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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