N-3 fatty acids metabolism in Atlantic salmon

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Abbreviations

ACO	Acyl-CoA oxidase
ASP	acid soluble products
BSA	Bovine serum albumin
cDNA	Complementary DNA
CoA	Coenzyme A
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
Elovl	Very long chain fatty acyl elongases
EPA	Eicosapentaenoic acid
FAD	Flavin adenine dinucleotide
FAME	Fatty acid methyl esters
GC	Gas-liquid chromatograph
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-pressure liquid chromatography
HUFA	Highly unsaturated fatty acids
L-15	Leibovitz's L-15 medium
LA	Linoleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
OA	Oleic acid
PBS	Phosphate buffer saline
PUFA	Polyunsaturated fatty acids
qPCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
TCA	Tricarboxylic acid

Abstract

***Background*:** The main human dietary source for n-3 highly unsaturated fatty acids (HUFAs) is fish and seafood. In farmed fish, n-3 HUFA content has been reduced because of increased pressure to replace fish oil by vegetable oils in aqua-feeds. Recently a strategy combining genetic selection with changes in diet formulations has been proposed to improve n-3 HUFA content in farmed Atlantic salmon. n-3 HUFA composition has been shown to be a heritable trait in Atlantic salmon. Further, genes involved in the HUFA biosynthetic pathway in Atlantic salmon can be regulated by fatty acids in the diet. Therefore, the aim of this study was to investigate if variation exists, in the expression of genes regulating the n-3 HUFA biosynthesis pathway, between different Atlantic salmon families. Further, we wanted to study the influence of different levels of oleic acid (OA, 18:1 n-9) and docosahexaenoic acid (DHA, 22:6 n-3) on n-3 fatty acid metabolism in hepatocytes.

***Result*:** Liver samples from 1044 salmon from 103 families (ca 10 fish per family), were analysed for Δ6fad gene expression. Of this, twelve families were analysed for EPA and DHA composition. The results showed that the gene expression levels of Δ6fad_b were differently expressed between the families. According to this difference, 6 families of Atlantic salmon were selected as "high expression families" and 6 families were selected as "low expression families". The levels of the n-3 HUFAs DHA and eicosapentaenoic acid (EPA, 20:5 n-3) although not significant, were slightly higher in "high expression families" than in the "low expression families". Atlantic salmon hepatocytes were isolated and pre-incubated with increasing ratios of DHA/OA (100 OA, 75OA/25DHA, 25OA/75DHA) in order to change the intracellular level of these fatty acids. Then the hepatocytes were incubated with radio-labelled [1-^{14}C] 18:3 n-3. After incubation the main radio-labelled products were the desaturation product 18:4 n-3 and the desaturation and

elongation products EPA, DHA and 22:5 n-3. Increasing OA content in incubation media led to a significant increase in the production of radio-labelled EPA. Radio-labelled DHA showed a tendency to be higher in the 100OA and 75OA/25DHA groups than in the100DHA and 25OA/75DHA groups.

Conclusions**:** The *in vivo* results showed that Atlantic salmon families have different expression levels of the Δ6fad gene which possibly influences the capacity to produce n-3 HUFA. The *in vitro* study results showed that pre-incubation of salmon hepatocytes with increasing ratios of OA may increase the EPA and DHA synthesis.

Key words**:** Elongases, desaturases, Atlantic salmon, n-3 fatty acids, DHA, EPA, gene expression,

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1. Introduction

Marine fish oils have traditionally been used as the only dietary lipid source for farmed salmon, since it contain high level of n-3 highly unsaturated fatty acids (HUFA) (Reviewed by Turchini et al. 2009). Fish oil can satisfy dietary requirements for the energy and essential fatty acids for optimal growth and health of farmed fish (Reviewed by Tocher 2003). Aquaculture industry is still highly dependent upon fish oil and fish meal. However, fish oil supply from capture fisheries cannot satisfy the increasing fish meal and fish oil demand from the aquaculture industry (Tacon et al. 2006; Gatlin et al. 2007). According to FAO (2012), global fish oil production decreased from 1.50 million tonnes in 1994 to 1.07 million tonnes in 2009. And analysis of the data for the last 15 years (1994-2009) indicates that global fish oil production from marine capture fisheries have been decreasing at annual average rates of 2.6 %. But aquaculture industry is currently the fastest growing food production sector in the world. Aquaculture production is projected to reach about 79 million tonnes in 2021, rising by 33 % over the period 2012-2021. It requires that the supply of feed inputs will also have to grow at similar ratios so as to meet demand. The rapidly growing aquaculture industry cannot continue to rely on finite stocks of marine fish as a supply of fish oil. In addition, this strong market demand for fish meal and fish oil lead to increasing market cost for these finite commodities.

Therefore, it is necessary to find cheap and sustainable alternatives to fish oil. Vegetable oils are considered as the only sustainable alternatives to fish oil today. The production of vegetable oils has increased considerably and the prices of the three major vegetable oils (soybean, rapeseed and sunflower oils) have historically been lower than the price of fish oil (Reviewed by Turchini et al. 2009).

Current research show that vegetable oils are readily digested and further

catabolised by fish as an energy source for growth (Bell et al. 2001; Regost et al. 2003; Stubhaug et al. 2007). However, vegetable oils do not contain n-3 HUFAs as marine fish oils. Vegetable oils are rich sources of n-6 and n-9 fatty acids, mainly linoleic acid (LA, 18:2 n-6) and oleic acid (OA, 18:1 n-9). Thus, dietary requirements for n-3 polyunsaturated fatty acids (PUFA) of the fish, limit vegetable oils as the single alternative to fish oil. The diets containing blends of vegetable oils and fish oil have been shown to give good fish growth in salmon farming (Ruyter et al. 2000a; Los & Murata 1998; Torstensen et al. 2005). However, the blending of vegetable oils and fish oil in the diets, leads to less of the very long chain n-3 fatty acids, and consequently lower nutritional value of the salmon fillets (Sargent et al. 2002; Bell et al. 2003 a; b).

In Norway, farmed Atlantic salmon have been selected for increased growth rate since 1975 (Gjedrem et al. 1991), together with improvements in nutrition and management. Recently, a study showed that deposition and/or retention of the dietary n-3 HUFA docosahexaenoic acid (DHA, 22:6 n-3) and eicosapentaenoic acid (EPA, 20:5 n-3) in the flesh, is a highly heritable trait ($h^2 = 0.77 \pm 0.14$) in salmon (Leaver et al. 2011). This suggests that families with high flesh n-3 HUFA content might have a good ability to retain/maintain/synthesize n-3 HUFA (Schlechtriem et al. 2007). Families with high level of flesh n-3 HUFA can be selected to maintain levels of flesh n-3 HUFA when fed diets formulated with vegetable oils and reduced proportions of fish oil (Schlechtriem et al. 2007). The use of selective breeding programs to enhance traits of commercial importance is becoming increasingly more common in aquaculture (Gjedrem & Baranski 2009).

Combining genetic selection with changes in commercial feed formulations (i.e., higher levels of inclusion of vegetable) may be a viable strategy to meet worldwide demand for farmed fish without compromising animal welfare or nutritional value (Morais et al. 2011). Information on the molecular genetics

and mechanisms of the n-3 HUFA biosynthetic pathway in salmon enables the possibility of optimizing the use of vegetable oils in the feed for Atlantic salmon. Previous studies from our group have shown that the capacity for biosynthesis of n-3 HUFA, and the gene expression of the Δ5 fatty acid desaturase (Δ5fad) and Δ6 fatty acid desaturase (Δ6fad) activities in salmon is reduced when fed high dietary levels of fish oil, while vegetable oils increase the capacities (Ruyter et al. 2003; Moya Falcon et al. 2005; Kjær et al. 2008). However, there is still a lot we do not know about optimal ratios of different oils which gives optimal production of DHA and EPA. Therefore, the main objective of this thesis was to find variations in Δ6fad gene expression and EPA and DHA level. Further, the second aim was to study how different cellular levels of DHA and OA influence the n-3 fatty acids metabolism in hepatocytes.

2. Literature review

2.1 Lipids and fatty acids

Lipids are hydrophobic compounds that are soluble in organic solvents. They mainly include triacylglycerols, phospholipids, wax esters and sterols. Fatty acids are an important constituent of most lipids. Triacylglycerols constitute a major class of neutral lipid and consist of three molecules of fatty acids esterified to the three alcohol groups of glycerol (Tocher et al. 2003). Phospholipids are a major class of polar lipids and phosphoglycerides are the most common of the phospholipids. Phosphoglycerides also consist of a glycerol with two fatty acid chains and characterized by a phosphate-containing polar hydroxyl group. In salmon, the main site of lipid storage is the flesh (myosepta) and visceral adipose tissue, while the liver plays a major role in processing of triacylglycerols and phosphoglycerides, including modification of fatty acid chain lengths and the degree of unsaturation (Sargent et al. 1993).

Fatty acids are usually divided into saturated, monounsaturated and polyunsaturated fatty acids according to their degree of unsaturation (number of ethylenic or "double" bonds). Saturated fatty acids lack double bonds, monounsaturated fatty acids possess only one double bond, whereas PUFA contain two or more double bonds. PUFA, having carbon chain lengths $\geq$ C20 and with $\geq$ 3 ethylenic bonds, are defined as HUFA (Henderson & Tocher 1987; Sargent et al. 2002; Tocher 2003). PUFA can be defined by the first double bond position relative to the methyl terminus of the fatty acids chain. n-3 means that the first double bond occurs between the third and fourth carbons from the methyl end of the fatty acids chain, whereas in the n-6 series the double bond is between the sixth and seventh carbons (Menon & Dhopeshwarkar 1982).

Triacylglycerols plays a major role for the storage in fish (Tocher et al. 2003). The phospholipids are structural components of biomembranes and have particularly important roles in fish and human nutrition (Reviewed by Sargent et al. 1999). In fish, DHA and EPA are the major HUFAs of cell membranes. DHA and EPA are both involved in maintaining cell membrane structure and function (Reviewed by Sargent et al. 1999). Especially, the retina and brain of fish are characteristically rich in DHA (Tocher & Harvie 1988). In human, DHA and EPA have many health beneficiary effects, in particular prevention of cardiovascular disease and they are fundamental to brain function (Kidd 2007).

2.2 Dietary lipids

In fish, dietary lipids are an important source of energy for regular growth, health, reproduction and body functions. High proportion of the dietary lipid is used for energy production, thus potentially sparing dietary protein for muscle growth. Improvements in growth, feed utilization efficiency and nutrient retention in fish fed energy-dense diets (Sargent et al. 2002). The lipid content of feed is important as a source of dietary energy, but is also fundamental in supplying essential fatty acids (Sargent et al. 2002). A fatty acid is commonly termed ‘essential fatty acid’ when its biosynthesis *de novo* is not possible; thus, it must be supplied within the diet (Reviewed by Turchini et al. 2009). Long-term absence of essential fatty acids from the diet leads to deficiency symptoms like reduced growth and increased mortality in fish (Reviewed by Glencross 2009). In general, 18:3 n-3 and/or 18:2 n-6 could satisfy the essential fatty acid requirements of freshwater fish, whereas the n-3 HUFA, DHA and EPA, are required to meet the essential fatty acid requirements of marine fish (Yone 1978; Watanabe 1982; Kanazawa 1985). Salmon is an anadromous species, which hatched in fresh water and then spends most of their life in the sea and returns to fresh water to spawn. Therefore, salmon require 18:3 n-3 and 18:2 n-6 as well as certain amounts of DHA and EPA as essential fatty acids

(Ruyter et al. 2000a; Ruyter & Thomassen 1999).

Dietary lipids in fish are largely composed of a combination of triacylglycerols and phospholipids, with sterols, free fatty acids, pigments and waxes (Reviewed by Glencross 2009). These dietary lipids break down into smaller components by lipases in order to be easily absorbed into the intestine. The digestion of lipids in fish is found along the entire digestive tract, including the stomach, pyloric caeca and proximal and distal intestines of most fish (Reviewed by Glencross 2009). There is limited information on the specifics of lipase functionality in fish. The main triacylglycerol lipase in salmon is a bile salt-activated lipase (Olsen & Ringo 1997). Dietary phosphoglycerides are presumably digested by pancreatic or intestinal phospholipases (Henderson & Tocher 1987; Sargent et al. 1989). The main products of lipid digestion in fish are free fatty acids from all classes of lipids. In addition, glycerol can be products from digestion of triacyglycerols. The majority of phosphoglycerides are digested to 1-acyl lysophosphoglyceride (Tocher 2010).

These digestion products are further solubilized or emulsified in bile salt micelles, followed by absorption into the cells of the digestive tissue (Smith et al. 1983). Long chain fatty acids appear to be mainly absorbed in the mid intestine, while middle chain fatty acids are absorbed in the pyloric caeca (Denstadli et al. 2004). Once assimilated into the cells, free fatty acids are activated with a Coenzyme A (CoA) molecule to get the resultant product, fatty acyl CoA (Reviewed by Glencross 2009). Then the fatty acyl CoA combines with assimilated acyl glycerols and lysophospholipids to re-synthesize triacylglycerols and phosphoglycerides (Sargent et al. 1989). This re-synthesize process is called re-esterification (Reviewed by Glencross 2009). This is the primary lipid biosynthetic process of dietary-derived lipids (Smith et al. 1983). The lipid that is not re-esterified could be catabolised by the process of β-oxidation or subjected to modification process such as elongation/desaturation

(Reviewed by Glencross 2009).

2.3 Fatty acid oxidation

The oxidation of fatty acids which starts at the β-carbon with removal of two carbon units is termed β-oxidation. Fatty acids are most commonly degraded in series of β-oxidation reaction (Schulz 2002; Tocher 2003).The β-oxidation of fatty acids occurs in two different organelles, the mitochondria and the peroxisomes (Tocher 2003). Heart, liver, and red muscle are the most important tissues involved in β-oxidation in fish (Henderson & Tocher 1987; Henderson 1996; Bilinski et al. 1970). Mitochondrial β-oxidation dominates in these tissues except in livers where the peroxisomal β-oxidation may represent significant amount of total β-oxidation (Crockett & Sidell 1993a; b). Peroxisomal β-oxidation shortens HUFAs, which are poor substrates for mitochondrial β-oxidation. After being oxidized in peroxisomes, the shortened fatty acids enter mitochondria where they are completely chain shortened in series of mitochondrial β-oxidation steps (Tocher 2003). Mitochondrial β-oxidation is an important source of energy for several tissues including liver, heart and red muscles (Frøyland et al. 2000) and provides acetyl-CoA to enter the tricarboxylic acid (TCA) cycle (Tran & Christophersen 2001; 2002).

Fatty acids need to be transported into the organelle and activated by conversion to fatty acyl-CoA thioesters with acyl-CoA synthetase before they enter the peroxisomal β-oxidation spiral (Schulz 2002) and mitochondrial β-oxidation spiral (Figure 1). In mitochondrial β-oxidation system, acyl-CoA is dehydrogenated to 2-trans-enoyl-CoA. Then enoyl-CoA hydratase catalyze the hydration of 2-trans-enoyl-CoA to L-3-hydroxyacyl-CoA, further the NAD^+-dependent dehydrogenation of L-3-hydroxyacyl-CoA to 3-ketoacyl-CoA. Finally, 3-ketoacyl-CoA is cleaved by thiolase forming acetyl-CoA (Schulz 2002).

Fatty acyl-CoA thioesters oxidized in peroxisomal *β*-oxidation also go through successive steps of dehydrogenation, hydration and thiolytic cleavage, the same as mitochondrial *β*-oxidation system (described above) except for the first step of dehydrogenation. In contrast to mitochondrial *β*-oxidation, the first dehydrogenation step involves the reduction of O_2 to H_2O_2 by acyl-CoA oxidase (ACO), whereas in mitochondria, the first dehydrogenation step transfers two hydrogens from FAD to $FADH_2$ (Lazarow & de Duve 1976).

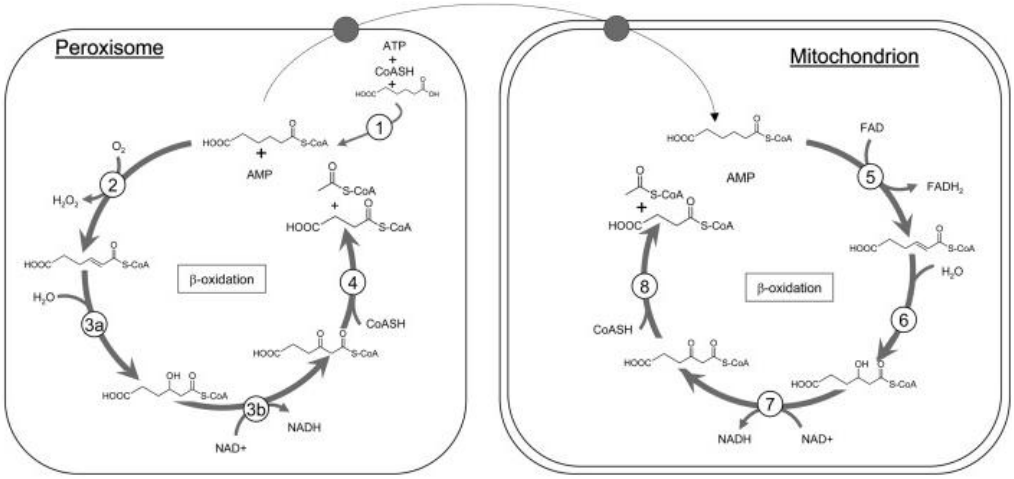

Figure 1: Overview of *β*-oxidation in mitochondria and the peroxisomes (Veiga et al. 2012). (1) acyl-CoA ligase; (2) ACO and peroxisomal acyl-CoA dehydrogenase; (3a) enoyl-CoA hydratase activity of multifunctional enzyme; (3b) 3-hydroxacyl: CoA dehydrogenase activity of multifunctional enzyme; (4) peroxisomal 3-keto-acyl-CoA thiolase; (5) mitochondrial acyl-CoA dehydrogenase; (6) enoyl: CoA hydratase; (7) 3-hydroxyacyl: CoAdehydrogenase; (8) mitochondrial3-ketoacyl-CoAthiolase.

Peroxisomal *β*-oxidation is also found in the biosynthesis pathway of HUFA. DHA is formed from tetracosahexaenoic acid (24:6 n-3) by one round of β-oxidation (Ferdinandusse et al. 2001). ACO are enzymes that catalyze the rate-limiting step in peroxisomal *β*-oxidation, the dehydrogenation step involving the reduction of O_2 to H_2O_2. ACO determine the substrate selectivity and capacities for *β*-oxidation (Crockett et al. 1993b).

2.4 Desaturases and desaturation

The lipid composition of organisms not only depends on the dietary lipid ingested, but also on the ability of tissues or organs to synthesise or transform these lipids through desaturation and elongation pathways (Clandinin et al. 1983; Holman 1986; Lands 1991).

Desaturases are enzymes that introduce unsaturated bonds (predominantly of the *cis* configuration) to a fatty acid chain. They do this by removing two hydrogen atoms (reviewed by Pereira et al. 2003). These enzymes are found almost universally in most living cells, where they help regulate the fluidity of membrane lipids, and also play a critical role in the biosynthesis of PUFAs (reviewed by Pereira et al. 2003). The desaturation reaction occurs in the endoplasmic reticulum of cells of particular tissues (Brenner 1974). Desaturation of fatty acids in fish is an aerobic process, it requires fatty acids esterified to CoA as substrate. Desaturases catalyze the removal of two hydrogen atoms and release a pair of electrons from the substrate. Simultaneously, two molecular water are produced by molecular oxygen and two pair of electrons (one pair of electrons obtained from NAD(P)H and transport by a short electron transport chain) (Brenner 1974).

Three types of desaturases are found in animals, and named based on their desaturation site from the carboxyl end of the fatty acids chain; Δ9, Δ6 and Δ5. Δ9 catalyzes synthesis of monounsaturated fatty acids. These enzymes introduce the first cis-double bond at the Δ9 position of the saturated fatty acid to synthesis monounsaturated fatty acids (Reviewed by Nakamura & Takayuki 2004). These monounsaturated products formed (16:1 n-7 and 18:1 n-9) have markedly lower melting points than their saturated precursors (16:0 and 18:0) (Tocher 2010). Hence, Δ9 fatty acid desaturase provides a means of regulating the fluidity of cell membranes in response to changing temperature.

Δ6fads and Δ5fads are required enzymes in the pathways for the biosynthesis of HUFA. Δ6fads and Δ5fads now have been isolated from Atlantic salmon (Hastings et al. 2004; Monroig 2010). Δ6fad introduce another double bond at the Δ6 position of the unsaturated fatty acid. According to the "Sprecher pathway" (Sprecher 2000) a Δ6fad catalyse the desaturation step involved in production of 22:5 n-6 from 20:4 n-6 and 22:6 n-3 from 20:5 n-3 (Figure 2). It is generally accepted that both the n-6 and n-3 fatty acids are metabolized by the same enzymes system (Sprecher 1981). Desaturases that introduce ethylenic bonds at five carbons from the carboxyl end of the molecule are termed Δ5. Δ5fad add a double bond at the Δ5 position of 20:4 n-3 to produce 20:5 n-3. True marine fish are unable to produce DHA at a physiologically significant rate, and this has been attributed to the lack of Δ5fad activity in some species, such as sea bream (Tocher & Ghioni 1999). In the anadromous Atlantic salmon, both Δ6fad and Δ5fad genes were highly expressed in intestine, liver and brain of salmon (Zheng et al. 2005a).

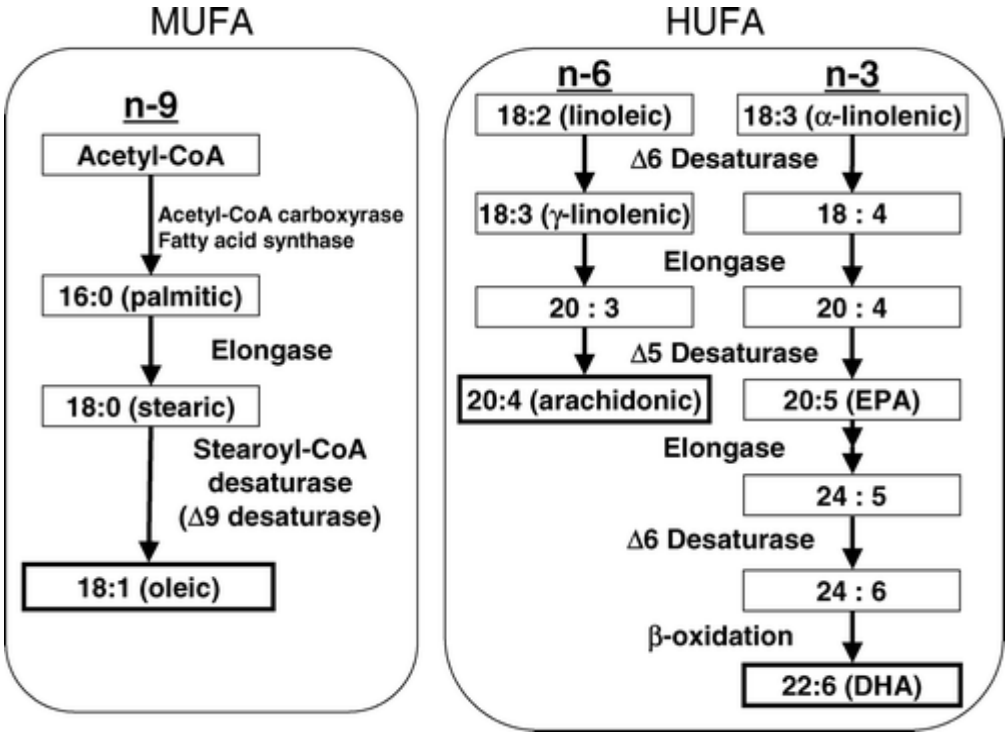

Figure 2: A simplified scheme showing the biosynthesis of HUFA, indicating pathways of elongation and desaturation (Nakamura & Nara 2004).

As mentioned above, it is generally accepted that both the n-6 and n-3 fatty acids are metabolized by the same enzymes system (Sprecher 1981), however the Δ6fad appear to have a preference for longer fatty acid chains and a higher level of unsaturation (Sargent et al. 1993). Therefore, the affinity for the Δ6fad is in the order 18:1 n-9 < 18:2 n-6 < 18:3 n-3. The 18:3 n-3 fatty acid could also compete with 24:5 n-3 for the Δ6fad (Ruyter & Thomassen 1999). Thus, the 20:5 n-3 can be converted to 24:5 n-3, which inhibit the desaturation of 18:3 n-3 for the Δ6fad (Ruyter & Thomassen 1999).

2.5 Elongase and elongation

In addition to desaturation, the synthesis of HUFA involves enzyme-mediated fatty acyl elongation. Synthesis of EPA is achieved by Δ6 desaturation of 18:3 n-3 to produce 18:4 n-3 that is elongated to 20:4 n-3 followed by Δ5 desaturation (Cook 1996). Synthesis of DHA from EPA has been suggested to precede via a C24 intermediate, requiring two successive elongations to 22:5 n-3 and then 24:5 n-3 (Sprecher et al. 1995).

In the HUFA biosynthetic pathway, the fatty acids are elongated into very long chain fatty acids in a four-step reaction cycle by membrane-bound enzymes (Jakobsson et al. 2006). The reaction process begins with the condensation of a long chain acyl-CoA molecule with malonyl-CoA (provides 2-carbon units), resulting in *β*-ketoacyl-CoA. Subsequent reactions include reduction to *β*-hydroxyacyl-CoA, dehydration to an enoyl-CoA, and a second reduction to yield the elongated acyl-CoA (Jakobsson et al. 2006).

The first step involves condensation of malonyl-CoA with acyl-CoA to yield carbon dioxide and a *β*-ketoacyl-CoA in which the acyl moiety has been elongated by two carbon atoms. The initial condensation reaction catalyzed by *β*-ketoacyl-CoA synthase is not only the substrate-specific step but also the rate

limiting step.

Very long chain fatty acyl elongases (Elovl) are believed to perform the first step (condensation) in the elongation cycle (Jakobsson et al. 2006). Two fatty acid elongases termed Elovl5 and Elovl2 are characterized as important enzymes in the biosynthesis of PUFAs in higher vertebrates (Leonard et al. 2004; Jakobsson et al. 2006). Gene sequences for similar elongase-like enzymes (Elovl2 and Elovl5) have recently been cloned from Atlantic salmon (Morais et al. 2009). Elovl are suggested to determine the substrate specificity and are the rate-limiting step in the elongation cycle (Jakobsson et al. 2006). Functional studies suggest that Elovl2 and Elovl5 have a substrate preference for PUFA. The Elovl5 is mainly involved in the elongation of C18-C20 PUFAs, while Elovl2 elongates C20-22 HUFAs, but not C18 PUFAs (Leonard et al. 2002).

2.6 Dietary fatty acids influence the HUFA biosynthesis

Animals in general lack the Δ12fads and Δ15fads, which are required for the production of linoleic acid (18:2 n-6) and α-linolenic acid (18:3 n-3). As mentioned earlier, these fatty acids are considered to be essential and must be derived from the diet (reviewed by Pereira et al. 2003). Thus the dietary lipid composition may modulate the ability of species to desaturate fatty acids in order to adjust and maintain the n-3 HUFA content in tissues (Brenner 1981a; b; Buzzi et al. 1996; Tocher et al. 1997).There is currently been considerable interest in the nutritional regulation of Δ6fad gene expression and its activity, with the aim of determining which of the fatty acids in vegetable oils that can be utilized to substitute fish oil (Mourente & Dick 2002; Sargent et al. 2002). Although the detailed mechanisms behind regulation of the desaturases and elongases is not clear, the activity of the desaturation/elongation pathway is increased when Atlantic salmon are fed high levels of vegetable oils rich in

C18 fatty acids compared to salmon fed n-3 HUFA (Zheng et al. 2005b). Higher Δ6fad mRNA levels were measured in the liver and red muscle of Atlantic salmon fed diets in which 75 % of fish oil had been replaced by a blend of rapeseed, palm, and linseed oils in a 3.7:2:1 ratio compared to fish fed HUFA-rich diets containing only fish oil as a lipid source (Zheng et al. 2005a; b). It has also been suggested that an increase in n-3 HUFA and reduced HUFA substrate levels may have a feedback inhibition effects on enzymes of fatty acid desaturation in the liver (Tocher et al. 2003; Thomassen et al. 2012). Altered dietary fatty acid composition possibly directly affects gene expression. The changes in gene expression may lead to altered activity of the HUFA biosynthetic pathway, and further also result in changes in tissue fatty acid composition.

3. Materials and methods

3.1 Materials

Table 1: Chemicals and equipment

Chemicals and equipment	**Producer**
PureLink™ Pro 96 RNA Purification Kit	Invitrogen, CA, USA
AffinityScript QPCR cDNA Synthesis Kit	Agilent Technologies, CA, USA
Radio-labelled 18:3 n-3	American Radio-labelled Chemicals, St. Louis, USA
Ethylene glycol tetra-acetic acid (EGTA)	AppliChem, Darmstadt, Germany
Hewlett Packard 6890 gas-liquid chromatograph (GC)	Avondale, PA, USA
Precellys 24	Bertin Technologies, Montigny-le-Bretonneux, France
SPECTROstar Nano	BMG LABTECH,Ortenberg, Germany
Kontes Pellet Pestle	Daigger, IL, USA
scintillation fluid	Ecoscint A, GA, USA
Leibovitz's L-15 medium	Gibco Life Technologies, MD, USA
Primers	Invitrogen Ltd, Paisley, UK
DNaseI	Invitrogen, Auckland, NZ
Triton-X 100	Merck, Darmstadt, Germany
Sodium chloride (NaCl)	Merck, Darmstadt, Germany
Benzene	Merck, Darmstadt, Germany
Hexane	Merck, Darmstadt, Germany
NanoDrop 1000 Spectrophotometer	NanoDrop Technologies, DE, USA
Six-well cell culture plates	Nunc™, Denmark
TRI-CARB 1900 TR scintillation counter	Packard Instrument, IL, USA
RNeasy® Mini Kit	Qiagen, Valencia, CA, USA
Radio-active flow detector A100	Radio-matic Instruments & Chemicals, FL. USA
SYBR Green-I Master	Roche Applied Science, Germany

Light Cycler 480	Roche Diagnostics Gmbh, Germany
ID-BP ×70 column	SGE, Melbourne, Australia
Non radio-labelled DHA	Sigma, St. Louis, USA
Non radio-labelled OA	Sigma, St. Louis, USA
Total Protein Kit	Sigma, St. Louis, USA
Sucrose	Sigma, St. Louis, USA
HEPES	Sigma, St. Louis, USA
Foetal bovine serum (FBS)	Sigma, St. Louis, USA
Glutamine	Sigma, St. Louis, USA
Trypan blue	Sigma, St. Louis, USA
Penicillin	Sigma, St. Louis, USA
Streptomycin	Sigma, St. Louis, USA
Bovine serum albumin (BSA)	Sigma, St. Louis, USA
Phosphate buffer saline (PBS)	Sigma, St. Louis, USA
Ethylene diamine tetra-acetic acid (EDTA)	Sigma, St. Louis, USA
Sodium hydroxide (NaOH)	Sigma, St. Louis, USA
Flavin adenine dinuleotide (FAD)	Sigma, St. Louis, USA
Peroxidase	Sigma, St. Louis, USA
2',7'-dichlorofluorescine	Sigma, St. Louis, USA
Dimethoxypropane	Sigma, St. Louis, USA
Palmitoyl CoA	Sigma, St. Louis, USA
96-well EIA/RIA plates	Sigma, St. Louis, USA
Tris base	Sigma, St. Louis, USA
Hydrochloric acid (HCl)	Sigma, St. Louis, USA
Laminin	Sigma, St. Louis, USA
Dithiothreitol (DTT)	Sigma, St. Louis, USA
Chloroform	VWR International, PA, USA
Methanol	VWR International, PA, USA
Collagenase type 1	Worthington Biochemical CLS, N.J., USA

3.2 In vivo study

3.2.1 Fish and experimental design

Fish for this *in vivo* study were obtained from Salmo Breed. Liver samples were selected from 1044 salmon from 103 families, ca 10 fish per family. Fish were anaesthetized with metacain (MS-222) and their weights and lengths determined. They were killed with a blow to the head, and the livers were removed from fish and stored at - 70 ℃.

The liver samples (1044 samples) were used for analysis of the level of Δ6fad_b gene expression. From the result of the Δ6fad_b gene expression, salmon families were selected as "high expression families" or "low expression families". Further, from this 60 fish were selected for ACO measurement and ACO gene expression (30 fish from "high expression families" and 30 fish from "low expression families", 5 families from each group, 6 fish per family). And from this 60 fish of similar weight were selected for fatty acid composition analysis and comparing Δ6fad_b expression between high and lowΔ6fad_b expression families.

3.2.2 Tissue RNA isolation

Total RNA from salmon liver tissue was isolated using PureLink™ Pro 96 RNA Purification Kit. This kit enables isolation of RNA from 96 samples in one plate. Approximately 30 mg liver tissue were homogenized in 800 µl PureLink™ Pro 96 Lysis Buffer using Precellys 24 for 2 × 20 seconds with a break at 5000 rpm. The samples were centrifuged at 2600 × g for 10 minutes. Thereafter, 250 µl of the supernatant was added 250 µl 70 % ethanol and transferred to a to a filter plate. The plate was centrifuged at 2100 × g, for 3 minutes at room temperature. 500 µl wash buffer was added and the plate centrifuged again at 2100 × g for 3 minutes at room temperature. RNA attached to the filter was treated with

DNaseI to remove genomic DNA by incubating with 80 µl DNaseI solution for 15 minutes at room temperature. The plate was centrifuged at 2100 × g for 3 minutes at room temperature. The RNA was washed twice by adding 700 µl wash buffer and centrifuged at 2100 × g for 3 minutes at room temperature. The second centrifugation was prolonged to 10 minutes. RNA was eluted by adding 50 µl RNase-free water and centrifugation at 2100 × g for 3 minutes at room temperature. The RNA samples were kept at - 70 ℃ until analysis.

3.2.3 RNA measurements

Concentration and purity of RNA isolated from liver tissue were evaluated using NanoDrop 1000 Spectrophotometer. Nucleic acids absorb light mainly at wavelength 260 nm, proteins at 280 nm, therefore 260/280 is the most important ratio to value nucleic acid purity. 260/230 is a secondary evaluation of purity, since other contaminants absorb near 230 nm.

3.2.4 Purification of RNA

Some of the samples were purified by precipitation due to low 260/280 ratio. The RNA samples were added 0.1 volumes ethanol (100 %) and 3 volumes sodium acetate (3M, pH 4.8). After 30 minutes incubation at room temperature, the samples were centrifuged for 10 minutes at 4°C at 10,000 × g. Thereafter the supernatant were removed and the pellets were dried for 10 minutes. The RNA pellet was resolved in RNase-free water.

3.2.5 cDNA synthesis

cDNA was made from 500 ng RNA in a 10 µl reaction volume by using AffinityScript QPCR cDNA Synthesis Kit. RNA (500 ng) was added RNase-free water to a final volume of 3 µl. Thereafter 5 µl first strand master mix (2 ×), 1.5 µl oligodT and 0.5 µl AffinityScript RT/RNase Block enzyme mix was added. The cDNA synthesis was run in a polymerase chain reaction

(PCR) machine under the following conditions: 25°C for 5 minutes to allow primer annealing, 42 °C for 45 minutes to synthesize cDNA, and 95 °C for 5 minutes to terminate cDNA synthesis reaction. The cDNA was stored at - 20 °C until analysis.

3.2.6 QPCR analysis

The reaction mix for qPCR consisted of 4 µl diluted (1:10) cDNA, 0.5µl forward primer, 0.5 µl reverse primer (final concentration of 0.5 µM), and 5 µl SYBR Green-I Master. A standard curve was included for each primer pair to evaluate the primer efficiency. All samples were analysed in parallels. The qPCR reaction was run on a Light Cycler®480 under the following conditions: Preincubation at 95 °C for 5 minutes, amplification with 45 cycles at 95 °C for 15 seconds and 60 °C for 1 minutes, melting curve at 95 °C for 5 seconds and 65 °C for 1 minutes, cooling at 40 °C for 10 seconds.

Table 2: Sequence of primes. All sequences are presented as 5'-3'

Gene	Accession no.	Direction	Primer sequence
Δ6fad_b	GU207400	Forward	TGACCATGTGGAGAGTGAGGG
		Reverse	AACTTTTGTAGTACGTGATTCCAGCT
ACO	DQ364432	Forward	CCTTCATTGTACCTCTCCGCA
		Reverse	CATTTCAACCTCATCAAAGCCAA
etif	DW542195	Forward	CAGGATGTTGTTGCTGGATGGG
		Reverse	ACCCAACTGGGCAGGTCAAGA

3.2.7 Lipid extraction

Total fat was extracted from liver tissue according to the Folch extraction (Folch et al. 1957). Approximately 60 mg liver tissue was homogenized in 50

ml chloroform/methanol (2:1, v/v) with butylated hydroxytoluene 2 mg/ml and 6 ml 0.9 % NaCl for 60 seconds with a knife homogenizator. The homogenized sample was added 6 ml 0.9 % NaCl and then homogenized for 5 seconds. The homogenate was filtered by flowing through a cotton filter inside at funnel into a graded cylinder. The graded cylinder was capped in case of evaporation and kept in - 40 ℃ freezer to the next day. The methanol phase and protein was carefully removed, and 20 ml of the chloroform phase were transferred to a weighted beaker for measuring fat content. The remaining chloroform phase was stored at - 20 ℃ until fatty acid analysis. The chloroform phase in the beaker was dried at < 60 ℃ until the organic solvent evaporated and then transferred to oven at 102 ℃ for 30 minutes. The final weight of the beaker was subtracted from the start weight to get the total fat weight. The total fat content was calculated as: $\%\,\text{fat} = \frac{\text{g fat} \times 100}{\frac{\text{I} \cdot \text{U}}{37.5}}$, g fat is total fat weight in beaker, I is the weight of sample in g, U is the volume of chloroform phase in the beaker in ml, 37.5 is total volume of chloroform solvent in ml.

3.2.8 Fatty acid methylation

The remaining chloroform phase stored at - 20 ℃ (described above) was thawed and chloroform was evaporated at 60 ℃ with nitrogen overflow. The residual lipid extract was trans-esterificated to fatty acid methyl esters (FAME) in 2 ml benzene, 2 ml metanolic-HCl and 0.2 ml dimetoxypropan, as described by Mason and Waller (1964). FAME was extracted and purified by using 2 ml hexan and neutralized by adding 4 ml 6 % $NaHCO_3$. The upper phase containing the organic phase was transferred to a new glass vial and the organic solvent evaporated at 60 ℃ with nitrogen overflow. The dried lipid sample was then dissolved in 1 ml hexane prior to gas-liquid chromatography (GC) analysis.

3.2.9 Fatty acid composition

FAME was analyzed by Hewlett Packard 6890 GC equipped with a ID-BP ×70 column (60 m × 0.25 mm i.d., 0.25 μm film thickness). FAME was introduced by split injection (50:1) at 300 ℃. Helium was used as carrier gas and temperature was 50 ℃ for 1.2 minutes, increased from 50 ℃ to 170 ℃ at 4 ℃/minute, and increased to 200 ℃ at 0.5 ℃/minute, then to 300 ℃ at 10 ℃/minute. Separated fatty acid composition was detected by flame ionization detector (FID) at 300 ℃. Peak integration was performed using HP Chem Station software. The relative quantity of each fatty acid present was determined by measuring the area of the peak corresponding to a particular fatty acid, and then identification of major peaks was made by comparing the retention times with those of fatty acid methyl ester standards of GLC-85.

3.2.10 Determination of peroxisomal *β*-oxidation: ACO activity

Approximately 50 mg liver tissue was homogenized in 250 μl homogenization buffer(0.25 M sucrose, 15 mM HEPES, 1.0 mM EDTA and 1.0 mM EGTA) using Kontes Pellet Pestle. The samples were diluted by using equal amount of 0.4 % Triton-X 100 and then centrifuge at 800 ×g for 15 minutes at 4 ℃.

Spectrophotometric assay of ACO was determined by measuring production of H_2O_2, which was coupled to oxidation of 2', 7'-dichlorofluorescine in a reaction catalyzed by peroxidase (Small et al., 1985). The reaction mixture contained 0.5 M Tris (pH 8.5), 60 mg/ml bovine serum albumin (BSA), 9.8 μg peroxidase, 0.05 M 2', 7'-dichlorofluorescine, 1.5 mM FAD. Prepared samples were added to this mixture, and then 6 mM palmitoyl-CoA was used to start the reaction. 2', 7'-dichlorofluorescine and FAD were prepared daily and protected from light. The reaction was performed in 96-well EIA/RIA plates and measured with SPECTROstar Nano at 502 nm for 180 seconds at room temperature. Results were corrected by substrate blank.

The ACO activity was calculated as:

$$\text{ACO activity (nmol / min / ml)} = \frac{\Delta A_{502}/\text{min}}{260\,(\text{mM}^{-1} \times \text{cm}^{-1}) \times \text{d}} \times \frac{\text{Vt}}{\text{Ve}} \times \text{D} \times 1000$$

ΔA_{502} is the increasing absorbance during 3 minutes at 502 nm, 260.6 ($mM^{-1} \times cm^{-1}$) is extinction coefficient of 2', 7'-dichlorofluorescine at 502 nm, d is the diameter of reaction well in cm, Vt is the total reaction volume in ml, Ve is the enzyme sample volume in ml, D is the dilution factor of the enzyme sample.

The activities per mg of protein (specific activity) in liver homogenates were calculated as:

$$\text{ACO Activity (nmol/ min/ mg protein)} = \frac{\text{ACO Activity (nmol/ min/ ml)}}{\text{Concentration of protein in enzyme sample (mg/ml)}}$$

The activities per g of liver (nmol / min / g liver) were calculated as:

$$\text{ACO Activity (nmol/ min/ g liver)} = \frac{\text{ACO Activity (nmol/ min/ ml)} \times \text{V}}{\text{W}}$$

W is the weight of tissue sample in g, V is the volume of homogenization buffer in ml.

3.2.11 Protein measurements

Protein concentration in the liver tissue homogenates was measured by using a total protein kit (Micro Lowery/ Peterson's modification), which rely on the method of Lowry et al. (1951) and modified by Peterson (1977). Standards (BSA) were prepared by diluting 400 µg/ml of BSA in water. Sodium chloride was added to a concentration of 0.1 M to eliminate interference, then 0.15 % DOC and 72 % TCA were added to precipitate proteins. Precipitated proteins were resolved in Lowry reagent solution and added Folin and Ciocalteu's Phenol Reagent. Absorbance of the colored solution was read with SPECTROstar Nano at 500 nm.

3.3 In vitro study

3.3.1 Isolation of hepatocytes

Two experimental fish, Atlantic salmon (of approximately 300 g), were used for isolation of hepatocytes. The fish came from fish lab of Norwegian University of life sciences. Fish were anaesthetized with 2 g/l metacain (MS-222), and then 0.1 ml heparin (5000 IU/ml) was inserted into the back vein to inhibit thrombosis. The hepatocytes were prepared by a two step collagenase perfusion procedure described by Seglen (1976) and modified by Dannevig and Berg (1985). Briefly, after the abdominal cavity was exposed, aportal cannula was inserted into the portal vein on the underside of the liver. The heart was cut open, so that the perfusion liquid from the liver could be transported out. The liver was perfused with EDTA perfusion solution (25 mM EDTA, 143 mM NaCl and 6.7 mM KCl) for approximately 10 minutes until most of the blood was removed from the liver. Further, the liver was perfused with a collagenase solution which contained 143 mM NaCl, 6.7 mM KCl, 1 mM $CaCl_2$ and 0.1 % collagenase type 1 (125-200 U/mg) for approximately 20 minutes. The liver was then transferred into Leibovitz's L-15 mediumand gently shaken until cells were loosen. The suspension of cells was filtered through a 100 μm nylon filter, then washed two times in L-15 medium and collected by centrifugation at 800 × g for 2 minutes. The cells were resuspended in growth medium (L-15 medium containing 0.375 mg/ml bicarbonate, 2 mM glutamine, 10 mM HEPES, 50 U/ml penicillin, 50 μg/ml streptomycin and 10 % foetal bovine serum (FBS)). 100 μl 0.4 % trypan blue was added to 100 μl cell suspension to assess cell viability and count them in a Burkes counting chamber. The cells were diluted in growth medium to 2 million cells/ml and seeded onto 20 laminin coated plates (Figure 3) and incubated at 13 ℃ to let the cells attach to the bottom.

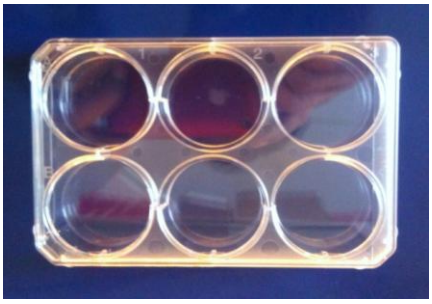

Figure 3: Six-well cell culture plates (Latif 2010). 20 plates (total 120 wells) were used in this cell experiment.

3.3.2 Incubation of hepatocytes with fatty acid

The hepatocyte cultures were preincubated at 13 ℃ for 24 hours prior to initiation of the experimental conditions. Hepatocytes at a density of ca. 5×10^5 cells/cm^2 were incubated with OA and DHA in different ratios. Specifically, the four fatty acid combination were 100 µM OA, 25 µM OA/75 µM DHA, 75 µM OA/25 µM DHA and 100 µM DHA. Each fatty acid combination treatment was performed in 25 wells. In addition 20 wells got a control treatment, added albumin (0.12 g/ml) instead of fatty acid. The fatty acids OA and DHA were added to the medium in the form of their potassium salts bound to BSA (2.7/1, molar ratio). Briefly, 25 mg fatty acid was dissolved in 1 ml chloroform (to 76 mM) and evaporated under a stream of nitrogen at 50-60 °C or 37-40 °C (OA at 50-60 °C and DHA at 37-40 °C). 3 ml of 0.1 M preheated NaOH was slowly added. The fatty acid-NaOH solution was then transferred to 12 ml preheated phosphate-buffered saline (PBS)-albumin which contained 1.88 g albumin. The Fatty acids on albumin were adjusted to pH = 7. The fatty acids were added to the L-15 medium containing 0.375 mg/ml bicarbonate, 10 mM HEPES, 1 % penicillin-streptomycin and 10 % FBS. The incubation was performed with a final concentration of 100 µM of Fatty acids at 13 ℃ for 66 hours. The cells for gene expression were washed two times in PBS and harvested in

QIA-shredder columns. The cells for ACO activity measurement and fatty acid analysis were washed two times in PBS with 1 % albumin, and one time in PBS, and harvested in 1 ml PBS and stored at - 70 °C.

3.3.3 RNA isolation

RNA isolation from hepatocytes was carried out by using an RNeasy® Mini Kit. The cells were lysed in 350 µl RLT buffer with 0.04 M DTT in multiwell-plates. The lysate were homogenized by pipetting the lysate into QIA shredder columns and centrifuged at 13000 rpm for 2 minutes. The upper parts of the columns were taken away and the suspension were added 350 µl 70 % ethanol to provide appropriate binding conditions for the total RNA to bind to the RNeasy silica membrane when the samples were transferred to RNeasy columns and centrifuged at 13000 rpm for 15 seconds. Thereafter, the samples were incubated in 80 µl DNase mix (10 µl Dnase I + 70 µl RDD buffer) for 15 minutes at room temperature to remove the DNA in the samples. Then the DNasewas eliminated by a second wash with Buffer RW1. Contaminants in the samples were efficiently washed away in 500 µl RPE buffer with ethanol and centrifuged at 13000 rpm for 15 seconds. 40 µl RNase free water was added to the RNA and centrifuged at 13000 rpm for 1 minute to elute RNA. After RNA isolation, the total RNA concentrations were determined by spectrophotometry (NanoDrop 1000 Spectrophotometer).

3.3.4 ACO activity analysis

Approximately 20 µl hepatocytes in 180 µl PBS was homogenized using Kontes Pellet Pestle. The samples were diluted by using equal amount of 0.4 % Triton-X 100 and then centrifuge at 800 × g for 15 minutes at 4 ℃.

Spectrophotometric assay of ACO in hepatocytes was determined by measuring production of H_2O_2, which was coupled to oxidation of 2', 7'-dichlorofluorescine in a reaction catalyzed by peroxidase (Small et al.

1985). Prepared 20 µl samples were added to the reaction mixture (described in ACO measurement *in vivo* study), and then 6 mM palmitoyl-CoA was used to start the reaction. The reaction was performed in 96-well EIA/RIA plates and measured with SPECTROstar Nano at 502 nm for 180 seconds at room temperature. The ACO activity was calculated as described above (ACO measurement *in vivo* study).

3.3.5 Lipid extraction and analysis of fatty acid composition

Total lipid was extracted from cell pellets according to Folch et al. (1957) using chloroform/methanol (2:1, v/v) containing 0.03 % butylated hydroxytoluene. 0.9 % NaCl was added in two rounds, to get the volume ratio of chloroform, methanol and water in solution to approximately 8:4:3. Following mixing and separation of the phases by centrifugation at 3000 rpm for 10 minutes at 4 ℃, the chloroform layer was pipetted off and dried under nitrogen before being redissolved in chloroform/ methanol (2:1, v/v) containing 0.03 % butylated hydroxytoluene.

The chloroform phase was dried under nitrogen and trans-methylated overnight with 2 ml benzene, 2 ml metanolic-HCl and 0.2 ml dimetoxypropan as described by Mason and Waller (1964). Next day, FAME was extracted and purified by using 2 ml hexan neutralized by adding 4 ml 6 % $NaHCO_3$. The FAME was separated according to degree of unsaturation and chain length by GC and the fatty acids were identified and calculated as described above.

3.3.6 Incubation with radio-labelled fatty acids

After stimulation of hepatocytes with OA and DHA, the cells for *β*-oxidation and metabolism were washed two times in PBS with 1 % albumin, and one time in L-15. Further hepatocytes at density of ca. 5×10^5 cells/cm^2 were incubated with 7 µM (final concentration) radio-labelled 18:3 n-3 ([1-^{14}C] 18:3 n-3). The

radio-labelled fatty acid was added to the medium in the form of its potassium salt bound to BSA, prepared as described above. 20 wells (5 wells from each OA/DHA treatment) were incubated with this isotope at 13 ℃ for 24 hours in 2.5 ml L-15 medium (containing 0.375 mg/ml bicarbonate, 10 mM HEPES, 1 % penicillin-streptomycin) without FBS.

After incubation with [1-^{14}C] 18:3 n-3 for 24 hours, the media were harvested for measuring *β*-Oxidation, the cells were washed in PBS with 1 % albumin and in PBS without albumin and then were harvested in 1 ml of PBS for analysis of elongation and desaturation products by high-pressure liquid chromatography (HPLC).

3.3.7 *β*-Oxidation

The capacity of *β*-oxidation was measured by determination of the radio-labelled products, acid soluble products (ASP) and CO_2 as described by Christiansen et al. (1976).

The production of CO_2 was measured by transferring 1.4 ml of the medium to glass vials sealed with a rubber stop and a central well containing a filter paper. CO_2 released from the acidified medium by addition of 0.3 ml 1 M $HClO_4$ to the trap flasks, was trapped by injecting 0.3 ml phenylethylamine/methanol (1:1 v/v) to the filter paper for 1 hour at room temperature. Then the filter papers were placed in vials and added 10 ml of scintillation fluid for scintillation counting.

The amount of radio-labelled ASP was determined by adding 0.5 ml ice cold 2 M $HClO_4$ to 1 ml cell media and incubated on ice for 1 hour, then the samples were centrifuged at maximum speed for 10 minutes at 4 ℃ and 100 µl of the supernatant was collected for scintillation counting. A standard curve

were prepared by measuring radio-activity in 0 µl, 5 µl, 10 µl, 20 µl, 40 µl, 80 µl, respectively, of the [1-^{14}C] 18:3 n-3 fatty acids.

3.3.8 High-pressure liquid chromatography (HPLC)

After total lipid was extracted from cells as described above, 50 µl of the chloroform phase was transferred into scintillation vials containing 5 ml of scintillation fluid to measure radio-activity in an aliquot of the total lipid with a TRI-CARB 1900 TR scintillation counter. Results were corrected for counting efficiency and quenching of ^{14}C under exactly these conditions. The rest of chloroform phase was dried under nitrogen.

FAMEs were prepared as above which is then analyzed in high-pressure liquid chromatography as described by Narce et al. (1988). The mobile phase was acetonitrile/H_2O (85:15 by volume) at 30 ℃ and with flow rate 1 ml/minute. The column used was a reversed-phase Symmetry 3.5 µm C18 HPLC column from Waters was used. Radio-activity level in the different fatty acids was measured in a radio-active flow detector A100. The radio-active fatty acids were identified by comparing the retention time of samples with the retention volumes of known fatty acid standards.

3.4 Statistical analysis

For all data in the *in vivo* study, except for gene expression and correlation, the independent t-test in SPSS (IL, USA) was used. Correlation between DHA + EPA and fat content (or body weight) was analyzed by Pearson correlations, followed two-tailed test to determine significant differences. All data in the *in vitro* study were subjected to one-way ANOVA for the factor "treatment" (ratio of OA/DHA), and differences were ranked by Ducan's multiple range test. We used the software package UNISTAT (London, England). The significance level was set to $P \leq 0.05$.

4. Results

4.1 In vivo trial

4.1.1 Δ6fad_b gene expression in liver tissue from fish in different families

Screening 103 families for their expression of the Δ6fad_b gene showed that the levels of Δ6fad_b gene expression were greatly different between the families. The family with the highest expression had almost 25 times higher expression of the gene than the family with the lowest expression (Figure 4).

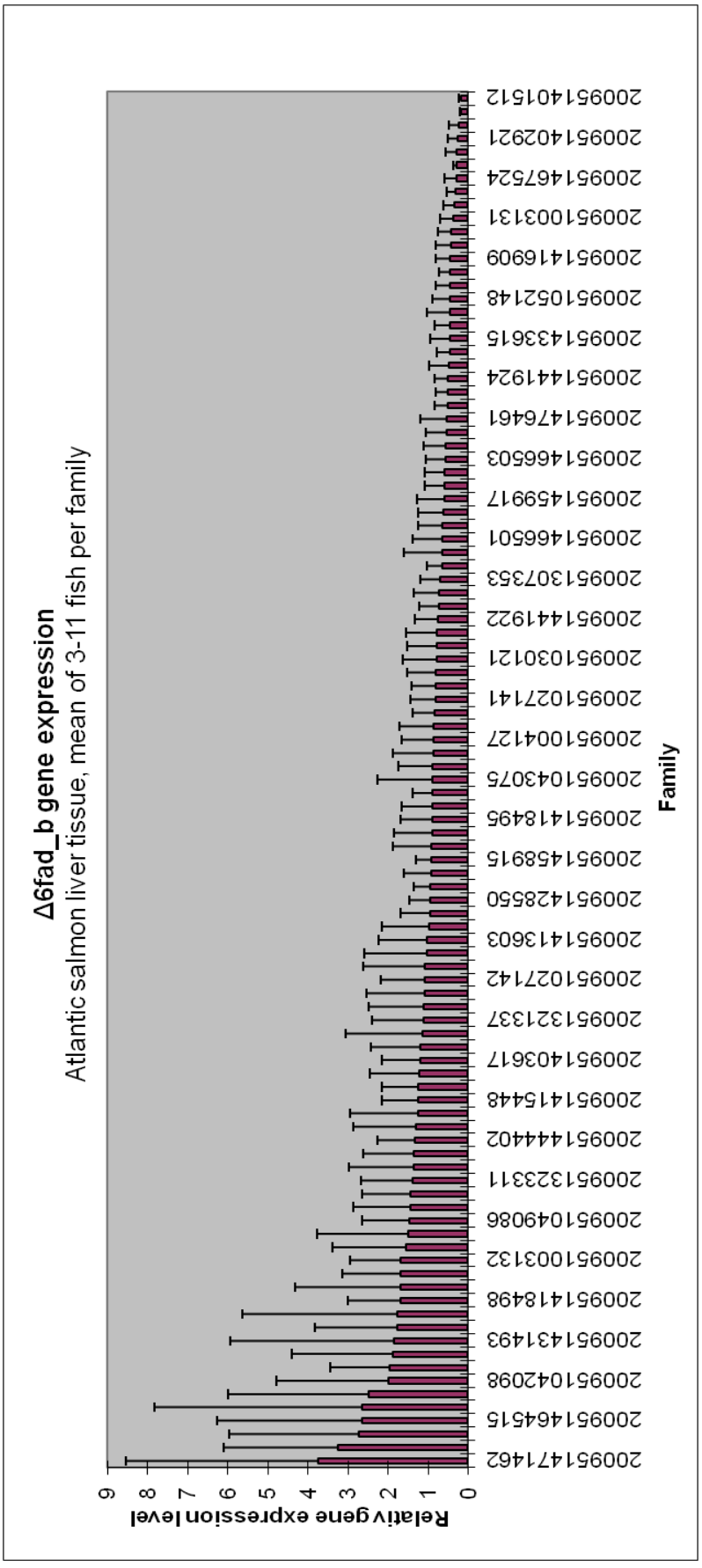

Figure 4: Relative gene expressions of Δ6fad_b in salmon liver. The gene expression of Δ6fad_b is normalized to the reference gene *etif*. The values are expressed as the mean of all fish in each family and with ± SEM.

When comparing the Δ6fad_b gene expression level in fish with similar weight from the families selected as "high expression families" and "low expression families", we could see that the relative gene expression of "high expression families" were about thirteen fold higher than "low expression families" (Figure 5).

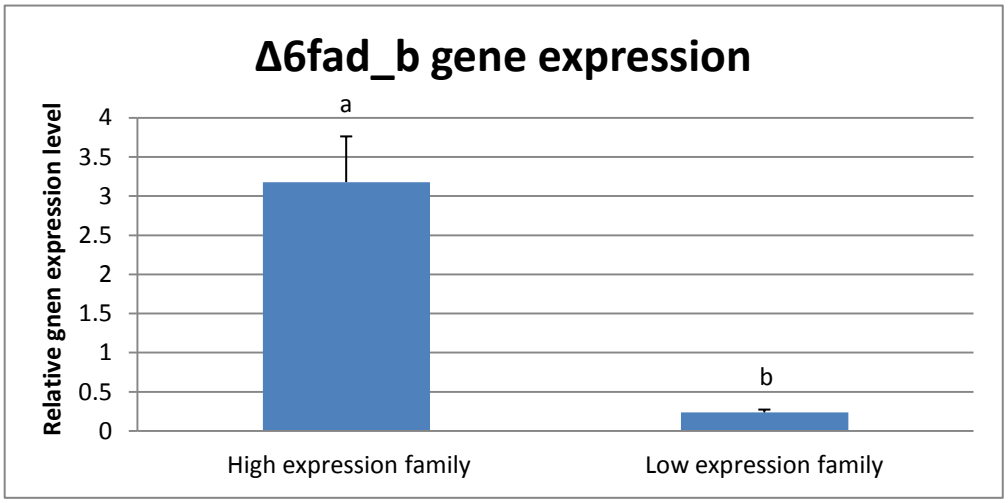

Figure 5: Relative gene expression level of Δ6fad_b in liver of the "high expression families" and "low expression families". The gene expression of Δ6fad_b is normalized to the reference gene etif. Data are means ± SEM (n = 14 for high expression families, n = 16 for low expression families). Different letters indicate statistical differences ($P \leq 0.05$).

4.1.2 Correlation between the percentage of DHA + EPA and fat content in liver tissue

Percentages of DHA + EPA of total fatty acids in salmon liver tissue showed a strong linear relationship with fat content in liver tissue, decreasing with increasing fat content (Figure 6). This clear negative relationship was found in both "high expression families" and "low expression families".

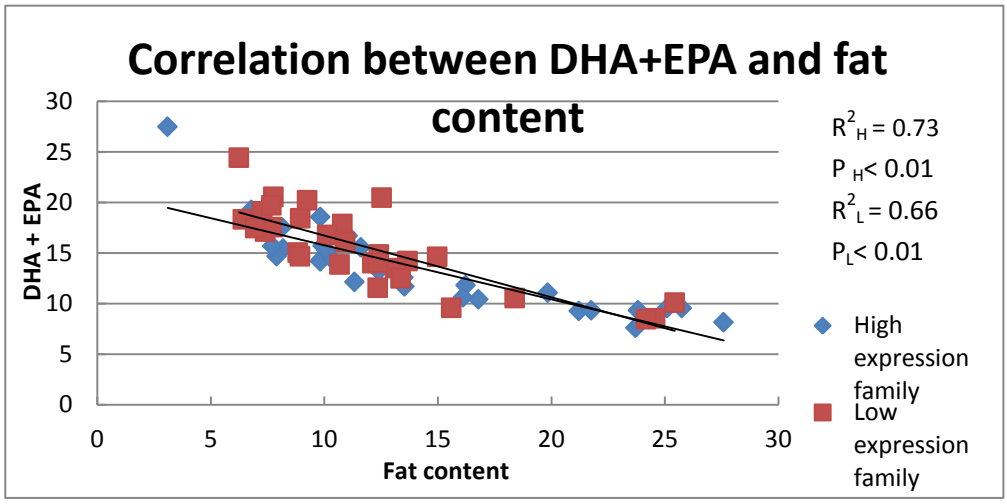

Figure 6: Correlation between DHA + EPA (percent of total fatty acids) and fat content (% fat) in salmon liver of the "high expression families" and the "low expression families". Liver samples analyzed from 5 fish in each family.

The ratio DHA + EPA / fat content in the families with high expression were about 30 % higher than in the families with low expression (Figure 7), however this was not statistically significant.

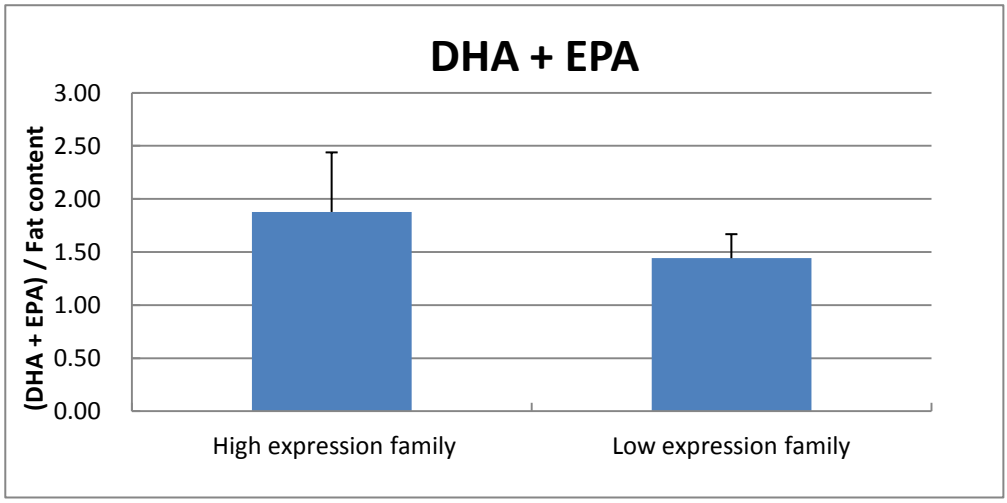

Figure 7: The ratio of DHA+EPA (percent of total fatty acids) / fat content (% fat) in the "high expression families" and the "low expression families". Data are means ± SEM (n = 14 for high expression families, n = 16 for low expression families). Different letters indicate significant differences ($P \leq 0.05$).

4.1.3 Correlation between the percentage of DHA + EPA and body weight of fish

Percentages of DHA + EPA of total fatty acids in "high expression families" showed a negative correlation with individual fish weight, decreasing with increasing weight (Figure 8). However, no correlation was found between individual weight of fish and percentages of DHA + EPA in "low expression families".

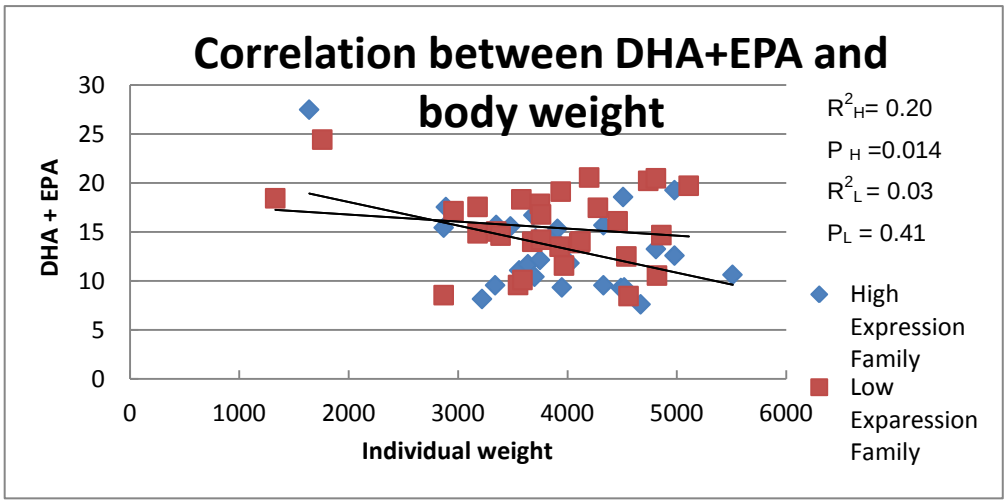

Figure 8: Correlation between DHA + EPA (percent of total fatty acids) and body weight (g) of the "high expression families" and the "low expression families". Liver samples analyzed from 5 fish in each family.

4.1.4 Fatty acid compositions in liver tissue from fish in different families

Fatty acid compositions in liver tissue of the "high expression families" and "low expression families" are shown in Table 3. The levels of the n-3 long chain fatty acid DHA and EPA were slightly higher in "high expression families" than "low expression families", although not significant. In addition, the livers from fish of the "high expression families" contained significantly higher of ∑saturated fatty acids than the fish of the "low expression families".

Table 3: Comparison of fatty acid compositions of livers in similar body weight salmon between "high expression families" and "low expression families". The quantity of each fatty acid is given in percent of total fatty acids. Data are means ± SEM (n = 14, 16). Different letters indicate significant differences ($P \leq 0.05$).

Fatty acids (% of total)	**High expression families**	**Low expression families**
C 14:0	2.5 ±0.07[a]	2.2 ±0.05[b]
C 16:0	9.0 ±0.40	7.9 ±0.27
C 18:0	3.8 ±0.16	3.3 ±0.14
∑ **Saturated**	15.3 ±0.53[a]	13.4 ±0.36[b]
C 16:1 n-7	2.7 ±0.10	2.7 ±0.13
C 18:1 n-7	3.0 ±0.03	2.8 ±0.13
C 18:1 n-9	30.0 ±0.79	31.1 ±0.78
C 18:1 n-11	1.9 ±0.14	1.9 ±0.10
C 22:1 n-7	1.8 ±0.04	1.8 ±0.04
C 20:1 n-9	5.9 ±0.18	6.3 ±0.12
C 22:1 n-9	0.5 ±0.02	0.5 ±0.02
C 22:1 n-11	2.9 ±0.09	3.0 ±0.11
∑ **Monounsaturated**	48.7 ±1.11	49.3 ±0.98
C 18:2 n-6	7.7 ±0.16	7.9 ±0.12
C 18:3 n-3	2.7 ±0.06	2.8 ±0.06
C 20:4 n-3	1.4 ±0.05	1.4 ±0.05
C 20:2 n-6	1.6 ±0.06	1.7 ±0.04
C 20:4 n-6	0.8 ±0.05	0.8 ±0.06
C 20:5 n-3	4.6 ±0.21	4.5 ±0.22
C 22:5 n-3	1.6 ±0.08	1.8 ±0.07
C 22:6 n-3	10.5 ±0.64	10.3 ±0.61
∑ **Polyunsaturated**	31.0 ±0.69	31.5 ±0.76
∑ n-3	20.7 ±0.79	20.8 ±0.78
∑ n-6	10.0 ±0.16	10.5 ±0.13
EPA + DHA	15.0 ±0.84	14.8 ±0.82

4.1.5 ACO activity in liver tissue from fish in different families

The ACO activity was not significantly different between the "high expression families" and the "low expression families", being 17.93 ± 1.20 nmol × min^{-1} × g^{-1} liver and 16.56 ± 1.22 nmol × min^{-1} × g^{-1} liver respectively (Figure 9).

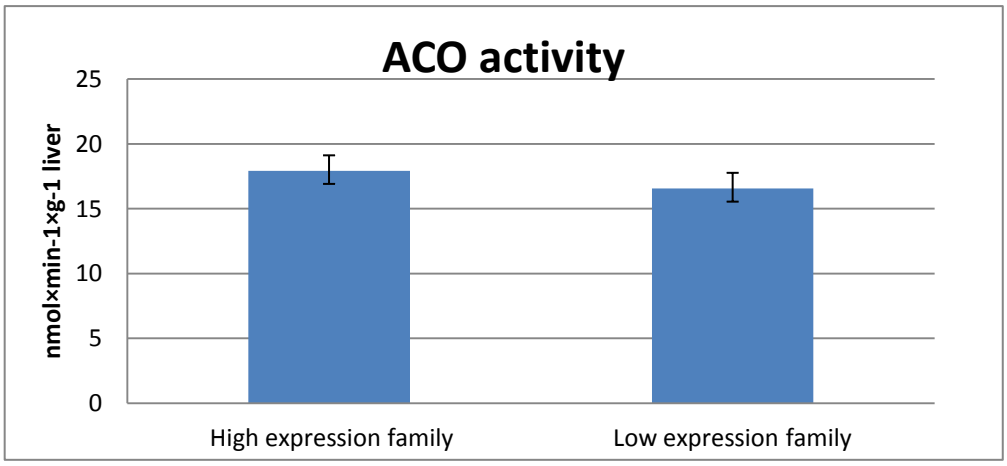

Figure 9: ACO activity in salmon liver tissue of the "high expression families" and "low expression families". The results is displayed here as nmol/min/g liver tissue. Data are presented as means ± SEM. Different letters indicate significant differences ($P \leq 0.05$).

4.1.6 ACO gene expression in liver tissue from fish in different families

The ACO gene expression was not significantly different between the "high expression families" and the "low expression families", being -0,68 ± 0.33 and -0,61 ± 0.23 respectively (Figure 10).

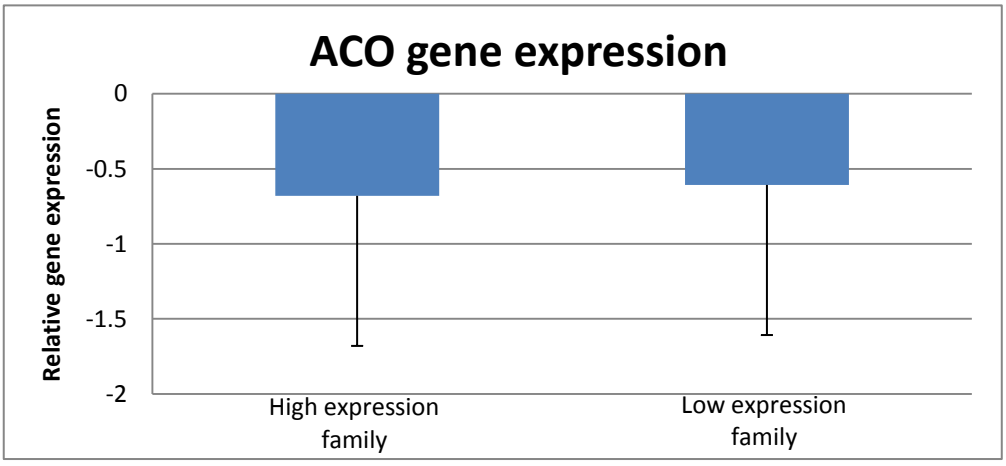

Figure 10: Relative gene expression level of ACO in liver of the "high expression families" and "low expression families". The gene expression of ACO is normalized to the reference gene etif. Data are means ± SEM. Liver samples analyzed from 5 fish in each family. Different letters indicate statistical differences ($P \leq 0.05$).

4.2 In vitro trial

4.2.1 Fatty acid compositions in hepatocytes

The fatty acid compositions in hepatocytes (Table 4) reflected the ratio of OA and DHA given to the hepatocytes. Incubation of hepatocytes with increased level of OA led to increased intracellular level of OA and its elongation product 20:1 n-9. The percentage of DHA in hepatocytes also increased with increasing level of this fatty acid supplemented in the cultivation media. The percentage of EPA and DHA increased from 17.5 % in the 100OA group to 20 % in the 75OA/25DHA group and to approximately 22 % in 25OA/75DHA and 100DHA groups.

Table 4: Fatty acid compositions in hepatocytes stimulated with OA and DHA. The quantity of each fatty acid is given in percent of total fatty acids. Data are means ± SEM (n = 5).

	100OA	**75OA/25DHA**	**25OA/75DHA**	**100DHA**
C 14:0	1.11 ±0.12	1.0 ±0.04	1.1 ±0.01	1.1 ±0.02
C 16:0	10.2 ±0.37^{a}	10.2 ±0.08^{a}	10.9 ±0.05^{b}	11.2 ±0.13^{b}
C 18:0	5.2 ±0.08^{a}	5.1 ±0.04^{a}	5.5 ±0.04^{b}	5.5 ±0.03^{b}
∑ Saturated	16.5 ±0.55^{a}	16.4±0.14^{a}	17.4 ±0.08^{b}	17.8 ±0.17^{b}
C 16:1 n-7	3.00 ±0.05^{a}	3.0 ±0.04^{a}	2.8 ±0.06^{b}	2.9 ±0.06^{ab}
C 18:1 n-9	35.1 ±0.24^{a}	34.1 ±0.11^{b}	30.9 ±0.06^{c}	30.3 ±0.26^{d}
C 18:1 n-7	2.9 ±0.04^{a}	2.9 ±0.02^{a}	2.9 ±0.01^{a}	3.0 ±0.03^{b}
C 20:1 n-9	5.0 ±0.07^{a}	4.7 ±0.02^{b}	4.1 ±0.02^{c}	4.2 ±0.09^{c}
C 22:1 n-7	0.5 ±0.07	0.5 ±0.02	0.6 ±0.02	0.6 ±0.02
∑ Monounsaturated	46.5 ±0.25^{a}	45.2 ±0.16^{b}	41.4 ±0.12^{c}	41.0 ±0.38^{c}
C 18:2 n-6	6.7 ±0.06	6.7 ±0.03	6.8 ±0.02	6.7 ±0.10
C 18:3 n-3	1.6 ±0.04^{a}	1.6±0.00^{a}	1.7 ±0.02^{b}	1.6 ±0.04^{a}
C 20:2 n-6	1.7 ±0.02^{a}	1.8 ±0.06^{b}	1.8 ±0.01^{b}	1.9 ±0.02^{b}
C 20:3 n-6	1.0 ±0.02^{a}	1.0 ±0.01^{a}	0.9 ±0.02^{b}	1.0 ±0.03^{ab}
C 20:4 n-6	2.1 ±0.05^{a}	2.2 ±0.03^{ab}	2.3 ±0.01^{b}	2.2 ±0.06^{ab}
C 20:3 n-3	0.4 ±0.01^{a}	0.5 ±0.01^{b}	0.5 ±0.01^{b}	0.5 ±0.01^{b}
C 20:5 n-3	1.6 ±0.01^{a}	1.7 ±0.01^{b}	2.0 ±0.02^{c}	2.0 ±0.02^{d}
C 22:5 n-3	0.8 ±0.02^{a}	0.8 ±0.02^{a}	1.0 ±0.03^{b}	1.0 ±0.03^{b}
C 22:6 n-3	16.0 ±0.28^{a}	18.1 ±0.09^{b}	20.0 ±0.07^{c}	20.2 ±0.26^{c}
∑ Polyunsaturated	32.0 ±0.36^{a}	34.5 ±0.14^{b}	37.1 ±0.11^{c}	36.9 ±0.27^{c}
Sum n-3	20.4 ±0.29^{a}	22.7 ±0.10^{b}	25.5 ±0.09^{c}	25.3 ±0.26^{c}
Sum n-6	11.6 ±0.08^{a}	11.8 ±0.07^{bc}	11.9 ±0.02^{c}	11.7 ±0.05^{ab}
EPA+DHA	17.5 ±0.28^{a}	19.8 ±0.10^{b}	21.9 ±0.06^{c}	22.2 ±0.28^{c}

4.2.2 ACO activity in hepatocytes

The ACO activity in hepatocytes incubated with fatty acid combinations are as show in Figure 11. The ACO activity was increased in response to increased proportion of DHA stimulation. Hepatocytes incubated with 75OA/25DHA had significantly lower ACO activity than hepatocytes incubated with 100DHA.

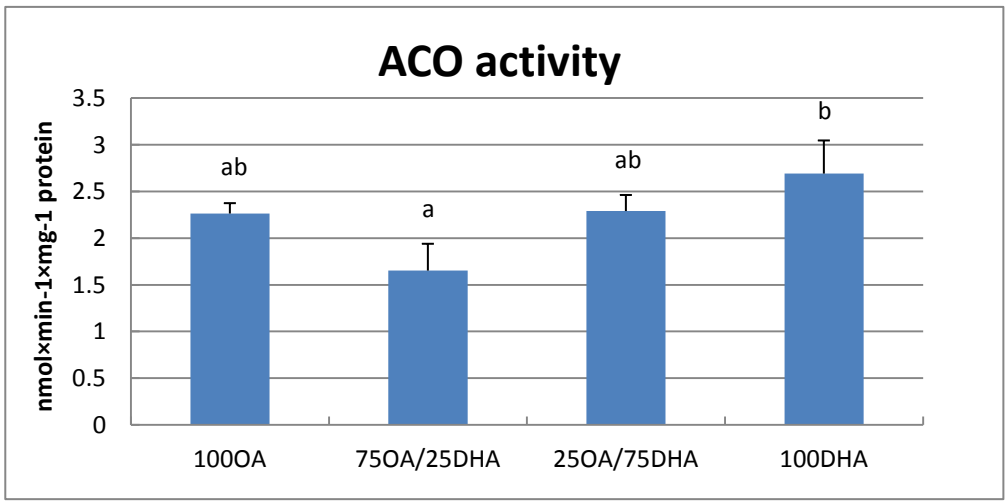

Figure 11: ACO activity in salmon hepatocytes. The hepatocytes were stimulated with one of four fatty acid combinations; 100OA, 75OA/25DHA, 25OA/and 100DHA. ACO activity is displayed as nmol/min per mg protein. Data are presented as means ± SEM (n = 5). Different letters indicate significant differences ($P \leq 0.05$).

4.2.3 ACO gene expression in hepatocytes

The relative expression of the ACO gene is shown in Figure 12. The hepatocytes incubated with 25OA/75DHA had significantly lower expression of ACO than hepatocytes added 100DHA. The ACO gene expression showed a tendency to increase in response to increased proportion of DHA stimulation.

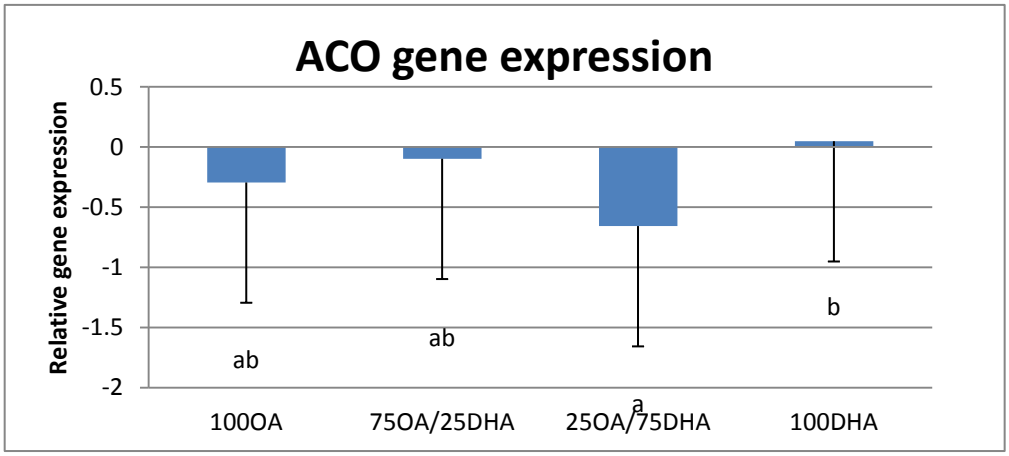

Figure 12: Relative gene expression of ACO in hepatocytes stimulated with one of four fatty acid combination; 100OA, 75OA/25DHA, 25OA/and 100DHA. The gene expression level of ACO is normalized to the gene expression of the reference gene *etif*. Data are presented as means ± SEM (n = 5). Different letters indicate significant differences ($P \leq 0.05$).

4.2.4 Δ6fad_b gene expression in hepatocytes

The relative expression of Δ6fad_b is shown in Figure 13. The hepatocytes incubated with high proportion of OA had significantly higher expression of Δ6fad_b than hepatocytes incubated with high proportion of DHA.

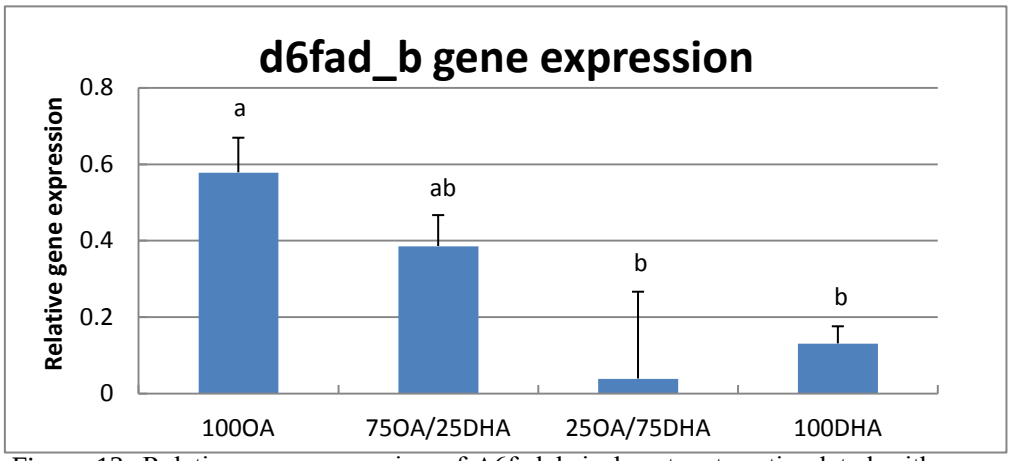

Figure 13: Relative gene expression of Δ6fad_b in hepatocytes stimulated with one of four fatty acid combination; 100OA, 75OA/25DHA, 25OA/and 100DHA. The gene expression of Δ6fad_b is normalized to the reference gene *etif*. Data are presented as means ± SEM (n = 5). Different letters indicate significant differences ($P \leq 0.05$).

4.2.5 Metabolism of [1-^{14}C] 18:3 in hepatocytes

Approximately 70 % of the added radio-labelled substrate was taken up by the cells, while approximately 30 % remained in the culture media. Table 5 show that there were no significant differences in the total nmol radio-labelled fatty acids taken up by the cells per mg protein between the different groups. More than 99 % of the fatty acids taken up were found in cellular lipids and less than 1 % was found in the β-oxidation product CO_2.

Table 5: Distribution of total nmol radio-activity in the cells and β-oxidation products, after incubation of hepatocytes with [1-^{14}C] 18:3 n-3 for 24 hours.

	100OA	**75OA/25DHA**	**25OA/75DHA**	**100DHA**
Total cellular uptake (nmol mg^{-1} protein)	1.38 ±0.04	1.35 ±0.17	1.09 ±0.09	1.42 ±0.23
Cellular lipids (nmol mg^{-1} protein)	1.38 ±0.04	1.34 ±0.16	1.09 ±0.09	1.41 ±0.23
CO_2 (nmol mg^{-1} protein)	0.005 ±0.0004	0.008 ±0.0012	0.006 ±0.0005	0.007 ±0.0009

Data are presented as mean ± SEM (n = 5). Different letters indicate statistical differences ($P \leq 0.05$).

The main metabolic products found in the hepatocytes were the desaturation product 18:4 n-3 and the desaturation and elongation products DHA, EPA and 22:5 n-3. Minor amounts of the "dead end" elongation product 20:3 n-3 were also found.

Figure 14 show that approximately 50 % of the radio-activity in cellular lipids was recovered as un-metabolised [1-^{14}C] 18:3 n-3 substrate. Although not statistically significant, more un-metabolised 18:3 n-3 substrate was recovered with increased DHA level in hepatocytes.

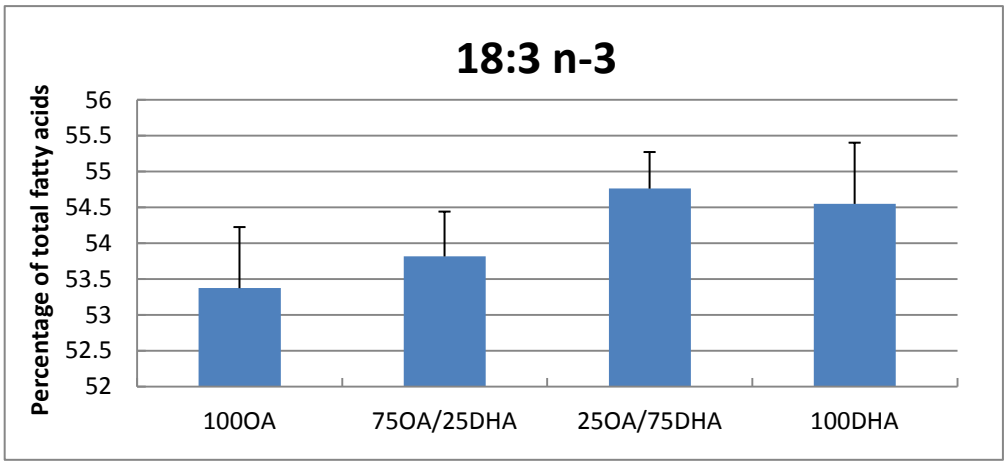

Figure 14: Level of radio-labelled 18:3 n-3 in hepatocytes cultured in one of four fatty acid combination (100OA, 75OA/25DHA, 25OA/and 100DHA) and stimulated with [1-^{14}C] 18:3 n-3 for 24 hours. The quantity of fatty acid is given as percent of total radio-labelled fatty acids. Data are presented as mean ± SEM (n = 5).

Approximately 2 % of the total recovered radio-labelled fatty acids were found as the Δ6 desaturation product of 18:3 n-3, namely 18:4 n-3 (Figure 15). There were no significant differences between the treatment groups in the formation of radio-labelled 18:4 n-3, although, there was a tendency to higher radio-labelled 18:4 n-3 production in the 100OA group than the groups supplemented with DHA in the culture media.

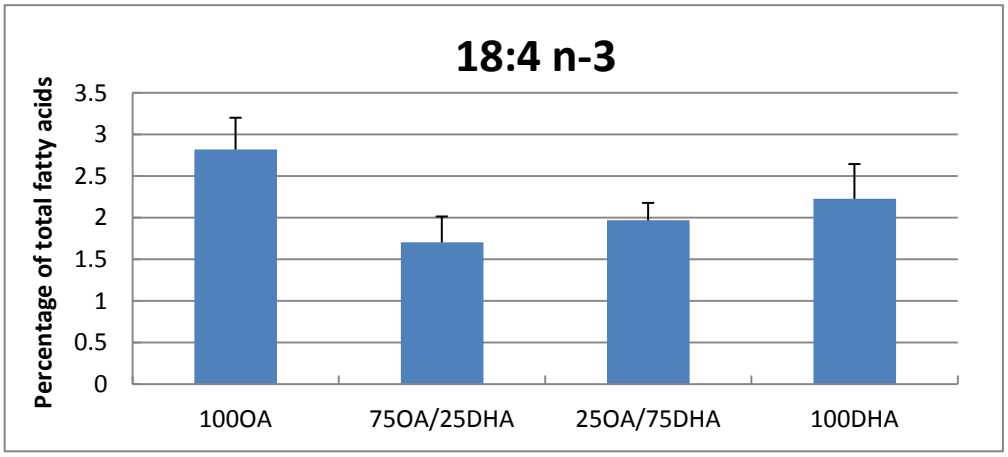

Figure 15: Level of radio-labelled 18:4 n-3 in hepatocytes cultured in one four fatty acid combination 100OA, 75OA/25DHA, 25OA/and 100DHA, and stimulated with [1-^{14}C] 18:3 n-3 for 24 hours. The quantity of fatty acid is given in percent of total radio-labelled fatty acids. Data are presented as mean ± SEM (n = 5).

Increasing DHA content in incubation media led to an increase in this fatty acid in hepatocyte lipids which again led to a significant reduction in the production of radio-labelled EPA (Figure 16).Radio-labelled EPA production from [1-^{14}C] 18:3 n-3 decreased from approximately 13 % in the 100OA incubated hepatocytes to approximately 11 % in the 75OA/25DHA incubated hepatocytes and 10 % in the 25OA/75DHA incubated hepatocytes and 8 % in 100DHA hepatocytes.

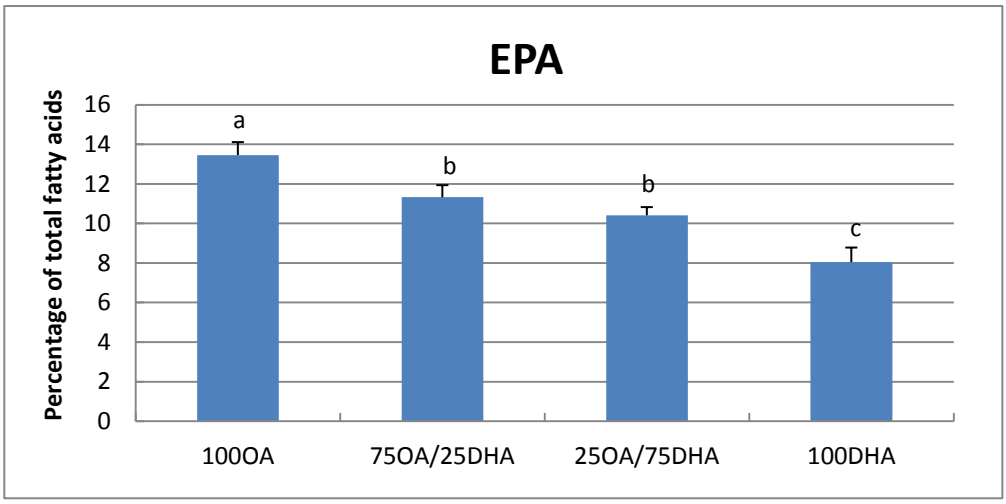

Figure 16: Level of radio-labelled EPA in hepatocytes cultured in one of four fatty acid combination 100OA, 75OA/25DHA, 25OA/and 100DHA, and stimulated with [1-^{14}C] 18:3 n-3 for 24 hours. The quantity of fatty acid is given in percent of total radio-labelled fatty acids. Data are presented as mean ± SEM (n = 5). Different lowercase letters indicate statistical differences ($P \leq 0.05$).

One-way ANOVA showed that the overall effects of the four fatty acid treatments were not significant on n-3 fatty acids production of radio-labelled DHA (Figure 17). However, when comparing OA and DHA stimulation, the hepatocytes showed a tendency to higher percentage of radio-labelled DHA in 100OA and 75OA/25DHA incubation than 100DHA and 25OA/75DHA incubation.

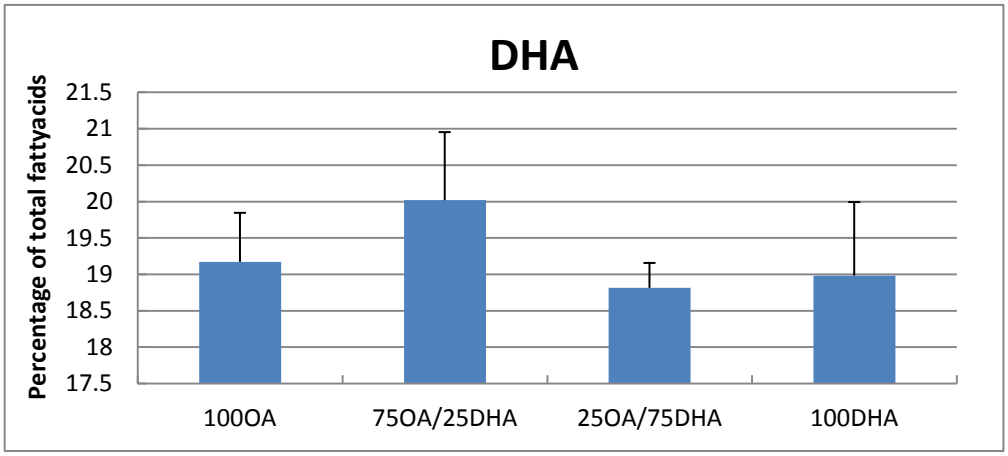

Figure 17: Level of radio-labelled DHA in hepatocytes cultured in one of four fatty acid combination 100OA, 75OA/25DHA, 25OA/and 100DHA, and stimulated with [1-^{14}C] 18:3 n-3 for 24 hours. The quantity of fatty acid is given in percent of total radio-labelled fatty acids. Data are presented as mean ± SEM (n = 5).

Although not statistically significant, there is a tendency to decreased production of radio-labelled 22:5 n-3 with increasing percentage of DHA in the hepatocyte culture media (Figure 18). Percentage of radio-labelled 22:5 n-3 decreased from approximately 1.75 % in the 100OA incubated hepatocytes to approximately 1.5 % in the 75OA/25DHA and 25OA/75DHA incubated hepatocytes, to approximately 1.1 % in the 100DHA incubated hepatocytes.

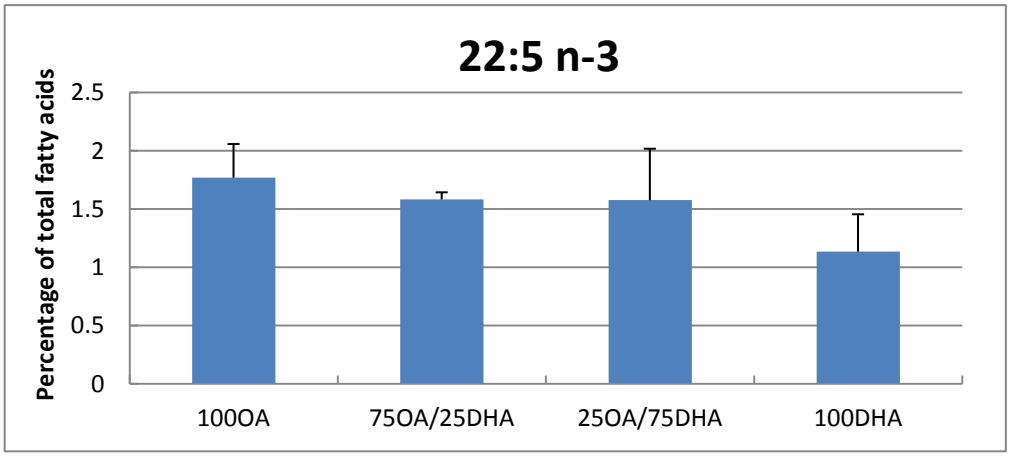

Figure 18: Level of radio-labelled 22:5 n-3 in hepatocytes cultured in one of four fatty acid combination 100OA, 75OA/25DHA, 25OA/and 100DHA, and stimulated with [1-^{14}C] 18:3 n-3 for 24 hours. The quantity of fatty acid is given in percent of total fatty acids. Data are presented as mean ± SEM (n = 5).

Decreasing OA content in the incubation media was reflected in graded increased percentages of radio-labelled 20:3 n-3 (Figure 19). Percentage of radio-labelled 20:3 n-3 increased from approximately 9 % in the 100OA incubated hepatocytes to approximately 12 % in the 75OA/25DHA and 25OA/75DHA incubated hepatocytes, to approximately 15 % in the 100DHA incubated hepatocytes.

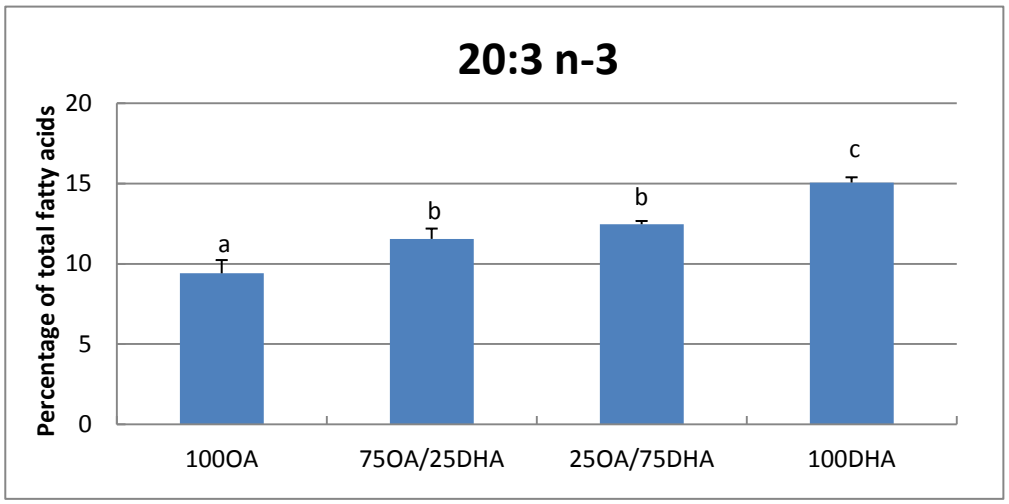

Figure 19: Level of radio-labelled 20:3 n-3 in hepatocytes cultured in one of four fatty acid combination 100OA, 75OA/25DHA, 25OA/and 100DHA, and stimulated with [1-^{14}C] 18:3 n-3 for 24 hours. The quantity of fatty acid is given in percent of total fatty acids. Data are presented as mean ± SEM (n = 5). Different lowercase letters indicate statistical differences ($P \leq 0.05$).

4.2.6 *β*-oxidation in hepatocytes

Very small amount of the total uptake of [1-^{14}C] 18:3 n-3 was *β*-oxidised (Figure 20). Percentages of *β*-oxidized [1-^{14}C] 18:3 n-3 relative to uptake in 100OA incubated cells was significant lower than in cells supplemented with DHA. Approximately 0.39 % of [1-^{14}C] 18:3 n-3 taken up by the cells was *β*-oxidized in 100OA incubated cells, and approximately 0.58 % was *β*-oxidized in the different DHA supplemented cells.

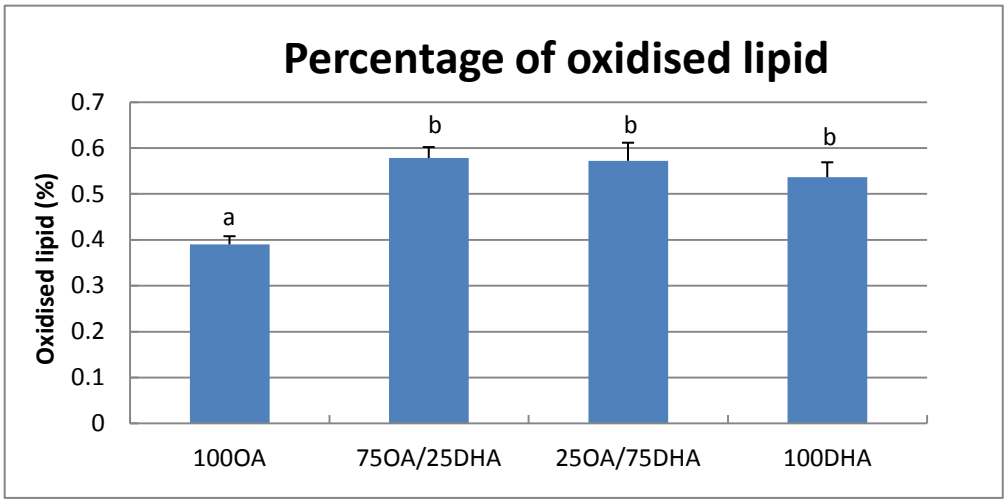

Figure 20: Relative amount of [1-^{14}C] 18:3 n-3 β-oxidized compared with total amount of [1-^{14}C] 18:3 n-3 taken up during the 24 hours incubation time. Data are shown as means ± SEM (n = 5). Different letters indicate statistical differences ($P \leq 0.05$).

5. Discussion

5.1 In vivo trial

Previous dietary studies with Atlantic salmon have demonstrated that fatty acid compositions of fish tissues is determined mainly by the type of dietary lipid ingested, and by the ability of the individual fish species to modify that dietary input via pathways of desaturation and elongation (Bell et al. 1993; 1995). According to Schlechtriem et al. (2007), irrespective of diet, variation exists in the content of n-3 HUFA in individual salmon. It means that, when individual fish are feed the same dietary fatty acids, there are some variations in the content of n-3 HUFA between the individuals. This variation may be due to differences in desaturation and elongation capacities in the n-3 HUFA pathway between the different individuals. Leaver et al. (2011) has further shown that flesh n-3 HUFA composition is a highly heritable trait ($h^2 = 0.77 \pm 0.14$), which indicate that high contents of n-3 HUFA may vary between families. Our goal was to identify whether there are differences in the n-3 HUFA level in different families. Further to see if there are differences in the capacities to produce n-3 HUFA. Because of the high costs for measuring fatty acid composition, we have used gene expression of Δ6fad as an indirect marker of capacity to produce n-3 HUFA when screening all the selected salmon families.

Δ6fad is regarded as the main enzyme in the n-3 HUFA biosynthesis pathway, and Δ6fad activity is far greater than Δ5fad activity in salmon (Hastings et al. 2004). Three Δ6fad genes (Δ6fad_a, Δ6fad_b and Δ6fad_c) are cloned from salmon (Monroig et al. 2010). Δ6fad_b showed highest expression level when Atlantic salmon hepatocytes are stimulated with fatty acids like OA (previous unpublished results from our group). Therefore, it was chosen to analyse the expression of Δ6fad_b gene when screening 100 selected salmon families for capacities to produce n-3 HUFAs in this *in vivo* study.

The results of our *in vivo* study showed that the levels of Δ6fad_b gene expression in liver were different between the different Atlantic salmon families used in our trial. This result may indicate that there are differences between different families in their capacities to produce EPA and DHA in agreement with the study of Leaver et al. (2011) showing that n-3 HUFA is a highly heritable trait in Atlantic salmon. Our data may indicate that differences in Δ6fad activity between families may be involved. However, it is not known which enzymes in the n-3 HUFA pathway that is responsible for the difference. This cannot be stated with certainty based on our data, and needs to be further elucidated by actual enzyme measurement and determination of fatty acid composition in all the families in addition to the gene expression data. Due to high costs, it was only possible to select 12 of the 100 families for analyses of fatty acid composition. According to the difference found in Δ6fad_b gene expression, 6 families of Atlantic salmon were selected as "high expression families" and 6 families were selected as "low expression families" and analysed for fatty acid composition.

There were no clear differences in fatty acid compositions between high expression families and low expression families when all fish in both groups were analysed. There were 5 fish from each family analysed, 30 fish in total per selected group. These fish varied markedly in both size and fat content in the liver. We found that the EPA and DHA compositions in the high and low families were significantly negatively correlated with the fat content of the liver. We therefore decided to compare the EPA and DHA content after correction for the fat content. After exclusion of the fat content effect, the proportions of DHA and EPA tended to be higher in the families with the high expression of Δ6fad than in the families with low expression of Δ6fad, although not significant. It indicates that increased proportion of DHA and EPA may be a result of high expression of Δ6fad. However this result was not

significant and it is necessary with analyses of more families in order to conclude as to whether there is a connection between gene expression of Δ6fad and the EPA and DHA content in the fish. Several of the livers in our trial had a fat percentage between 15-20 %, which indicate development of fatty livers in some of the analysed families, which may have an impact of the result since more fatty livers were found in the high expression families than in the low expression families. Several factors prior to sampling of the fish may have caused the accumulation of fat in the livers. Today it is common with 70 % vegetable oil in fish diets and this may markedly influence the liver fat content. According to Ruyter et al. (2006) and Kjær et al. (2008), high levels of plant oils in the diet for Atlantic salmon lead to higher accumulation of fat in the liver than compared to the livers of fish fed a fish oil diet, resulting in decreased relative deposition of EPA and DHA in the liver. This may be caused mainly by a selective accumulation of 18:2 n-6 and 18:1 n-9. This agrees with Olsen and Skjervold (1995), showing that the percentage DHA and EPA in the fillet became gradually reduced with increasing fish age and fat content. In addition, they also found that body weight is another reason which can explain the variation found in n-3 fatty acids in farmed salmon. Therefore, we used regression analysis in order to find the correlation between DHA and EPA with body weight. We found that the percentages of DHA and EPA in the high desaturase families had a negative correlation with individual fish weight, increasing with decreasing weight. However, no correlation was found between individual weight of fish and percentages of DHA and EPA in the low expression families. High expression families compared with low expression families, in our study, have higher average body weight. The regression analysis result may indicate that individual body weight influence the DHA and EPA content in the high expression families.

The peroxisomal ACO is an important enzyme in the last steps in production of DHA (Ferdinandusse et al. 2001) and was therefore analysed in our

selected extreme groups. There were however, no significant differences in ACO activity and ACO gene expression between high expression families and low expression families. However, tendency of higher ACO activity and ACO gene expression were seen in the high expression families, indicating that high Δ6fad expression and higher tendency of ACO activity and ACO gene expression in high Δ6fad_b expression families may result in higher activity of the n-3 HUFA biosynthetic pathway, leading to increased production of DHA and EPA. The tendency towards higher DHA and EPA in the high Δ6fad_b expression families in our experiment is in agreement with this explanation. However as also stated above, analyses of more fish in more families is necessary in order to make a sound conclusion.

In conclusion this *in vivo* study indicates that families have different Δ6fad gene expressions which possibly influence the capacity to produce n-3 HUFA. This may open a way to increase DHA and EPA content in salmon by genetic selection.

5.2 In vitro study

Ruyter and Thomassen (1999) found that Atlantic salmon hepatocytes possess the ability to desaturate and elongate C18 n-3 fatty acids to the n-3 HUFAs. In our trial, we pre-incubated hepatocytes with OA and DHA and could then use hepatocytes as a model system to investigate how differences in endogenous level of OA and DHA influence the n-3 fatty acid metabolic pathway. OA and DHA were selected as representatives for vegetable oil and fish oil, respectively.

In this *in vitro* study, we found that pre-incubation of salmon hepatocytes with different ratios of OA and DHA prior to incubation with radio-labelled 18:3

n-3 had effect on the fatty acid composition in hepatocytes. Hepatocytes incubated with increased levels of OA had increased percentages of OA and its elongation product 20:1 n-9 in cellular lipids. The percentage of DHA in hepatocytes was also increased in cells where this fatty acid was supplemented in the cultivation media. Our results from fish hepatocytes agree with findings from previous *in vivo* and *in vitro* studies, which show that tissue fatty acid or hepatocytes compositions in salmon is influenced to a huge extent by the fatty acids supplemented to the diet or to the cell culture medias (Ruyter & Thomassen 1999; Ruyter et al. 2000b; Moya Falcon et al. 2004; Kjær et al. 2008). It was a further aim of our study to evaluate how different endogenous levels of DHA and OA influence n-3 fatty acid metabolism.

The composition radio-labelled fatty acids in the hepatocytes after incubation with [1-^{14}C] 18:3 n-3 was determined by HPLC separation of different fatty acids. The main radio-labelled products were the desaturation product 18:4 n-3 and the desaturation and elongation products EPA, DHA and 22:5 n-3.

Recovery of unmetabolised [1-^{14}C] 18:3 n-3 substrate in the cells tended to decreased with increased percentage of OA in the cells, indicating that more of the radio-labelled 18:3 n-3 substrate has been converted to other metabolic products in the groups incubated with OA than in those incubated with high levels of DHA. There were no significant differences in the level of [1-^{14}C] 18:4 n-3 produced in the hepatocytes of the various treatment groups, although the 100OA group tended to have the highest level of radio-labelled 18:4 n-3. This indicate that the rate of the first step in the conversion of 18:3 n-3 to DHA by Δ6 desaturation to form 18:4 n-3 was increased by increased proportion of OA and decreased proportion of DHA in the culture media. This result is consistent with Ruyter and Thomassen (1999). In addition, this result fit the expression of Δ6fad_b in our *in vitro* study that was stimulated by increased level of OA in the incubation medium.

There was further a significant higher production of radio-labelled 20:5 n-3 from [1-^{14}C] 18:3 n-3 with increasing OA in the cells. An explanation for this significant higher radio-labelled EPA in the OA groups was probably due to an increased Δ6fad_b activity. Elongation reactions are not usually considered as major control steps in fatty acid metabolism (Thomassen et al. 2012). Neither is Δ5fad regarded as the main rate limiting enzyme in the n-3 HUFA biosynthesis pathway in salmon (Hastings et al. 2004). Therefore, increased Δ6fad activity, as a main regulation enzyme of HUFA biosynthesis, may be the most important factor explaining the elevated EPA production in our trial, which agrees with the findings of Alimuddin et al. (2005).

Although there were no significant differences in percentage of radio-labelled DHA produced between cells incubated with OA and DHA, there was a tendency to higher production of radio-labelled DHA in the high OA groups than in the high DHA groups. This result is in agreement with a previous study where it was shown that vegetable oil can stimulate Δ6fad gene expression (Thomassen et al. 2012; Skrzypski et al. 2009; Vagner & Santigosa 2011).

The gene expression analysis of ACO and measurement of ACO enzyme activity show tendency to increase with increased DHA stimulation of hepatocytes. These results agree with previous findings in an *in vivo* study, showing both increased ACO enzyme activity and ACO gene expression when fed high DHA levels (Kjær et al. 2008). Also other earlier studies with Atlantic salmon are showing that hepatic *β*-oxidation activity is affected by the type of input fatty acid (Torstensen & Stubhaug 2004). According to Torstensen and Stubhaug, the total *β*-oxidation activity of [1-^{14}C] 18:3 n-3 was higher in hepatocytes stimulated with DHA than stimulated with OA. Therefore, OA probably reduce fatty acid *β*-oxidation leading to decreased energy production.

In summary, the results from this *in vitro* study indicates that pre-incubation of salmon hepatocytes with increasing ratios of OA may increase the EPA and DHA synthesis. The expression of Δ6fad_b in our *in vitro* study is stimulated by increased level of OA in the incubation medium.

Concluding remarks

The *in vivo* study indicates that families have different Δ6fad_b gene expression, which possibly influences the capacity to produce n-3 HUFA. When comparing high and low expression families, there were a tendency of higher DHA and EPA content in liver tissue in high gene expression families. This may indicate that it is possible to produce Atlantic salmon families with an enhanced capacity to produce DHA and EPA content through selective breeding of families with high Δ6fad gene expression. However, analyses of more families are necessary to certificate this connection.

The *in vitro* study indicates that increasing level of OA may increase the EPA and DHA synthesis in salmon hepatocytes. The expression of Δ6fad_b in our *in vitro* study is stimulated by increased level of OA in the incubation medium.

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