NUTRIGENOMIC STUDY OF LIPID METABOLISM IN ATLANTIC SALMON (*SALMO SALAR* L.)

- THE EFFECTS OF DIETARY PLANT OIL INCLUSIONS

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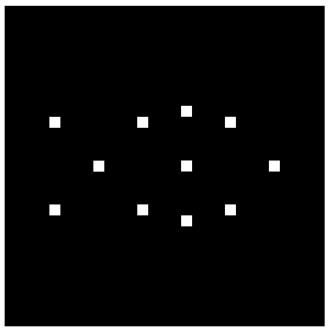
Dissertation for the degree philosophiae doctor (PhD)

at the University of Bergen

April 2006

“Every experiment proves something. If it doesn’t prove what you wanted it to prove, it proves something else”

Prof. Anon



NIFES

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Preface

The present work was conducted at NIFES during the years 2001 to 2005, and was financially supported by RAFOA ("Researching Alternatives to Fish Oils in Aquaculture", Q5RS-2000-30058 funded by EU, The Fifth Framework Programme). I would like thank Professor Øyvind Lie, the director at the Institute during this period, for being a co-supervisor at the end and for his encouragements during my stay at NIFES.

First of all I would like to thank my supervisors, Dr. Bente Torstensen and Dr. Ivar Hordvik, but also Dr. Gro Ingunn Hemre. This thesis could not have been finished without their support. Professor Øyvind Lie initiated the research project, and Dr. Bente Torstensen interest in molecular biology made me come here to explore the field of lipid nutrition research. A great thank to Dr. Ivar Hordvik which greatly contributed to the development of the molecular biology methods applied in this study. I would also thank Dr. Pål Olsvik, Kai Kristoffer Lie, Dr. Monica Sanden and Dr. Ernst Morten Hevrøy for "all those molecular discussions". A special thanks to Pål Olsvik for evaluating those reference genes and also for being a great guy! One can always come by your office.

The researchers and institute IMB, NRC, Halifax, Canada need a special thanks for their contribution. I had a fantastic stay with you, and I would very much like to thank Dr. Sue Douglas, Dr. Stephen Tsoi, Susanne Penny, Dr. Dominic Nanton and Dr. Santosh Lall for their help and hospitality during my stay at NRC.

Dr. Dominic Nanton and Dr. Ingunn Stubhaug, thank you for all those valuable discussions within the field of lipid nutrition. Eva Mykkeltvedt, thank you for all your contributing hours of methodological development, without you, this could not have been done!

I am grateful for PhD students and colleagues at NIFES for good companionship. A special thanks to Mari, Heidi and Arne as well as my room mates Anita and Monica. All you friends, and family, thank you for putting up with me during those frustrated days! Bjarte: thank you for being; a good husband, friend, father and a "molecular discussion" partner. Thank you to all of my friends who have helped me in getting out of the office and letting me forget the thesis. Siri, bless your memory! Signild and Therese, thank you for being a great inspiration at all times. All those not mentioned are thanked even more, you are all valuable! Special thanks go to Ingrid and Odd Arne, for all the help with Nikolai. You are great!

Nikolai, my son, now it is your time!

Bergen, 2006

Ann-Elise Olderbakk Jordal

Abbrevations

ACAT = acetyl CoA acetyltranferase
ACD = acyl-CoA dehydrogenase
ACS = acyl-CoA synthetase
AOX = acyl-CoA oxidase
CM = chylomicron
COX = cyclooxygenase
CPT I and II = carnitine palmitoyltransferase I and II
ECH = enoyl-CoA hydratase
FABP = fatty acid binding protein
FAS = fatty acid synthetase
HDL = high density lipoprotein;
HNF-1/4 = hepatic nuclear factor-1/4
HSL = hormone sensitive lipase
ILBP = intracellular lipid binding protein
LCAT = Lecithin cholesterol acyltransferase
LCFA = long chained fatty acids (Gene Ontology Browser- carbon chain length: C_{12}-C_{18}) (in this thesis especially 18:1n-9, 18:3n-3, 18:2n-6 and 16:0)
LDL = low density lipoprotein
LPL = lipoprotein lipase
LXR = liver X receptor
NF-κβ = nuclear factor kappa-β
PC/E/I = phosphatidyl choline/ethanolamine/inositole
Protein kinase C = PKC
PLC = phospholipase C
PPAR = peroxisome proliferator activated receptor
PPRE = peroxisome proliferator response element
Q-PCR = quantitative RT- PCR
RXR = retinoid X receptor
SAM = Significance Analysis of Microarrays
SCD = Δ9 fatty acid desaturase
SREBP = sterol regulatory element binding protein
TAG = triacylglycerol
VLCFA = very long chain fatty acids; all acids with 20 carbons or more (in this thesis especially 20:5n-3 (EPA), 22:6n-3 (DHA), and 20:4n-6 (ARA))
VLDL = very low density lipoprotein.
6PF-2-K/Fru-2,6-P(2)ase = 6 phosphofructo 2 kinase/fructose 2,6 biphosphatase

Abstract

The two dietary trials conducted in the present study (Papers I-III) used dietary rapeseed oil fed in a regression design and a full plant oil blend replacement for fish oil, respectively. The metabolic response of Atlantic salmon was examined in relation to gene expression (Paper I-III), lipid storage (Paper II and III) and fatty acid oxidation (Paper II). Furthermore, intracellular fatty acid uptake and transport (Paper II) and lipoprotein metabolism (Paper III) was studied. In order to examine gene expression of lipid metabolic genes thorough technical validation and quality control of microarray studies (Paper I) and evaluation of reference genes for Q-PCR were prioritised (Paper IV).

Dietary rapeseed oil induced Δ5 fatty acid desaturase mRNA expression (Paper I), and lipogenic enzyme activities were partially increased for Atlantic salmon fed high dietary inclusion of plant oils (Paper III). Dietary long term partial rapeseed oil inclusion resulted in reduced expression of several mitochondrial transport proteins, transcription factors, co-activators and signal transducers (Paper I). The expression of these proteins is known to be indirectly influenced by dietary fatty acids mediated through changes in membranes phospholipids compositions.

Partial dietary rapeseed oil inclusion had no impact on FABP3 or FABP10 gene expression in Atlantic salmon liver or muscle tissues (Paper II). Nonetheless, a tendency for decreased FABP3 protein expression with decreasing inclusion of dietary rapeseed oil was observed. Overall, liver and muscle tissues of Atlantic salmon seem to express several FABPs, possibly linked to different metabolic functions. Relative FABP3 mRNA levels dominated in both red and white muscle tissues. Red muscle appeared to express higher levels of FABP3 than white muscle and heart. Liver FABP10 mRNA appeared to be expressed at high levels compared to liver FABP3. Modest changes in liver and muscle FABPs mRNA levels between different life stages were observed.

Liver TAG stores, plasma lipid and LDL levels were significantly affected by dietary plant oil replacement in Atlantic salmon during a long term feeding experiment (Paper III). Current results indicate that high dietary plant oil inclusion increase liver TAG stores and decrease plasma lipid levels possibly through decreased VLDL synthesis. The expression of liver PPARγ increased prior to seawater transfer followed by a decrease, and then another increase towards the final sampling (22 months) which was correlated with increased liver TAG stores.

mRNA expression of Atlantic salmon apolipoproteins seem to be regulated by dietary fatty acids (Paper I). Yet complex post translational mechanisms for lipoprotein assembly are believed to occur in Atlantic salmon as in mammals (Paper I and III).

Through thorough evaluation of potential systemic and technical variation (Paper I and IV), the experimental design chosen enabled us to measure dietary and lifecycle gene expression variations in a system showing extrinsic and intrinsic variability (Paper I- III).

List of papers

Paper I **Ann-Elise O. Jordal, Bente E. Torstensen, Stephen Tsoi, Douglas R. Tocher, Santosh P. Lall, Susan E. Douglas** (2005) Dietary rapeseed oil affects the expression of genes involved in hepatic lipid metabolism in Atlantic Salmon (*Salmo salar* L.). Journal of Nutrition, 135: 2355-2361

Paper II **Ann-Elise O. Jordal, Ivar Hordvik, Maurice Pelsers, David Bernlohr, Bente E. Torstensen** FABP3 and FABP10 in Atlantic salmon (*Salmo salar* L.), – general effects of dietary fatty acid composition, and life cycle variations. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology (in press)

Paper III **Ann-Elise O. Jordal, Øyvind Lie and Bente E. Torstensen** Complete replacement of dietary fish oil with a vegetable oil blend affect liver lipid and plasma lipoprotein levels in Atlantic salmon (*Salmo salar* L.)
Aquaculture Nutrition (submitted)

Paper IV **Pål A. Olsvik, Kai K. Lie, Ann-Elise O. Jordal, Tom Ole Nilsen, Ivar Hordvik** (2005) Evaluation of potential reference genes in real- time RT-PCR studies of Atlantic salmon BMC Molecular Biology 2005, 6:21 17 November 2005

In the following these four papers are referred to in the text by their roman numerals. In the case where a paper has yet not been published, the style of the manuscript is that required by the Journal.

Contents

1. Introduction

The long standing interest of fish lipids is due mainly to the fact that fish lipids are rich in n-3 very long chained fatty acids (VLCFA) which have particularly important roles in fish and human nutrition (Tocher, 2003).

At present, 30 % of globally available fish oils, a variable resource, is used for breeding of cultivated fish species (Waagbo et al, 2001). Waagbo and co-workers (2001) estimated, based on available fish oil resources and fish oil consumption in aquaculture, a lack of fish oils for feed production by 2005. Consequently, sustainable alternatives must be found, that do not compromise fish health and product quality. This implies that the choice of dietary oils should meet the fatty acid requirements in salmonids, in order to prevent negative effects on fish health. The requirements for salmonids are 1% of the total dietary energy for both n-3 and n-6 fatty acids (NRC, 1993; Sargent et al., 1995). In order to maintain high product quality and optimise the beneficial health effects for human consumption, the alternative diet should maintain a relatively high level of n-3 VLCFAs for storage in Atlantic salmon flesh. As these VLCFAs have been shown to have beneficial effects on cardiac diseases, levels of triacylglycerol (TAG) in plasma, blood pressure and inflammatory responses (Kris-Etherton et al., 2003).

The strongest candidates to fish oil for dietary inclusion in Atlantic salmon diets are plant oils. In general, plant oils do not contain VLCFA, but high levels of long chained fatty acids (LCFAs) especially of 18 carbons length. Plant oils also tend to have higher levels of n-6 LCFAs compared to marine oils. Marine oils are known for their high levels of polyunsaturated VLCFAs of the n-3 and n-6 family such as 20:5n-3 and 22:6n-3 and 20:4n-6, as well as monounsaturated VLCFAs, such as 20:1n-11 and 22:1n-11.

In general, Atlantic salmon primarily use proteins and lipids as energy sources with 10-20 % of the energy produced originating from lipids (Van den Thillart, 1986). The major fate of lipids in fish is for the storage, and provision of metabolic energy provided through β-oxidation of fatty acids (Frøyland et al., 2000; Sargent et al., 1989; Stubhaug et al, 2005a). Furthermore, cellular metabolic responses are believed to be highly dependent on selective uptake and transport of fatty acids, and it is important to study whether these mechanisms are affected by dietary oil replacements. Finally, dietary polyunsaturated VLCFAs of the n-3 and n-6 family, present in fish oil diets, will also be distributed to virtually every cell in the body with effects on membrane composition and function, eicosanoid synthesis, cell signalling and regulation of gene expression (Jump, 2002a). Consequently, the lack of dietary VLCFAs may mediate effects on these processes in fish fed the 100% plant oil diets. In general

different cells respond differently to changes in dietary VLCFA composition as determined by their cell-specific lipid metabolism and expression of fatty acid-regulated transcription factors

The recent generation of Atlantic salmon genomic data has helped to increase our knowledge of this species biology, and makes nutrigenomic surveys possible. Nutrigenomic studies in aquaculture are a new and growing field of science. These studies will enable us to understand how different dietary fatty acids or their metabolites mediate regulation of gene expression through direct or indirect mechanisms. When examining the potential effects through different life stages one may possibly understand more on how different important lipid metabolic regulators and transport proteins mediate specific metabolic responses to alternative dietary lipids.

2. Aims of the study

- to screen how dietary fatty acids influence the expression of genes involved in the liver lipid metabolism of Atlantic salmon (Papers I, II and III)

- to measure the effects of dietary fatty acid composition, and life stage variations on the expression of fatty acid transport proteins FABP3 and FABP10 in metabolic active tissues of Atlantic salmon (Paper II)

- to measure the effects of dietary fatty acid composition and life stage variations on PPARγ in liver of Atlantic salmon (Paper III)

- to study the transport of lipids and lipoprotein metabolism when fed different dietary fatty acids (Paper III)

- to evaluate recently established methods used for nutrigenomic studies in Atlantic salmon (Papers I and IV: small scale microarray and reference genes for use in Q-PCR)

3. Background

3.1 Lipid metabolism in Atlantic salmon

Lipid homeostasis can be defined as the balance between lipid uptake, transport, storage, biosynthesis, metabolism and catabolism (Tocher, 2003).

3.1.1 Extracellular transport of dietary lipids

The transport of lipids and other lipid soluble components from the intestine to peripheral tissues is predominantly mediated by lipoproteins (Figure 1). Chylomicrons (CM) and very-low density lipoproteins (VLDL) are synthesised in the intestines and transport re-esterified TAG, polar lipid, cholesterol and sterol esters to various tissues (Babin and Vernier, 1989). CMs and VLDL may transport lipids from the intestine to liver, either through the hepatic vein or through the lymph system, as it is not established whether fish has a lymph system or not (Henderson and Tocher, 1987; Babin and Vernier, 1989; Press and Evensen, 1999). In order to facilitate fatty acid uptake, all lipoproteins, except low density lipoprotein (LDL) which is taken up by endocytosis (Gjoen and Berg, 1993), binds to specific receptor on the cell membrane (Schneider, 2002). Fatty acids are then hydrolysed from TAG and taken up by the cell through the action of lipoprotein lipase (LPL). LPL is expressed in numerous tissues of rainbow trout (*Oncorhynchus mykiss*) (Babin and Vernier, 1989). VLDL synthesised in liver transports endogenously synthesised TAG to extra hepatic tissues (Jonas, 2002). Intermediate density proteins (IDL) and LDL arise from metabolic transformation of VLDL in circulation. LDL is the transporter of cholesterol from liver to peripheral tissues (Babin and Vernier, 1989). Whereas nascent high density lipoprotein (HDL), synthesised in liver and intestine, remove excess cholesterol from cells and transport it to liver. HDL is the most common lipoprotein in salmonids (Babin and Vernier, 1989; Lie et al., 1993; Torstensen et al., 2001), and contains high levels of the fatty acid 22:6n-3 in the phosphatidyl choline (PC) and neutral lipid fraction (Lie et al., 1993).

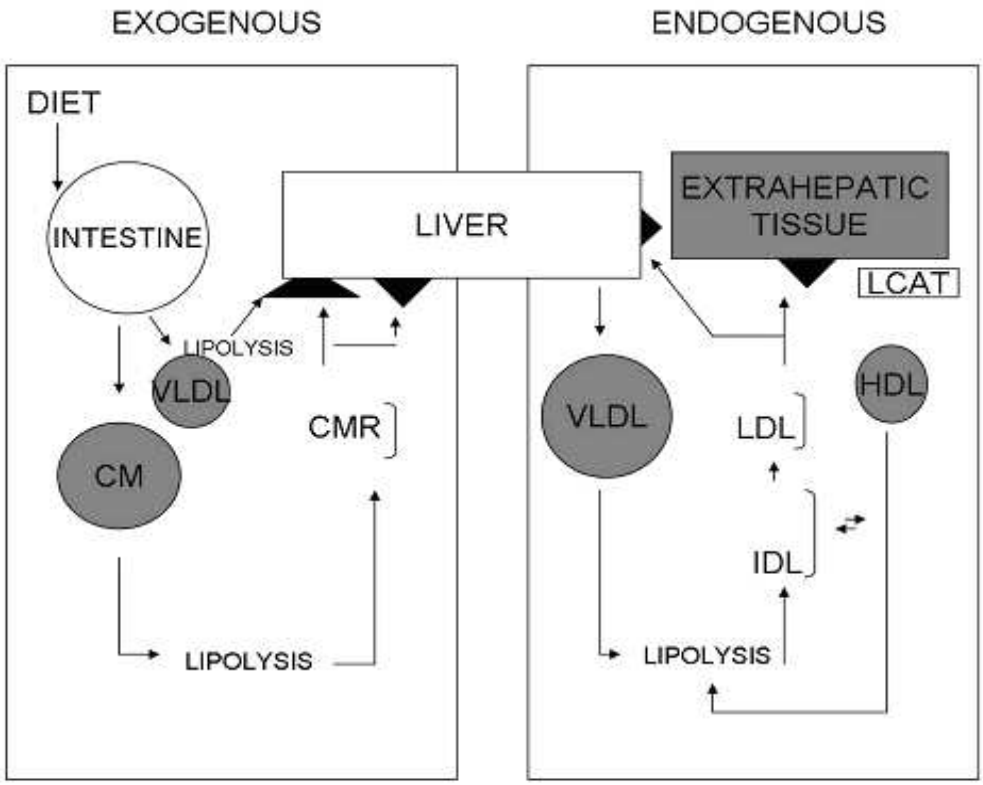

Figure 1. Schematic overview of extracellular transport of lipids.

The figure was modified from (Schneider, 2002). See text for further explanations.

The fatty acid composition of dietary lipid influence the fatty acid composition of PC, the major phospholipids, and the neutral lipid fraction in VLDL, LDL and HDL (Lie et al., 1993). Neutral lipids were clearly more influenced by diet than PC. Overall, VLDL, LDL, and HDL fatty acid compositions have been shown to be decreasingly affected by dietary fatty acid composition (Torstensen et al., 2000; Torstensen et al., 2001). As also seen when Atlantic salmon were fed dietary rapeseed oil, HDL was high in 22:6n-3 irrespective of diet, whereas VLDL was highly influenced by dietary fatty acid composition (Torstensen et al., 2004).

Rainbow trout VLDL are mainly composed by apolipoprotein (apo) B, but also contain apo A-II, and apo Cs (Babin and Vernier, 1989). LDL contains apo B, whereas the main apolipoprotein in HDL is apo A-I, followed by A-II. LPL is activated in the presence of HDL, suggesting that Apo C-II, the activating co-factor of LPL, is present in HDL as in VLDL. Atlantic salmon liver and muscle express apo A-I precursor (Powell et al., 1991) and its expression was suggested in small intestine (Zannis et al., 1983). The presence of apo A-I in HDL activates lechitin cholesterol acyltransferase (LCAT), an enzyme involved in cholesterol metabolism (Fielding et al., 1972).

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3.1.2 Organ specific responses to dietary fatty acids

The major fate of dietary fatty acids in fish is for the storage, and provision of metabolic energy in the form of ATP provided through β-oxidation of fatty acids (Frøyland et al., 2000; Sargent et al., 1989; Stubhaug et al., 2005a). Red muscle has the highest capacity for total β-oxidation, followed by liver and white muscle (Frøyland et al., 1998; Frøyland et al., 2000; Stubhaug et al., 2005a). Red muscle store more lipids within myoseptas (adipocytes) than white muscle (Zhou et al., 1995). Recently, intracellular lipid droplets were visualized, using light microscopy, in close association with mitochondria within both Atlantic salmon muscle tissues (Nanton et al., 2005). Lipid levels in these tissues were not significantly different between fish fed fish oil and plant oil diets.

Lipid droplets were observed in the intestine and liver of rainbow trout (Caballero et al., 2002) and Atlantic salmon (Ruyter et al., 2006). Lipid droplet formation in liver of salmonids was indicated as being higher when given dietary plant oil instead of fish oil. Nevertheless, the liver has a higher polar lipid /neutral lipid ratio compared to white and red muscle in rainbow trout (Weber et al., 2003) and Atlantic salmon (Torstensen et al., 2004).

In general, fatty acid specificity in uptake and intracellular transport as well as elongation and desaturation (Bell et al., 2001; Bell et al., 2002) and β-oxidation (Frøyland et al., 2000; Torstensen et al., 2000) is believed to affect the fatty acid composition of membranes and deposit lipids within cells.

N-3 VLCFAs, either supplied through diet, or endogenously synthesised, are then likely to affect intracellular non-esterifed fatty acids or their CoA thioester levels, which in turn affects transcription and signal transduction factors that will have impact on several regulatory mechanisms (Jump, 2002a).

3.1.3 Liver lipogenesis and VLDL assembly

Liver is quantitatively the principal site of lipogenesis, the biosynthetic reactions for the formation of new endogenous lipid (Tocher, 2003). The pathway of lipogenesis is similar to those in other vertebrates (Sargent et al., 1989). The ultimate carbon source for biosynthesis of new lipids is acetyl-CoA formed in mitochondria. The key pathway in lipogenesis is catalysed by cytocolic fatty acid synthethase (FAS). This enzyme complex, responsible for producing fatty acids *de novo*, was first purified from the liver of plaice (*Pleuronectes platessa*) (Wilson and Williamson, 1970). All four cytosolic dehydrogenases which generate NADPH for fatty acid biosynthesis has been shown to be active in livers of rainbow trout (Baldwin and Reed, 1976) coho salmon (*Oncorhynchus kisutch*) (Lin et al., 1977a and b) and Atlantic salmon (Arnesen et al., 1993; Sanden et al., 2003; Torstensen et al., 2004).

Atlantic salmon fed diets containing 100% rapeseed oil show significantly decreased glucose-6-phosphate dehydrogenase (G6PDH) and increased NADH- isocitrate dehydrogenase (ICDH) activity compared with fish fed fish oil (Torstensen et al., 2004). In rainbow trout, low levels of 18:3n-3 exerted a stimulatory effect on all the lipogenic enzymes assayed with the exception of FAS, and increased amounts of the same LCFA showed some inhibition of lipogenic activities (Alvarez et al., 2000). 20:5n-3 and 22:6n-3 showed a similar effect, although the former strongly inhibited FAS activity while the latter showed greater potential to inhibit acyl-CoA carboxylase (ACC) and G6PDH.

Fish are capable of modifying both dietary fatty acids and the fatty acid products of exogenous synthesis by desaturation and elongation (Sargent et al., 1989). Desaturation of fatty acids in fish takes place in the endoplasmatic reticulum of cells of certain tissues and is catalysed by multicomponent systems comprising NAD(P)H cytochrome b_5 reductase, cytochrome b_5 and terminal fatty acid desaturase enzymes (Brenner, 1974). The essential LCFAs, 18:2n-6 and 18:3n-3 can be desaturated, by Δ5 and Δ6 fatty acid desaturase, and elongated by elongase, to form physiologically essential 20 and 22-C VLCFAs, 20:4n-6, 20:5n-3 and 22:6n-3 (Figure 2). Atlantic salmon fed diets containing plant oils show significantly increased desaturation and elongation activity compared with fish fed fish oil (Bell, 1997; Tocher et al., 1997; Tocher et al., 2000; Bell et al., 2001; Tocher et al., 2001; Bell et al., 2002; Zheng et al., 2005a). Recently, the clones of Atlantic salmon Δ6 fatty acid desaturase (Zheng et al., 2005b), Δ5 fatty acid desaturase and elongase (Hastings et al., 2004) were functionally characterised. Lower amounts of desaturation and elongation products in primary hepatocytes from Atlantic salmon fed 75% plant oil instead of fish oil may indicate lower Δ9 fatty acid desaturase (SCD) activity (Stubhaug et al., 2005b).

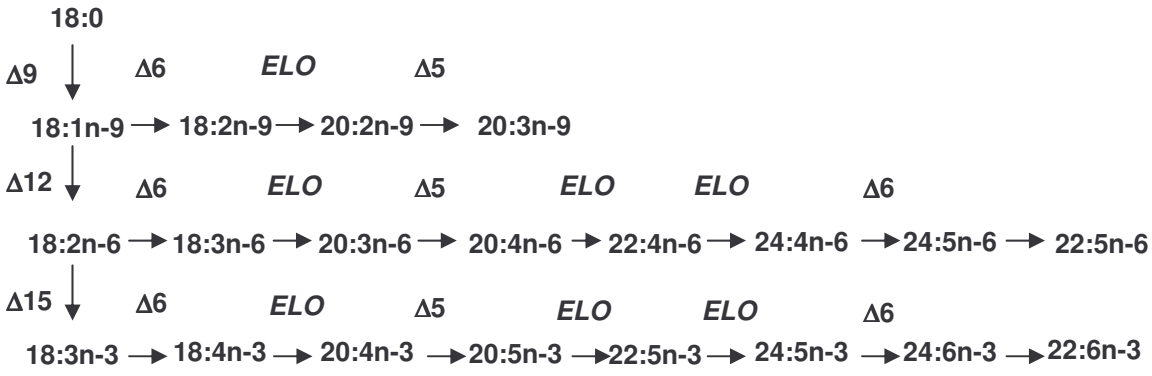

Figure 2. Desaturation and elongation pathways for fatty acids. The figure was modified from (Sprecher, 1992). See text for further explanations. 22:5n-6 and 22:6n-3 is made from shortening of 24:5n-6 and 24:6n-3 respectively through peroxisomal β-oxidation.

In total, liver lipid biosynthesis which leads to VLDL assembly, include *de novo* fatty acid synthesis, elongation and desaturation, as well as TAG, phospholipids and cholesterol ester synthesis (Shorten

and Upreti, 2005). TAG synthesis is said to be the rate limiting step in lipoprotein biosynthesis (Bostrom et al. 1988). Most dietary fatty acids enter the TAG storage pool before emerging as VLDL in hepatocytes of mammals (Gibbons et al., 2000). *In vitro* studies have shown that primary hepatocytes from Atlantic salmon incubated with 20:5n-3 accumulated significantly more cellular lipid than cells treated with 18:1 n-9 and 22:6n-3 (Vegusdal et al., 2005). Further, *in vivo* studies reports a significantly higher accumulation of lipid in the liver of Atlantic salmon fed 100% sunflower oil compared with 100% fish oil diets (Ruyter et al., 2006) as also seen for red drum (Tucker et al., 1997) and rainbow trout (Caballero et al., 2002).

3.1.4 Phospholipids and membrane function

Apart from being components in VLDL, phospholipids are components of cell membranes (Shorten and Upreti, 2005). Where they function in regulation of uptake of molecules, impart rigidy in the membrane as well as they play a role in cell recognition and signalling. Overall, the properties of phospholipids are greatly affected by their fatty acid composition. PC account for approximately 50% of the phospholipids synthesised in hepatocytes (Stubhaug et al., 2005b). PC is the main component in liver cellular membranes, and is dominated by the fatty acids 16:0, 20:5n-3 and 22:6n-3 in fish (Bell and Dick, 1991). Phosphatidyl inositols (PI) are rich in 20:4n-6 and 20:5n-3 in fish and mammals (Irvine, 1982; Bell and Dick, 1990), and are thus believed to be involved in eicosanoid metabolism in fish (Bell et al., 1986; Bell et al., 1994). The synthesis of prostaglandins by cyclooxygenase (COX) is well characterised (Smith and DeWitt, 1996). In rainbow trout mRNA from both the constitutively expressed, COX-1, and inducible form, COX-2, has been cloned (Zou et al. 1999).

Plant oil diets have been shown to affect membrane composition in kidney in rainbow trout (Hvattum et al., 2000) and in the liver and intestine of Atlantic salmon (Ruyter et al., 2006). In the latter study, the percentages of 20:5n-3 and 22:6n-3 were considerably lower, while the percentages of 20: 4n-6 and 20:4n-3 was higher in fish fed plant oil compared with fish oil diets.

3.1.5 Fatty acid catabolism

Fatty acid catabolism or β-oxidation is a cellular process believed to be fuelled mainly by extracellular fatty acids (Gibbons et al., 2000). The β-oxidation of fatty acids occur in mitochondria and peroxisomes (Mannaerts et al., 1979; Foerster et al., 1981; Reddy and Mannaerts, 1994) and the reactions are believed to be the same for fish as for mammals (Henderson, 1996) (Figure 3). The rate limiting step for peroxisomal β-oxidation is thought to be acyl-CoA oxidase (AOX) (Inestrosa et al., 1979), an enzyme with high activity in Atlantic salmon (Frøyland et al., 1998). The key point in regulation of mitochondrial β-oxidation is thought to be the inhibition of carnitine palmitoyltransferase- I (CPT-I) by malonyl-CoA, the product of the rate limiting enzyme in lipogenesis, ACC, both in mammals and Atlantic salmon (McGarry et al., 1978; McGarry and Foster,

1979; McGarry and Foster, 1980; Frøyland et al., 1998). CPT-I, localised at the outer mitochondrial membrane, catalyse acyl-CoA to acyl-carnitine transfer (Murthy and Pande, 1987) before the acyl carnitine complex cross the inner mitochondrial membrane via the carnitine: acetylcarnitine translocase (Pande, 1975). Finally, in the mitochondrial matrix, CPT-II, located at the inner mitochondrial membrane, catalyses the transfer of acyl residues from carnitine to CoA to form acyl-CoA thioesters which then enter the β-oxidation spiral (Murthy and Pande, 1987). Atlantic salmon CPT-II activity dominated in red muscle, whereas in the liver and white muscle CPT-I and CPT-II activities were similar (Frøyland et al., 1998). Furthermore, liver but not white muscle CPT-I and CPT-II activities were affected by dietary treatments with rapeseed oil in juvenile brown trout (*Salmo trutta*) (Turchini et al., 2003). A partial cDNA sequence for CPT-I was cloned and its messenger RNA (mRNA) expression in liver, white and red skeletal muscles, heart, intestine, kidney and adipose tissue of trout has been characterised (Gutieres et al., 2003).

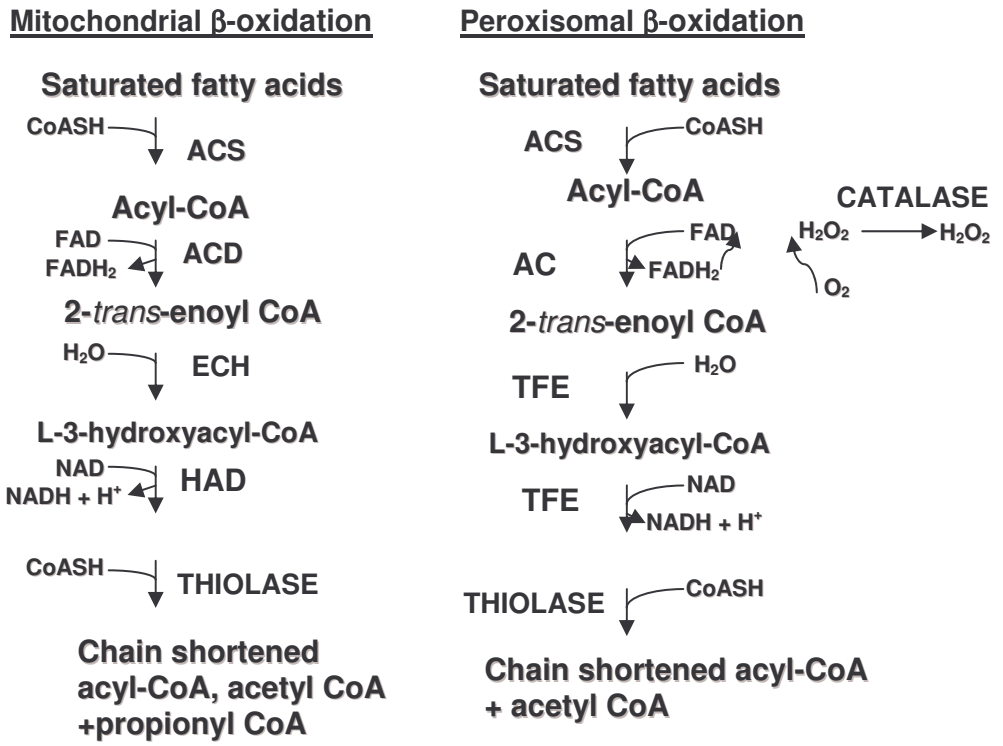

Figure 3. Schematic overview of the β-oxidation reactions, which occur within mitochondria and peroxisomes.

Acetyl –CoA synthethase (ASC) catalyse the first step, where acyl-CoA is attached to the saturated fatty acids. In mitochondria acyl-CoA dehydrogenase (ACD) ensures production of 2-trans enoyl-CoA whereas the process is catalysed by AOX in peroxisomes. In mitochondria, 2-trans enoyl-CoA hydratase (ECH) catalyses the formation of L-3 hydroxyacyl-CoA, whereas trifunctional enzyme (TFE) perform the same catalytically step in peroxisomes. Peroxisomal TFE also catalyse the formation of 3-ketoacyl-CoA. 3-hydroxyacyl-CoA dehydrogenase (HAD) catalysed the same reaction in mitochondria. Finally, thiolase catalyses the formation of chain shortened acyl-CoA and acetyl-CoA in peroxisomes and mitochondria. In general, oxidation of unsaturated fatty acids includes several enzymes which participate in removal or moval of double bonds.

3.2 Uptake and intracellular transport of fatty acids

3.2.1 Models for cellular fatty acid uptake

Whether the nature of cellular fatty acid uptake is active or passive is still debated (Berk and Stump, 1999; Hajri and Abumrad, 2002). When fatty acids are transported over cellular membranes through passive transport, they may be protonated at the extracellular side which permit the fatty acid to integrate in to the phospholipid bilayer and flip flop across the cell membrane (Hamilton and Kamp, 1999). The hypothesis that fatty acids traversed the membrane by facilitated transport was recently tested for rainbow trout, with results suggesting that the uptake of LCFA were protein mediated, at least in white and red muscle (Richards et al., 2004).

There are several models for protein mediated cellular fatty acid uptake and transport in mammals (Figure 4) (Koonen et al., 2005). Fatty acid binding proteins (FABPs) are believed to enhance uptake of fatty acids in to cells by increasing their concentration gradient, in order to minimise unbound fatty acids in the cells (Schaap et al., 2002). Intracellular mammalian FABP3 and FABP4 [1] types are membrane active, meaning that they exchange fatty acids with membranes via collisional transfer (Storch et al., 2002). FABP1 however exchanges fatty acids by diffusional transfer, and do not interact with membrane (Thumser and Storch, 2000) (Figure 4, arrow 1). The interaction of fatty acid translocase/CD36 (FAT/CD36) and plasma membrane fatty acid binding protein ($FABP_{pm}$) in protein-mediated LCFA transport accelerates the dissociation of LCFA from albumin (Figure 4, arrow 2), and then delivers fatty acids to the intracellular FABPs. FAT/CD36 have also been proposed to interact with fatty acid transport protein (FATP) in mediating LCFA transport (Stahl et al., 2001) (Figure 4, arrow 3), and to mediate mitochondrial acyl-CoA uptake (Campbell et al., 2004). FATP may also transfer LCFA directly across the plasma membrane (Figure 4, arrow 4). The first identified FATP family member, FATP1 (Schaffer and Lodish, 1994) exhibited intrinsic acyl-CoA synthetase activity (ACS) with a broad specificity for both LCFA and VLCFA (Coe et al., 1999b; Herrmann et al., 2001; Hatch et al., 2002; Hall et al., 2003). The FATP produced acyl-CoA is then transported intracellulary with acyl-CoA-binding protein (ACBP). ACBP have been shown to transport fatty acid-CoAs to the nucleus (Helledie et al., 2002; Petrescu et al., 2003) and are suggested as a transport protein for TAG synthesis (Kannan et al., 2003).

Recent knockout studies in mice (*Mus musculus*) gave no gross phenotypical changes when deleting particular FABP genes (Haunerland and Spener, 2004) or FATP or FAT/CD36 genes

[1] The various FABPs will be referred to only by their gene name as introduced by Hertzel and Bernlohr (2000) to avoid confusion based on the initial terminology which named FABPs after the tissue where they were first were found. FABP3 is used for heart and muscle FABPs, FABP4 for adipocyte FABP, and FABP1 and 10 for liver FABPs in mammals and fish respectively.

(Koonen et al., 2005) indicating that several of these proteins may participate in mediating fatty acid uptake and transport within cells.

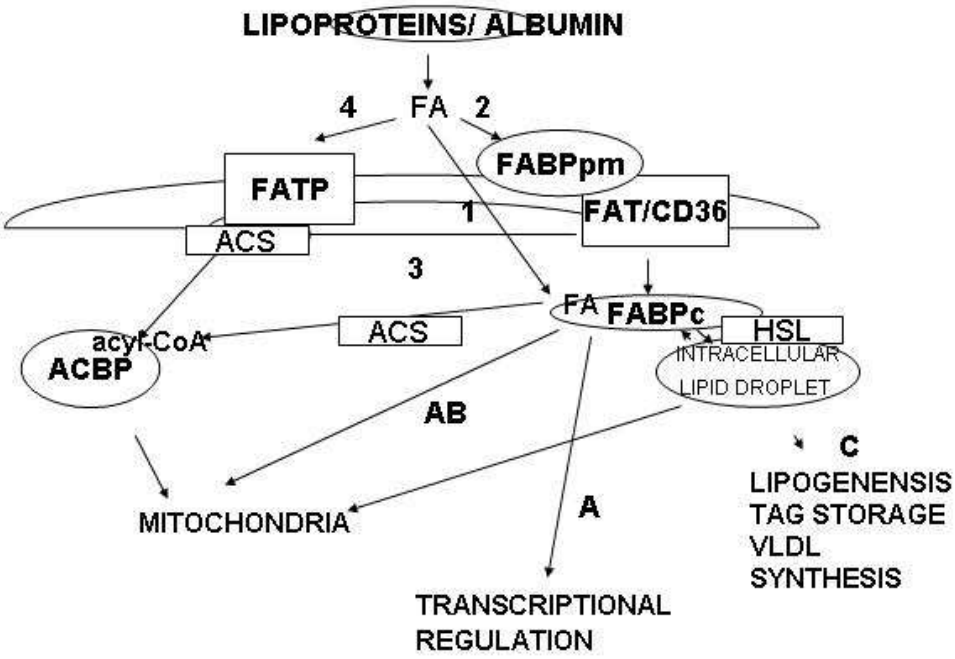

Figure 4. A schematic overview over current models for cellular fatty acid uptake and transport.

The figure was modified from (Koonen et al., 2005). The numbers 1–4 illustrate the proposed routes of long chained fatty acid (LCFA) uptake. See text for further explanations. The letter A indicates results from knock-out and *in vitro* studies for the FABP1 form, whereas the letter B and C indicate functional implications from knock out studies for FABP3 and FABP4, respectively.

3.2.2 Fatty acid binding proteins

Functionally, FABPs are believed to be involved in lipid transport and metabolism (Veerkamp and Maatman, 1995; Veerkamp et al., 1991; Spener et al., 1989; Ockner et al., 1972). The control of tissue specific expression of FABPs is poorly understood, but it often reflects the tissues lipid metabolising capacity (Haunerland and Spener, 2004). Structurally, FABPs encode ~15 kDa proteins of the common tertiary fold forming a β-barrel (Hertzel and Bernlohr, 2000). The β-barrel is formed by two orthogonal five stranded β-sheets, and create an internal water filled cavity with ~50% polar amino acids, for the internalisation of hydrophobic ligands as fatty acids. All FABPs bind LCFAs and VLCFAs, but differences in ligand selectivity, binding affinity and binding mechanism exist (Hanhoff et al., 2002) rooted in subtle structural differences between the four subfamilies of intracellular lipid binding proteins (iLBPs) (Table 1).

Table 1. FABP characterisation in to subfamilies and their tissue specific expression in mammals and several fish species. ILBPs which have not been described in fish were left out form this table. These are fabp6, fabp8 and fabp9. For more details see paper II.

iLBP type	Gene name	Expression in mammalian cells [1]	Ligands	Fish species	Tissue specific expression
Subfamily I					
CRBP I-IV		Ubiquitous in mammalian cells	Retinol	Tuna[2] Zebrafish [3]	Eye Intestine, liver (crbp1 and 2) Brain, ovary, testis (crbp 1)
CRABP I and II		Ubiquitous in mammalian cells	Retinoic acids	Japanese pufferfish [4] Zebrafish[5]	Ubiquitous (CRABP1) Muscle, testes and skin, heart, ovary, brain.
Subfamily II					
L-FABP	fabp1	Liver, intestine, kidney, lung and pancreas	LCFAs, acyl CoA, home	Catfish[6]	Intestine
Lb-FABP	fabp10	Fish and bird and amphibian liver, and bird skeletal muscle	LCFAs	For more details, see Table 2	
Subfamily III					
I-FABP	fabp2	Intestine and liver	LCFAs	Zebrafish[7]	Intestine, brain, liver, muscle, testis, heart, skin ovary
Subfamily IV					
H-FABP	fabp3	Heart, skeletal muscle, brain, kidney, lung, mammary, placenta, testis ovary, stomach	LCFAs	Antarctic icefish[8] Shark[9] Mummichog[10] Lamprey[11] Zebrafish [12] Rainbow trout [13]	White and red muscle, heart, kidney, brain Liver Liver, gills, gonads Liver Ovary, liver, heart, muscle brain Heart
A-FABP	fabp4	Adipose tissues, macrophages, liver	LCFAs	Antarctic icefish[8]	Spleen, white and red muscle, heart, kidney, brain
E-FABP	fabp5	Skin, adipose tissue, lung, brain, heart, skeletal muscle, testis, retina kidney, liver	LCFAs	Catfish [14]	Liver
B-FABP	fabp7	Brain, glia cells and retina	LCFAs, 22:6n-3	Zebrafish [15]	Brain, liver, testis, intestine, heart, muscle

[1](Hertzel and Bernlohr, 2000) [2] (Nishiwaki et al. 1990), [3] (Liu et al. 2004b; Liu et al. 2005) [4] (Kleinjan et al. 1998) [5] (Sharma et al. 2003) [6] (Di Pietro et al.1997) [7] (Pierce et al. 2000; Sharma et al. 2004) [8] (Vayda et al., 1998) [9](Medzihradszky et al., 1992) [10] (Bain, 2002) [11] (Baba et al., 1999) [12] (Liu et al., 2003a) [13] (Ando et al., 1998) [14] (Di Pietro and Santome, 1996) [15] (Liu et al., 2004a; Liu et al. 2003b)

3.2.2.1 Fatty acid binding proteins of subfamily I

FABP1 and FABP10 are believed to be paralogue genes (Di Pietro et al., 1997; Di Pietro et al., 1999). FABP1 and FABP10 originated by ancient gene duplication, before the fish tetrapod divergence, approximately 694 million years ago. Functionally, FABP1 was suggested, through *in vitro* studies, as a signalling molecule conveying messages to the nucleus, thus being the co-activator of peroxisome proliferator activated receptor (PPAR) (Wolfrum et al., 2001) (Figure 4). Knock out studies in mice indicate that FABP1 is involved in β-oxidation, which results in less TAG storage (Newberry et al., 2003; Erol et al., 2004). Recently, a physiological role for FABP1 in influencing liver bile acid metabolic phenotype was suggested through knock out studies in mice (Martin et al., 2005).

Tissue specific expression of FABP10 mRNA has been determined for zebrafish (*Brachydanio rerio*) (Denovan-Wright et al., 2000) and Tsaiya duck (*Anas platyrhynchos*) (Ko et al., 2004). FABP10 expression was shown in skeletal muscle (Ko et al., 2004) and liver (Denovan-Wright et al., 2000; Ko et al., 2004). Higher liver FABP10 gene expression was observed in egg-laying Tsaiya ducks than in the prelaying ducks indicating a possible *in vivo* function in fatty acid uptake for liver lipid metabolism. No specific function has been suggested for fish FABP10 so far. However, chicken (*Gallus gallus*) FABP10, which bind the same ligands and exhibit similar relative affinity for fatty acids as catfish FABP10 (Beringhelli et al., 2001), was suggested as a bile acid binding protein based on crystal structures and amino acid alignments (Nichesola et al., 2004; Nolan et al., 2005).

The binding cavity of FABP1 allows binding of two fatty acid molecules in opposite orientation (Thompson et al., 1997). As for FABP1, *in vitro* binding studies using isolated and characterised FABP10 from argentine toad (*Bufo arenarum*) (Di Pietro et al., 2001; Di Pietro et al., 2003) and lungfish (*Lepidosiren paradoxus*) (Di Pietro and Santome, 2001) has confirmed a broad binding specificity (Table 2). Lungfish FABP10 binds two molecules in the same orientation, whereas catfish (*Rhamdia sapo*) FABP10 only binds one fatty acid (Di Pietro et al., 1997). Lungfish FABP10 preferentially binds large ligands, but has higher affinity for mono and polyunsaturated fatty acids than saturated fatty acids. FABP1 preferably binds monounsaturated and n-3 fatty acids (Hanhoff et al., 2002). High FABP10 binding affinity for bile salts and 18:1n-9 is seen for lungfish (Di Pietro and Santome, 2001) and catfish (Di Pietro et al., 1997). The fabp1 gene harbours a fully functional peroxisome proliferator response element (PPRE) (Schachtrup et al., 2004), whereas no PPRE has been detected in fabp10 genes thus far.

3.2.2.2 Fatty acid binding proteins of subfamily IV

Through knock out studies in mice FABP3 have been implicated in the fatty acid metabolism of heart and skeletal muscle and connected to reduced rates for fatty acid oxidation in skeletal muscle (Binas et al., 1999). This has lead to the theory that FABP3 deliver LCFA from sarcolemma through the cytoplasm to the outer mitochondrial membrane, which is the site for ACS (Hertzel and Bernlohr, 2000). ACS then converts LCFA to acyl CoA making it available for either TAG synthesis or mitochondrial β-oxidation (Koonen et al., 2005).

FABP4 knock out mice exhibit increased fat mass, decreased lipolysis, increased muscle glucose oxidation and attenuated insulin resistance (Hertzel et al., 2005). The observed reduction in lipolysis has been connected to the ability FABP4 has to activate hormone sensitive lipase (HSL) (Figure 4) (Shen et al., 1999). Thus the lack of FABP4 leads to a down regulation of lipid export from adipocytes (Coe et al., 1999a). HSL have also been suggested to provide fatty acids for oxidation in muscle (Jeukendrup et al., 1998).

Both FABP3 and FABP4 were both found in the heart and muscle tissues of Antarctic teleosts and associated with the duplicate functions these tissues have for utilising and storing lipids (Vayda et al., 1998). FABP3 proteins have been identified in heart of rainbow trout (Ando et al., 1998), and in liver, gills and gonads in mummichog (*Fundulus heteroclitus*) (Bain, 2002). FABP3 liver specific expression have also been observed in elephant fish (*Callorhynchus callorhynchus*) (Cordoba et al., 1998), shark (*Halaetunus bivius*) (Medzihradszky et al., 1992) and lamprey (*Entosphenus japonicus*) (Baba et al., 1999). Tissue specific gene expression studies identified FABP3 expression in the ovaries, liver, heart, muscle and brain of zebrafish (Liu et al., 2003a). Functionally, FABP3 in elephant fish liver is believed to participate in metabolising fat (Cordoba et al., 1998), whereas zebrafish FABP3 has been implicated in the process of liver lipogenesis (Liu et al., 2003a). FABP4 like proteins were identified in liver of elephant fish (Cordoba et al., 1998) and shark (Medzihradszky et al., 1992) and were believed to function in storage of lipids as an energy source.

Preferential binding for n-6 fatty acids was observed for FABP3 (Hanhoff et al., 2002) and FABP3 also binds 18:1n-9 and 16:0 with a higher affinity than both FABP1 and FABP4 proteins (Zimmerman et al., 2001). *In vitro* binding essays conducted on FABP3s in Antarctic icefish (*Chaenoceptalus aceratus*) and striped bass (*Morone saxatilis*) detected no significant differences in binding capacity (Londraville and Sidell, 1995; Londraville and Sidell, 1996) (Table 2). However, cold acclimation of striped bass caused an increase in the FABP3 concentration of aerobic skeletal muscle, perhaps to increase fatty acid flux through β-oxidation (Londraville and Sidell, 1996).

The fatty acids, 18:0, 18:1n-9, 18:2n-6 18:3n-3 and 20:4n-5 and 16:0 gave a 2-3 fold increase, of *in vitro* FABP3 mRNA expression in cardiac muscle cells compared with control (van der Lee et al.,

2000; Chang et al., 2001). However, the fabp3 gene harbours a non-functional PPRE (Schachtrup et al., 2004). The 5' upstream sequence of the zebrafish fabp3 gene has a hepatic nuclear factor-1 (HNF-1) response element, but no PPRE (Liu et al., 2003a). HNF-1 null mice exhibited enlarged fatty livers and alterations in the expression of genes involved in fatty acid synthesis and peroxisomal, but not mitochondrial β-oxidation and fatty acid transport, mediated by FABP1 (Akiyama et al., 2000).

Table 2. Characterisation of *in vitro* binding affinity of FABP3 and FABP10 in lower vertebrates.

Species	Gene	Tissue specific expression	High affinity for	Low affinity for	Reference to publication
Antarctic icefish	fabp3	Heart White muscle red muscle spleen, kidney brain	No difference in K_d detected for 16:0, 16:1n-7, 18:1n-9, nor 22:6n-3		(Vayda et al., 1998) (Londraville and Sidell, 1995)
Striped bass	fabp3	Muscle	No significant differences in binding affinity between 16:0,16:1n7, 18:1n-9, 22:6 n-3, 20:5n-3 and 18:0		(Londraville and Sidell, 1996)
Lungfish	fabp10	Liver	Fatty acyl-CoAs Lysophospholipids retionoids, bilirubin bile salts, 18:2n-6, 18:3n-3, 16:1n-7, 18:1n-9 20:4n-6	16:0 18:0 20:0 Prostaglandins Palmitoyl-carnitine Peroxisome proliferators	(Di Pietro and Santome, 2001)
Shark	fabp10	Liver	18:2n-6 Retinoic acids Lysophosphatidylcholine	16:0 18:1n-9 20:4n-6	(Cordoba et al., 1999)
Catfish	fabp10	Liver	16:0 18:0 and 18:1n-9	16:1n-7 18:2n-6 18:3n-3 20:0 20:4n-6 20:5n-3	(Di Pietro et al., 1996; Di Pietro et al., 1997)
Toad	fabp10	Not listed	16:1n-7. 18:1n-9 18:2n-6, 18:3n-3, 20:4n-6 Fatty acyl CoAs Lysophospholipids Retionoids Bile salts	16:0 18:0 20:0 Prostaglandins Palmitoyl-carnitine	(Di Pietro et al., 2003)
Chicken	fabp10	Liver	Decreasing affinity for 16:0, 18:1n-9 and retinoic acid		(Beringhelli et al., 2001)

3.3 Nutrigenomics

3.3.1 General overview

Nutrigenomics can be defined as the study on how nutrients, in this case fatty acids or their metabolites, can act directly or indirectly, through signal transduction pathways, on transcription factors and thereby affecting the transcription of genes, also known as gene expression (Muller and Kersten, 2003; Jump, 2002b) (Figure 5). Nutrigenomics is believed to promote increased understanding of how nutrition influences metabolic pathways and homeostatic control (Muller and Kersten, 2003).

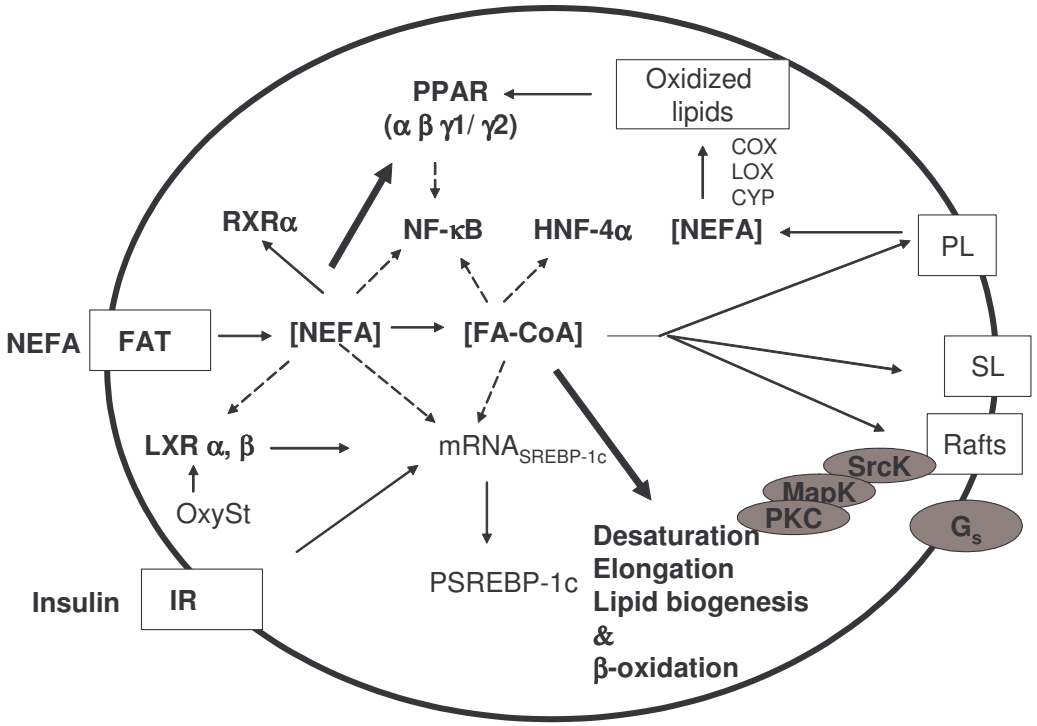

Figure 5. Schematic overview of the multiple mechanisms involved in the fatty acid regulation of transcription factor activity and abundance. The figure was modified from (Jump, 2002a). Solid arrows represent activation, dashed lines represent inhibition. Non-esterified fatty acids (NEFA) or their co-esters (FA-CoA) are believed to affect gene expression directly, or be metabolised according to their destined intracellular fate. Different dietary fatty acids may also mediate transcriptional regulation through changes in membrane composition, which alters signal transduction pathways and eicosanoid synthesis. See text for further explanations.

Fish oil diets, with their high degree of VLCFAs, have been shown to induce fatty acid oxidation and glukoneogenesis, while suppressing fatty acid synthesis through the action of transcription factors in mammals (Berger et al., 2002; Jump, 2002b). It has recently become evident that VLCFAs exerts many effects on gene transcription which are not mediated by a single subfamily of orphan nuclear receptors –PPARs. VLCFAs can affect expression of many different genes through the action of

transcriptional factors as hepatic nuclear factor -4α (HNF-4α), nuclear factor κB (NFκB), retinoid X receptor α (RXRα), sterol regulatory element binding protein-1c (SREBP-1c) and liver X receptors (LXRα and LXRβ) (Jump, 2002b). In a recent review Jump and co-workers (2005) argues against the role of LXR α as a target for VLCFA control of gene transcription.

Furthermore, dietary VLCFAs can alter membrane phospholipid composition and impact eicosanoid synthesis and signal transduction pathways. Dietary n-6 VLCFA stimulates, whereas n-3 VLCFA inhibit eicosanoid synthesis and signalling, and NF-kB activation (Jump, 2002a). Dietary n-3 fatty acids increase the amount of 20:5n-3, 22:5n-3 and 22:6n-3 in lipid rafts, which are membrane micro domains that contribute to signal transduction (Ikonen, 2001). These lipid rafts contain G-proteins and members of the SRC kinase family which participate in mediating signal down streaming signalling events that result in transcriptional activitation (Jump, 2002a). G-proteins acts through cAMP signalling and the SRC kinase family through calcium signalling, ERK activation and other downstream signalling events (Jump, 2004; Stulnig et al., 1998; Webb et al., 2000; Liang et al., 2001; Stulnig et al., 2001). It was suggested that the physical properties of the membrane bilayer affect the activity of protein kinase C (PKC), and that increased PC unsaturation increases PKC activity (Slater et al., 1994). In general, phospholipase C (PLC) hydrolyses phosphatidylinositiol-4-5-bisphospate to produce 1,4,5- inositol triphosphate and diacylglycerol, both substrates for PKC (Gomez-Fernandez et al., 2004). PKC has been shown to control PPARα expression in rat (*Rattus norvegicus*) (Yaacob et al., 2001) and human (Blanquart et al., 2004) *in vitro* hepatocyte assays.

3.3.2 Transcriptional activation

The general model for activation of orphan nuclear receptors, PPARs and LXR, is that they form heterodimers with RXR, and bind to specific nucleotide sequences (response elements) in the promoter regions of a large number of genes (Table 3). PPREs in gene promoters, direct repeats of the hexanucleotide sequence AGGTCA, are also referred to as DR-1 response elements (Cherkaoui-Malki et al., 2001). The nuclear receptor undergoes conformation changes after ligand binding, which results in co-repressor removal. Transcriptional activation is then enabled through recruitment of co-activators and histone acetylation (Pegorier et al., 2004). Yet, recent studies have shown that PPAR activities also results in gene repression through trans-repression of among others NFκB response elements in genes which do not harbour a PPRE (Tan et al., 2005).

HNF-4α or PPARα, which can compete for binding to direct repeat (DR)-1 elements is believed to mediate the effect polyunsaturated fatty acids has on liver glukoneogenesis (Berger et al., 2002). Homeodimerised HNF-4α is also known regulate apolipoprotein synthesis (Jump, 2004).

The most studied nuclear orphan receptors PPARs appear to be activated by different fatty acids and eicosanoids as well as hypolipidemic agents that control the transcription of lipid metabolism genes (Dreyer et al., 1993; Keller et al., 1993; Yu et al., 1995; Forman et al., 1996; Schoonjans et al., 1996b; Kliewer et al., 1997; Krey et al., 1997; Desvergne and Wahli, 1999). There are three PPAR subtypes (α, β, and γ) which all exhibit tissue specificity (Braissant et al., 1996; Lemberger et al., 1996; Desvergne and Wahli, 1999). All PPARs binds to polyunsaturated fatty acids with micromolar affinity, whereas PPARα binds to a wide range of saturated fatty acids (Xu et al., 1999a), which may suggest a role in the regulation of lipid metabolism of saturated fatty acids (Kliewer et al., 1997 ; Gottlicher et al., 1992; Gottlicher et al., 1993; Xu et al, 1999a). PPARα and PPARγ play critical roles in the catabolism and storage of fatty acids, whereas PPARβ, also referred to as δ, has been implicated in cholesterol transport (Oliver et al., 2001; Vosper et al., 2001) and muscle lipid β-oxidation (Dressel et al., 2003; Luquet et al., 2003). PPARα is also reported to regulate genes involved in fatty acids transport, glucose metabolism, and ketogenesis as well as Δ5 and Δ6 and Δ9 desaturation (Jump, 2002b) (Table 2). PPARγ2 knock out mice studies (Gavrilova et al., 2003; Wolf, 2004) and *in vitro* studies (Schadinger et al., 2005) suggest that PPARγ2 regulates triglyceride homeostasis. Liver PPARγ induced LPL and fatty acid transporters as well as inhibited NF-κb function and thus expression of cyclooxygenase- 2 (COX-2) (Hwang, 2000). PPARγ has also been implicated in mitochondrial biogenesis in mice (Mootha et al., 2003), and in mediating enzymatic response to fibrates in Atlantic salmon (Ruyter et al., 1997).

PPARs have recently been described in several fish species, including zebrafish (Ibabe et al., 2002; Ibabe et al., 2005a; Ibabe et al., 2005b), gray mullet (*Mugil cephalus*) (Ibabe et al., 2004), sea bass (*Dicentrarchus labrax*) (Boukouvala et al., 2004), plaice and sea bream (*Sparus aurata*) (Leaver et al., 2005). The tissue expression profile of PPARs in sea bass is similar to that observed for mammals, where PPARγ predominate in adipocytes, PPARα are mainly expressed in liver and PPARβ appears to be ubiquitously expressed (Boukouvala et al., 2004). In plaice and seabram PPARγ are expressed at levels comparable to PPARβ in several tissues and exhibit a wider expression profile than in other vertebrates (Leaver et al., 2005). Marine PPARs have also been shown to heterodimerise with RXR on DR-I response elements, in both mammalian and piscine genes (Boukouvala et al., 2004; Leaver et al., 2005). 18:1n-9 was identified as the most effective fatty acid activator of sea bream and plaice PPARα (Leaver et al., 2005), whereas sea bass PPARα and β were significantly induced by 18:2n-6 and 18:3n-3 (Boukouvala et al., 2004). Thus, marine PPARα and β were suggested to share similar functions as their mammalian counterpart, namely β-oxidation. All marine PPARγs were poorly activated by fatty acids (Boukouvala et al., 2004; Leaver et al., 2005), and VLCFAs induced transcription in sea bream only (Leaver et al., 2005). Hence, PPARγ show a different fatty activation profile compared with mammals, which are activated by monounsaturated fatty acids (Kliewer et al., 1997). The observed activitation profile may result from observed sequence differences in the ligand

binding domain (LBD). To summarise, marine PPARγs were suggested to exhibit different functions, from PPARγs in mammals (Leaver et al., 2005).

Through the action of PPRE in the gene promoters of CPT- I and II, enoyl-CoA hydratase (ECH), catalase and AOX (Zhang et al., 1992; Mascaro et al., 1999; Kassam et al., 2000; Berger et al., 2002; Girnun et al., 2002; Tachibana et al., 2005), VLCFAs are believed to indirectly induce fatty acid oxidation when supplemented in diet of mammals. Increased expression of the genes listed above, with the exception of catalase, was observed in liver of rats fed a fish oil diet compared with a palm oil diet using Northern blot analysis (Ide et al., 2000). Increased expression of liver CPT-II and ECH, but not CPT-I was observed when mice were fed fish oil diet enriched with 22:6n-3 and 20:5n-3 induced expression compared to a diet lacking VLCFA was observed using microarray analysis (Berger et al., 2002) (Table 3).

SREBP-1c, a basic helix loop-helix leucine zipper family transcription factor (Osborne, 2000) binds sterol regulatory elements (SRE) in promoters and induce genes involved in fatty acid synthesis, (Table 3) including ACC, fatty acid synthases and SCD (Mater et al., 1999). It was also demonstrated that mouse Δ5 and Δ6 fatty acid desaturases are subject to dual regulation of gene expression by PPARα and SREBP-1c (Matsuzaka et al., 2002). Based on the identification of SREBP-1c as a key regulator of Δ6 fatty acid desaturase, liver SREBP-1 c was suggested to regulate phospholipid synthesis rather than TAG synthesis (Nakamura and Nara, 2002). This was argued on the basis that VLCFAs are the main substrates for phospholipid synthesis, not the latter.

In general, fatty acid regulation of SREBP-1c may not involve direct fatty acid binding (Figure 5) but rather control the nuclear abundance of SREBP-1c, as it has been shown that long chained n-3 and n-6 fatty (20:5n-3 and 22:6n-3) acids suppress the cellular level of $mRNA_{SREBP\text{-}1c}$ in rat liver as well as the cellular level of precursor and nuclear forms (Kim et al., 1999; Mater et al., 1999; Xu et al., 1999b; Yahagi et al., 1999). SREBP-1c is also required for the insulin mediated induction of liver fatty acid synthesis and triglyceride homeostasis (Foretz et al., 1999; Azzout-Marniche et al., 2000; Osborne, 2000; Schultz et al., 2000; Vaulont et al., 2000).

Table 3. Genes involved in lipid metabolism in mammals, their regulation by fish oil, and their response elements.

Function	Gene	Response Elements [1]	Regulation by fish oil[2]	Cited reference
β-OXIDATION	cpt-I	PPRE (1,2)	-	(Mascaro et al., 1999; Berger et al., 2002)
	cpt-II	PPRE (1, 2)	+	(Berger et al., 2002; Barrero et al., 2003)
	ech	PPRE (1, 2)	+	(Berger et al., 2002) (Zhang et al., 1992; Kassam et al., 2000; Tachibana et al., 2005)
	catalase	PPRE (1, 2)		(Girnun et al., 2002; Tachibana et al., 2005)
	carnitine acyltransferase	PPRE (1)	+	(Berger et al., 2002)
	acd	PPRE (1,2)	-	(Schoonjans et al., 1995; Berger et al., 2002; Tachibana et al., 2005)
	Trifunctional enzyme	PPRE (1,2)		(Nicolas-Frances et al., 2000; Tachibana et al., 2005)
	acat	PPRE (1)		(Tachibana et al., 2005)
	aox	PPRE (2)		(Tugwood et al., 1992)
Fatty acid synthesis	acs	SRE (1,2)	+	(Berger et al., 2002; Jump, 2002b; Sone et al., 2002)
	fas	SRE (1,2)	-	(Magana and Osborne, 1996; Berger et al., 2002; Jump, 2002b)
	scd	SRE (1,2) PPRE (2)	-	(Miller and Ntambi, 1996; Lefevre et al., 2001; Berger et al., 2002)
	elongase	SRE (1)	+	(Moon et al., 2001; Berger et al., 2002)[3]
	Δ5 fatty acid desaturase	SRE, PPRE (1)		(Matsuzaka et al., 2002)
	Δ6 fatty acid desaturase	SRE, PPRE(1)		(Matsuzaka et al., 2002)
Transport	fabp1	PPRE (1,2)		(Schachtrup et al., 2004; Tachibana et al., 2005)
	fabp3	PPRE (2)		(Schachtrup et al., 2004)[4]
	fabp4	PPRE (2)		(Schachtrup et al., 2004)
	Fabp5	PPRE(1,2)	+	(Berger et al., 2002; Schachtrup et al., 2004)[5]
	fat/cd36	PPRE (1,2[5])	+	(Berger et al., 2002; Sato et al., 2002)
	apoA-I	PPRE (2)		(Vu-Dac et al., 1994)
	apoA-II	PPRE (2)		(Vu-Dac et al., 1995)
	apoB	PPRE (1)	+	(Berger et al., 2002; Jump, 2002b)[7]
	apoC-I	PPRE (1)	-	(Berger et al., 2002; Jump, 2002b)[7]
	apoC-II	PPRE (1)	+	(Berger et al., 2002; Jump, 2002b)[7]
	apoE[6]	PPRE (1,2)	+	(Galetto et al., 2001; Berger et al., 2002)
	lpl	PPRE (2)		(Schoonjans et al., 1996a)

[1] The responseelements are either presumed by gene expression analysis (1) or identified through characterization or functional analysis of promoter areas (2)
[2] Reference made to the microarray study by Berger (Berger et al., 2002).
[3] Is assumed to have a SRE
[4] Has a non functional PPRE
[5] Identified as not having a PPRE
[6] Not believed to exist in salmonids
[7] Involved in VLDL secretion and activated through HNF-4α

4. Methodological considerations

The two methods used for measuring gene expression, microarray (Paper I) and Q-PCR (Papers I-IV) were carefully evaluated according to experimental and methodological standards available at the time of the current studies. These methods share some potential pitfalls which may contribute to technical and systemic variation. Firstly, high technical RNA quality as well as sequence information quality is needed to assure high reproducibility and reliability of the obtained data. Furthermore, good experimental setups are needed to ensure control over technical variation, especially for microarray analysis (Paper I). It is also essential to control intra and inter plate variability in Q-PCR (Papers II and III). The experimental design chosen should also minimise the systemic variation, by ensuring valid reference and normalisation strategies. For microarray analysis it is also important to ensure optimal quality analysis. For both methods high statistical power for the statistical analysis must be assured. Q-PCR analyses were done on individual tissue samples from Atlantic salmon fed different diets (Papers I-III). For microarray analysis pooling of samples has been recommended to ensure high statistical power when references are made on a group level (Peng et al., 2003) (Paper I). The number of pooled samples was according to the central limit theorem to ensure normal distribution, low SD, and thus minimising genetic variation (Zar, 1999).

In microarray screening, the use of MIAME standards ensured recommended documentation of experimental details and results (Paper I). High reproducibility and repeatability were ensured by the use of high quality RNA and recommended RNA quantity (Garosi et al., 2005). Flour flips were used to control for systemic variance, which may be introduced through different labelling intensities and scanning properties of the fluorofores (Churchill, 2002).RNA purification strategies were optimised to ensure high quality RNA for both methods measuring gene expression. RNA purity measurements were within limits recommended when using nuclease free water, and all quantity measurements at A_{260} were done within the linear range (Ambion, 2005). Prior to Q-PCR and microarray analysis (Papers I-III) gel-electrophoresis was conducted with the aim to measure total RNA integrity (results not shown). Recent discoveries indicate that these 28S:18S rRNA ratio measurements alone not are indicative of high RNA quality (Ambion, 2004). Thus suggestions are made on defining RNA quality as the sum of RNA integrity and purity. Finally, for our experimental setup, validation of RNA quality was also accomplished through assessment of reference gene expression stability (Perez-Novo et al., 2005) (Papers II and III, see Appendix, Table 1).

EST sequenced cDNA used for microarray construction and thus analysis, only provides information from a unique stretch of cDNA within a coding region of the gene. The initial information on the cDNA identity was based on BLAST searches (http://cbr-

rbc.nrc.cnrc.gc.ca/reith/salmon/salmon.html). It should be noted that identification of these gene sequences depends on the number of sequences available for BLAST searches. Consequently, their identities have recently been verified by the use of several online sources (http://www.ncbi.nlm.nih.gov/BLAST/ and http://www.salmongenome.no/cgi-bin/sgp.cgi).

In Q-PCR, high quality information on the nucleotide sequence used for primer and probe design may ensure analytical precision. Thus, we decided to further examine genes which were fully cloned, Δ5 fatty acid desaturase, FABP3 and PPARγ, or assessed through fully sequenced ESTs, namely FABP4 and 10. Primers and probes were designed based on exon-exon boundaries, to assure specificity to mRNA and minimise genomic DNA contamination. For more details on the primers and probes designed for the reference genes see Paper IV. All primer and probe assays constructed using Assay by Design (Applied Biosystems) functioned satisfactorily. In fact, no mispriming was present as verified using gel electrophoresis, which detected single PCR products of expected amplicon size (results not shown).

Inter and intra plate assay variation was assessed for our Q-PCR production life cycle assays (Papers II and III), ascertaining that variation between Ct values from individual samples were within the realistic range, between 2 and 4% (Pfaffl, 2004). In our experimental protocol, % deviation around mean (n=3 per sampling for FABP assays and n=6 for PPARγ assays) between and within plates was less than 4 % at all samplings within all Q-PCR assays through production lifecycle (Appendix, Figure 1-3). Furthermore, the between sampling control also varied less than 4 % for all assays. This control was total RNA from one fish from the second sampling, assayed using all Q-PCR assays, on plates from all the other samplings. This established that the life cycle changes in FABP and PPARγ relative gene expression were not due to technical variation (Papers II and III). Additionally, it was ensured that the same sample size and RNA quantity (±5%) was used in all assays. This has been described as a part of a good normalisation strategy (Huggett et al., 2005).

Differences in PCR efficiency would result in the variation of Ct-values, using the threshold method (Bustin et al., 2005). Consequently, the same interassay dependent threshold values were set throughout the production lifecycle Q-PCR experiments. In order to assure optimal control over PCR efficiency, dilution curves for all assays were run on every second plate at each sampling. PCR efficiency ranged form 97-100%, with the exception of red and white muscle tissue sampled from fish in seawater samplings (Appendix, Table 2). Yet, this lowered efficiency did not seem to introduce analytical imprecision, indicated by low interassay variation in Ct values for these samplings (Appendix, Figure 1-3). Moreover, there seem to be a correlation between inadequate PCR efficiency and low A_{260}/A_{230} ratios due to PCR poisoning (Paper IV). This is probably a consequence of the use of DNAse.

18S rRNA was earlier recognised as “the gold standard” (Ambion, 2006). However, recently criticism against the use of 18S rRNA has been addressed (Bustin and Nolan, 2004). In addition 18S rRNA has shown relatively high expression variability when evaluated for Atlantic salmon studies (Paper IV) (Ingerslev et al., 2006). Hence, indicating that care should be taken when using 18S rRNA as an endogenous control for gene expression studies. Some reservations should also be taken in to consideration in the quantification step. Since the high expression of 18S rRNA compared to mRNA may, when used in ratio models, result in the introduction of statistical bias (Hocquette and Brandstetter, 2002). This has lead to the use of 18S rRNA as a potential quality control in the present study, in order to confirm results using $EF1A_A$ as the main reference gene. Thus, when relative gene expression was calculated, both 18S rRNA and $EF1A_A$ were used at all times. Normally, 18S rRNA confirmed results using $EF1A_A$ (muscle data Paper II, and for paper III, see Appendix, Figure 4, Paper I, see Appendix Figure 5 and 6). 18S rRNA was used as the endogenous control when calculating relative liver Δ5 fatty acid desaturase mRNA expressions after 22 and 42 weeks of feeding (Paper I). However, relative gene expression of liver Δ5 fatty acid desaturase, when using $EF1A_A$ as the denominator, was also significantly increased when Atlantic salmon were fed 75% rapeseed oil compared to fish oil (Appendix Figure 5 and 6). There where however differences in gene expression patterns of liver FABPs through production life cycle, dependent on the endogenous control used (Paper II).

The thorough technical validation of microarray data with regard to labelling and hybridisation has been addressed earlier (Paper I). Systemic variation was less focussed on. First of all, data pre-processing, involving image analysis and normalisation, are required to reliably quantify the fluorescence intensities (Goldstein and Delorenci, 2005). In the present study a combination of fixed circle segmentation, and gridding and flagging of spots for image analysis were used. According to Goldstein and Delorenci (2005), this constitutes the best available technique at the present time. Furthermore, the use of primary scans to asses the basis of analysis, and secondary scans to correct the intensity of spots with saturation, ensured reliable intensity ranges and thus optimal scanning procedures (Lyng et al., 2004). Channel dependent global normalisation gave the same adjustment to each spot on the array without regard to individual spot features or location (Goldstein and Delorenci, 2005). We also used quadruplicate spotting of all spots on the array including quality controls in the form of empty spots and housekeeping genes. A five point concentration gradient of chlorophyll synthethase from *Arabidopsis thaliana* was also spotted four times to asses binding of our internal control. Therefore, this enabled us to visually control our normalised results for spatial dependent imbalances. Furthermore, restricted coverage arrays seems problematic to normalise properly with other available methods, due to violations of assumptions made for the methods *lowess/loess*, print tip, and the use of control sequences and housekeeping genes. Consequently, the global strategy seems to

be most appropriate for these types of arrays. It should be stated that the normalisation resulted in channel dependent intensity plots which go through origio (results not shown).

T-test based models have been recommended for statistical analysis in cDNA microarray experiments where replication exists for two conditions (Cui and Churchill, 2003). Methods which estimate the false discovery rate (FDR), as SAM, have been advised for use in nutrigenomics studies (Garosi et al., 2005). Consequently, we tested the gene specific t-test ($p<0.01$) and the SAM analysis (Paper I). SAM analysis offers higher stability in estimating variance from each gene, and the approach for multiple testing by using FDR (Cui and Churchill, 2003). FDR is the proportion of false positives among the genes identified as being differentially expressed and is a post data measure of confidence, rather than a significance level. It should be noted that the number of differential expressed genes were reduced using SAM instead of a t-test with a 99% confidence limit.

Since, there is a correlation between the PCR efficiency and the Ct-value, only software with PCR efficiency correction, i.e. REST (Pfaffl et al., 2002) and Q-Gene (Muller et al., 2002; Simon, 2003) were used. The Q-Gene method was used in Papers I-III. Results from statistical analysis examining whether diet induced differences in gene expression (Papers I and II) were supported by results using statistical randomisation with pair-wise permutations in REST (results not shown).

When analysing tissue samples using relative quantification one should be aware of the fact that the variability of gene expression measurements may be influenced by local variation in reference gene expression stability, due to tissue and cell-specific factors (Bustin and Nolan, 2004). In a recent letter to Nature low induction levels were addressed as a source of extrinsic variability (Volfson et al., 2005). Extrinsic variability, thus meaning effects of random fluctuations in the environment, or the effect of regulatory inputs that are common to multiple genes, have been shown to be a significant component in gene expression variability. Thus, the high standard deviation seen in the present studies (Papers I-III) is believed to be due to high extrinsic variability possible exacerbated by tissue specific factors. Gene expression measurements are also influenced by intrinsic sources of variability due to different physiological state, age, sex and genetic polymorphism (Lettieri, 2006). Variation between tanks might also explain variability in white muscle FABP3 expression relative to $EF1A_A$ after 3, 16 and 22 months of feeding (Paper II). No tank specific effects were seen for FABP3 expression in red muscle or FABP10 expression in liver during the production life cycle experiments (Paper II). Liver FABP3 mRNA levels after sea water transfer, and PPARγ expression after 6 (both diets) and 16 months (plant oil diet) varied due to observed variation between tanks (Paper III). Thus, extrinsic variability is in this fish nutrition experiments were not the only source of variability.

However, the present identification of a valid reference, $EF1A_A$, for data normalisation (Paper IV) is believed to have assured accurate, reproducible and biologically relevant mRNA quantification (Paper I, see Appendix, Figure 5 and 6, and Papers II and III) (Bustin, 2004). There is a growing awareness regarding the fact that these snap-shot gene expression measurements of the dynamic cells, although analytically precise, should be used in concert with other analytical methods to validate biologically significance. However, it should be stated that there is reason to believe that small changes in gene expression for regulatory proteins and transcription factors do have biological relevance.

To summarise, the experimental design used for microarray screening was chosen to ensure low genetic variation and high statistical power when comparisons were done at a group level (Paper I). The use of several quality controls and a high degree of technical replicates enabled us to evaluate systemic and technical variation. The strategy for labelling and scanning procedures as well as image analysis have been evaluated and found advisable for use in this experimental analysis. The choice of normalisation strategy and statistical analysis were as recommended for use in nutrigenomical surveys using gene focussed arrays. The data set met MIAME standards.

The endogenous controls used for the experimental setups were thoroughly evaluated for use in Atlantic salmon (Paper IV). The present identification of a valid reference, $EF1A_A$ for data normalisation enabled us to assure accurate, reproducible and biologically relevant mRNA quantification (Papers I-III). The use of 18S rRNA assays to calculating relative gene expression of liver PPARγ and FABPs in muscle verified results using $EF1A_A$ as an endogenous control (Papers II and III). Minimal analytical variation, as evaluated by interplate, intraplate and intrasampling controls, was observed for all Q-PCR assays run on individual fish samples (Papers II and III).

5. General discussion

5.1 Nutrigenomics in aquaculture nutrition

Nutrigenomics can be defined as the use of systems biology i.e. genomics, transcriptomics, proteomics and metabolomics, but also bioinformatics in nutrition research (van Ommen and Stierum, 2002). For aquaculture nutrition research, the integration of systems biology has just started. Since the exploration of the genomes of several aquacultured fish species, among these Atlantic salmon, are quite recent compared to mammals.

The present use of a gene focussed array to study lipid metabolism in Atlantic salmon should include the study of transcription factor pathways mediating nutrient gene interactions (Paper I). It should also explore the expression of regulatory proteins which mediate metabolic processes meaning transport proteins and known signal transducers. In order to further elucidate possible mechanisms for transcriptional activation, but also study selective fatty acid uptake which are believed to have impact on the regulation of cellular lipid metabolism. Overall, studies of metabolic end products and metabolic processes should be used in concert with microarrays to evaluate the biological meaning of these snap shot gene expression measurements. In the present study, established methods were used to asses' dietary, tissue and lipoprotein fatty acid composition (Papers I-III) (Torstensen et al., 2004 and 2005). For both dietary trials liver and lipoprotein lipid class composition were determined (Paper III) (Torstensen et al., 2004). Measurements of lipid catabolism in white- and red muscle and liver were also included in the present study (Stubhaug, 2005; Stubhaug 2005a). Further, enzymatic assays were used to address dietary implications on lipogenesis in Atlantic salmon (Paper III). Protein assays were used to asses' translational patterns for muscle FABP3 which correlated with mRNA expression (Paper II).

Several specific challenges characterise aquaculture nutrition research. One specific challenge is the use of *in vitro* primary cell culture studies versus *in vivo* experiments. Especially, as several fatty acids vary in a system under homeostatic control in an *in vivo* experimental design such as a feeding experiment varying the dietary oil source. The *in vitro* designs are often focussed on the study of the effect of one specific fatty acid. A second challenge when using dietary trials to examine the *in vivo* response, are ambient temperature and light. Under low induction levels, as seen in nutrigenomic *in vivo* assays, extrinsic variability, meaning the effect of random fluctuations in the environment and regulatory inputs on multiple genes simultaneously, is common in gene expression measurements (Volfson et al., 2005). *In vivo* gene expression measurements are also influenced by intrinsic sources of variability due to different physiological state, age, sex and genetic polymorphism (Lettieri, 2006). In addition, reference gene variability may be introduced when using tissue samples for relative gene

expression measurements (Tricario et al., 2002). Furthermore, as studies of nutritional effects *in vivo* are studies of a well balanced homeostasis one may find few biomarkers. In these studies one may expect to define a fine tuned metabolic change mediated by long term effects of dietary fatty acid composition (van Ommen and Stierum, 2002: Jump, 2002a). This introduces methodological and technological challenges in the field of systems biology as a whole. Overall, one has to conclude that the nutrigenomical survey used in the present study constitutes the beginning of a new field of aquaculture nutrition studies.

5.2 Extracellular lipid transport

Overall, liver and lipoprotein fatty acid composition in Atlantic salmon (Paper III) was highly influenced by dietary fatty acid composition. This is in line with previously reported results (Lie et al., 1993; Torstensen et al., 2000; Torstensen et al., 2004). HDL had generally high levels of 22:6n-3 irrespective of dietary fatty acid level. Thus agreeing with the high phospholipids levels compared to the other lipoproteins, as seen earlier (Lie et al., 1993).

The quantitative impact of altered lipogenic enzyme activity on liver and plasma lipid levels is considered to be minor in Atlantic salmon, especially when fed high levels of dietary lipid (Arnesen et al., 1993). This is also in agreement with observations done in other fish species (Sargent et al., 1989). However, feeding Atlantic salmon a 100% plant oil replacement diet increased liver TAG stores, and decreased plasma lipid levels, possibly through decreased VLDL synthesis (Paper III). These results were consistent with the higher lipid accumulation in the liver of Atlantic salmon fed a diet containing 100% sunflower oil, compared with a fish oil diet at 5°C (Ruyter et al., 2006). TAG lipase, which mobilises fatty acids from endogenous TAG for VLDL synthesis (Gibbons and Wiggins, 1995; Gilham et al., 2005), has been identified in liver of Antarctic ice fishes (Sidell and Hazel, 2002). Winter flounder TAG lipase, was printed on the array (Paper I). The cDNA, synthesised from the isolated Atlantic salmon mRNA, seemed not to hybridise to the spotted winter flounder cDNA. This may have been due to poor interspecies hybridisation efficiency as previously examined (von Schalburg et al., 2005). The recent published work by Ruyter and co-workers (2006) suggested alternative explanations for increased lipid deposition, compared to the ones given in Paper III. Low water temperature was suggested to reduce the activity of enzymes involved in the esterification of fatty acids in to both neutral and polar lipids for VLDL production (Ruyter et al., 2006). Low water temperature was observed at the last sampling (Paper III) and may therefore potentially result in increased lipid deposition in the liver. Ruyter and co-workers (2006) also suggested that the lack of dietary n-3 VLCFA may imply a reduction in VLDL synthesis. Especially as 22:6n-3 has been shown to mainly be esterified in to the polar lipids of VLDL, thus low 22:6n-3 leading to liver TAG accumulation *in vitro* (Vegusdal et al., 2005). Vegusdal (2005) also suggest that dietary FO reduces

TAG secretion from Atlantic salmon hepatocytes and that 20:5n-3 mediates this effect. 20:5n-3 has also been shown to inhibit secretion of TAG when studied in cultured rat hepatocytes (Nossen et al, 1986). Frøyland and co-workers (1997) reported decreased plasma TAG induced by 20:5n-3 whereas 22:6n-3 had no effects on plasma TAG levels in rats. Both in the study by Ruyter (2006) and in the present study (Paper III) low dietary levels of dietary n-3 VLCFA were reported in the plant oil based diets. Although within recommendations for both dietary 20:5n-3 and 22:6n-3 (NRC, 1993; Sargent et al., 1995) these plant oil diets seem to introduce liver TAG accumulation. Common for these *in vivo* experiments are high dietary levels of 18.1n-9, as well as high dietary lipid loads. Ruyter and co-workers (2006) reported that the higher fat accumulation seemed to be mainly caused by a selective accumulation of 18:2n-6 and 18:1n-9. 18:2n-6 is the most prominent dietary fatty acid in the 100% sunflower oil diets. It should be stated that the reported trends are somewhat contrary to what could be expected based on published *in vitro* studies in Atlantic salmon (Vegusdal et al., 2005, Stubhaug et al., 2005b) and rat hepatocytes (Nossen et al., 1986). These studies indicate that 18:1n-9 increase secretion of TAG. However, *in vivo* feeding experiments are more balanced regarding nutrient composition and the system is under constant homeostatic control compared to an *in vitro* experiment with cultured hepatocytes being exposed to high concentrations of a single fatty acids. Thus, there is reason to believe that the dietary induced response in the *in vivo* system under study may be more complex than assumed based on *in vitro* studies.

In the present experiment differences in growth rate after 22 months of feeding can not be excluded as a possible factor explaining increased liver TAG levels in plant oil fed compared to fish oil fed Atlantic salmon (Paper III). However, it was not observed any growth differences after 14 months of feeding when increased liver TAG levels in plant oil fed compared to fish oil fed Atlantic salmon. Consequently, growth alone can not be the sole explanation for increased liver TAG levels in plant oil fed fish.

Dietary regulation of liver apo-AI and c-II gene expression was observed in the present study (Paper I). Thus agreeing with results recently reported for Atlantic salmon (Kleveland et al., 2005) and earlier studies performed using rodents (Berger et al., 2002; Hatahet et al., 2003). Hence, it may suggest the presence of PPREs in these Atlantic salmon genes as identified for human apo A-I (Vu-Dac et al., 1994). The presence of PPREs has also been suggested in apo C-II genes (Berger et al., 2002; Jump, 2002b).

The observed induction of apolipoproteins apoA-I and c-II when the fish were fed the 75% rapeseed oil diet compared to 100% fish oil (Paper I) appears to be correlated with an observed decrease in total plasma HDL protein content for Atlantic salmon fed the same diets (Torstensen et al., 2004). Whereas the plant oil blend diet induced no differences in HDL protein content (Paper III), but did give significantly reduced plasma LDL levels. Thus in Atlantic salmon these apolipoproteins may be regulated by complex post transcriptional and translational mechanism as observed in mammals

(Fojo et al., 1986; Hoeg et al., 1986). Apo A-I, the predominant protein associated with HDL, undergo co-translational proteolytic processing. The protein also undergoes post-translational conversion of pro apo A-I to mature apo A-I prior to cellular secretion. Moreover, it has been suggested that linkage of lipids to apolipoproteins may play a critical role in apolipoprotein and lipoprotein metabolism (Hoeg et al., 1986). Thus, there is clearly a need for further studies to elucidate mechanisms for the regulation of the apolipoprotein synthesis in Atlantic salmon liver cells.

5.3 Fatty acid uptake and intracellular transport

Phylogenetic analysis of FABP sequences suggest that the mammalian FABP1s and the FABP10 forms expressed in liver of Atlantic salmon (Papers I and II) and zebrafish (Denovan-Wright et al., 2000) are clearly products of distinct genes. Based on distance matrices, FABP10 was suggested to have diverged from FABP1 by gene duplication approximately 679 million years ago (Schaap et al., 2002). Furthermore, FABP3 co-group with the h8 forms identified in Antarctic ice fish (Vayda et al., 1998) and the characterised FABP3 mRNA widely expressed in zebrafish (Liu et al., 2003a). With high node support FABP3 seems distinct from FABP4 in vertebrates. All h6-FABPs co-group with vertebrate FABP4s. Thus the Atlantic salmon h6-form, identified in the macrophage cDNA library (http://cbr-rbc.nrc.cnrc.gc.ca/reith/salmon/salmon.html) and used for microarray screening (Paper I) was suggested to be of the FABP4 isotype. Hence, confirming suggestions made for heart muscle FABP4 in Antarctic teleost (Vayda et al., 1998). Whether the predicted FABP4 from zebrafish (Figure 6) is a duplicate genetic form of the suggested FABP4 isotype, is still unknown at the present time. Yet, there is no indication of expression of a similar isoform in salmonidae protein databases sequences (http://www.salmongenome.no/cgi-bin/sgp.cgi). Duplicate iLBP genes have been found for FABP7 (Liu et al., 2004a) and CRBPI and II (Liu et al., 2005) and is suggested for FABP3 in zebrafish (Liu et al., 2003a). Thus the presence of duplicate FABPs genes in salmonids may not be excluded, as actionopterygian (ray finned) fish is known to have a duplicated genome compared to all land vertebrates (Meyer and Schartl, 1999).

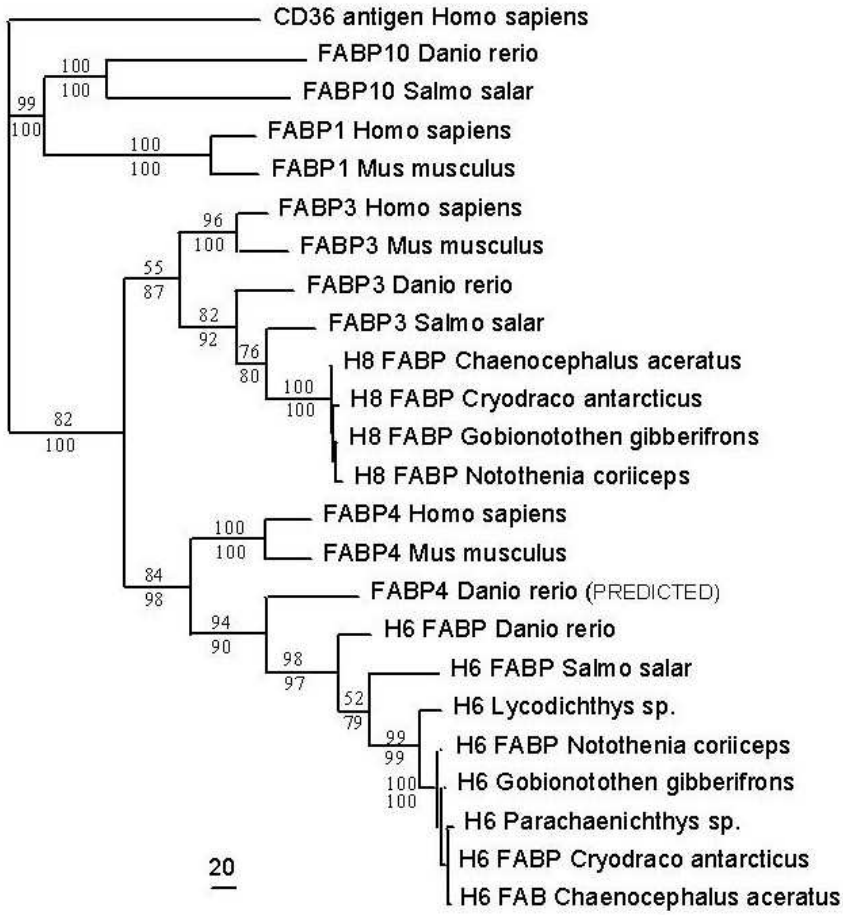

Figure 6. **One most parsimonious tree resulting from the unweighted parsimony analysis of 24 FABP and the CD 36 antigen nucleotide sequences from vertebrates.**
Sequences were aligned in ClustalX with gap: gap extension cost 6:4,transitions:transversion cost 0.5. The analysis was based on 520 sequence positions with a minimum of missing data included. This topology was not sensitive to different sequence alignments (ti:tv ratio 0.5 for all alignments; gap opening: gap extension costs 15:6.66 (default), 10:4, 10:10, 6:4, 6:6), or to the inclusion of a distantly related outgroup sequence (CD36 antigen sequence from the human genome). Bootstrap support values are given above (outgroup included) and below (outgroup excluded) each internal branch. The scale given is 20 substitutions.

Overall, all FABP's used for dietary mRNA expression profiling (Papers I and II) appear to be FABP like, as their predicted amino acid sequence return the characteristic β-barrel structure (Figure 7). The predicted Atlantic salmon FABP3 protein was 133 amino acids long, had a deduced molecular weight of 14630.5 g/mol and a theoretical pI of 5.52 (Paper II). The reported protein sequence length and theoretical pI of Atlantic salmon FABP3 were identical to that found for rainbow trout (Ando et al., 1998). The deduced molecular weight of rainbow trout FABP3 was 14531 g/mol. The Atlantic salmon FABP3 showed high amino acid sequence identity to FABP3 from other species (For more details se Paper II). Atlantic salmon FABP10 cDNA encodes a 126 amino acid protein with a deduced molecular weight of 14023.2 g/mol and a theoretical pI of 8.52. Its characteristic pI and protein residue identity to other FABP10s clearly suggest the existence of a salmon FABP10 isoform (For more details se Paper II).

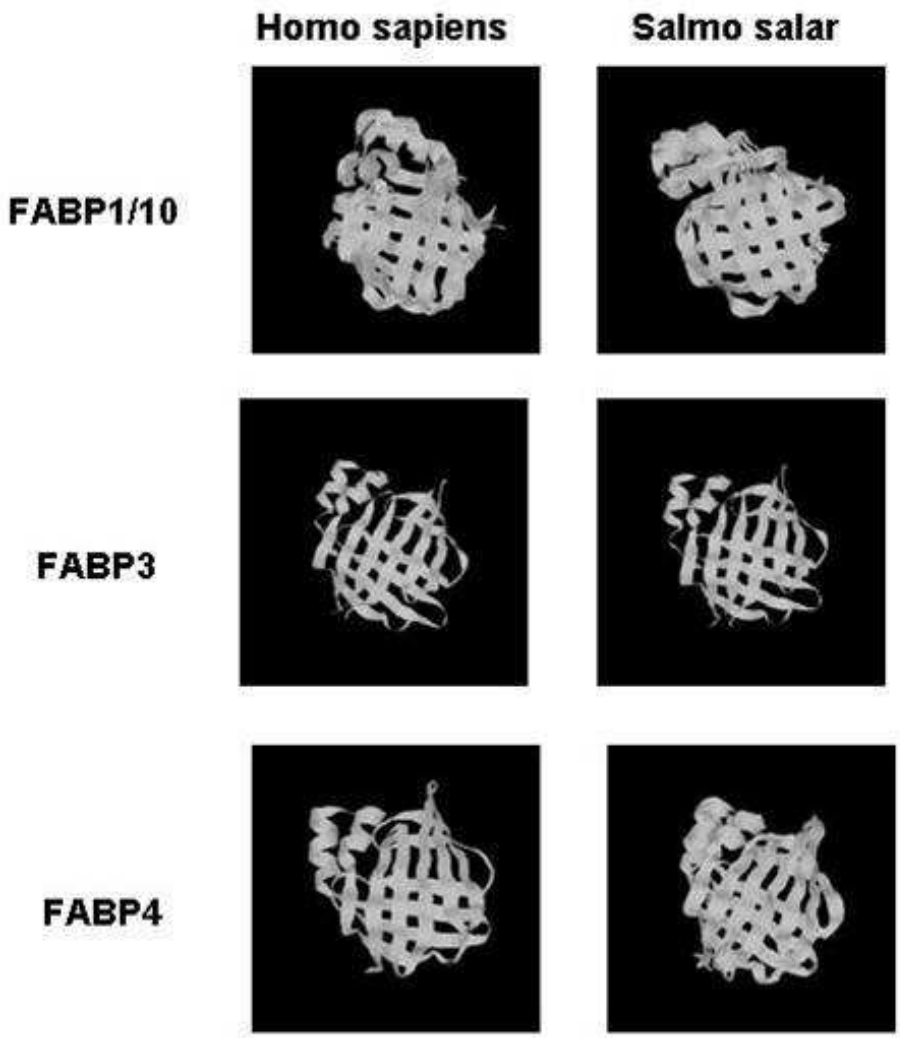

Figure 7. Ribbon diagram of FABPs. Ribbon diagrams of all FABPs were created with the use of RasMol v2.6 software Ribbon diagrams for Atlantic salmon FABPs were based on predicted protein sequences submissed to Swiss model.

FABP3 (Paper II), FABP4 (Paper I) and FABP10 (Papers I and II) mRNA expression in muscle and liver (FABP4, liver only) were not affected by dietary fatty acids in the present study. FABP3 gene expression results were in co-ordance with protein expression studies for FABP3 (Paper II). Thus, there were no significant differences in muscle FABP3 protein or gene expression between Atlantic salmon fed 75% rapeseed oil and 100% fish oil. Nonetheless, protein expression studies suggested that muscle FABP3 were influenced by the degree of dietary rapeseed oil inclusion. This was based on an observed tendency for decreased FABP3 protein expression with decreasing inclusion of dietary rapeseed oil. Thus, not excluding the possibly that dietary fatty acids may influence FABP3 mRNA

expression, as seen earlier in rat muscle cells *in vitro* (van der Lee et al., 2000; Chang et al., 2001). Especially, since it has been suggested that the control over FABP3 expression occurs predominantly at the level of transcription initiation (Zhang and Haunerland, 1998). Moreover, these dietary specific adaptations may suggest that FABP3 mediate selective fatty acid uptake and transport. However, it has been concluded that limited fluctuations in muscle FABP3 content is unlikely to have a major effect on muscle fatty acid uptake and utilization (Luiken et al., 2003). Thus, the small fluctuations seen could not be used to conclude that Atlantic salmon muscle FABP3 is a transport protein connected to β-oxidation, as suggested for mammals (Veerkamp and Vanmoerkerk, 1993; Binas et al., 1999).

In a comparison of human and rainbow trout FABP3s it was argued that amino acid substitutions within the binding site of rainbow trout FABP3 resulted in amino acids with similar characteristics and size to human FABP3 (Ando et al., 1998). In addition, all side chains of these amino acids were argued as being located far away from the bound fatty acid molecule in rainbow trout FABP3. Hence, it was thought to be very unlikely that the binding site and binding behaviour of trout FABP differed in any way from that of the human protein. FABP3 identified in the heart muscle of rainbow trout confer 98% sequence identity to Atlantic salmon FABP3 (Figure 8). The characteristic β-barrel formed by 10 β-sheets closed off by a helix turn helix is highly conserved and there are no differences between salmonids within described positions in the binding cavity (positions: 34, 50, 85, 91 and 125). Thus, there is reason to assume similar binding specificity and behaviour for Atlantic salmon and human FABP3. *In vitro* binding studies for FABP3 have shown high binding affinity for 18:1n-9 (Zimmerman et al., 2001) and n-6 fatty acids in mammals (Hanhoff et al., 2002). 18:1n-9 was a prominent fatty acid in the rapeseed oil diet, and 18:2n-6 was also present at high levels in the rapeseed oil diets (Paper II). Thus, these fatty acids may mediate differential protein expression of Atlantic salmon muscle FABP3, as a response to selective uptake mechanisms.

18:1n-9, 18:2n-6 as well as 18:3n-3, also present at high levels in the rapeseed oil diets, has all been shown to induce expression of FABP3 mRNA *in vitro* in cultured rat myoblasts (Chang et al., 2001). Thus, there is a possibility that several fatty acids mediate differential FABP3 gene expression, in Atlantic salmon muscle. Assuming that models of regulation of muscle FABP3 expression on a transcriptional level holds for Atlantic salmon as for the insect, desert locust (*Schistocerca gregaria*) (Zhang and Haunerland., 1998).

```
                    ....|....| ....|....| ....|....| ....|....| ....|....|
                             10         20         30         40         50
S.salar_FABP3       MAEAFAGTWN LKDSKNFDEY MKALGVGFAT RQVGGMTKPT TIIEVAGDTV
O.mykiss_FABP3      MAEAFAGTWN LKDSKNFDEY MKALGVGFAT RQVGGMTKPT TIIEVAGDTV
Clustal Consensus   ********** ********** ********** ********** **********

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                             60         70         80         90        100
S.salar_FABP3       TLKTQSTFKN TEISFKLGEE FDETTADDRK VKSLITIDGG KMVHVQKWDG
O.mykiss_FABP3      TLKTQSTFKN TEISFKLGAE FDETTADDRK VKSLITIDGG KMVHVQKWDG
Clustal Consensus   ********** ******** * ********** ********** **********

                    ....|....| ....|....| ....|....| ...
                            110        120        130
S.salar_FABP3       KETTLVREVS GNALERTLTL GDVVSTRSYV KAE
O.mykiss_FABP3      KETTLVREVS GNALELTLTL GDVVSTRSYV KAE
Clustal Consensus   ********** ***** **** ********** ***
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Figure 8. Clustal X alignment of the amino acids sequences of rainbow trout and Atlantic salmon FABP3s, identified in heart and white muscle tissue, respectively. The sequences confer 98% sequence identity as only two out of 133 fatty acids are different. The amino acids are not believed to be directly involved in ligand binding.

Atlantic salmon FABP3 may function as a transport protein connected to liver β-oxidation, as suggested for elephant fish liver FABP3 (Cordoba et al., 1998). Atlantic salmon FABP3 may also function as a transport protein for lipogenesis, as suggested for zebrafish (Liu et al., 2003a). However, the function of Atlantic salmon liver FABP3 is presently unknown. The high expression of FABP3 in Atlantic salmon muscle, compared to the expression of muscle FABP3 in zebrafish (Liu et al., 2003a) could imply that these gene transcripts have distinct functions. However, no further intra tissue comparisons will be done as relative expression analysis was used in the present study.

It is presently unknown whether the lack of correlations between Atlantic salmon liver β-oxidation and FABP3 expression may be influenced by the high degree of peroxisomal β-oxidation in this tissue (Stubhaug, 2005). Studies have shown that FABP1 expression and peroxisomal oxidation was induced by 18:1n-9, in the presence of an inhibitor of CPT-I activity (Kaikaus et al., 1993). Thus, indicating that selective fatty acid transport to peroxisomes may occur. All together, further characterisation of potential response elements in Atlantic salmon liver fabp3 would reveal more details on its tissue specific regulation. HNF-1 response elements may be present in Atlantic salmon fabp3 as seen for zebrafish (Liu et al., 2003a). HNF-1 has been implied to have a role in peroxisomal, and not mitochondrial β-oxidation (Akiyama et al., 2000). The HNF-1α null mice constructed for this study also exhibited enlarged fatty livers, thus implying that HNF-1 had a function in lipogenesis.

Lipid metabolism in fish has been shown to be more dependent on season than on growth and temperature (Kiessling et al., 1991, Pelletier et al., 1993; Nordgarden et al., 2003). Recently, total β-oxidation capacity of liver, red and white muscle was also found to be more affected by changes in

energy demand, due to life stage, and less by temperature, growth rate and dietary fatty acids (Stubhaug, 2005). In mammals, the muscular FABP3 content is related to the fatty acid oxidation capacity of the tissue (Haunerland and Spener, 2004). Although modest changes in white muscle FABP3 mRNA levels between the different life stages were observed, higher white muscle FABP3 mRNA expression before seawater transfer than at late seawater samplings coincided with changes in total β-oxidation capacity (Paper II) (Stubhaug, 2005). Red muscle FABP3 expression increased between the first sampling in fresh water and the first sampling in sea water followed by a decreased expression level at late sea water samplings. This coincided with life stage variations in red muscle total β-oxidation capacity (Stubhaug, 2005).

Overall, the use of a reference gene strategy when examining tissue samples has received some criticism as tissue samples are known to contain numerous cell types (Bustin and Nolan, 2004; Tricarico et al, 2002). Teleost liver cells contain hepatocytes, endothelial cells, hepatic stellate cells, Kuppner cells and bile ductules cells (Akiyoshi and Inoue, 2004). Atlantic salmon is known to store lipid within myoseptas (adipocytes) in both red- and white muscle (Zhou et al., 1995). We reported variable reference gene expression stability during the production life cycle, especially for white muscle and liver (Appendix, Table 1). Significant variation in expression of housekeeping genes have been shown when using biopsies (Tricario et al., 2002). Consequently, relative mRNA expression measurements may be influenced by tissue complexity. Overall, the study of short-lived mRNA in prolonged dietary studies may need further evaluation as transcriptomes are prone to rapid turnover based on external and internal stimuli (Fan et al., 2002).

The high expression of Atlantic salmon liver FABP10 compared to liver FABP3 (Paper II) may indicate an important function in metabolism of one or several ligands. FABP10 is expressed in the liver of catfish and lungfish (Di Pietro and Santome, 2001; Di Pietro et al., 1996). Lungfish (Di Pietro and Santome, 2001) and catfish (Di Pietro et al., 1997) FABP10s exhibit a broad binding specificity. However, both FABPs have higher affinity for bile salts than fatty acids. Thus, this may indicate a potential role for fish FABP10 in the metabolism of bile salts, as recently suggested for chicken FABP10 (Nichesola et al., 2004; Nolan et al., 2005). This function was also suggested for FABP1 in mice through *in vivo* knock out studies (Martin et al., 2005).

Lungfish FABP10 and mammalian FABP1 both have higher affinities for unsaturated than saturated fatty acids (Di Pietro and Santome, 2001; Hanhoff et al., 2002). The relative affinity of fatty acids for chicken FABP10 and FABP1 are however remarkably different (Beringhelli et al., 2001). Chicken FABP10 binds fatty acids in a similar manner as catfish FABP10. FABP10s from lungfish and catfish binds 18:1n-9 with high affinity (Di Pietro et al., 1996; Di Pietro and Santome, 2001).

It has been shown in the present study that partial dietary rapeseed oil replacement does not change Atlantic salmon FABP10 gene expression *in vivo*.

Recently, two different mammalian type – $FABP_{pm}$ s was identified in rainbow trout white and red muscle, together with the observation that specific inhibition of a presently unidentified FAT/CD36 reduced palmitate uptake in to muscle cells (Richards et al., 2004). Therefore, one may suggest that salmonids resemble mammals in mechanisms for fatty acids uptake. In mice fed fish oils enriched in 22:6n-3 and 20:5n-3, FAT/CD36 was up regulated in liver (Berger et al., 2002). FAT/CD36 was also activated by a short term high fat diet in human skeletal muscle, contrary to $FABP_{pm}$ (Cameron-Smith et al., 2003). Thus, further studies of selective fatty acid uptake in Atlantic salmon should consider the potential complexity in this process.

4.4 Nutrigenomics

Gene candidates differentially expressed by all means of Significance Analysis of Microarrays (SAM) analysis were VLDL receptor (recently renamed vitellogenin receptor), long chain ASC3 (*M. glutinosa*), acyl carrier protein, Δ5 fatty acid desaturase, NFκβ P105 subunit, nucleic acid binding factor, PKC Δ and the cAMP dependent protein kinase, but also enolase, retinol binding protein II and CTP synthase (Paper I). Nevertheless, the fish oil diet appeared to repress expression of genes involved in lipogenesis and lipoprotein synthesis, as well as induce expression of genes involved in gluconeogenesis and β-oxidation, relative to the 75% rapeseed oil diet (Table 4). Thus, confirming microarray screening results from experiments with mice (Berger et al., 2002) and results from earlier studies in mammals (Jump, 2002b). It should be stated that the 75% rapeseed oil diet was selected because the body weight of the fish fed 100% rapeseed oil seemed notably lower than for the fish fed 100% fish oil after 22 weeks. And since the aim of the study was to measure gene expression patterns caused by differences in dietary fatty acid composition and not (suspected) reduced feed intake and growth effects in fish fed 100% rapeseed oil diets (For more details, see Paper I).

No physiological or biochemical measurements within the present study was performed to support results indicating differential gene expression for genes involved in gluconeogenesis. Yet, nutritional regulation of 6 phosphofructo 2 kinase/fructose 2,6 biphosphatase (6PF-2-K/Fru-2,6-P(2)ase) mRNA expression in gilthead sea bream has been observed (Meton et al., 2000).

Atlantic salmon fed diets containing plant oil show significantly increased desaturation and elongation activity compared with fish fed fish oil (Bell, 1997; Tocher et al., 1997; Tocher et al., 2000; Bell et al., 2001; Tocher et al., 2001; Bell et al., 2002; Zheng et al., 2005a). As also suggested in the present study by higher Δ5 fatty acid desaturase expression (Paper I) and accumulation of 20:5n-3 and 22:6n-3 in liver independent of dietary oil source (Torstensen et al., 2004). Altogether, dietary regulation suggests the presence of response elements in genes of Atlantic salmon fatty acid desaturases and

elongase as suggested for mammals (Moon et al., 2001; Berger et al., 2002; Matsuzuka et al., 2002). However, no response elements have yet been identified in promoters of the characterised Atlantic salmon elongase and fatty acid desaturase genes (Hastings et al., 2004; Zheng et al., 2005b).

Recently 22:6n-3, but also 20:5n-3, was reported to be significantly lower in the phospholipids fraction of livers and intestines of Atlantic salmon fed sunflower oil compared to fish oil (Ruyter et al., 2006). This is in line with previous reported results indicating that plant oil diets negatively affect the levels of 22:6n-3 in membranes (Rosjo et al., 1994; Hvattum et al., 2000). No chemical analysis confirms differences in the fatty acid composition of membrane phospholipids in the present *in vivo* study (Paper III). However, fatty acid composition analyses of livers from Atlantic salmon fed dietary plant oils were significantly lower in 22:6n-3 and 20:5n-3 than fish fed fish oil diets after 22 months of feeding. Liver in Atlantic salmon has a high polar lipid /neutral lipid ratio (Torstensen et al., 2004). Thus it is reason to argue that long term feeding using dietary plant oil reduce the degree of membrane unsaturation, or more specific membrane phospholipids levels of 22:6n-3 and 20:5n-3. Furthermore, *in vitro* studies showed a reduced incorporation of 20:5n-3 and 22:6n-3 into phospholipids in hepatocytes when Atlantic salmon had been fed a blend of 75% plant oils (Stubhaug et al., 2005b).

PKC Δ and cAMP dependent protein kinase but also PI specific PLC gene expression was lower in liver of Atlantic salmon fed the 75% rapeseed oil diet (Paper I). Overall, it may be expected that a change in the fatty acid composition of membrane phospholipids could alter the expression of genes involved in signal transduction. This is argued based on studies which indicate that changes in the degree of membrane polyunsaturation alter the endogenous substrates for membrane associated phopholipases (deJonge et al., 1996). This, in turn results in altered enzymatic products, and change in the downstream signalling cascades, as activation of distinct protein kinase C isoenzymes. Overall, studies in mammals indicated that PKC activity increased with increasing degree of PC and phospatidyl ethanolamine (PE) unsaturation (Slater et al., 1994). The molecular species of 18:1n-9-22:6n-3 in PE has been shown to exhibit the largest activation of PKC (Stillwell and Wassall, 2003). Preferential incorporation of 22:6n-3 in to PC and relatively high incorporation in to PE in primary hepatocytes isolated from Atlantic salmon was observed (Stubhaug et al., 2005b). These data are consistent with earlier *in vivo* studies suggesting that 22:6n-3 is incorporated in to PE and PC of rainbow trout liver (Chen and Claeys, 1996).

Finally, lipid rafts have been shown to be enriched in 22:6n-3 (Jump, 2004). Thus, dietary long term effects may change G-protein signalling, which thereby may mediate an indirect negative effect on gene regulation, through the action of cAMP signalling (Jump, 2002a). In conclusion, changes in signal transduction pathways would be expected to change the expression of several nuclear receptors and transcription factors (Paper I).

Furthermore, PI specific PLC activity have been shown be dependent on chain length and degree of lipid unsaturation of membrane phospholipids (Lehto and Sharom, 2002). Long term feeding of a partial plant oil replacement diet (Paper I) may have induced a decrease in molecular species of PI, containing 20:5n-3, as shown for Atlantic salmon kidney when fed dietary soybean oil (Hvattum et al., 2000). This may in turn suggest a decrease in prostaglandin production, as seen earlier (Bell et al., 1993; Tocher et al., 2003). However, as COX-2 from rainbow trout was present on the array but returned no results, no indications can be made in the current study.

Finally, PKC and calcium signalling may also be involved in the process of VLDL secretion. Since it has been shown that hepatic VLDL secretion can be related to changes in hepatocyte levels of calcium and PKC (Bjornsson et al., 1998). Calcium antagonists and prostaglandins was also shown to inhibit VLDL associated TAG secretion in primary cultures (Nossen et al., 1987; Bjornsson et al., 1992). Thus, signal transduction pathways may also participate in regulation of what seems to be a result of decreased VLDL accosiated TAG secretion in Atlantic salmon fed plant oils for 22 months (Paper III).

Dietary fish oil induced expression of genes involved in mitochondrial β-oxidation, cpt-II and ech, in Atlantic salmon (Paper I). This may indicate the presence of PPREs in their promoter regions, as previously identified for these genes in mammals (Mascaro et al., 1999; Barrero et al., 2003). However, no statistical significant differences in total liver β-oxidation capacity between fish fed the 75% rapeseed oil and 100% fish oil diet was observed in the present study (Stubhaug et al., 2005a).

Overall, the fish oil induced expression of several mitochondrial membrane proteins, may indicate differential mitochondrial biogenesis in Atlantic salmon (Paper I), as seen for mice fed dietary 20:5n-3 (Totland et al., 2000). The water temperature at the sampling selected for microarray screening was low (4 °C). Thus, one may expect mitochondrial biogenesis as a response to thermal acclimation, as shown earlier for rainbow trout muscle tissue (Guderley and St-Pierre, 2002). Further, an increased polyunsaturation of phospholipids in the mitochondrial membrane is regarded to be a direct response to cold water temperature (Guderley, 2004). This process may possibly be aggravated by reduced dietary VLCFA. Moreover, the integration of the many processes leading to the formation of functional mitochondria does involve control mechanisms at the level of gene action and interaction (Attardi and Schatz, 1988). Nuclear genes specify all the enzymes in the mitochondrial matrix and encode all the components of the protein and RNA import machinery. Hence, this may suggest dietary induced differential gene transcription of mitochondrial transport proteins. Finally, differential protein expression might also be expected to take place. Since, it previously has been shown that the affinity for membrane proteins correlates to chain length and degree of saturation of mitochondrial membrane lipids (Daum, 1985).

Whether the process of mitochondrial biogenesis in Atlantic salmon involves the PPARγ as seen in mice (Mootha et al., 2003), is presently unknown. It can neither be concluded as to whether concomitant differential PPARγ and catalase expression indicate that liver PPARγ is connected to peroxisomal β-oxidation, as suggested earlier (Ruyter et al., 1997). Earlier studies have shown that 22:6n-3, 20:5n-3 and 20:4n-6 significantly induced expression of sea bream PPARγ *in vitro* (Leaver et al., 2005). Thus, there is a possibility that these dietary fatty acids may influence Atlantic salmon PPARγ expression *in vivo*. However, liver PPARγ was not differentially expressed in Atlantic salmon fed 100% plant oils compared with 100% fish oil (Paper III). This agrees with the observed lack of fatty acid specificity for plaice PPARγs using *in vitro* transactivation assays (Leaver et al., 2005). In general, recent studies indicate that piscine PPARα have a similar activation profile to that of mammals (Leaver et al., 2005). Hence, it may suggest that Atlantic salmon PPARα may be involved in transcriptional regulation of genes involved in liver lipid catabolism, as observed for mammals (Jump, 2002b).

The expression of PPARγ in livers of Atlantic salmon increased prior to sea water transfer followed by a decrease, and then another increase towards the final sampling which correlated with increased liver TAG stores (Paper III). Further studies are needed to elucidate mechanisms behind this correlation. Especially since PPARγ have been implicated to have another function in fish (Leaver et al., 2005) than in rodents (Gavrilova et al., 2003; Wolf, 2004; Inoue et al., 2005; Schadinger et al., 2005), thus in mediating liver TAG homeostasis.

No gene markers for the lipogenic enzymes involved in the production and regeneration of NADPH, nor enzymes catalysing *de novo* fatty acid synthesis, were present on the microarray (Paper I) (Table 4). Nonetheless, FAS, malic enzyme (ME), G6PDH and 6-phosphogluconate dehydrogenase (6PGDH) were assayed by their enzyme activity (Paper III). The dietary regulation of lipogenic enzymatic activity measured in the present study was dependent on enzyme, as seen earlier (Torstensen et al., 2004). Except for ME, the enzyme activity was higher in livers from Atlantic salmon fed 100% plant oil diet compared to the 100% fish oil fed group, although not always statistically significant. The 6PDGH response was the opposite of the response found when Atlantic salmon had been fed rapeseed oil for 42 weeks (Torstensen et al., 2004). Furthermore, FAS activity was actually repressed in Atlantic salmon fed plant oil compared with fish oil after 16 months of feeding (Paper III). This may be consistent with findings in rainbow trout, in which low levels of 18:3n-3 did not stimulate FAS enzymatic activity (Alvarez et al., 2000). High lipid dietary load appears to repress lipogenic activity in several fish species (Sargent et al., 1989) and as a consequence of this dietary responses may be less profound.

Table 4. Designated gene names for amplicons printed on the microarray.
Gene names written in bold were differentially expressed using SAM analysis.

Gene name	
Transport	
Apo A-I-i	FABP10
Apo C-II	FABP4
VLDL receptor	Apo A-I-ii
	Apo B
Mitochondrial markers	
Long chain ACS3 (*M. glutinosa*)	Long-chain ACS1
Translocase of outer mitochondrial membrane	Translocase of outer mito membrane (tom70)
Acyl carrier protein	Mitochondrial solute carrier protein
Outer mitochondrial membrane translocase	Malate dehydrogenase
ECH (*M.mizolepsis*)	ECH (*D. rerio*)
CPT-II	Cytochrome oxidase III
	NADPH-Ubiquionine Oxidoreductase
	Translocase of inner mitochondrial membrane
	ACAT
	Carnitine acyl transferase (*H.hippoglossus*)
	ATP synthase lipid binding protein P3
Peroxisomal markers	
Catalase	Electron transfer flavoprotein
Desaturation and elongation	
Δ5 fatty acid desaturase	Elongase
SCD	
Nuclear receptors and transcription regulators	
PPARγ	PPAR- β1
NFκβ P105 subunit	Nuclear receptor subfamily 0.Group B
Nucleic acid binding factor	SREBP (*D. rerio*)
P300/CBP associated factor (P. americanus)	Transcription factor BTF3
Nuclear receptor subfamily 0. Group B	Cellular nucleic acid binding protein
Transcription factor AP-1 (jun)	
Others	
PKC Δ	Cyclooxygenase 1 (*O. mykiss*)
PKC (cAMPdept)	Cyclooxygenase 2 (*O. mykiss*)
LPL	Triacylglycerol lipase (*P. americanus*)
Bilesalt dependent lipase (P. americanus)	Bilesalt dependent lipase (*S. viviparus*)
PLC PI specific (P. americanus)	PLC (*P. americanus*)
Enolase	Arachidonate-5-lipoxygenase
Retinol binding protein 2	Prostaglandin D synthase (*P. americanus*)
CTP synthase	Fructose bisphosphate aldolase B (*P. americanus*)
Eggshell protein	Succinyl-CoA synthetase
6PF-2-K/Fru-2,6-P(2)ase	Phosphatidylcholine-sterol-acyltransferase
	3-hydroxy-3methylglutaryl coenzyme A reductase
	Phosphoprotein phosphatase
	Phosphatidylinositol 3-kinase
	Glutathione S-transferase
	Phosphatidylserine specific phospholipase A1 alpha
	1-phosphatidylinositol phosphodiesterase (*D. rerio*)

Conclusions

Dietary fatty acids and their influence on genes involved in Atlantic salmon liver lipid metabolism

Partial dietary fish oil replacement using 75% rapeseed oil induced expression of liver Δ5 fatty acid desaturase mRNA in Atlantic salmon both after 22 and 42 weeks of feeding (Paper I). The introductions of high dietary levels of plant oils change the degree of membrane fatty acid unsaturation. This explains the observed reduction of gene expression for several mitochondrial transport proteins, transcription factors, co-activators and signal transducers known to be indirectly influenced by dietary fatty acids in salmon fed plant oil compared with fish oil diets.

Partial dietary rapeseed oil replacement modestly reduced Atlantic salmon liver PPARγ gene transcript levels compared to fish fed 100% fish oil (Paper I). Liver PPARγ expression was not affected by changes in dietary fatty acid composition when Atlantic salmon were fed a diet containing a blend of plant oils compared to fish fed 100% fish oil (Paper III).

mRNA expression of Atlantic salmon apolipoproteins appears to be regulated by dietary fatty acids (Paper I). Yet, complex post translational mechanisms for lipoprotein assembly are believed to occur in Atlantic salmon as in mammals (Paper I and III).

Dietary fatty acid composition and its effect on FABP expression, and life cycle changes in FABP expression

Partial dietary rapeseed oil replacement had no impact on FABP3 and FABP10 gene expression Atlantic salmon liver, nor red or white muscle tissues (Paper II). When examining dietary effects on protein expression, a tendency for decreased muscle FABP3 protein expression with decreasing inclusion of dietary rapeseed oil was observed. Thus, there is reason to believe that LCFAs present in excess in the rapeseed oil diet mediate differential expression of FABP3 in Atlantic salmon red and white muscle tissues.

Overall liver and muscle tissues appeared to express several FABPs possibly linked to different metabolic functions. Relative FABP3 mRNA levels dominated in both red and white muscle tissues. Red muscle appeared to express higher levels of FABP3 than white muscle and heart.

Liver FABP10 mRNA appeared to be expressed at high levels compared to liver FABP3. FABP10 expression seems to be modestly affected by life cycle changes, although mean relative FABP10 expression at the first sampling was higher than mean relative expression at all subsequent samplings. Red muscle FABP3 expression increased between the first sampling (October) in fresh water and the first sampling in sea water (June) followed by a decreased expression level at late sea

water samplings (August and January). Modest changes in liver and white muscle FABP3 mRNA levels between different life stages were observed.

Dietary fatty acids and their effect on transport of lipids and lipoprotein metabolism and PPARγ expression at different life stages

The fatty acid composition of liver and plasma lipoproteins reflected the dietary fatty acid composition. HDL had generally high levels of 22:6n-3 irrespective of dietary fatty acid level (Paper III). Further, liver TAG stores, plasma lipid and LDL levels was significantly affected by dietary fatty acid composition in Atlantic salmon during the long term feeding experiment. High dietary plant oil inclusion increased hepatic TAG stores and decreases plasma lipid levels, possibly through decreased VLDL synthesis. Plasma HDL levels were not affected by dietary plant oil replacement.

The expression of liver PPARγ increased prior to seawater transfer followed by a decrease, and then another increase towards the final sampling (22 months). The last increase was correlated with increased liver TAG stores. Overall, this may indicate that PPARγ has a role in liver lipid metabolism.

Evaluation of recently established methods in nutrigenomics

The experimental design used for microarray screening was chosen to ensure low genetic variation and high statistical power when comparisons were done at a group level (Paper I). The use of several quality controls and a high degree of technical replicates enabled us to evaluate systemic and technical variation. The strategy for labelling and scanning procedures as well as image analysis have been evaluated and found advisable for use in this experimental analysis. The choice of normalisation strategy and statistical analysis were as recommended for use in nutrigenomical surveys using gene focussed arrays. The data set met MIAME standards.

The endogenous controls used for the experimental setups were thoroughly evaluated for use in Atlantic salmon (Paper IV). The present identification of a valid reference, $EF1A_A$ for data normalisation enabled us to assure accurate, reproducible and biologically relevant mRNA quantification (Papers I-III). The use of 18S rRNA assays to calculating relative gene expression of PPARγ and FABPs in muscle verified results using $EF1A_A$ as an endogenous control (Papers II and III). Minimal analytical variation, as evaluated by interplate, intraplate and intrasampling controls, was observed for all Q-PCR assays run on individual fish samples (Papers II and III).

Further Perspectives

Some of the areas that still need further investigation are presented below.

1. *In vitro* studies, both in liver and in other tissues, gives us the opportunity to investigate specific mechanisms in a more refined manner than studies performed in the whole animal. Fatty acids are known to activate transcriptional factors, which when studied in a simplified system could increase the basic knowledge on their activation profiles. It is also important to combine techniques both at the molecular and biochemical level to asses whether metabolic processes infer on gene expression patterns and vice versa.
2. The further full characterisation of several Atlantic salmon lipid metabolic genes, especially those involved in oxidation, lipid biogenesis and fatty acid transport and uptake may be needed. This approach may also be used to identify gene transcript variants, and modes of alternative splicing. But also to reveal presence of response elements in promoter regions to be able to say more about their regulation. The cDNA clones obtained, may further be used as internal controls for absolute quantification studies, applied together with updated methods for RNA quality and quantity determination.
3. Several intracellular fatty acids transport proteins in Atlantic salmon should be studied to reveal whether these proteins have fatty acid specificities, and to further elucidate their potential function. The FABP4 clone, identified through the present study, may be of special interest as its study may reveal details related to lipid storage. Further, details of ligand binding affinity for those FABPs examined here may be studied using *in vitro* replacement essays.
4. To further investigate VLDL assembly in Atlantic salmon, as this information may be used to elucidate the optimal dietary strategy when using plant oils. Furthermore, studies should be designed to investigate mechanisms for the regulation of apolipoprotein synthesis in Atlantic salmon liver cells.
5. Furthermore, the use of intervention based microarray screening may provide results on the dietary effect on fish health and lipid metabolism. Also, as there is no direct correlation between protein and mRNA expression in an organism, there is a clear need for further knowledge on a proteome level. This may clearly establish further knowledge on mechanisms regulating lipid metabolism in Atlantic salmon.
6. Together with cell culture studies, the use of mutant knock out fish models may become an invaluable tool. In combination with gene silencing in cell-cultures, the use of knock out fish will greatly contribute to the generation of detailed molecular pathways showing how nutrients regulate gene and protein expression.

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Appendix

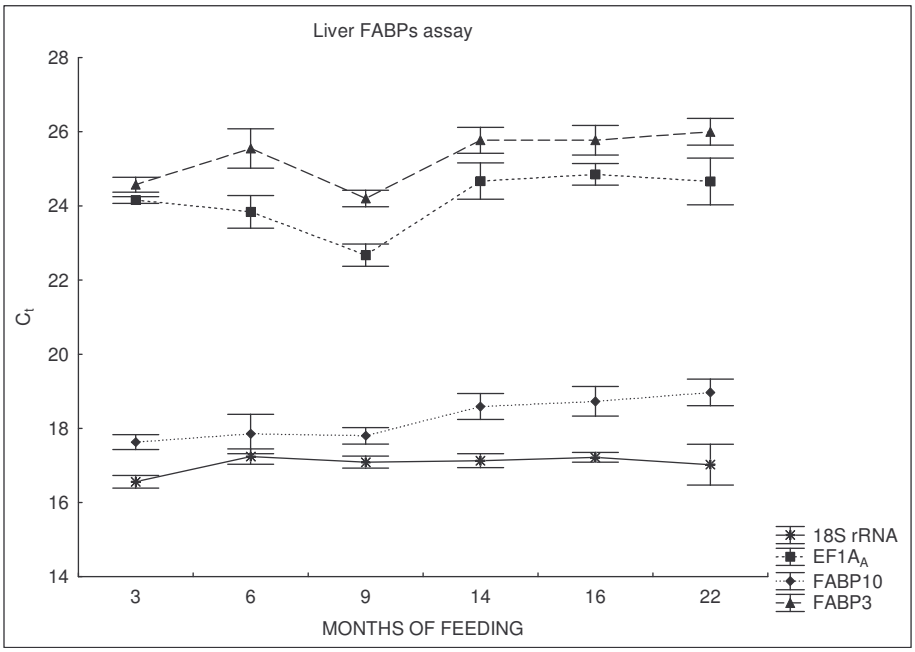

Figure 1 Assay variation during lifecycle for the liver FABP experiment. Through lifecycle the C_t value of endogenous control used for all analysis, $EF1A_A$, varied 4 % in distribution about mean value. Inter and intra plate variation was between 0.5 and 3 % for all assays.

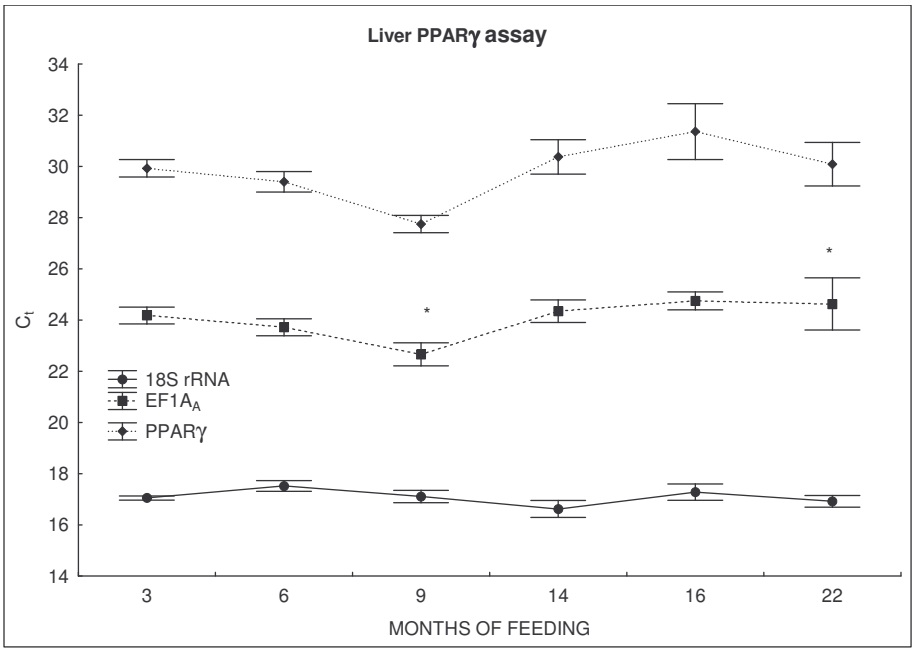

Figure 2 Assay variation during lifecycle for the liver PPARγ experiment. Through lifecycle the C_t value of endogenous control used for all analysis, $EF1A_A$, varied 4 % about mean value. Specifically, the distribution about mean value for $EF1A_A$ was 2% for the first four samplings, and 4% for the last. * denotes that variation in distribution about mean value, where higher than for PPARγ. No intra plate controls were applicable for this experimental setup. Ct values exhibited an inter plate specific variance about mean value between 0.5 and 4% at all life stages for all assays.

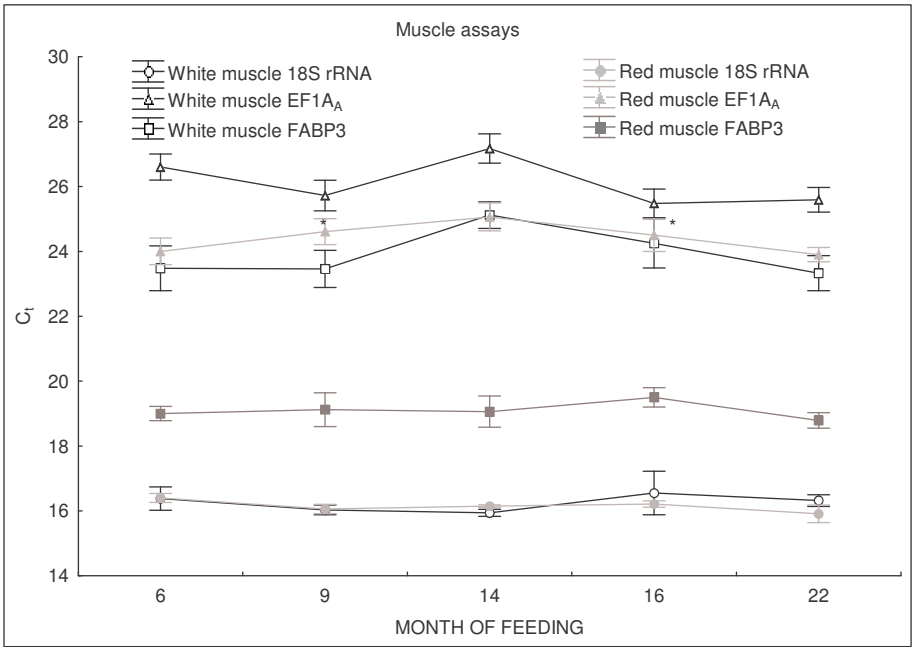

Figure 3. Assay variations during lifecycle for the muscle FABPs experiments. The measured percentage variation about mean value was between 0.5-4 % per sampling for the two endogenous controls. Through lifecycle the C_t value of endogenous control used for all analysis, EF1A$_A$, varied 3 % in white muscle, and 2 % in red muscle. * denotes that variation in distribution about mean value, where higher than for FABP, however only 2% .For all essays, inter- and intra- plate assay variation were less than 4 %.

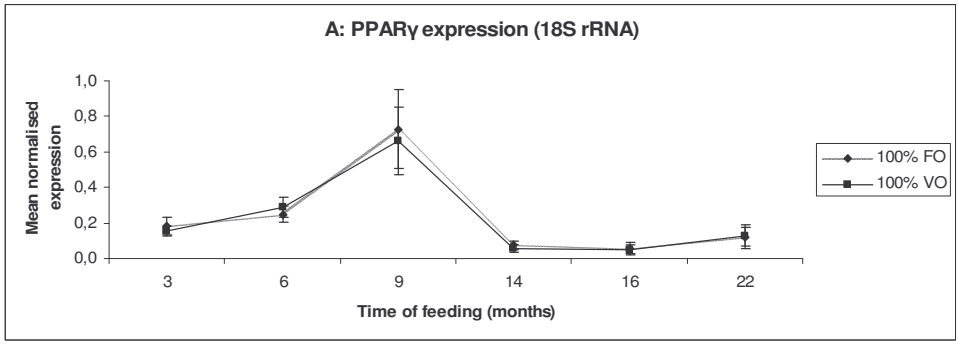

Figure 4. PPARγ expression at different life stages.
Mean normalised expression of PPARγ fed dietary fish oil or plant oil at different life stages, when using 18S rRNA as an endogenous control.

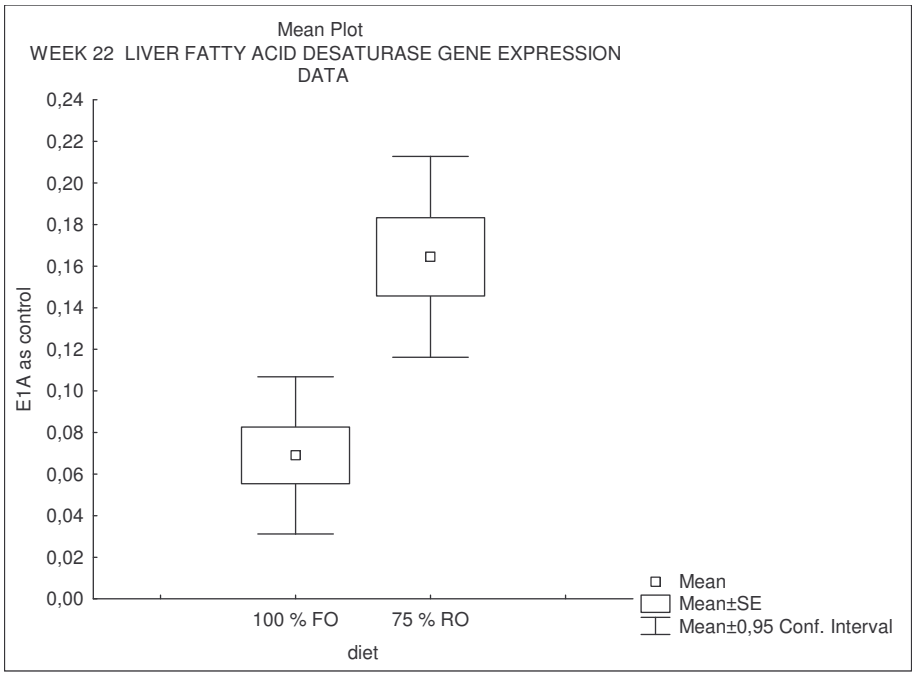

Figure 5; Relative expression of liver Δ 5 fatty acid desaturase after 22 weeks of feeding, normalised to $EF1A_A$. Results from Q-PCR analysis, using liver Δ5 fatty acid desaturase (gi:18958527) specific primers and probe, on individual liver samples from 75% rapeseed oil and control (100 % fish oil) after 22 weeks of feeding. Δ5 fatty acid desaturase gene expression were normalised using $EF1A_A$ in Q gene relative normalisation software, and were presented as mean±SE.MWU and K- S test were used as statistical tests (n=5 and n=6). Δ5 fatty acid desaturase mRNA expression increased significantly in liver of Atlantic salmon fed 75 % rapeseed oil (MWU; (p=0,006170); K-S test (p<.01)), as the average relative expression were 2.4 times higher than in control.

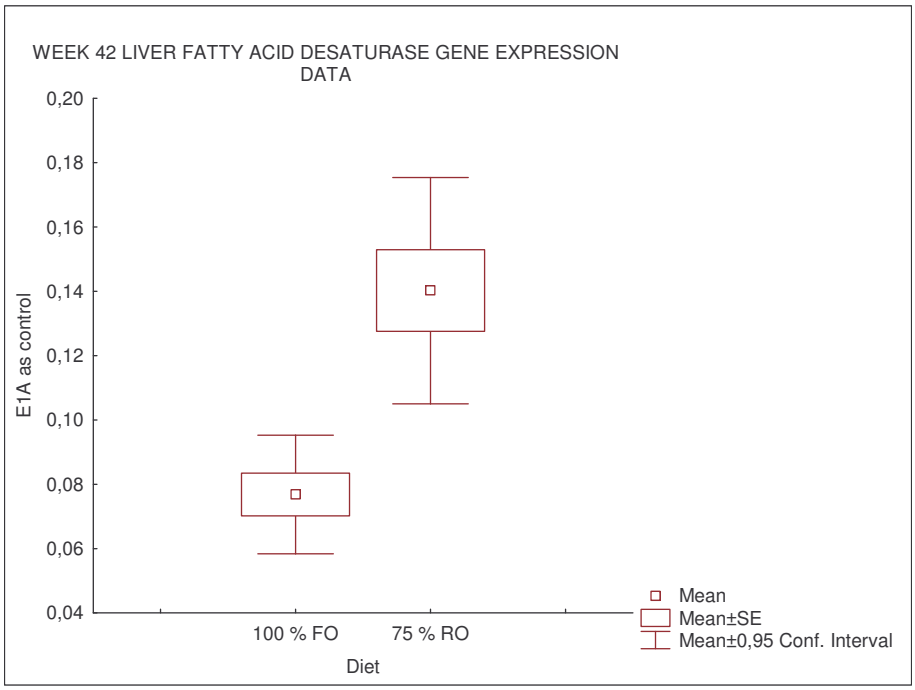

Figure 6; Relative expression of liver Δ 5 fatty acid desaturase after 42 weeks of feeding, normalised to $EF1A_A$. Results from Q-PCR analysis, using liver Δ5 fatty acid desaturase (gi:18958527) specific primers and probe, on individual liver samples from 75% rapeseed oil and control (100 % fish oil) after 42 weeks of feeding. Δ5 fatty acid desaturase gene expression were normalised using $EF1A_A$ in Q gene relative normalisation software, and were presented as mean±SE.MWU and K- S test were used as statistical tests (n=5). Δ5 fatty acid desaturase mRNA expression increased significantly in liver of Atlantic salmon fed 75 % rapeseed oil (MWU; (p=0,016310); K-S test (p<.05)), as the average relative expression were 1.8 times higher than in control.

Table 1. Evaluation of the reference gene expression stability during FABP3 studies (Paper II). The M-value listed was obtained using GeNorm and is indicative of the reference gene expression stability.

Sampling	Reference gene					
	18S rRNA			$EF1A_A$		
	Liver	Red muscle	White muscle	Liver	Red muscle	White muscle
	M-value					
3	0.35			0.6		
6	0.40	0.35	0.5	0.35	0.45	0.7
9	0.30	0.45	0.4	0.35	0.40	0.45
14	0.40	0.40	0.45	0.50	0.40	0.4
16	0.40	0.40	1.0	0.45	0.45	0.75
22	1.0	0.40	0.50	0.80	0.25	0.4
All samplings	0.75	0.50	0.95	0.70	0.55	0.85

Table 2. RT-PCR efficiency as measured by the slope of dilution curves for all gene specific assays analysed in Papers II and III. The absolute value for the slope and its corresponding approximate efficiency in percent is given for all gene specific assays in liver and muscle of Atlantic salmon.

Month of feeding	Tissue								
			Liver			Red muscle		White muscle	
	PPARγ	$EF1A_A$	FABP3	FABP10	$EF1A_A$	FABP3	$EF1A_A$	FABP3	$EF1A_A$
	Slope/ approx efficiency								
3	3.37/99	3.38/97	3.34/99	3.32/100	3.33/99				
6	3.35/99	3.34/99	3.41/97	3.32/100	3.34/99	3.32/100	3.33/99	3.32/100	3.32/100
9	3.39/97	3.37/99	3.33/99	3.34/99	3.34/99	3.33/99	3.36/99	3.52/>95	3.39/97
14	3.34/99	3.34/99	3.38/97	3.39/97	3.39/97	3.43/<95	3.43/<95	3.34/99	3.45/>95
16	3.35/99	3.35/99	3.34/99	3.32/100	3.32/100	3.46/>95	3.46/>95	3.58/>95	3.58/>95
22	3.38/97	3.34/99	3.37/99	3.39/97	3.38/97	3.34/99	3.35/99	3.58/>95	3.56/>95