscle) of Atlantic salmon fed the four different diets (FO, RO, EPA and DHA), 0 h post-mortem. Each histobar represents the mean value ± SE of one dietary group (n = 3). Different letters denote significant differences with the other dietary groups.

diets may have caused impairment of the lysosomal function as well.

Analyses of fatty acid and lipid class compositions of the muscle CLF immediately post-mortem showed that, in general, the composition of the muscle CLF reflected the diets. This was not surprising, since we (Thomassen & Røsjø, 1989) and several other groups (Bell et al., 2003; Einen, Tomter, & Fjæra, 2002; Rosenlund et al., 2001; Torstensen et al.,2004, 2005; Nanton et al., 2007; Pratoomyot, Bendiksen, Bell, & Tocher, 2008) have shown that fatty acid composition of Atlantic salmon muscle lipids is closely influenced by dietary fatty acids. To the best of our knowledge, however, this is the first time that fatty acid composition of the muscle CLF influenced by dietary lipids is described.

In the present study, replacement of fish oil (FO) by rapeseed oil (RO) significantly increased the percentages of oleic (18:1 n-9), linoleic (18:2 n-6), linolenic (18:3 n-3) and arachidonic (20:4 n-6) acids in the muscle CLF, while percentages of very long chain n-3 FAs, EPA and DHA, were less affected: the percentage of EPA in the muscle CLF of salmon fed the RO diet was not different from the FO group and the percentage of DHA was only 6% lower. This is most probably related to the CLF fraction being dominated by phospholipids (about 65%). It is generally accepted that very long chain n-3 FAs, and especially DHA, are preferentially incorporated in phospholipids and that phospholipids are less influenced by dietary fatty acid composition as compared to triacylglycerols (Brodtkorb, Rosenlund, & Lie, 1997; Olsen & Henderson 1997). This is supported by a small and not statistically significant increase in DHA found in muscle CLF of salmon fed the DHA diet, containing four times more DHA than the FO diet. Similarly, the percentage of EPA was found to be only three times higher in the muscle CLF of salmon fed the EPA diet, when compared to the FO group, although the percentage of this FA was six times higher in the EPA diet. These results may suggest that a saturation point had been reached, at least in the DHA group, where the muscle CLF of salmon belonging to this group would not incorporate more of this FA. Thus, the influence on lysosomal FA composition seemed to have been less than originally expected, based on the relatively extreme n-3 content in the EPA and DHA diets. It seemed that the percentage of DHA in the FO diet (12%) was almost sufficient to reach the "maximum" level in the muscle CLF.

Our microscopic observations did not show any differences in the percentage of myofibre–myofibre detachments between the FO and RO groups. Replacing fish oil (FO) by rapeseed oil (RO) in the diet of Atlantic salmon thus does not have any negative impact on muscle integrity at the myofibre–myofibre level. Taylor et al. (2002) suggested that salmon fillet texture is determined by the level of myofibre–myofibre detachments. Accordingly, our results agree with Regost, Jakobsen, and Rørå (2004), who revealed no difference in texture measured instrumentally between Atlantic salmon fed an RO diet and a capelin oil diet, both diets being comparable to the RO and FO diets used in the present study.

In salmon fed the EPA or DHA diet, a tendency towards a higher rate of myofibre–myofibre detachments until 24 h post-mortem and a significantly higher rate in the EPA group in general was seen. Our results form the present study and the ones from Kjær et al. (2008) and Todorcevic et al. (2008) strongly suggested an increased oxidative stress in the EPA and DHA groups. Histological lesions of the muscle due to lipid peroxidation have already been observed (Brownell & Engel, 1978; García-Buñuel, 1984; Wada, Hatai, & Kubota, 1991). Our results also agree with Izquierdo et al. (2005), who noticed a softer flesh texture in seabream fed diets with PUFA levels comparable to our extreme diets EPA and DHA. They attributed this softer texture to the higher amount of PUFAs and lower amount of saturated FAs in the diet/muscle of those fish, compared to the control, as was the case in the present study.

Analyses of myofibre–myocommata detachments did not show any differential trend in time or between diets. This agrees with Bahuaud et al. (2008), who did not find any changes in percentage of myofibre–myocommata detachments between 0h and 6 days of storage in Atlantic salmon, and Taylor et al. (2002) and Ofstad et al. (2006) before 5 days in Atlantic salmon and 7 days in wolffish. Bahuaud et al. (2008) also did not notice any differences in myofibre–myocommata detachments between super-chilled and control Atlantic salmon, although differences in myofibre–myofibre detachments between the two treatments were clear until 48 h post-mortem.

Microscopic observations of muscle longitudinal sections showed more contracted myofibres in the DHA and to some extent in the EPA fed salmon, 96 h post-mortem, compared to the FO group. This is in accordance with Mørkøre (2006), who showed a higher contraction rate of fillets of Atlantic cod fed a diet where fish oil was replaced at 40% by soybean oil, compared to cod fed a 100% fish oil diet. In the study, Mørkøre suggested that this difference might be due to accelerated energy depletion in the cod fed the soybean oil diet, since she found a linear relationship between *rigor* contraction and ATP depletion. She explained this faster ATP depletion by a reduced ability to cope with stress during the slaughtering process or a different enzymatic activity for fish fed other lipid sources than fish oil. This statement agrees with our results on cathepsins, where we found significant differences between the EPA/DHA groups and the FO/RO groups in cathepsin B+L, cathepsin B and cathepsin L activities as well as cathepsin B relative gene expression in muscle homogenate.

Cathepsins belong to a large family of lysosomal cysteine proteases playing a major role in the post-mortem degradation of mammalian and fish muscle. Fragility of the lysosomal membrane is known to facilitate the release of these degrading enzymes into

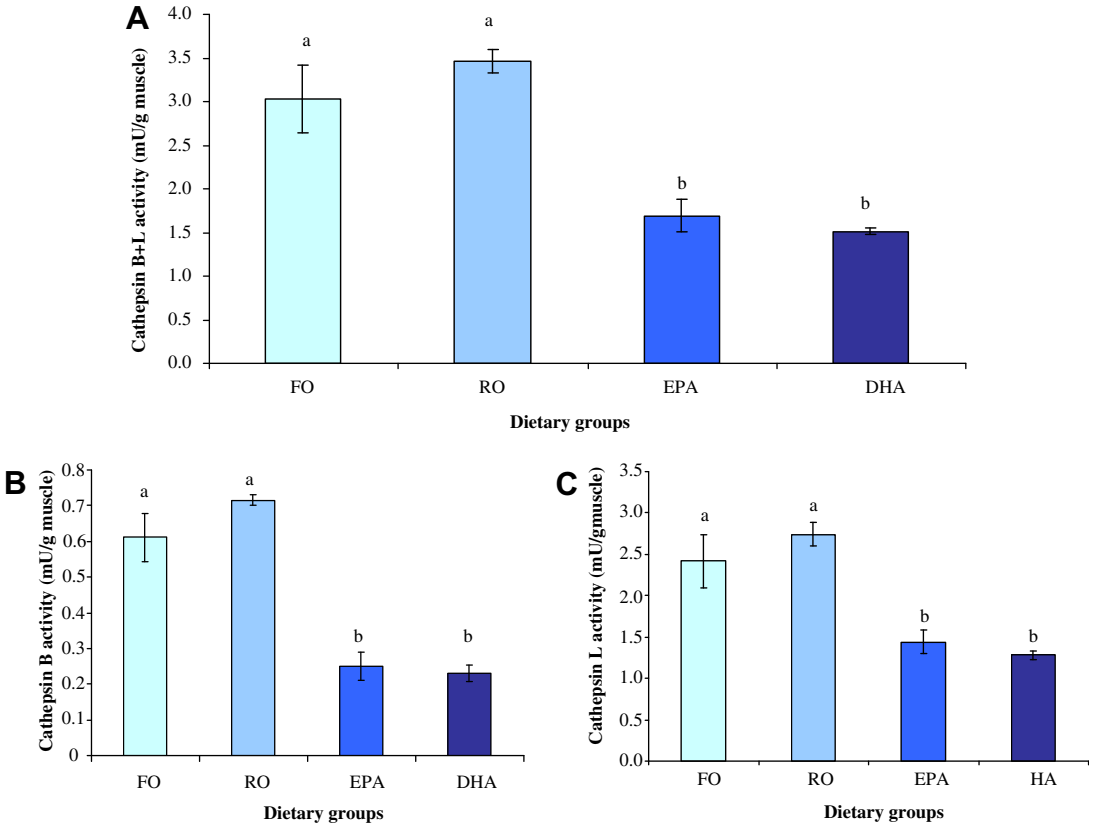

Fig. 9. (A) Cathepsin B + L activity (mU/g muscle) in the muscle homogenate; (B) Cathepsin B (mU/g muscle) in the muscle homogenate; (C) Cathepsin L (mU/g muscle) in the muscle homogenate of Atlantic salmon fed the four different diets (FO, RO, EPA and DHA), 0 h post mortem . Each histobar represents the mean value ± SE of one dietary group (n = 3). Different letters denote significant differences with the other dietary groups.

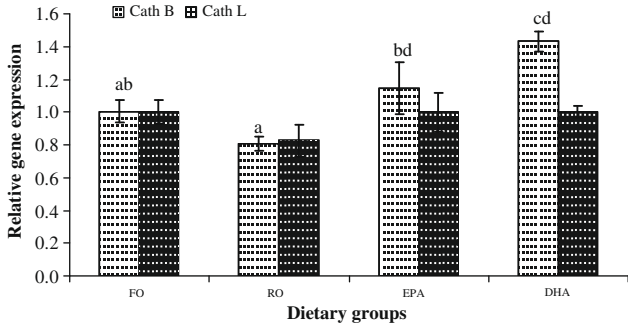

Fig. 10. Relative gene expression of cathepsin B and cathepsin L in muscle of Atlantic salmon fed the four different diets (FO, RO, EPA and DHA), 0 post-mortem. Each histobar represents the mean value ± SE of one dietary group (n = 3). Different letters denote significant differences with the other dietary groups for either cathepsin B or cathepsin L.

the cytosol and the catabolism of cell components (Allison, 1975; Köhler, 1991; Moore, 1988; Moore & Lowe, 1985). Measuring cathepsin activity in the lysosomal fraction of fish muscle has been previously used and is a precise and efficient mean to evaluate fish flesh degradation (Aoki & Ueno, 1997; Bahuaud et al., 2008). A decrease of cathepsin activity in the muscle lysosomal fraction thus indicates a fragility and breakage of the lysosomal membrane, leading to the release of proteolytic enzymes, and eventually, the start of muscle proteolysis. Besides, measuring cathepsins in muscle homogenate immediately post-mortem may also highlight the level of potential proteolysis in fish (Chéret, Hernandez-Andres, Delbarre-Ladrat, de Lamballerie, & Verrez-Bagnis, 2006; Chéret et al., 2007; Mommsen, 2004; Salem, Kenney, Rexroad, & Yao, 2006). An increase of cathepsin total activity in the muscle homogenate can thus indicate higher potential muscle degradation.

According to this, we obtained surprising and paradoxical results of our cathepsin measurements in the present study:

- On one hand, we observed lower cathepsin B+L activity in the muscle CLF of the EPA and DHA groups than in the FO and RO groups, 0 h post-mortem. This would agree with our results obtained on fatty acid composition of the muscle CLF showing very high percentages of PUFAs in the EPA and DHA groups, leading to higher lipid oxidation measured at least in the mitochondria fraction (Kjær et al., 2008; Todorcevic et al., 2008), thus a potential fragility of the lysosomal membrane, and eventually the release of proteolytic enzymes, cathepsins, into the cytosol. This phenomenon could thus have explained the higher rate of myofibre–myofibre detachments found in these groups.
- On the other hand, we also found less cathepsin B + L activity in the muscle CLF supernatant of the EPA and DHA groups than of the FO and RO groups. Less cathepsin activity in this fraction would indicate that less enzyme had been released, leading to less potential muscle degradation. These results, however, did not agree with the higher rate of myofibre–myofibre detachments observed in the EPA and DHA groups.
- Finally, we obtained significantly less cathepsin B + L, cathepsin B and cathepsin L activities in the muscle homogenate (total activity) of the salmon fed the EPA or DHA diet. This strongly suggests that these diets led to a reduction in vivo of cathepsin B and cathepsin L activities, and a lower potential for muscle degradation than in the FO and RO groups which would have

been expected. Once again, these results did not agree with the rate of myofibre–myofibre detachments. However, the lower cathepsin B + L, cathepsin B and cathepsin L activities found in salmon fed the EPA or DHA diet may result from an impairment of the lysosomal function or a disturbed biosynthesis of these enzymes (Kharbanda, McVicker, Zetterman, & Donohue, 1995, 1996), due to oxidative stress (Sargent, Tocher, & Bell, 2002). Headlam, Gracanin, Rodgers, and Davies (2006) also showed that cathepsins are inactivated by protein hydroperoxides. In the present study, production of hydroperoxides due to oxidative stress in the EPA and DHA fed salmon, could thus explain the lower cathepsin B and cathepsin L activities in the muscle, even with a higher relative gene expression of cathepsin B in both groups, compared to the FO and RO fed salmon.

Consequently, according to our results in the present study, total or released cathepsin B and cathepsin L activities did not seem to be directly responsible for the observed muscle degradation, as their total activity did not correlate with the rate of myofibre–myofibre detachments. This underlines the complexity in muscle degrading processes and the necessity for further research in this area. In the present study, other proteases as calpains may have been involved (Chéret et al., 2007; Delbarre-Ladrat, Verrez-Bagnis, Noel, & Fleurence, 2004). Results from the same study as the present one, on mitochondrial function from liver and adipose tissue of Atlantic salmon (Kjær et al., 2008; Todorcevic et al., 2008), do, however indicate that lipid peroxidation and related protein denaturation should also be considered.

It was interesting to note that the level of cathepsin L activity was dominant when compared to cathepsin B in the muscle homogenate, since almost four times as much cathepsin L than cathepsin B activity was found in all groups. These results agree with Yamashita and Konagaya (1991), who showed that in salmon, cathepsin L was dominant in proteolytic degradation compared to cathepsin B, which had a lower hydrolysis rate.

The unexpected effects of dietary lipids on the in vivo level of cathepsin B + L, cathepsin B and cathepsin L activities obtained in the present study demonstrate for the first time an impact of dietary lipid composition on lysosomal proteases. Alizadeh, Smit-McBride, Oltjen, and Hjelmeland (2006) showed that oxidative stress increases mRNA expression of cathepsin B and cathepsin L in the retinal pigment epithelium of mice, with consequent degeneration of the retina. Accordingly and in agreement with our results discussed above, oxidative stress could thus be responsible for the increased relative gene expression of cathepsin B found in the EPA and DHA groups in the present study. This higher gene expression would then be the response to an increased need to get rid of more damaged materials due to oxidative stress. In rainbow trout, Salem et al. (2006) showed that during spawning and post-spawning, the relative gene expression of cathepsin D was not correlated with the relative activity of cathepsin D, but that the relative expression of cathepsin L was correlated with the relative activity of cathepsin L. Our gene expression study did not correlate with the lower cathepsin activity in the EPA and DHA groups. Thus, it seems that regulation of cathepsin activity is a complex system and further investigation is needed.

5. Conclusion

The present study showed an influence of dietary level and types of n-3 FAs on the degradation and stability of Atlantic salmon muscle post-mortem. The observed myofibre–myofibre detachment rate did not, however, correlate with cathepsin total activity or activity released from broken lysosomes. Other explanations, such as the action of other proteases such as calpains, or more probably protein denaturation due to lipid peroxidation, have to be considered.

Our study further revealed an effect of dietary lipid on cathepsin B and cathepsin L total activities in white muscle. Feeding Atlantic salmon with relatively extreme diets containing high levels of EPA or DHA, led to significantly reduced cathepsin B and cathepsin L activities, suggesting a possible impairment of the lysosomal function, a disturbance in biosynthesis, or more likely an inactivation of these enzymes due to production of hydroperoxides during oxidative stress. Cathepsin B and cathepsin L gene expression did not correlate with the enzyme activity measurements. However, an interesting effect of dietary lipid composition on the relative gene expression of cathepsin B was observed, suggesting an increase of the expression due to oxidative stress. The present study showed that dietary lipids can influence salmon muscle structure integrity, muscle lysosome composition, and lysosomal cathepsin activity and gene expression, but also underlined the complexity of the mechanisms of salmon muscle proteases and the importance of further studies.

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