Transcriptional Regulation in Salmonids with Emphasis on Lipid Metabolism:

In Vitro and *In Vivo* Studies

AnnaLotta Schiller Vestergren
Faculty of Natural Resources and Agricultural Sciences
Department of Food Science
Uppsala

Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2014

Acta Universitatis agriculturae Sueciae
2014:89

Cover: “Kokanee: *In the Moment*”, Shelley Hocknell Zentner, 2010
Printed with permission of the artist (oil on canvas)
(www.shelleyhocknell.com)

ISSN 1652-6880
ISBN (print version) 978-91-576-8126-3
ISBN (electronic version) 978-91-576-8127-0

Print: SLU Service/Repro, Uppsala 2014

Transcriptional Regulation in Salmonids with Emphasis on Lipid Metabolism: *In Vitro* and *In Vivo* Studies

Abstract

Fish is a vital source of valuable omega-3 (n-3) fatty acids (FA) in the human diet. With declining commercial fisheries, aquaculture fish constitute a growing proportion of human consumption. Sustainable development of aquaculture requires that the fish feed used is not solely based on fish meal and oil (FO), but also contains increasing levels of vegetable oil (VO). The replacement of FO with VO influences FA composition in fish tissues by decreasing n-3 long-chain polyunsaturated fatty acids (LCPUFAs) and the nutritional value for humans. Accordingly, the last decade of salmonid research has focused on increasing the amount of n-3 LCPUFAs in fish fed VO diets *e.g*. addition of bioactive compounds. This thesis examined the potential effects of bioactive compounds on lipid metabolism in salmonids.

Genes involved in transcriptional regulation, uptake, β-oxidation, elongation and desaturation were shown to be affected by addition of bioactive compounds in both *in vivo* and *in vitro* experiments. Effects on FA composition were also observed, but no clear effect on docosahexaenoic acid (DHA) content.

The discrepancies between increased gene expression of target genes in the desaturation and elongation cascade and the actual lack of response in FA content of eicosapentaenoic acid and docosahexaenoic acid may be the result of a combination of feedback regulation and post-transcriptional regulation, such as RNA silencing through microRNA (miRNA) repression.

This thesis describes the miRNA transcriptome in liver tissue of Atlantic salmon post-smoltification and the tissue distribution of selected miRNAs in nine different somatic tissues of juvenile Atlantic salmon (*Salmo salar*) for the first time. The results expand the number of known Atlantic salmon miRNAs and provide a framework for understanding the n-3 LCPUFA pathway in Atlantic salmon.

Keywords: β-oxidation, desaturation, elongation, isomiR, microRNA, *Oncorhynchus mykiss, Salmo salar*, transcription factors

Author's address: AnnaLotta Schiller Vestergren, SLU, Department of Food Science, Uppsala BioCentrum, P.O. Box 7051, 750 07 Uppsala, Sweden
E-mail: AnnaLotta.Schiller.Vestergren@ slu.se

Dedication

To my Father, for inspiring me, for giving me my curiosity, my stubbornness and for always believing in me …

In memory of my Mother

Life is made up of small pleasures. Happiness is made up of those tiny successes. The big ones come too infrequently. And if you don't collect all these tiny successes, the big ones don't really mean anything.

Norman Lear, American television producer, born 1922

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Vestergren, A.L.S., Trattner, S., Pan, J., Johnsson, P., Kamal-Eldin, A., Brännäs, E., Moazzami, A.A., Pickova, J. (2013). The effect of combining linseed oil and sesamin on the fatty acid composition in white muscle and on expression of lipid-related genes in white muscle and liver of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture International* 21(4) 843-859.

II Schiller Vestergren, A., Wagner, L., Pickova, J., Rosenlund, G., Kamal-Eldin, A., Trattner, S. (2012). Sesamin modulates gene expression without corresponding effect on fatty acids in Atlantic salmon (*Salmo salar* L.). *Lipids* 47(9), 897-911.

III Schiller Vestergren, A., Trattner, S., Mráz, J. Ruyter, B., Pickova, J. (2011). Fatty acids and gene expression responses to bioactive compounds in Atlantic salmon (*Salmo salar* L.) hepatocytes. *Neuroendocrinology Letters* 32(Suppl. 2), 41-50.

IV Trattner, S., Vestergren A.S. (2013). Tissue distribution of selected microRNA in Atlantic salmon. *European Journal of Lipid Science and Technology* 115(12), 1348-1356.

V Schiller Vestergren, A., Trattner, S., Pickova, J. Hepatic microRNA Profile in mature Atlantic salmon (*Salmo salar* L.) (manuscript submitted).

The contribution of AnnaLotta Schiller Vestergren to the papers included in this thesis was as follows:

I Participated in planning the gene expression studies and experimental work, together with the supervisors. Performed the laboratory work and evaluation and analysis of the gene expression data. Mainly responsible for preparation of the manuscript.

II Participated in planning the gene expression studies and experimental work, together with the supervisors. Performed the laboratory work and evaluation and analysis of the gene expression data. Together with the co-authors, was responsible for preparation and writing of the manuscript.

III Participated in planning the gene expression studies and experimental work, together with the supervisors. Performed the laboratory work and evaluation and analysis of the gene expression data. Mainly responsible for preparation and writing of the manuscript.

IV Responsible for planning the study and experimental work, together with the supervisors. Participated in the collection of samples for RNA extraction and performance of the laboratory work. Performed the evaluation of the next generation sequencing results and was responsible for preparing and writing the manuscript, together with the co-supervisor.

V Responsible for planning the study and experimental work, together with the supervisors. Participated in the collection of samples for RNA extraction and completion of the laboratory work. Performed the evaluation of the next generation sequencing results and was responsible for preparing and writing the manuscript.

Abbreviations

ACO	Acyl-CoA oxidase
ALA	α-linolenic acid (18:3n-3)
ARA	Arachidonic acid (20:4 n-6)
BLAST	Basic local alignment search tool
CD36	Cluster of differentiation 36
cDNA	Complementary DNA
CPT1	Carnitine palmitoyltransferase 1
Δ5FAD	Delta 5 fatty acid desaturase
Δ6FAD	Delta 6 fatty acid desaturase
DHA	Docosahexaenoic acid (22:6 n-3)
DPA	Docosapentaenoic acid (22:5n-3)
EF1α	Elongation factor 1a
EFA	Essential fatty acid
ELOVL	Elongase of very long chain fatty acids (four different transcripts)
EPA	Eicosapentaenoic acid (20:5n-3)
ER	Endoplasmic reticulum
ES	Episesamin
ETiF	Eukaryotic translation initiation factor 3
FA	Fatty acid
FO	Fish oil
G	Genistein
LA	Linoleic acid (18:2n-6)
LCPUFA	Long chain polyunsaturated fatty acids
LDL	Low-density lipoprotein
LO	Linseed oil
LPA	Lipoic acid
LXRα	Liver X receptor α
miRNA	MicroRNA
MUFA	Monounsaturated fatty acids

n-3	Omega-3
n-6	Omega-6
n-6/n-3	n-6/n-3 PUFA
NUOR	NADH-ubiquinone oxidoreductase
PL	Phospholipid
PPAR	Peroxisome proliferators-activated receptor
PPRE	Peroxisome proliferator response element
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
PUFA	Polyunsaturated fatty acid
RISC	RNA induced silencing complex
RPL2	RNA polymerase II polypeptide
RXR	Retinoid-X-receptor
S	Sesamin
SAFA	Saturated fatty acid
SD	Standard deviation
SR-B1	Scavenger receptor class BI
SREBP	Sterol regulatory element-binding protein
TAG	Triacylglycerol
TTA	Tetradecylthioacetic acid
UTR	Untranslated region
VO	Vegetable oil

1 Introduction

Gene expression deals with differences in expression of genes, as a response to external and/or internal stimuli, in which daily environmental influences and feeding habits play a crucial role. Atlantic salmon (*Salmo salar* L) and rainbow trout (*Oncorhynchus mykiss*) are among the most popular fish species in the Western diet and their content of n-3 long-chain polyunsaturated fatty acids (LCPUFA) is of great importance for human health.

In this thesis, the underlying molecular regulation mechanisms of n-3 LCPUFA biosynthesis were studied, with the emphasis on post-transcriptional regulation in salmonids. The aims were to contribute to future optimization of the content of n-3 LCPUFA in salmonids through enhanced activity of the desaturation and elongation pathway and to enable sustainable use of vegetable oils (VO) in aquaculture while maintaining the beneficial lipid composition for human consumption.

1.1 Lipid metabolism in salmonids

Lipids and fatty acids (FA), together with proteins, are the major macronutrients in the diet of salmonids (Leaver *et al.*, 2008a; Tocher, 2003; Torstensen *et al.*, 2000). They act as a source of essential FAs and energy, as well as functioning as a carrier of other lipid-soluble compounds such vitamins and pigments.

Lipids are a diverse group of compounds that are classified depending on their insolubility in water. There are basically two classes of lipids – neutral and polar lipids. Neutral lipids primarily include triacylglycerols (TAG), diacylglycerols, monoacylglycerols and sterols, which mostly serve as storage and sources of energy. Neutral lipid composition, particularly TAG composition, reflects changes made in dietary FA composition (Torstensen *et al.*, 2001; Lie *et al.*, 1988). Polar lipids are mainly phospholipids (PL), which

are predominantly incorporated into membrane structures (Tocher, 1990; Tocher & Dick, 1990a). Phospholipids to some degree, also reflect the polyunsaturated fatty acid (PUFA) composition of the diet, but shorter dietary PUFA, such as α-linolenic acid (18:3n-3, ALA) and linoleic acid (18:2n-6, LA), are normally elongated and desaturated prior to incorporation into PL.

1.1.1 Polyunsaturated fatty acids

In PUFA, the position of the first double bond is important for the nomenclature. If the first double bond is present next to the third carbon atom from the methyl end of the carbon chain, the FA is classified as an n-3 FA, while if it is next to the sixth carbon atom, the FA is classified as an n-6 FA.

Two PUFAs are essential in salmonids as in all vertebrates, namely ALA and LA. In salmonids, dietary LA can be metabolized to its longer chain derivate, arachidonic acid (ARA, 20:4 n-6), and ALA can be converted to eicosapentaenoic acid (EPA, 20:5n-3) and further on to docosahexaenoic acid (DHA, 22:6 n-3) through a series of desaturation and elongation steps. It has been shown that growth can be significantly improved in salmonids by inclusion of dietary n-3 LCPUFAs (reviewed in Tocher, 2010; Ruyter *et al.*, 2000).

1.2 Aquaculture

Aquaculture is one of the fastest-growing animal food-producing sectors and, in the next decade, the total production from both capture and aquaculture is expected to exceed that of beef, pork and poultry (FAO, 2012). Roughly 50% of fish for human consumption are now farmed and this portion will continue to grow (FAO, 2012; FAO, 2010). However, if aquaculture is to continue to expand, the availability of sustainable and quality aquafeeds must increase.

Aquafeeds are generally used for feeding omnivorous fishes, carnivorous fishes and crustacean species. Fish living primarily on phytoplankton do not require any other forms of feeding and only use limited amounts of commercial aquafeed (FAO, 2006).

Aquafeeds have traditionally been based on fish meal and fish oil (FO) with high levels of n-3 LCPUFAs from pelagic fisheries (Regost *et al.*, 2004). The amount of FO consumed in the aquaculture sector has grown threefold since 1992 and today 90% of all FO produced goes to aquafeeds (FAO, 2012; Tacon, 2005). Accordingly, the development of aquaculture has been heavily dependent on the availability of FO. The global production of fishmeal and FO has remained stable or even shown a decline while aquaculture production has increased. It is therefore important to find a viable alternative to FO as the lipid

source in fish feed. Substantial efforts have been made to find alternative sustainable solutions, of which the use of vegetable oils (VO) as a replacement for FO in aquafeed formulations has been shown to be an accessible alternative (reviewed in Nasopoulou & Zabetakis, 2012; Tacon & Metian, 2008; Powell, 2003).

1.2.1 Effects of vegetable oils in salmonid culture

Studies of Atlantic salmon have shown that VO can replace FO in fish feed to a large extent without compromising growth, flesh astaxanthin levels or mortality rate (Sanden *et al.*, 2011; Torstensen *et al.*, 2005b; Bell *et al.*, 2003a; Rosenlund, 2001; Ruyter & Thomassen, 1999). The taste of salmon is known to vary depending on the composition of the salmon feed. Sensory analyses have shown that fillets from salmon fed a mixture of VO have roughly the same taste and aroma as fillets from salmon fed a diet including FO, but have a somewhat less characteristic marine taste (Sanden *et al.*, 2011; Torstensen *et al.*, 2005b). Similar results have been reported for rainbow trout, in which fillet pigmentation is highly affected by different VO dietary inclusion levels and shelf-life of the refrigerated product increases (Turchini *et al.*, 2013b).

However, the tissue FA composition of fish has been shown to be highly sensitive to differences in diet lipid composition (Turchini *et al.*, 2013b; Torstensen *et al.*, 2009; Torstensen *et al.*, 2004; Torstensen *et al.*, 2000; Tocher & Dick, 1990a). The most severe effect from a human health perspective is the decreased nutritional value as a result of reduced fillet content of n-3 LCPUFA, *e.g*. EPA and DHA (Rosenlund, 2001). Major scientific efforts to find alternatives to FO and still maintain as high an n-3 LCPUFA content as possible in fish fillet have been undertaken (Turchini *et al.*, 2013a; Turchini *et al.*, 2013b; Mráz *et al.*, 2012; Ruxton *et al.*, 2005; Robin *et al.*, 2003; Ackman, 1996).

Salmonids have the capacity to convert ALA to EPA and DHA, a capacity which is stimulated in fish fed VO compared with fish fed FO (Kjær *et al*., 2008; Buzzi *et al.*, 1996). According to Ruyter *et al.* (2000), the capacity for conversion of ALA to EPA and DHA is 20% lower in hepatocytes from Atlantic salmon fed FO than from Atlantic salmon fed linseed oil. Despite being quite efficient at converting ALA to EPA and DHA, in Atlantic salmon (Torstensen *et al.*, 2004; Bell *et al.*, 2003b) and rainbow trout (Thanuthong *et al.*, 2011; Turchini *et al.*, 2007b), the change from FO to VO formulation in aquafeed causes a total net reduction in fish body content of EPA and DHA.

1.2.2 Strategies to restore LCPUFA levels in salmonids

Different strategies have been applied to achieve satisfactory nutritional levels of EPA and DHA in farmed fish fillets after reducing the amount of FO used in fish feed.

- In order to ensure that the levels of saturated FAs (SAFAs), monounsaturated fatty acids (MUFAs) and PUFAs in VO-based feed are at roughly the same level as in FO-based feed, a mixture of linseed oil, rapeseed oil and palm oil can be used (instead of single VO) (Torstensen *et al.*, 2005b).
- By introducing a finishing diet period immediately preceding slaughter, the EPA and DHA content in the muscle can be increased (Mráz, 2012; Turchini *et al.*, 2007a; Rosenlund, 2001).
- Addition of bioactive compounds has been shown to promote the ability of fish to convert ALA to EPA and DHA (Trattner *et al.*, 2008a; Kennedy *et al.*, 2007a; Kleveland *et al.*, 2006a).
- Selective breeding for heritable traits associated with EPA and DHA composition has been evaluated (Berge *et al.*, 2014; Berge *et al.*, 2013; Leaver *et al.*, 2011; Morais *et al.*, 2011; Olesen *et al.*, 2003; Gjedrem, 1997).

The focus in this thesis is on use of bioactive compounds as supplements to VO-based diets to possibly counteract the observed decrease in n-3 LCPUFA in fish tissues. In addition, transcriptional and post-transcriptional regulation of lipid metabolism were studied. As a first step, a screening of microRNA (miRNAs) in Atlantic salmon was carried out. The overall objective of this part of the work was to find an approach to interact with the lipid metabolism by manipulating the miRNAs, similarly to other therapeutics targeting miRNAs. By determining the miRNA status in Atlantic salmon, identifying their functions and finding an approach to manipulate these miRNAs, the aim was to open up for new possibilities for sustainable production of fish rich in n-3 LCPUFA.

1.3 Bioactive compounds

Bioactive compounds are naturally occurring constituents present in small amounts in plant products and lipid-rich foods that provide health benefits beyond the basic nutritional value of the product (Kris-Etherton *et al*., 2002). Many of these substances affect lipid metabolism and/or exhibit antioxidative properties. A number of bioactive substances, *e.g*. sesamin, episesamin (ES),

tetradecylthioacetic acid (TTA) and lipoic acid (LPA), have been reported to affect lipid metabolism and/or FA composition in rainbow trout, Atlantic salmon and pacu (*Piaractus mesopotamicus*) (Trattner *et al.*, 2008a; Moya-Falcón *et al.*, 2004).

1.3.1 Sesamin

Sesamin is an oil-soluble lignan found in sesame seed and oil. During the refining process of sesame oil, episesamin is formed from sesamin. Sesame lignans are well studied in mammals and are reported to have significant effects on lipid metabolism. They have been shown to increase β-oxidation (Jeng & Hou, 2005; Ashakumary *et al.*, 1999), affect elongation and desaturation of FAs (Fujiyama-Fujiwara *et al.*, 1995) and lower serum levels of triacylglycerols and cholesterol (Jeng & Hou, 2005; Kushiro *et al.*, 2002; Kamal-Eldin *et al.*, 2000). Ide *et al.* (2001) also showed that sesamin can decrease the hepatic activity and messenger RNA (mRNA) expression of enzymes involved in FA synthesis. The lipid-modulating effects of sesamin may be mediated via the activation of peroxisome proliferator-activated receptors (PPARs) and the inhibition of sterol regulatory element-binding protein-1 (SREBP-1) (Ide *et al.*, 2004; Ide *et al.*, 2003; Ashakumary *et al.*, 1999).

Sesamin/episesamin and TTA have been shown to increase β-oxidation products and the levels of DHA in rainbow trout muscle (Trattner *et al.*, 2008a) and to affect the expression of delta-5 fatty acid desaturase (*Δ5FAD*) and delta-6 fatty acid desaturase (*Δ6FAD*), carnitine palmitoyl transferase 1 (*CPT1*), *PPARα* and *PPARγ* in Atlantic salmon hepatocytes (Trattner *et al.*, 2008b).

Kushiro *et al.* (2002) showed that sesamin is metabolized faster than episesamin in rat liver and that episesamin is more effective than sesamin in increasing the activity and gene expression of FA oxidation enzymes. Yasuda *et al*. (2012) showed a difference in metabolism of sesamin and episesamin in human liver microsomes, resulting in different biological effects.

1.3.2 Lipoic acid

Another bioactive compound of interest for fish feed is LPA. It is a potent antioxidant with one lipophilic and one lipophobic part (Kozlov *et al.*, 1999; Lykkesfeldt *et al.*, 1998). LPA is synthesized in the mitochondria by lipoic acid synthase as part of the *de novo* synthesis of FA (Hiltunen *et al.*, 2010; Morikawa *et al.*, 2001; Wada *et al.*, 1997) and has been shown to be active in cellular energy metabolism (reviewed in Bast & Haenen, 2003).

Huong & Ide (2008) and Yi & Maeda (2006) demonstrated that LPA can decrease the PL and TAG concentrations and the cholesterol concentration in

serum and liver of rodents. A dose-dependent decrease in both gene expression and activity of the enzymes involved in FA synthesis and in the elongation and desaturation cascade was observed in that study. LPA has been shown to affect the FA composition of fish muscle towards higher levels of EPA (Trattner *et al.*, 2007).

Feeding a combination of sesamin and LPA to rats has been shown to decrease the activity and mRNA levels of hepatic lipogenic enzymes in a synergistic fashion. The strong effect of sesamin on hepatic FA oxidation enzymes is reported to be antagonized by LPA (Ide *et al.*, 2012). The latter study showed that even though sesamin and LPA had a very similar effect on both mRNA level and activity of lipogenic enzymes, only sesamin had any effect on the transcription factor *SREBP-1c*.

1.3.3 Genistein

Genistein is a phytoestrogen formed after hydrolysis of the isoflavone genistin found abundantly in soybean. Genistein is known to exhibit antioxidative and hormone-like effects (Yuan *et al.*, 2007). Genistein inhibits the oxidation of low-density lipoprotein (LDL) in human blood (Safari & Sheikh, 2003) and studies on mice have shown that hepatic FA synthase, β-oxidation and *CPT1* activities are significantly lower after genistein supplementation (Ae Park *et al.*, 2006). However, genistein has also been shown to act as a potential ligand for *PPARα*, enhancing the expression of genes involved in lipid catabolism through activation of *CPT1* in human cell lines, a finding which is somewhat contradictory (Kim *et al.*, 2004). Other studies have shown that genistein treatment has dose-dependent toxic effects on zebrafish embryos (Kim *et al.*, 2009; Sassi-Messai *et al.*, 2009).

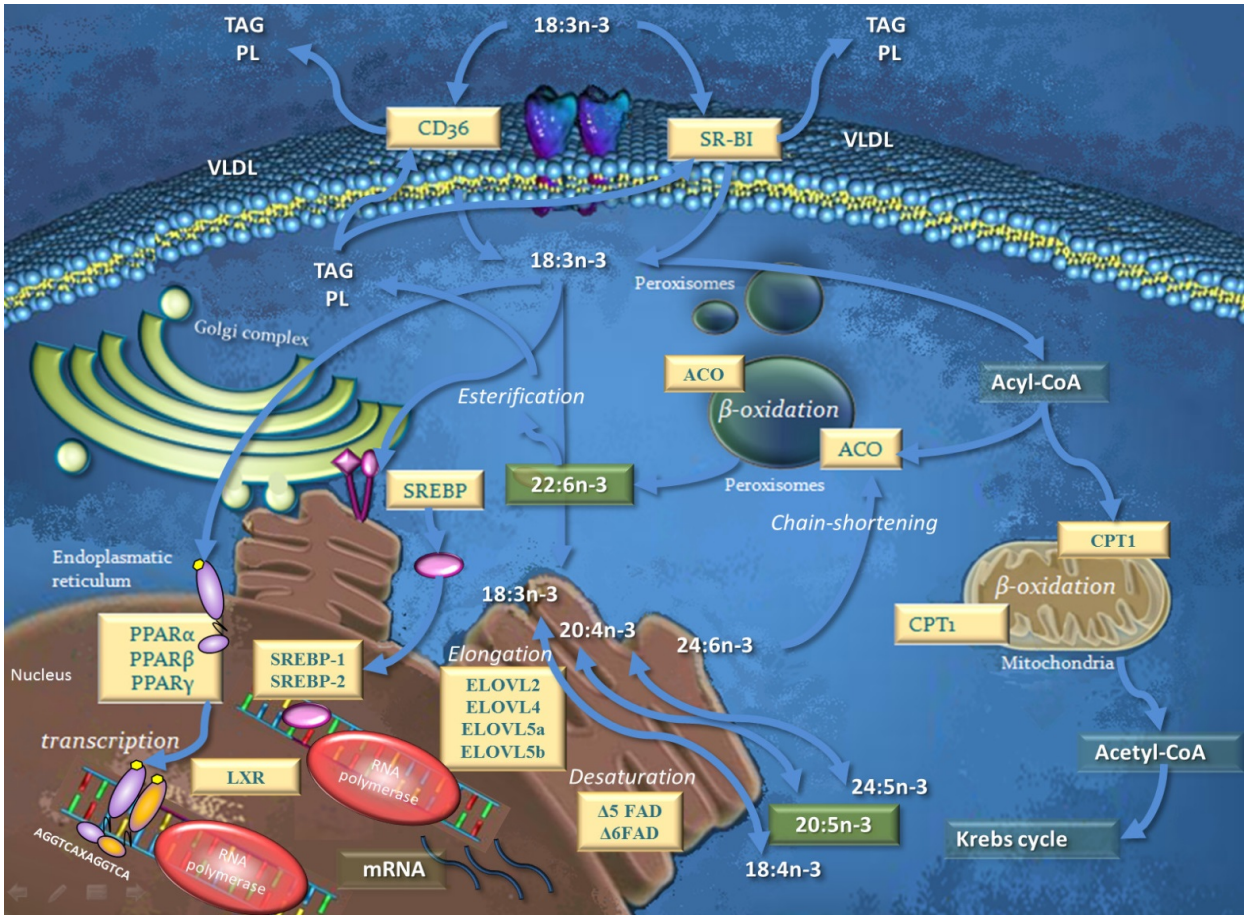


Figure 1. Schematic drawing of the lipid metabolism and potential cellular outcome of ALA (18:3n-3). The different genes evaluated in this thesis are marked with yellow boxes and the metabolic processes are written in italics. For gene abbreviations, see *Table 4*. PL = Phospholipids; TAG = triacylglycerols, VLDL = very low-density lipoprotein. (Modified after Trattner *et al.*, 2008c).

2 Lipid metabolism

Lipid metabolism consists of a mixture of metabolic processes that generate energy and primary metabolites from FAs and processes that create biologically important molecules (EPA and DHA) from essential FAs (*Figure 1*). In turn, dietary FAs act as regulators of gene transcription and consequently steer enzyme activity of the same processes (Jump & Clarke, 1999; Hesketh *et al*., 1998).

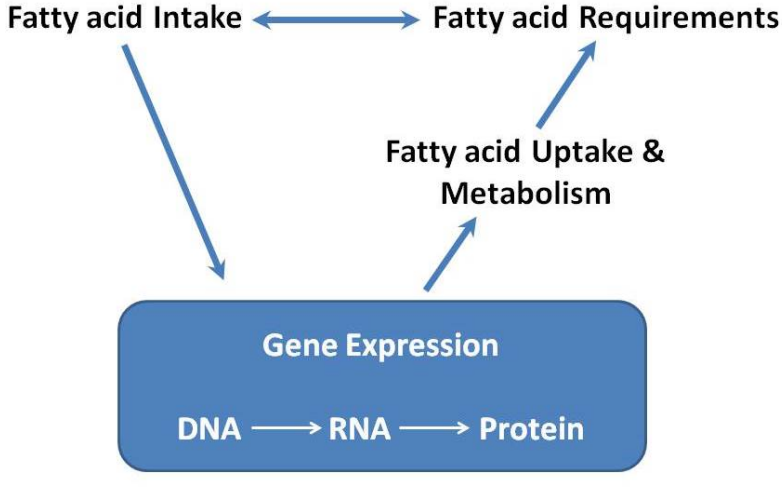


Figure 2. Inter-relationships between fatty acid homeostasis and gene expression (Modified after Hesketh *et al.*, 1998).

The regulation of lipid homeostasis in salmonids (*Figure 2*) is a complex balance between *e.g*. lipid uptake, transport, storage, energy utilization and biosynthesis. Each single process needs to be controlled independently and also in conjunction with other processes (Tocher, 2003). Dietary FAs may alter the

amount of functional protein expressed in these processes through a range of transcriptional, post-transcriptional and post-translational mechanisms. FAs and their metabolites can influence gene expression regulation either indirectly by activating different transcription factors in the cytoplasm or directly by entering the nucleus by themselves or in association with ligand-activated transcription factors.

2.1 Uptake & transport

The liver is the crossing point for the exogenous and endogenous transport of lipids. The dominant mechanism by which lipids are taken up into cells is through binding of lipoproteins to cell surface trans-membrane lipoprotein receptors. The expression levels of genes encoding proteins involved in the uptake and intracellular transport of FAs in Atlantic salmon are affected by the replacement of dietary FO with VO (Torstensen *et al.*, 2009; Stubhaug *et al.*, 2005a).

2.1.1 Scavenger receptor class B, type I

High-density lipoprotein receptor scavenger receptor class B, type 1 (*SR-B1*) is one of the most important cell surface lipoprotein receptors. The expression of *SR-BI* is controlled by a complex matrix of hormones, FAs and other nutrients. In turn *SR-BI* is involved in lipid uptake from the diet and is responsible for regulating lipid levels (Malerød *et al.*, 2002).

Kleveland *et al.* (2006b) cloned and characterized SR-BI in Atlantic salmon. Several transcription factors such as *SREBP*, Liver X receptor (*LXR*) (reviewed in Rhainds & Brissette, 2004), hepatocyte nuclear factor 4α (*HNF4α*), *PPARα* and *PPARγ* (Malerød *et al*., 2003) have been shown to be involved in the regulation of *SR-BI* expression in humans and rodents.

2.1.2 CD36

CD36 is a free FA transporter and a membrane receptor capable of taking up modified forms of LDL and FAs. *CD36* can also bind HDL. *PPARγ* is a positive regulator of *CD36* in rodents. Actually CD36 is a shared target of *LXR*, pregnane X receptor (*PXR*) and *PPARγ* (Zhou *et al.*, 2008; Zhou *et al.*, 2006). Gene expression levels of *CD36* are affected by changes in diet formulation. Significant downregulation in salmon white muscle has been seen after feeding a VO-based diet compared with a FO-based diet. This indicates that VO lower FA uptake in fish compared with FO (Torstensen *et al.*, 2009).

2.2 Desaturation and elongation of LCPUFA

The sequential chain of desaturation and elongation steps converting n-6 and n-3 FA precursors into LCPUFAs has been well described for both rainbow trout and Atlantic salmon (Tocher, 2003; Tocher *et al.*, 1989) and is suggested in earlier studies to involve the same two enzyme families – elongases of very long fatty acids (*ELOVL*s) and the fatty acyl desaturases (*FAD*) (Ruxton *et al.*, 2005; Cook & McMaster, 2002). Enzyme affinity, especially that of FAD, is higher for n-3 FA than for n-6 FA (Tocher & Dick, 1990a; Tocher & Sargent, 1990; Tocher *et al.*, 1989) and the relative activity in each of the steps in the reaction cascade in *Figure 3* decreases with increased chain length (Tocher, 2003). The majority of LCPUFA synthesis takes place in the endoplasmic reticulum (ER), with only the last chain-shortening step taking place in the peroxisomes (Sprecher, 2000).

2.2.1 ELOVL and FAD

Δ6FAD and *Δ5FAD* are actively expressed in both rainbow trout (Buzzi *et al.*, 1997; Buzzi *et al.*, 1996; Tocher *et al.*, 1989) and Atlantic salmon (Tocher & Dick, 1990b), enabling both species to elongate and desaturate ALA and LA to DHA and ARA, respectively. The genes for *Δ5FAD* (Hastings *et al.*, 2004) and *Δ6FAD* (Zheng *et al.*, 2005a) have been cloned from Atlantic salmon and functionally characterized. Four genes have been identified as coding for Δ5 and Δ6 desaturase in Atlantic salmon (Monroig *et al.*, 2010).

Buzzi *et al.* (1997) and (Tocher, 1990) showed that the formation of DHA in rainbow trout and Atlantic salmon, respectively, does not primarily involve Δ4 desaturation of DPA (22:5n-3), but rather proceeds through a final round of elongation and desaturation followed by peroxisomal β-oxidation (the Sprecher pathway) (Step I in *Figure 3*). However, this paradigm has recently been revised and it is now clear that another pathway exists for DHA synthesis from EPA, involving a Δ4 desaturation of DPA (reviewed in Monroig *et al.*, 2013; Li *et al.*, 2010b) (Step II in *Figure 3*). Morais *et al.* (2012a) did clone and functionally characterize *Δ4FAD* from Senegalese sole (*Solea senegalensis*).

However, Tu *et al.* (2012) demonstrated that there is an alternative n-3 LCPUFA elongation pathway, including a Δ8 desaturase that via elongases forms 20:3n-3 from ALA and then a Δ6/Δ8 desaturase to form 20:4n-3 in barramundi (*Lates calcarifer*), bypassing the first *Δ6FAD* desaturation step forming 20:3n-3. After desaturation of 20:3n-3, the pathway continues with the usual *Δ5FAD* desaturation (Step III in *Figure 3*). Monroig *et al.* (2011) showed the ability for Δ8 desaturation (capability to introduce double bonds into 20:3n-3 at the Δ8 position) in Atlantic salmon and rainbow trout, among other fish species.

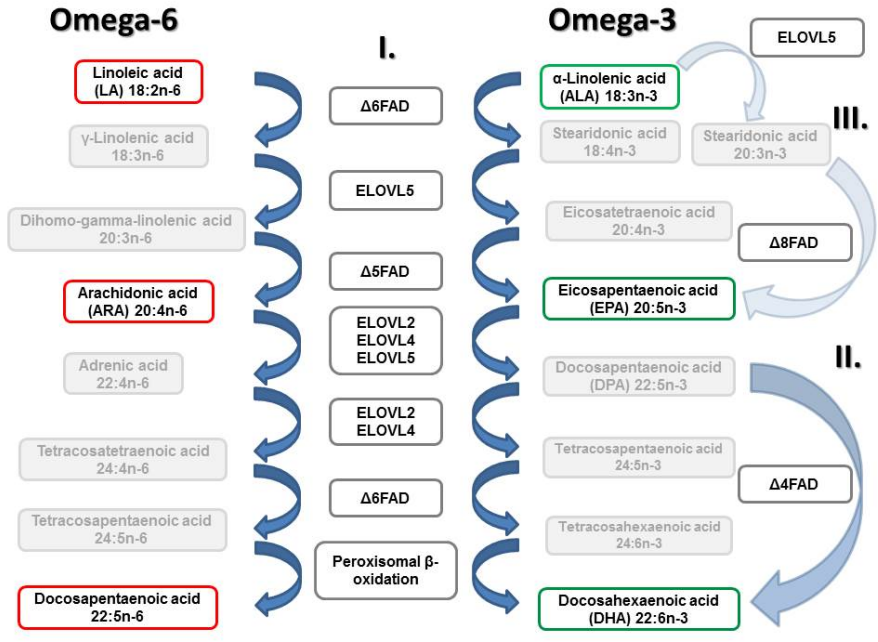


Figure 3. Elongation and desaturation pathway of n-6 and n-3 fatty acids. (Adapted from (Carmona-Antoñanzas *et al.*, 2011; Monroig *et al.*, 2011; Sprecher, 2000; Voss *et al.*, 1991); modified after (Trattner, 2009).

ELOVL5a (Hastings *et al.*, 2004), *ELOVL5b* and *ELOVL2* (Morais *et al.*, 2009) have been cloned and functionally characterized in Atlantic salmon. Atlantic salmon *ELOVL5a* and *ELOVL5b* were found to elongate C18 and C20 PUFA, and *ELOVL2* to elongate C20 and C22 PUFA. All three *ELOVL*s showed predominant expression in the intestine and liver, followed by the brain. Elongase expression was shown to be under differential nutritional regulation, with transcript levels of *ELOVL5b* and *ELOVL2*, but not of *ELOVL5a*, significantly increased in liver of salmon fed VO compared with salmon fed FO.

ELOVL4 has been shown to be a critical enzyme in the biosynthesis of both saturated and polyunsaturated very long-chain fatty acids having chains ranging from C26 to C40. *ELOVL4* has been isolated and functionally characterized in Atlantic salmon. *ELOVL4* has been shown to elongate C20 and C22 PUFA and to be able to convert EPA and DPA to 24:5n-3, an intermediate substrate for DHA biosynthesis (Carmona-Antoñanzas *et al.*, 2011). In terms of tissue distribution, *ELOVL4* mRNA transcripts are most abundant in eye, brain and testes.

The activity of the desaturation/elongation pathway is inhibited in salmonids having an adequate supply of n-3 LCPUFA in their natural diet. It has been shown that the desaturation and elongation cascade is under feedback regulation affected by the concentration of end products (EPA and DHA), as well as the availability of substrate FAs (LA and ALA) (Tocher *et al.*, 2003a). The desaturation and elongation of ALA have been shown to increase when salmonids are fed a diet containing VO rather than FO (Bell *et al.*, 2001; Tocher *et al.*, 2001). Several studies have demonstrated that the expression of Δ6FAD mRNA is lower in salmon fed FO compared with VO (Leaver *et al.*, 2008b; Zheng *et al.*, 2005a; Zheng *et al.*, 2005b). Furthermore, when dietary FO was replaced with VO, LCPUFA biosynthesis was shown to be regulated in a genotype-specific manner. In lean fish compared with fatty fish, Δ5FAD, Δ6FAD and ELOVL2 were upregulated, which was also reflected in the liver FA composition (Morais *et al.*, 2012b).

2.3 β-oxidation

The β-oxidation of FAs takes place in both mitochondria and peroxisomes, but the mitochondrial β-oxidation is quantitatively more important and can use a wide range of different FAs as substrate (Henderson, 1996). Mitochondria and peroxisome β-oxidation pathways have been shown to exhibit broad chain length specificity for different FAs (Henderson & Sargent, 1985). β-oxidation occurs in peroxisomes for FA chains that are too long to be processed directly in the mitochondria, but peroxisomal β-oxidation ceases at octanyl-CoA. Very long chain FAs (greater than C22) undergoes initial oxidation in peroxisomes, followed by final oxidation in mitochondria. The expression levels of genes encoding proteins involved in the β-oxidation of FAs in Atlantic salmon, *e.g.* acyl-CoA oxidase (*ACO*), *CPT1* and *CPT-2*, have been shown to be negatively affected by the replacement of dietary FO with VO (Torstensen *et al.*, 2009).

2.3.1 Carnitine palmitoyl transferase 1

For mitochondrial β-oxidation to occur, FAs need to reach the mitochondrial inner membrane space. *CPT1* is a mitochondrial enzyme positioned in the outer mitochondrial membrane that is responsible for the formation of acyl carnitines by catalyzing the transfer of the acyl group of a long-chain fatty acyl-CoA from coenzyme A. This allows for subsequent movement of the acyl carnitine from the cytosol into the inner membrane space of mitochondria.

2.3.2 Acyl-CoA oxidase

Peroxisomal β-oxidation requires a specific set of enzymes. Peroxisomal acyl-CoA oxidase (*ACO*) is the first and rate-limiting enzyme of peroxisomal β-oxidation (Kleveland *et al.*, 2006a; Ruyter *et al.*, 1997; Varanasi *et al.*, 1996). β-oxidation in the peroxisome starts with the use of ACO for transport of the activated acyl group into the peroxisome (Pagot & Belin, 1996).

3 Gene regulation of lipid metabolism

The regulation of all processes from production through activation and deactivation to degradation of proteins involves regulation of gene expression, directly or indirectly, at one point or another. The regulation of gene expression can occur at any step ranging from DNA-RNA transcription to post-translational modification of protein. During transcription, the chromatin arrangement and changes in DNA structure influence accessibility of promoter sequences and activation and activity of transcription factors, and determine whether genes are transcribed (Gräff *et al.*, 2011; Schneider & Grosschedl, 2007). There are three possible outcomes for fully transcribed mRNA in the cytoplasm: it may be translated to produce protein, it may be immobilized or inactivated and/or it may be degraded.

3.1 Genome duplication

The salmonid genome is complex due to an additional genome duplication that is believed to have occurred 96 million years ago (Berthelot *et al.*, 2014; Ohno, 1999; Allendorf & Utter, 1976). Tetraploidizations, or genome duplications, are important evolutionary events which were responsible for large increases in genome size and diversity early in vertebrate evolution (Ohno *et al*., 1968).

In salmonids, around half of all protein coding loci have remained as functioning duplicates, but the diploidization process has not come to an end (Berthelot *et al.*, 2014; Hordvik, 1998; Young *et al.*, 1998; Allendorf, 1978; Bailey *et al.*, 1978). Consequently, a specific locus in one species may still have four alleles, while in another species it may be converted to a pair of isoloci (*e.g*. pair of duplicate loci having gene products with identical constitution and electrophoretic mobility; (Waples, 1988). Several gene duplicates have been cloned and described for salmonids (Morash *et al.*, 2010; Evans *et al.*, 2008; Leaver *et al.*, 2007; McKay *et al.*, 2004; Hordvik, 1998; Kavsan *et al.*, 1993; Ohno *et al.*, 1968). The existence of such duplicates

makes it even more difficult to determine the intriguing gene regulation mechanisms in these species.

In contrast to the continuing diploidization process going on in protein coding genes, miRNA genes have almost all been retained as duplicate copies (Berthelot *et al.*, 2014). These authors identified 241 miRNA loci in the rainbow trout genome, of which 233 (97%) were present in duplicate copies, while eight loci only displayed one member of the ohnologous pair (3%).

3.2 Circadian control

In mammalian liver, most metabolic pathways, including both lipid and cholesterol metabolism (reviewed in Panda *et al.*, 2002), are under circadian control, meaning that they display an endogenous, cyclic fluctuation of about 24 hours (Reppert & Weaver, 2002). Betancor *et al.* (2014) showed that specific genes relating to lipid metabolism and homeostasis are under circadian control in the liver of Atlantic salmon. The mechanisms involve interacting positive and negative transcriptional feedback loops that drive periodic rhythms of the RNA and protein levels. In mammals, the coordination between these loops has been shown to be governed by the orphan nuclear receptors, *e.g. REV-ERB 1α* (reviewed in Reppert & Weaver, 2002) and tissue-specific post-transcriptional regulation factors, specifically several miRNAs (Du *et al.*, 2014; Shende *et al.*, 2014; Chen *et al.*, 2013; Shende *et al.*, 2011; Gatfield *et al.*, 2009b). *REV-ERB 1α* has recently been cloned in the liver of Atlantic salmon (Betancor *et al.*, 2014), but *REV-ERB 1α* in fish seems not to participate in exactly the same way in the circadian control mechanism as in mammals.

3.3 Transcription factors

One way for dietary FAs to influence gene expression is by controlling the activity or abundance of central transcription factors (Jump *et al.*, 2005). A transcription factor is a protein that binds to a specific promoter sequence -30, -75 and -90 base pairs (bp) upstream of the transcription start site in the promoter region, and by doing so controls the transcription of genetic information from DNA to mRNA. This function is performed single-handedly or in a complex with other proteins that promote (as an activator) or block (as a repressor) the binding of RNA polymerase.

Many transcription factors have been identified as targets for FA regulation, including *PPAR*s, *SREBP*s, hepatic nuclear factors (*HNF*s), retinoid X receptor (*RXR*) and *LXR*. Some of these are examined in more detail below.

3.3.1 Peroxisome proliferator-activated receptors

One nuclear receptor family that influences transcription according to nutritional state is the *PPAR* family (Issemann & Green, 1990). *PPAR*s respond to changes in lipid and glucose homeostasis (reviewed in Schoonjans et al., 1996). *PPAR*s are ligand-activated by FAs and eicosanoid metabolites. They are not only able to bind to the promoter region of genes involved in the metabolism of their ligands, and thereby regulate gene expression, but they also serve as intracellular receptors (reviewed in Kersten, 2008). The DNA-binding domain, which plays an important role in the binding of *PPAR* to the promoter region, has been characterized in Atlantic salmon (Ruyter *et al.*, 1997).

There are three subtypes of *PPAR*s in salmonids, *PPARα*, *PPARβ/δ* and *PPARγ*, with specific tissue and developmental patterns of expression. The *PPAR* subtypes can be activated by a variety of ligands without showing any particularly strict ligand specificity. In rainbow trout, *PPARα* expression is upregulated by SAFA, MUFA, ALA, ARA and DHA and downregulated by EPA (Coccia *et al.*, 2014). Similarly, Morash and McClelland (2011) showed that a LCPUFA-rich diet upregulated both *PPARα* and *PPARβ*.

As a result of the additional genome duplication event in salmonids, four genes coding for four different subtypes of *PPARβ/δ* have been identified in Atlantic salmon (Leaver *et al.*, 2007). Furthermore, two subtypes of *PPARγ* that differ in length, stability and presumably in ligand preferences have been described in Atlantic salmon (Andersen *et al.*, 2000; Ruyter *et al.*, 1997). *PPARα* is considered to be the main inducer of β-oxidation (Leaver *et al.*, 2006). However, *PPARα*, *PPARβ/δ* and *PPARγ* have all been shown to target genes coding for the β-oxidation enzymes, *CPT1* and *ACO*, and by doing so shift FAs away from esterification and storage, resulting in a decrease in EPA and DHA in liver and white muscle of rainbow trout and Atlantic salmon (Torstensen *et al.*, 2009; Du *et al.*, 2004; Ruyter *et al.*, 1997). Both *PPARα* and *PPARγ* have been shown to induce transcription of the transmembrane fatty acid transporter *CD36* and *SR-B1* (Torstensen *et al.*, 2009; Malerød e*t al*., 2003; Poirier *et al*., 2001; Motojima *et al*., 1998). *PPARγ* is present in two forms, *PPARγ* long, expressed in liver and involved in the regulation of FA metabolism, and *PPARγ short*, suggested to be present in Atlantic salmon adipocytes and involved in adipocyte differentiation (Todorčević *et al.*, 2008; Vegusdal *et al.*, 2003; Ruyter *et al.*, 1997).

The expression of *PPARβ/δ* is reported to be significantly downregulated in Atlantic salmon fed VO compared with FO fed fish (Torstensen *et al*., 2009). However, transcription regulators may respond differently to alternative plant-based feeds depending on genotype. In a study where diet formulation was

changed to VO, both *PPARα* and *PPARβ* were downregulated in lean fish, but this was not observed in fat salmon (Morais *et al.*, 2012b).

3.3.2 PPARγ coactivator-1

PPARγ coactivator-1 (*PGC-1*) has been demonstrated to interact with the *PPARγ* receptor, as well as with other members of the nuclear receptors. *PGC-1* plays a role in the regulation of energy homeostasis, lipid metabolism and fat deposition in mammals and lower vertebrates (Lemoine *et al.*, 2010). *PGC-1* greatly increases the transcriptional activity of *PPARγ* (reviewed in Aranda & Pascual, 2001). *PGC-1α* is also reported to be a potential marker for meat quality in pigs (Lefaucheur *et al.*, 2004). *PGC-1* has been shown to interact with *CD36* and *CPT-1*, resulting in increased FA transport and β-oxidation in rodents fed a 30% FO diet (Feillet-Coudray *et al.*, 2013).

PGC-1α has been cloned and characterized in a cyprinid species (*Schizothorax prenanti*). Here PGC-1α transcription levels in fish muscle seem to be positively correlated with intramuscular fat content (Li *et al.*, 2012b).

3.3.3 Sterol regulatory element-binding proteins

Another group of key regulators of lipid and cholesterol metabolism are the *SREBP*s, which are attached to the nuclear envelope or bound in ER (reviewed by Jump *et al.*, 2005). The FA levels, both intracellular and membrane, are under constant supervision by *SREBP* and are coordinated with *de novo* lipid biosynthesis (Horton *et al.*, 2002). In the nucleus, *SREBP* binds to the sterol regulatory element DNA sequence found in control regions of the target genes. This binding leads to the initiation of transcription (Osborne & Espenshade, 2009). Binding site for *SREBP*s has been identified in the promoter region of salmon *Δ6FAD* (Zheng *et al.*, 2009) and Minghetti *et al.* (2011) identified and characterized two *SREBP* genes in salmon that are homologous to mammalian *SREBP-1* and *SREBP-2*. The latter study also showed that both *Δ5FAD* and *Δ6FAD* regulate *SREBP-1* and that n-3 LCPUFAs, EPA and DHA downregulate *SREBP-1* expression in Atlantic salmon.

Replacement of dietary FO with VO in Atlantic salmon upregulate *SREBP-2* and, as a result, increased expression of genes coding for cholesterol biosynthesis (Leaver *et al.*, 2008b; Taggart *et al.*, 2008). As with *PPAR*s, *SREBP*s may respond differently to dietary changes depending on genotype. If diet formulation changes to VO, *SREBP-1* was upregulated in lean fish, but no similar effect could be seen in fat salmon (Morais *et al.*, 2012b).

3.3.4 Liver X receptors

Liver X receptors are transcription factors regulated by sterols and in turn regulate key target genes in cholesterol catabolism, storage, absorption and transport, as well as *de novo* FA synthesis. The gene coding for *LXR* was cloned and characterized in salmonids by Cruz-Garcia *et al.* (2009).

In a similar fashion to *PPAR*, ligand binding causes dissociation of the *LXR* from the co-repressors, followed by translocation from the cytoplasm to the nucleus where *LXR-RXR* binds to the *LXR* response elements in the promoter of the target genes, resulting in transcription initiation. In humans post-transcriptional regulation by miRNAs and post-translational modifications such as phosphorylation have been shown to finely tune *LXRα* target gene selectivity (Zhong *et al.*, 2013; Torra *et al.*, 2008). *ACO* and *ELOVL5* are possible direct targets of *LXR*, suggesting that salmon *ELOVL5* may be regulated in a different way than mammalian *ELOVL5*, an indirect target of *LXR*, reacting to *LXR*-dependent increases in *SREBP-1*. *LXR-SREBP-1c* pathway plays an important regulatory role in hepatic biosynthesis of LCPUFAs (Minghetti *et al.*, 2011; Zheng *et al.*, 2004).

The expression of *LXR* seems to depend on environmental changes, with *LXR* mRNA levels significantly higher in seawater fish than in freshwater fish and young parr tending to have a much higher expression rate than two year-old adult salmon and that diet changes from FO to VO affect adult fish more than pre-smolt fish (Cruz-Garcia *et al.*, 2009).

Replacing FO with VO in aquafeed causes a decrease in cholesterol content and an increase in phytosterols (Pickova & Mørkøre, 2007), which can have disruptive effects on cholesterol metabolism in salmonids. Substitution of FO with plant products induces genes of cholesterol and FA metabolism (Leaver *et al.*, 2008b), which partly may be caused by *LXR* (Plat *et al.*, 2005), since it is unclear whether phytosterols can induce *LXR* expression in the same way as cholesterol. A downregulating effect on *LXR* expression in rainbow trout fed VO has been shown (Cruz-Garcia *et al.*, 2011).

4 Post-transcriptional regulation of lipid metabolism

Post-transcriptional regulation is the control of gene expression at the RNA level, after transcription and before gene translation. Included within the post-transcription concept are regulation mechanisms such as modulation of the activity of RNA binding proteins, alternative splicing, RNA degradation, addition of poly(A) tail, processing, RNA editing and exportation from the nucleus to the cytoplasm, removal of the 5-prime cap from mRNA and finally regulation of the actual translation. All of these are involved in modifying the stability and distribution of the mRNA, ultimately affecting the outcome of the gene expression machinery.

During the last decades the picture of gene regulation has become even more complex with the discovery of epigenetic regulation. The four major components of epigenetic regulation are promoter methylation, histone modification, chromatin conformation changes and altered expression by non-coding RNAs, especially miRNAs (Moore, 2005; Bartel, 2004; Ambros, 2001).

The focus in this thesis is on miRNAs as a candidate for gene translation regulation.

4.1 MicroRNAs and gene silencing

MiRNAs are a family of short (approximately 21-25 nucleotides long) endogenous non-coding RNAs involved in a vast number of evolutionary conserved regulatory pathways (Bartel, 2009; Bartel, 2004; Lau *et al*., 2001). MiRNAs function as guide molecules in the post-transcriptional gene silencing process by base pairing with target mRNAs, which in turn leads to cleavage of mRNA or translational repression.

4.1.1 Background

The first miRNA was identified in 1993, when the gene *lin-4*, which controls the developmental timing in *Caenorhabditis elegans*, was shown not to code for proteins, but instead acted as 22nt RNA transcripts. This transcript regulated its target, lin-14, by base-pairing to the mRNA 3'-UTR with imperfect sequence complementarity (Lee *et al.*, 1993; Wightman *et al.*, 1993). This phenomenon was first thought to be unique for *C. elegans*, but the situation was reconsidered when a second miRNA, let-7, identified by Reinhart *et al.* (2000), was found to be conserved in several other species (Griffiths-Jones *et al.*, 2006), together with its target lin-41 (Pasquinelli *et al*., 2000).

Today, genes regulated by miRNAs and the miRNAs themselves have been identified in a wide range of vertebrates and plants and are believed to be present in all multicellular eukaryotes (Bartel, 2009) and responsible for more than 60% of the regulation of protein coding genes (Dweep *et al.*, 2011).

4.1.2 MicroRNA biogenesis

MicroRNAs are transcribed individually, in clusters or in conjunction with the protein that they regulate. They are located as individual (monocistronic) or (polycistronic) clusters and can be generated from either the sense or the antisense strand of the gene that codes them (*Figure 4*) (Lau *et al*. 2001).

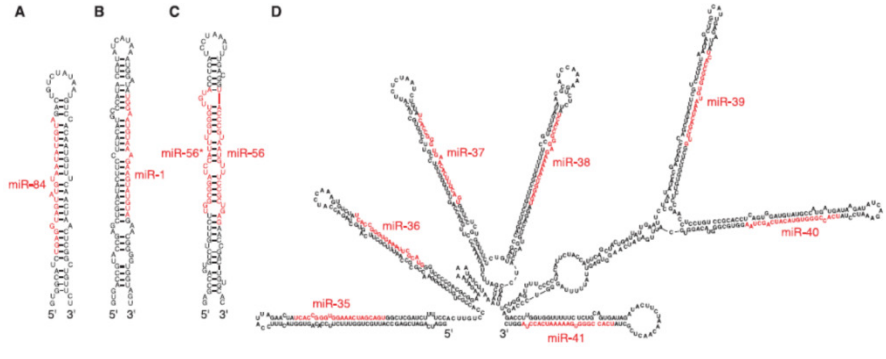


Figure 4. Examples of different secondary structures of miRNAs (red) and their flanking regions (black) (adapted after Lau *et al.*, 2001): A) miRNA residing on the 5′ arm of the fold-back structure, B) miRNA residing on the 3′ arm of the fold-back structure, C) two miRNAs cloned from both strands of the fold-back structure. A-C are examples of monocistronic located miRNAs and D) is a polycistronic miRNA cluster.

The synthesis of miRNA (*Figure 5*) occurs in two different cell compartments; the nucleus and the cytoplasm. MiRNAs are transcribed within the nucleus to form large precursors several kilobases long, called primary miRNAs (pri-

miRNAs) typically containing one to several characteristic stem loop structures (Kim, 2005).

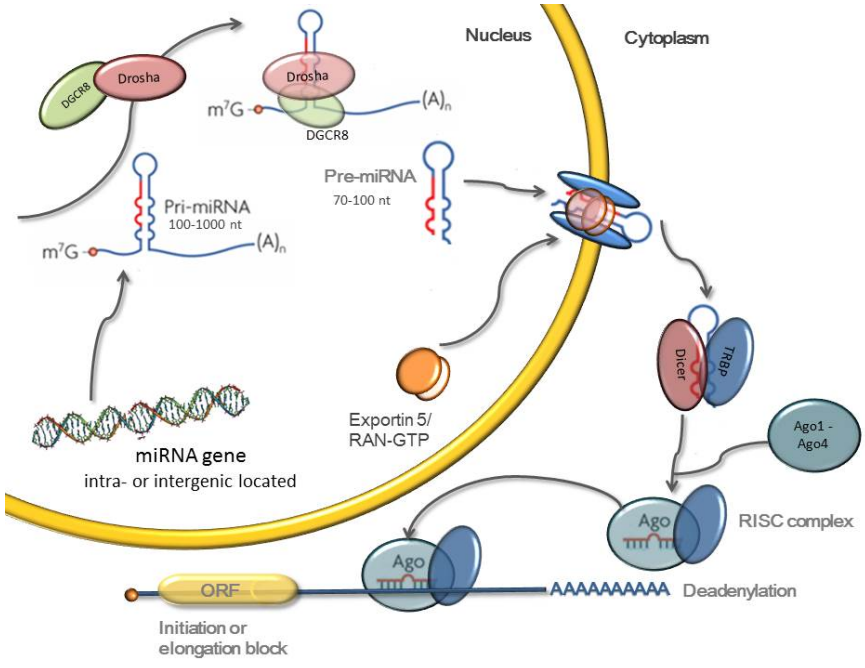


Figure 5. Biosynthesis of miRNA. The miRNAs are transcribed as primary transcripts (pri-miRNAs) by RNA polymerase II. Each pri-miRNA contains one or more hairpin structures that are recognised and processed by Drosha and DGCR8, generating a 70-nucleotide stem loop known as the precursor miRNA (pre-miRNA), which is actively exported to the cytoplasm by exportin-5. In the cytoplasm, the pre-miRNA is recognized by Dicer and TRBP. Dicer cleaves the precursor, generating a 20-nucleotide mature miRNA duplex. In general, only one strand is selected as the biologically active mature miRNA and the other strand is degraded. The mature miRNA is loaded into the RNA-induced silencing complex (RISC), which contains argonaute (Ago) proteins and the single-stranded miRNA. Mature miRNA allows the RISC to recognize target mRNAs through partial sequence complementarity with its target. The RISC can inhibit the expression of the target mRNA through two main mechanisms that have several variations: removal of the polyA tail (deadenylation), followed by mRNA degradation; and blockade of translation at the initiation step or at the elongation step or causing ribosome stalling. RISC-bound mRNA can be localized to sub-cytoplasmatic P-bodies, where they are reversibly stored or degraded (Modified after Inui *et al.*, 2010).

The processing of the pri-miRNA starts with the binding of DGCR8 to the pri-miRNA flanking sequences, followed by the positioning of the RNase III type endonuclease Drosha and the subsequent stem loop cleavage approximately one helical turn, or 11 bp, from the junction between the flanking sequences and the stem loop. This process generates a characteristic hairpin RNA

precursor called pre-miRNA (Lee *et al.*, 2003). The pre-miRNAs are roughly 65-70 nt long hairpins and are exported through the nucleus membrane into the cytoplasm by Exportin-5.

After entering into the cytoplasm, the pre-miRNAs are recognized and cleaved by Dicer, another RNase III enzyme removing the hairpin loop. The miRNAs are now RNA duplexes 22 nt in length.

Only one strand of the duplex strands (the miR strand) is loaded onto an argonaute protein (Ago). The other strand is degraded. Which of the two strands that is loaded onto Ago is somewhat unclear, but in general it is the strand with a less stable 5'end (fewer bindings) that enters into Ago. The RNA induced silencing complex (RISC) is formed and is now capable of binding to, and thereby repressing, target mRNA expression (Treiber *et al.*, 2012). The miRNA binds to the target mRNA 3′UTR region with imperfect complementarity except for a region in the miRNA (from 2^{nd} to 7^{th} nt) that creates an almost perfect match with the so-called seed region in the mRNA. The miRNAs are grouped into families based in similarities in seed region (Bartel, 2009). This short seed region is used in computational prediction of miRNA targets (Betel *et al.*, 2010; Xiao *et al.*, 2009; Shahi *et al.*, 2006; Krek *et al.*, 2005; Lewis *et al.*, 2005; Rehmsmeier *et al.*, 2004).

With the rise in next generation sequencing (NGS) platforms generating millions of reads, a new magnitude of variability in mature miRNA sequences has been observed. These sequence variants are referred to as isomiR. These are multiple mature sequences that have variations with respect to the reference miRNA sequence annotated in miRBase. In many cases, the miRNA* sequence and its isomiRs are also observed (Morin *et al.*, 2008).

4.1.3 Role of miRNA

The miRNAs have a profound impact on the development of all vertebrates. Knock-out animals lacking the Dicer enzyme responsible for processing the pre-miR into its mature form cannot live (Kloosterman & Plasterk, 2006; Ambros, 2004; Wienholds *et al.*, 2003). In mammals, miRNAs have been shown to be capable of regulating every aspect of cellular activity, including development and proliferation, differentiation, metabolism, viral infection, epigenetic modulation, apoptotic cell death and tumor genesis (Lin *et al.*, 2012; Bushati & Cohen, 2007; Esau & Monia, 2007; Bartel, 2004; Carrington & Ambros, 2003). One single miRNA can regulate more than 200 mRNAs and one single mRNA may be regulated by several different miRNAs (Dweep *et al.*, 2011). However, very few miRNA targets have actually been identified by biological methods.

Specific miRNAs have received attention due to their role as key metabolic regulators in mammals (Sacco & Adeli, 2012; Dávalos *et al.*, 2011; Fernández-Hernando *et al.*, 2011; Aoi *et al.*, 2010; Safdar *et al.*, 2009; Krützfeldt & Stoffel, 2006).

In fish, changes in miRNA have been documented during ontogeny (Mennigen *et al.*, 2013; Bizuayehu *et al.*, 2012), in egg (Ma *et al.*, 2012), larval and juvenile growth (Campos *et al.*, 2014) and in response to food ingestion (Mennigen *et al.*, 2012).

Even though miRNAs exhibit a high level of sequence conservation, the timing and location of miRNA expression is not strictly conserved. Variation in miRNA expression is more pronounced the greater the differences in physiology, and it is likely that changes in miRNA expression play a role in shaping the physiological differences produced during development (Ason *et al.*, 2006). One indication of this can be seen in rainbow trout, where Mennigen and his team studied selected liver-specific miRNAs (Mennigen *et al.*, 2014a; Mennigen *et al.*, 2014b; Mennigen *et al.*, 2013; Mennigen *et al.*, 2012). They expected both *miR-33* and *miR-122* to be linked to the regulation of cholesterol and lipid metabolism as well as glycose homeostasis in the same way as in mammals. However, they found that the metabolic consequences of *miRNA-122* inhibition differ between vertebrate species and that genes involved in hepatic lipid lipogenesis and β-oxidation are positively affected in rainbow trout but not in mammals, where inhibition of *miR-122* results in decreased expression of lipogenic genes.

Naturally occurring variation in miRNAs

Naturally occurring variation in miRNA genes or miRNA target sites may also contribute to normal phenotypic variations. Some of these phenotypic differences may affect economically important traits, such as that affecting muscle meatiness in Texel sheep (Clop *et al.*, 2011; Clop *et al.*, 2006). A single nucleotide polymorphisms (SNPs) located in the putative 3'UTR target sites of *miR-224* and the *miR-30* family have been shown to affect the transcription rate of genes and transcription factors involved in pig lipid metabolism, which can have an effect on lipid composition and pork quality (Bartz *et al.*, 2014; Stachowiak *et al.*, 2014). Peñaloza *et al*. (2013) suggested that SNPs in the flanking region of the myostatin gene of Atlantic salmon affecting the regulation of muscle development and growth might act through interfering with the highly conserved miRNA target site. The same phenomenon was later demonstrated by McFarlane *et al*. (2014) in mice. If this is also the case for lipid composition in Atlantic salmon, it might prove to be suitable for selective breeding and of commercial importance.

4.1.4 MicroRNAs in salmonids

The number of known fish miRNAs is not comparable to those for human and mouse, considering the conserved nature of miRNAs among different species. Today the miRBase database contains 1881 precursors and 2588 mature human miRNAs, compared with much lower number of entries from Atlantic salmon (371 precursors and 498 mature miRNAs) and no entries for rainbow trout. However, not all the Atlantic salmon and rainbow trout miRNAs identified to date have been uploaded onto the miRBase registry (Bekaert *et al.*, 2013; Salem *et al.*, 2010; Ramachandra *et al.*, 2008).

To the best of my knowledge, Andreassen *et al.* (2009) were the first to indicate that the Atlantic salmon genome contains conserved 7-mers in the 3'UTRs identical to miRNA target sequences, suggesting that miRNA and RNA silencing also play a role in controlling protein expression in *S. salar*. Using computer predictions, Andreassen *et al.* (2009) were able to identify four target motifs to complementary conserved miRNA families (*ssa-miR-101*, *ssa-miR-199*, *ssa-miR-144*, *ssa-miR-543*, *ssa-miR-446b-3-3p*, *ssa-miR-425-5*, *ssa-miR-731* and *ssa-miR-489*). Correspondingly, Ramachandra *et al.* (2008) were the first to clone and characterize rainbow trout miRNA. They identified 14 conserved miRNAs that were involved in regulation of maternal mRNA degradation during early embryogenesis. These 14 conserved miRNAs were included in the 54 miRNAs cloned and identified in a pooled sample consisting of nine somatic tissues from immature (~1-year-old) rainbow trout (Salem *et al*., 2010). The first more complete transcriptome analysis of 496 miRNAs in unfertilized eggs of rainbow trout was performed by Ma *et al.* (2012).

Barozai (2012) and (Reyes *et al.*, 2012) identified 102 and 307 mature miRNAs, respectively, belonging to 46 different miRNA families in Atlantic salmon from expressed sequence tag (EST) sequences based on bioinformatics approaches. These miRNAs were later identified by Bekaert *et al*. (2013) and Andreassen *et al.* (2013) using deep sequencing. All Atlantic salmon entries in miRBase version 21 so far have been made by Andreassen *et al*. (2013), but Bekaert *et al.* (2013) identified a total of 547 miRNA genes. However, all NGS studies on salmonids to date have mainly been conducted on egg or juvenile fish pre-smoltification (Andreassen *et al.*, 2013; Bekaert *et al.*, 2013; Ma *et al.*, 2012).

Identification and characterization of miRNAs expressed in the liver of mature Atlantic salmon and discovery of novel liver-predominant miRNAs would be an important step towards understanding the molecular mechanisms regulating hepatic LCPUFA synthesis.

5 Objectives

As Atlantic salmon is among the most popular fish species in the Western diet, the content of EPA and DHA in Atlantic salmon fillet and factors influencing these amounts are important. This thesis focuses on molecular regulation of lipid metabolism in Atlantic salmon with the main emphasis on n-3 LCPUFA biosynthesis. Understanding the molecular mechanisms behind transcriptional regulation of LCPUFA biosynthesis will enable optimization of the activity of the n-3 LCPUFA pathway to enable efficient and effective use of *e.g.* VO in aquaculture.

Specific objectives of the studies described in Papers I-V were to:

- Study the effect of minor compounds, often polar, from linseed oil in combination with sesamin on lipid metabolism in rainbow trout (Paper I)
- Study the effect of sesamin supplementation to vegetable oil-based diets on the expression of genes related to FA metabolism and on the FA composition in Atlantic salmon after *in vivo* trials (Paper II)
- Evaluate the effects of different bioactive compounds *in vitro* – a mixture of sesamin/episesamin, sesamin, lipoic acid and genistein, all of which are known to act as either antioxidants and/or influence lipid homeostasis in mammals (Paper III)
- Identify and evaluate potential endogenous control miRNA genes and compare expression of these and of other selected miRNAs in different tissues. The purpose was to create knowledge for coming larger studies, including treatments. The identification of specific miRNAs and evaluation of quantitative real-time polymerase chain reaction (qPCR) analysis of Atlantic salmon miRNAs may be of economic interest in

terms of *e.g.* feeding regimes and treatments of diseases in aquaculture of Atlantic salmon (Paper IV)

- To identify and sequence all major expressed miRNAs in the liver of Atlantic salmon post-smoltification using deep sequencing, in order to find out more about the expression and regulation of lipid related genes in the liver of Atlantic salmon. Such knowledge is important to understanding the mechanisms through which salmonids control and regulate the high lipid levels on which they are dependent for optimal growth (Paper V).

6 Material and methods

This chapter provides a short description of the material and methods used in the studies included in this thesis. For more details of the specific procedures, see Papers I-V.

6.1 Design of the experimental series

An overview of the materials tested, specific methods, software and techniques used in the three feeding trials and in the two microRNA studies are given in *Tables 1* and *2*, respectively.

In Paper I, rainbow trout with an average final weight of 73 g were fed vegetable oil mixtures with different combination linseed oil - commercial linseed oil (LO), purified linseed oil triacylglycerols (TAG) with the polar fraction removed and mixed linseed-sunflower oil (6:4 v/v) (MO). The effects of sesamin supplementation, content of α- and γ-tocopherols and FA composition were then evaluated, as well as gene expression of lipid related genes in liver and white muscle.

In Paper II, Atlantic salmon with an average final weight of 554 g were fed vegetable oil-based diets with different inclusions of sesamin. The diets used differed in n-6/n-3 fatty acid (FA) ratio (0.5 and 1) and sesamin content (high 5.8 g/kg, low 1.16 g/kg and no sesamin). The oils used in the feeds were a mixture of rapeseed, linseed and palm oil. The fish were fed for 4 months. The effects of sesamin supplementation on FA composition and expression of hepatic genes involved in transcription, lipid uptake, desaturation, elongation and β-oxidation in liver and white muscle were evaluated (*Table 1*).

Table 1. *Summary of experimental design and content for Papers I -III*

	Paper I	Paper II	Paper III
Species	Rainbow trout	Atlantic salmon	Atlantic salmon
Fish final weight	73 g	554 g	1300 g
Samples [a)]	Liver/white muscle	Liver/white muscle	Hepatocytes
Sample size [b)]	1.7 mg	1.7 mg	1.7 mg
Number of replicates	6[cd)]	6[cd)]	6[d)]
Environmental conditions	Non-chlorinated tap water 14.5 °C	Seawater at 12 °C	Seawater at 10 °C
Control diet[d)]	Commercial fish feed	Commercial fish feed	Commercial fish feed
Treatment	Sesamin	Sesamin/episesamin Sh = 5.8 g/kg feed Sl = 1.16 g/kg feed	Lipoic acid Sesamin/episesamin Genistein
Vegetable oil diet[e)]	Linseed oil (LO) Linseed oil Triacylglycerols (TAG) Mixed linseed-sunflower oil (6:4 v/v) (MO)	V0.5 = 0.5 n-6/n-3 FA V1 = 1.0 n-6/n-3 FA	
Measurements	Lipid analysis Gene expression Content of α- and γ-tocopherols	Lipid analysis Gene expression	Lipid analysis Gene expression
Target genes	*PPARα, PPARβ1A, PPARγ, CPT1, Δ6FAD, ACO*	*PPARα, PPARβ1A, PPARγ, PGC-1, SREBP-1, SREBP-2, LXR, CD36, SP-B1, ELOVL2, ELOVL5a, ELOVL5b, Δ5FAD, Δ6FAD, ELOVL4, ACO*	*PPARα, PPARβ1A, PPARγ, CD36, ELOVL2, ELOVL5a, Δ5FAD, Δ6FAD, ACO*
Housekeeping gene	*NUOR*	*NUOR*	*RPL2*

a) Only liver was tested in gene expression experiments.
b) For the gene expression studies only
c) All tests performed in triplicate
d) All diets contained the recommended levels of vitamins and minerals
e) Rapeseed, linseed and palm oil

In Paper III, hepatocytes were isolated from Atlantic salmon (1300 g) according to the two-step collagenase procedure (Kjær *et al.*, 2008; Dannevig & Berg, 1985; Seglen, 1976). The fish were kept in seawater at 10°C and fed a commercial diet prior to isolation of hepatocytes. Lipoic acid, genistein, episesamin and sesamin were added individually to the culture media of the

Atlantic salmon hepatocytes. An array of gene expression assays was designed covering transcription factors and genes coding for proteins/enzymes involved in lipid metabolism (for genes analyzed, see *Table 1*). The FA composition in Atlantic salmon hepatocytes was also analyzed.

Table 2. *Summary of experimental design and content for Papers IV & V*

	Paper IV	Paper V
Species	Atlantic salmon pre-smoltification	Atlantic salmon post-smoltification
Fish size	10 g (10 months old)	1 300 g
Samples	Liver, white muscle, red muscle, heart, brain, stomach, gills, intestine and kidney[a)]	Liver[b)]
Sample size[c)]	0.04 g tissue/individual ~1 g	0.17 g liver/individual~1 g
Number of replicates	3[d)]	6
Environmental conditions	Freshwater 'Dalälven' at 10 °C	Seawater at 12 °C
Diet[e)]	Commercial fish feed	Commercial fish feed
Treatment	Pilot study	Pilot study
Measurements	Next generation sequencing miRNA expression using qPCR	Next generation sequencing
Target genes	*ssa-let-7a, ssa-miR-16a, ssa-miR-16b, ssa-miR-194a, ssa-miR-22a, ssa-miR-22b, ssa-miR-27c, ssa-miR-26b, ssa-miR-92a-1, ssa-miR-122, ssa-miR-722, ssa-miR-21-1, ssa-miR-143-1*	
Endogenous control	*ssa-miR-27c*	

a) 1 g of somatic tissue were collected and mixed.

b) 1 g of liver tissue from each individual were collected and mixed.

c) 1 g of the total pool was used for NGS.

d) All tests performed in triplicate

e) All diets contained the recommended levels of vitamins and minerals

In Paper IV, the tissue samples used for miRNA analysis were collected from Atlantic salmon of approximately 10 g and 10 months of age and from the liver of six mature Atlantic salmon post-smoltification (*Table 2*). Since this was the first study to identify miRNA in Atlantic salmon, it was conducted as a pilot study, with one commercial diet fed to the fish.

The miRNA isolation and enrichment and complementary DNA (cDNA) libraries for two pooled samples from three individuals, each containing liver,

heart, brain, kidney, spleen, intestine, gill, white and red muscle and mature liver, and Illumina sequencing were constructed and executed by Vertis Biotechnology AG (Germany; http://www.vertisbiotech.com/).

Deep sequencing miRNA analysis was performed by our research group (unpublished results). Based on these unpublished data, miRNA candidates potentially suitable as endogenous controls in future expression studies were identified. In addition, the expression of certain miRNAs known to be related to lipid metabolism (*Table 2*) in different tissues of Atlantic salmon was investigated using a modified traditional TaqMan® assay specially designed for miRNAs in qPCR expression analysis.

In Paper V, all major miRNAs expressed in liver of Atlantic salmon at the post-smoltification stage were identified and sequenced using deep sequencing analysis of NGS data generated by the Illumina® HiSeq™ 2000 Sequencing System.

6.2 Lipid analysis

Total lipids from diets, tissue, cells and the medium were extracted using hexane:isopropanol (3:2 by vol.) (Hara & Radin, 1978).

Total lipids of muscle tissue and liver were separated into TAG and PL on thin-layer chromatography according to Pickova *et al.* (1997). Total lipids in the diets and the TAG and PL were methylated to fatty acid methyl esters (FAME) following the method described by Appelqvist (1968) and analyzed with gas chromatography according to Trattner *et al.* (2008a) (*Table 3*). The peaks were identified by comparing their retention times with a standard mixture.

6.3 Sesamin/episesamin analysis and tocopherol determinations

For the analysis of sesamin, episesamin, α- and γ-tocopherols in oils, feed and fish white muscle, lipids were dissolved in hexane and analyzed by high performance liquid chromatography (HPLC) using a similar system, column and conditions as described by Moazzami and Kamal-Eldin (2006). The concentrations of α- and γ-tocopherols and sesamin were determined by reference to authentic standards using the linear equation obtained from triplicate five-point standard curves.

Table 3. *Fatty acid composition (%) in the experimental diets or media used in Papers I-III*

	Average control	Paper I			Paper II		Paper III
	Fish oil	Linseed oil	Linseed oil triacylglycerols	Mixed linseed-sunflower oil	Low n-3/n-6	High n-3/n-6	Culture media
LA (18:2n-6)	3.20	21.2	21.0	36.5	14.6	15.3	3.70
ALA (18:3n-3)	1.85	36.5	36.8	22.8	27.5	13.1	1.00
ARA (20:4n-6)	0.40	-	-	-	0.10	0.10	2.40
EPA (20:5n-3)	8.15	0.16	0.15	0.16	1.1	1.3	0.90
DHA (22:6n-3)	9.95	0.38	0.37	0.38	1.6	1.8	1.20
SAFA	27.8	10.3	10.2	10.6	17.5	18.8	40.7
MUFA	34.3	23.0	23.5	25.2	34.0	47.0	22.3
n-3 PUFA	23.3	37.5	37.4	23.4	30.9	16.9	3.60
n-6 PUFA	6.20	22.5	36.8	21.3	15.4	15.9	6.90
n-6/n-3	0.27	0.60	1.57	0.57	0.50	0.94	1.92

SAFA saturated fatty acids (14:0, 16:0, 18:0); *MUFA* monounsaturated fatty acids (16:1n-7, 18:1n-9, 18:1n-7, 20:1, 22:1); *PUFA* polyunsaturated fatty acids

6.4 Gene expression analysis

Gene expression in liver (Papers I and II) and in white muscle (Paper I only) was investigated by qPCR using an array of target genes coding for enzymes involved in lipid homeostasis.

Table 4a. *Sequences of primers used to amplify housekeeping genes and corresponding GenBank accession numbers used in primer design*

Primer	Primer pair (5'-3')	Sequence	GenBank Acc. no
NUOR[a]	Forward	CAACATAGGGATTGGAGAGCTGTACG	DW532752
	Reverse	TTCAGAGCCTCATCTTGCCTGCT	
EF1-α[b]	Forward	CACCACCGGCCATCTGATCTACAA	AF321836
	Reverse	TCAGCAGCCTCCTTCTCGAACTTC	
RPL2[c]	Forward	TAACGCCTGCCTCTTCACGTTGA	CA049789
	Reverse	ATGAGGGACCTTGTAGCCAGCAA	
ETiF[c]	Forward	CAGGATGTTGTTGCTGGATGGG	DW542195
	Reverse	ACCCAACTGGGCAGGTCAAGA	

Abbreviations: RPL2 = RNA polymerase II polypeptide, EF1-α = elongation factor 1α, NUOR = NADH-ubiquinone oxidoreductase, ETiF = eukaryotic translation initiation factor 3. Already designed and validated in a) Bahuaud *et al*. (2010), b) Jorgensen *et al*. (2006), c) Castro *et al*. (2011).

Total RNA was isolated from fish liver and white muscle (Paper I only) using the spin purification method followed by DNase treatment. Total RNA was quantified and reverse transcription first strand cDNA was synthesized using the High-Capacity cDNA Archive kit. Real-time PCR analysis of the relative abundance of mRNA was assessed using Power or Fast SYBR® Green chemistry and gene-specific primers designed using available Atlantic salmon sequences from the online version of GenBank®(NCBI) (Trattner *et al.*, 2008c), using the Primer Express® software or copied from literature references.

Primers for qPCR analysis with corresponding GenBank accession numbers are listed in *Table 4a-c*. The same primers were evaluated and used for both Atlantic salmon and rainbow trout except for the *PPARγ*(long/short) reverse primer, which was redesigned for the rainbow trout study.

All samples were run simultaneously for each gene in triplicate, with a non-template control on each plate. A melt curve analysis was performed after each run to ensure that only a single product was amplified.

Table 4b. *Sequences of primers used to amplify transcription factors and corresponding GenBank accession numbers used in primer design*

Primer	Primer pair (5'-3')	Sequence	GenBank Acc. no
PPARα[a]	Forward	TCCTGGTGGCCTACGGATC	DQ294237
	Reverse	CGTTGAATTTCATGGCGAACT	
PPARβ1A[b]	Forward	GAGACGGTCAGGGAGCTCAC	AJ416953
	Reverse	CCAGCAACCCGTCCTTGTT	
PPARγ (long)[e]	Forward	CATTGTCAGCCTGTCCAGAC	AJ292963
	Reverse	TTGCAGCCCTCACAGACATG	
PPARγ (long/short)	Forward	CATTGTCAGCCTGTCCAGAC	AJ292963
	Reverse	ATGTGACATTCCCACAAGCA	
PGC-1α	Forward	CAACCACCTTGCCACTTCCT	FJ710605.1
	Reverse	CGGTGATCCCTTGTGGTCAT	
LXR[c]	Forward	GCCGCCGCTATCTGAAATCTG	FJ470290
	Reverse	CAATCCGGCAACCAATCTGTAGG	
SREBP-1	Forward	GACAAGGTGGTCCAGTTGCT	NM001195818
	Reverse	CACACGTTAGTCCGCATCAC	
SREBP-2[d]	Forward	TCGCGGCCTCCTGATGATT	NM001195819
	Reverse	AGGGCTAGGTGACTGTTCTGG	

Abbreviations: PPAR = peroxisome proliferator-activated receptor, PGC-1α = proliferator-activated receptor gamma coactivator 1 alpha, LXR = liver X receptor α, SREBP = sterol regulatory element binding protein. Already designed and validated in a) Jorgensen *et al.* (2006), b) Kleveland *et al.* (2006a), c) Cruz-Garcia *et al.* (2009), d) Minghetti *et al.* (2011). e) Used for rainbow trout in Paper I only.

Elongation factor 1a (*EF1α*), NADH-ubiquinone oxidoreductase (*NUOR*), eukaryotic translation initiation factor 3 (ETiF) and RNA polymerase II polypeptide (*RPL2*) were evaluated for their stability across all experimental variables and samples. The most stable reference gene was then chosen using the DataAssist software version 2.0. ΔCT was calculated by subtracting the CT for the reference gene from the CT for the gene of interest. The relative expression was then calculated by comparing the ΔCT values for fish fed the different experimental diets with fish fed the standard fish oil diet using the term $2^{-\Delta\Delta CT}$ and reported as arbitrary fold change units (Livak & Schmittgen, 2001).

Table 4c. *Sequences of primers used to amplify genes involved in uptake, β-oxidation, desaturation and elongation of FA and the corresponding GenBank accession numbers used in primer design*

Primer	Primer pair (5'-3')	Sequence	GenBank Acc. no
CD36[a]	Forward	GGATGAACTCCCTGCATGTGA	AY606034
	Reverse	TGAGGCCAAAGTACTCGTCGA	
SR-B1[b]	Forward	AACTCAGTGAAGAGGCCAAACTTG	DQ266043
	Reverse	TGCGGCGGTGATGATG	
ACO[b]	Forward	CCTTCATTGTACCTCTCCGCA	DQ364432
	Reverse	CATTTCAACCTCATCAAAGCCAA	
CPT1[a]	Forward	GTACCAGCCCCGATGCCTTCAT	AM230810
	Reverse	TCTCTGTGCGACCCTCTCGGAA	
Δ5FAD[a]	Forward	GAGAGCTGGCACCGACAGAG	AF478472
	Reverse	GAGCTGCATTTTTCCCATGG	
Δ6FAD[a]	Forward	AGAGCGTAGCTGACACAGCG	AY458652
	Reverse	TCCTCGGTTCTCTCTGCTCC	
ELOVL2[c]	Forward	CGGGTACAAAATGTGCTGGT	TC91192
	Reverse	TCTGTTTGCCGATAGCCATT	
ELOVL4[d]	Forward	TTGTCAAATTGGTCCTGTGC	HM208347
	Reverse	TTAAAAGCCCTTTGGGATGA	
ELOVL5a[c]	Forward	ACAAGACAGGAATCTCTTTCAGATTAA	AY170327
	Reverse	TCTGGGGTTACTGTGCTATAGTGTAC	
ELOVL5b[c]	Forward	ACAAAAAGCCATGTTTATCTGAAAGA	DW546112
	Reverse	CACAGCCCCAGAGACCCACTT	

Abbreviations: CD 36 = cluster of differentiation 36, SR-B1 = scavenger receptor class BI, ACO = acyl-CoA oxidase, CPT1 = carnitine palmitoyl transferase I, Δ5FAD = Δ5 desaturase, Δ6FAD = Δ6 desaturase, ELOVL = elongation of very long chain fatty acids gene. Already designed and validated in: a) Trattner *et al.* (2008c), b) Kleveland *et al.* (2006a), c) Morais *et al.* (2009), d) Carmona-Antoñanzas *et al.* (2011).

The RT-PCR assay for PGC-1α was designed using the cDNA sequence from rainbow trout.

6.5 MicroRNA analysis

Juvenile Atlantic salmon (53 g) approximately 10 months old were reared under standard conditions at the Älvkarleby research station. Five fish were sacrificed pre-smoltification and tissues were dissected and stored in RNALater until further miRNA isolation. From each fish, the liver, spleen, kidney, brain, heart, intestine, stomach, gill, red and white muscle were collected. A pool (Pool 1) of the different somatic tissues was constructed from three fish, with roughly 0.03 g taken from each tissue. A corresponding pool (Pool 2) of six liver samples from fish post-smoltification (1300 g) was

constructed for further miRNA extraction and cDNA library synthesis. The tissues in the two pools were ground under liquid nitrogen.

All miRNA isolation and enrichment and construction of cDNA libraries for Illumina sequencing were performed by Vertis Biotechnology AG, Germany (http://www.vertisbiotech.com/).

The RNA samples were separated on denaturing 15% polyacrylamide gel. As molecular mass standard, a mixture of oligonucleotides with size 19 nt and 29 nt was loaded. This mixture was also used as internal size marker in the RNA samples (*Figure 6A*). The small RNA fractions with a length of 19-29 bases were obtained by passive elution of the RNAs from the gels. The eluted miRNA was then precipitated with ethanol and dissolved in water.

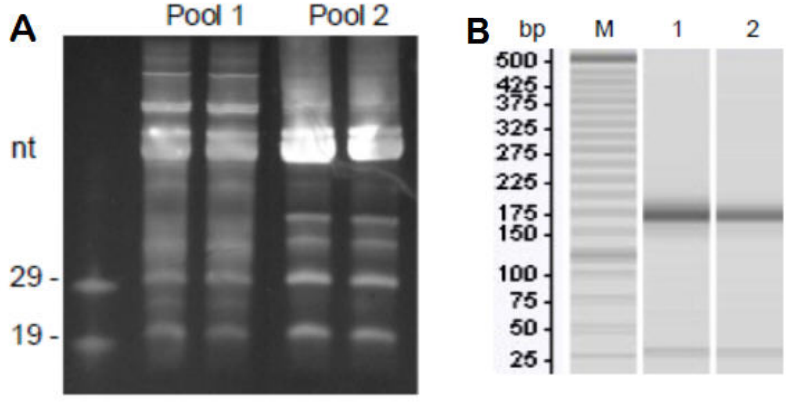


Figure 6. A) Separation of small RNA samples on denaturing 15% polyacrylamide gels for extraction of miRNA in the size range 19-29 nt and B) Analysis of PCR-amplified cDNAs on a Shimadzu MultiNA microchip electrophoresis system. M = 25 bp ladder.

6.6 Next Generation Sequencing

Illumina Sequencing-by-Synthesis enables discovery and profiling of microRNAs without prior genome annotation. The cDNA samples were pooled in equimolar amounts and the cDNA pool was sequenced on an Illumina® HiSeq™ 2000 Sequencing System (Illumina Inc., San Diego, CA) following the manufacturer's instructions at Vertis Biotechnology AG (Freising-Weihenstephan, Germany).

6.7 Computational methods

The dataset of small RNA were annotated to identify known miRNAs. Any miRNAs with conserved sequences matching previously discovered *S. salar* miRNAs were identified by a Basic Local Alignment Search Tool (BLAST)

search against MiRBase database version 21 (http://microrna.sanger.ac.uk/) using the CLCbio CLC Genomics Workbench for comparing the datasets against currently released miRNAs in miRBase v.21 using the default settings. Only data from the mature liver (Pool 2) were analyzed in this thesis.

After being classified into different categories based on sequence similarity, the remaining reads of the datasets were compared against currently released miRNAs of *Danio rerio*, *Cyprinus carpio*, *Hippoglossus hippoglossus*, *Takifugu rubripes*, *Ictalurus punctatus*, *Oryzias latipes*, *Paralichthys olivaceus* and *Tetraodon nigroviridis* in miRBase. Additional miRNAs identified were considered to be homologues to the published miRNAs if they had less than two mismatches, and were named accordingly.

Finally, all the annotated miRNAs were compared against the Atlantic salmon miRNAs identified by Barozai (2012), Bekaert *et al*. (2013) and Andreassen *et al*. (2013) and against miRNAs cloned and sequenced for rainbow trout (*O*. *mykiss*) (Ma *et al.*, 2012; Salem *et al.*, 2010; Ramachandra *et al.*, 2008) to identify conserved miRNAs (Griffiths-Jones *et al.*, 2008).

6.8 MicroRNA expression analysis

6.8.1 Candidates for endogenous controls

The expression of seven putative endogenous control genes (*ssa-let-7a*, *ssa-miR-16a*, *ssa-miR-16b*, *ssa-miR-194a*, *ssa-miR-22a*, *ssa-miR-22b* and *ssa-miR-27c*) was examined with regard to their tissue distribution and use as endogenous controls in microRNA expression studies.

By modification of the traditional TaqMan® assay concept by introduction of a target-specific stem-loop reverse transcription (RT) primer, it was possible to overcome the problem with the short length of mature miRNA without risking target specificity and precision in quantification. The primers used are presented in *Table 5*.

The stability and suitability of the miRNAs across all experimental variables and samples was tested using the DataAssist software version 2.0 (Applied Biosystems of Life Technologies, Foster City), where a low score indicates a stable control. The tissue distribution of miRNAs was tested using white muscle as reference tissue and *ssa-miR-27c* as the endogenous control gene.

6.8.2 Tissue distribution of selected miRNA

Expression of miRNA in gills, heart, brain, liver, stomach, spleen, kidney, red muscle, intestine and white muscle was investigated by qPCR using a selection of miRNA genes known to be involved in lipid homeostasis in mammals. The

miRNAs selected were *ssa-miR-26b*, *ssa-miR-92a-1*, *ssa-miR-122* and *ssa-miR-722*, which showed a high presence in liver, and *miR-21-1*, which was common in muscle (unpublished NGS data on juvenile Atlantic salmon pre-smoltification). In previous studies, *ssa-miR-143* has been connected to lipid metabolism in human and porcine adipose tissues (Wang *et al.*, 2011; Esau *et al.*, 2004) and was included in this study. In addition to its presence in liver, *ssa-miR-122* was chosen since documented connections to the metabolism of lipids have been reported (Esau *et al.*, 2006b).

6.9 Statistical analysis

All data in tables are presented as mean value ± standard deviation (SD). Differences between values were considered significant at $P \leq 0.05$. FAs were compared using the General Linear Model in SAS statistical software. The model included the fixed effect of treatment and random effect of individual. Correlation tests were performed using Minitab 15 statistical software. Relative expression of the different genes was determined and mean values and SD were calculated using StepOne™ software (ver. 2.2) and DataAssist software (ver. 2.0). The 95% confidence interval was calculated and used for statistical discrimination evaluation.

Table 5. *The miRNA primers used in Paper IV*

miRNA	miRNA sequence	TaqMan Q-PCR primer sequence
ssa-miR-722	UUUUGCAGAAACGUUUCAGAUU	TTTTGCAGAAACGTTTCAGATT
ssa-miR-122	UGGAGUGUGACAAUGGUGUUUG	TGGAGTGTGACAATGGTGTTTG
ssa-miR-194a	UGUAACAGCAACUCCAUGUGG	TGTAACAGCAACTCCATGTGG
ssa-miR-22a	AAGCUGCCAGCUGAAGAACUGU	AAGCTGCCAGCTGAAGAACTGT
ssa-miR-22b	AAGCUGCCAGUUGAAGAGCUGU	AAGCTGCCAGTTGAAGAGCTGT
ssa-miR-26b	UUCAAGUAAUCCAGGAUAGGUU	TTCAAGTAATCCAGGATAGGTT
ssa-miR-92a-1	UAUUGCACUUGUCCCGGCCUGU	TATTGCACTTGTCCCGGCCTGT
ssa-miR-16a	UAGCAGCACGUAAAUAUUGGAG	TAGCAGCACGTAAATATTGGAG
ssa-miR-16b	UAGCAGCACGUAAAUAUUGGUG	TAGCAGCACGTAAATATTGGTG
ssa-let-7a	UGAGGUAGUAGGUUGUAUAGUU	TGAGGTAGTAGGTTGTATAGTT
ssa-miR-143-1	UGAGAUGAAGCACUGUAGCUC	TGAGATGAAGCACTGTAGCTC
ssa-miR-21-1	UAGCUUAUCAGACUGGUGUUGGC	TAGCTTATCAGACTGGTGTTGGC
ssa-miR-27c	UUCACAGUGGUUAAGUUCUGC	TTCACAGTGGTTAAGTTCTGC

7 Summary of results

7.1 Lipid analysis

The results of changes in lipid composition as a result of different feeding diets in Papers I-III are presented in *Table 6* as percent unit increase or decrease in FA compared with the average FA content in fish fed the FO diet only. The data are shown without detailed statistical data, but significant differences are indicated. Detailed statistical data can be found in the individual papers.

In Papers I and II, the fat content in the white muscle was ~1.5% and ~1.6%, respectively, regardless of treatment, whereas the fat content in the liver in Paper II increased significantly from ~5% to 7-8% in fish with the highest level of sesamin supplementation.

The SAFA content in the white muscle triacylglycerol fraction in Paper I was lowered, but not significantly, in fish fed VO compared with fish fed FO, regardless of sesamin supplementation (*Table 6a*). In the PL fractions, there was an overall tendency for an increase in SAFA content when fish were fed the VO diet. However, there was still no clear significant increase except when fish were fed LO with supplementation of sesamin. In Papers I and III, the SAFA content in white muscle of fish fed FO and in the cell culture media was 21.8% and 20.1%, respectively.

In Paper II, the percentage of SAFA was significantly lower in both the triacylglycerol and PL fractions in liver and white muscle samples from fish fed VO compared with fish fed FO. The relative amount of SAFA in both triacylglycerols and PL fractions of the liver and white muscle of fish fed FO was on average 24.7%. The supplementation of sesamin to the VO diets decreased the amount of SAFA in the triacylglycerol fractions of both white muscle and liver (*Table 6a*) compared with an unsupplemented VO diet. In the liver, the amount of SAFA was lowered in a dose-dependent manner.

Table 6a. *Effect of bioactive compounds on saturated fatty acids (SAFA) in the triacylglycerol and phospholipid fractions (difference in percentage units of total identified, mean value) of white muscle (Papers I & II) and liver from fish fed a vegetable oil diet (Paper II only) compared with fish fed a fish oil (FO) diet (control) and in hepatocytes after incubation with the respective treatment (Paper III)*

	Feed	Tissue	Fraction	Bioactive compound supplementation						
				VO control[1)]	Sesamin	High sesamin	Episesamin	Lipoic acid	Low genistein	High genistein
Paper I[2)]	Linseed oil (LO)	White muscle	Triacylglycerols	-5.4	-3.9					
			Phospholipids	0.6^{ab}	3.2^{b}					
	Purified Linseed oil (TAG)	White muscle	Triacylglycerols	-4.5	-5.2					
			Phospholipids	-0.3^{ab}	0.6^{ab}					
	Mixed oil (MO)	White muscle	Triacylglycerols	-4.9	-3.9					
			Phospholipids	1.8^{ab}	1.6^{ab}					
Paper II[3)]	Low n-6/n-3	White muscle	Triacylglycerols	-7.8^{c}	-7.7^{bc}	-8.0^{c}				
			Phospholipids	-5.1^{c}	-3.8^{bc}	-4.4^{bc}				
		Liver	Triacylglycerols	-3.9^{b}	-6.5^{bcd}	-7.4^{cd}				
			Phospholipids	1.1	0.2	0.3				
	High n-6/n-3	White muscle	Triacylglycerols	-7.0^{bc}	-6.9^{b}	-7.6^{bc}				
			Phospholipids	-3.2^{b}	-3.5^{b}	-2.9^{c}				
		Liver	Triacylglycerols	-5.7^{bc}	-7.8^{cd}	-9.2^{d}				
			Phospholipids	-2.5^{b}	-3.3^{b}	-3.3^{b}				
Paper III	-	-	Culture media	-	-0.6	-	0.1	-0.5	-0.5	-0.1

1) Vegetable oil control diet without supplementation of bioactive compounds; 2) Rainbow trout; 3) Atlantic salmon

Table 6b. *Effect of bioactive compounds on monounsaturated fatty acids (MUFA) in the triacylglycerol and phospholipid fractions (difference in percentage units of total identified, mean value) of white muscle (Papers I & II) and liver from fish fed a vegetable oil diet (Paper II only) compared with fish fed a fish oil-based diet and in hepatocytes after incubation with the respective treatment (Paper III)*

	Feed	Tissue	Fraction	Bioactive compound supplementation						
				VO control[1)]	Sesamin	High sesamin	Episesamin	Lipoic acid	Low genistein	High genistein
Paper I[2)]	Linseed oil (LO)	White muscle	Triacylglycerols	1.6^{ab}	2.6^{b}					
			Phospholipids	2.2^{b}	2.5^{b}					
	Purified Linseed oil (TAG)	White muscle	Triacylglycerols	3.0^{b}	2.9^{b}					
			Phospholipids	2.2^{b}	3.0^{b}					
	Mixed oil (MO)	White muscle	Triacylglycerols	3.0^{b}	3.5^{b}					
			Phospholipids	3.2^{b}	2.1^{b}					
Paper II[3)]	Low n-6/n-3	White muscle	Triacylglycerols	-8.1^{d}	-7.8^{d}	-8.2^{d}				
			Phospholipids	2.4^{b}	2.2^{b}	2.4^{b}				
		Liver	Triacylglycerols	3.1	1.0	5.2				
			Phospholipids	-14.8^{c}	-14.6^{d}	-12.9^{c}				
	High n-6/n-3	White muscle	Triacylglycerols	4.5^{c}	3.8^{bc}	3.6^{b}				
			Phospholipids	5.9^{c}	6.1^{c}	5.9^{c}				
		Liver	Triacylglycerols	22.2^{c}	13.9^{b}	14.8^{b}				
			Phospholipids	-12.7^{c}	-12.4^{bc}	-11.2^{b}				
Paper III	-	-	Culture media	-	1.0	-	0.6	-1.5	0.1	-0.1

1) Vegetable oil control diet without supplementation of bioactive compounds; 2) Rainbow trout; 3) Atlantic salmon

Table 6c. *Effect of bioactive compounds on linoleic acid (LA, 18:2n-6) in the triacylglycerol and phospholipid fractions (difference in percentage units of total identified, mean value) of white muscle (Papers I & II) and liver from fish fed a vegetable oil diet (Paper II only) compared with fish fed a fish oil-based diet and in hepatocytes after incubation with the respective treatment (Paper III)*

	Feed	Tissue	Fraction	Bioactive compound supplementation						
				VO control[1)]	Sesamin	High sesamin	Episesamin	Lipoic acid	Low genistein	High genistein
Paper I[2)]	Linseed oil (LO)	White muscle	Triacylglycerols	9.4^{b}	8.1^{b}					
			Phospholipids	4.0^{a}	3.4^{bc}					
	Purified Linseed oil (TAG)	White muscle	Triacylglycerols	7.1^{b}	7.9^{b}					
			Phospholipids	3.5^{b}	3.2^{b}					
	Mixed oil (MO)	White muscle	Triacylglycerols	16.3^{c}	13.9^{c}					
			Phospholipids	7.0^{d}	6.3^{d}					
Paper II[3)]	Low n-6/n-3	White muscle	Triacylglycerols	8.7^{abc}	8.5^{bc}	8.8^{ab}				
			Phospholipids	4.7^{b}	4.5^{b}	4.8^{b}				
		Liver	Triacylglycerols	5.3^{b}	6.8^{a}	6.1^{ab}				
			Phospholipids	4.3^{c}	5.0^{ab}	5.2^{ab}				
	High n-6/n-3	White muscle	Triacylglycerols	8.4^{c}	8.5^{bc}	9.0^{a}				
			Phospholipids	6.6^{a}	6.4^{a}	6.5^{a}				
		Liver	Triacylglycerols	5.1^{b}	6.1^{ab}	6.2^{ab}				
			Phospholipids	4.7^{bc}	4.8^{ab}	5.3^{a}				
Paper III	-	-	Culture media	-	0.5	-	0.3	0.3	0.1	0.3

1) Vegetable oil control diet without supplementation of bioactive compounds; 2) Rainbow trout; 3) Atlantic salmon

Table 6d. *Effect of bioactive compounds on α-linolenic acid (ALA, 18:3n-3) in the triacylglycerol and phospholipid fractions (difference in percentage units of total identified, mean value) of white muscle (Papers I & II) and liver from fish fed a vegetable oil diet (Paper II only) compared with fish fed a fish oil-based diet and in hepatocytes after incubation with the respective treatment (Paper III)*

	Feed	Tissue	Fraction	Bioactive compound supplementation						
				VO control[1)]	Sesamin	High sesamin	Episesamin	Lipoic acid	Low genistein	High genistein
Paper I[2)]	Linseed oil (LO)	White muscle	Triacylglycerols	16.0^{d}	13.5^{c}					
			Phospholipids	8.8^{c}	7.7^{c}					
	Purified Linseed oil (TAG)	White muscle	Triacylglycerols	12.6^{c}	13.4^{c}					
			Phospholipids	8.5^{c}	7.9^{c}					
	Mixed oil (MO)	White muscle	Triacylglycerols	7.7^{b}	7.0^{b}					
			Phospholipids	5.5^{b}	5.0^{b}					
Paper II[3)]	Low n-6/n-3	White muscle	Triacylglycerols	17.4^{b}	17.0^{b}	17.9^{b}				
			Phospholipids	10.9^{b}	10.3^{b}	10.3^{b}				
		Liver	Triacylglycerols	8.5^{c}	11.1^{b}	9.8^{bc}				
			Phospholipids	3.5^{c}	4.2^{b}	4.6^{b}				
	High n-6/n-3	White muscle	Triacylglycerols	6.0^{c}	5.9^{c}	6.4^{c}				
			Phospholipids	5.3^{c}	5.0^{c}	5.1^{c}				
		Liver	Triacylglycerols	2.7^{d}	2.72^{d}	2.87^{d}				
			Phospholipids	1.3^{d}	1.51^{d}	1.69^{d}				
Paper III	-	-	Culture media	-	0.5	-	0.3	0.4	0.10	0.2

1) Vegetable oil control diet without supplementation of bioactive compounds; 2) Rainbow trout; 3) Atlantic salmon

Table 6e. *Effect of bioactive compounds on eicosapentaenoic acid (EPA, 20:5n-3) in the triacylglycerol and phospholipid fractions (difference in percentage units of total identified, mean value) of white muscle (Papers I & II) and liver from fish fed a vegetable oil diet (Paper II only) compared with fish fed a fish oil-based diet and in hepatocytes after incubation with the respective treatment (Paper III)*

				Bioactive compound supplementation						
	Feed	Tissue	Fraction	VO control[1)]	Sesamin	High sesamin	Episesamin	Lipoic acid	Low genistein	High genistein
Paper I[2)]	Linseed oil (LO)	White muscle	Triacylglycerols	-1.9^{a}	-1.7^{abc}					
			Phospholipids	-1.0	-0.7					
	Purified Linseed oil (TAG)	White muscle	Triacylglycerols	-1.5^{bc}	-1.4^{c}					
			Phospholipids	-0.6	-0.4					
	Mixed oil (MO)	White muscle	Triacylglycerols	-1.8^{ab}	-1.6^{abc}					
			Phospholipids	-0.4	-0.9					
Paper II[3)]	Low n-6/n-3	White muscle	Triacylglycerols	-2.7^{c}	-2.9^{cd}	-3.1^{d}				
			Phospholipids	-0.7^{b}	-0.9^{bc}	-1.2^{cd}				
		Liver	Triacylglycerols	5.3^{b}	6.8^{c}	6.1^{bc}				
			Phospholipids	4.3^{d}	5.0^{bc}	5.2^{bc}				
	High n-6/n-3	White muscle	Triacylglycerols	-3.1^{d}	-3.0^{cd}	-3.2^{d}				
			Phospholipids	-1.0^{c}	-1.0^{c}	-1.3^{d}				
		Liver	Triacylglycerols	5.1^{b}	6.1^{bc}	6.2^{bc}				
			Phospholipids	4.7^{cd}	4.8^{bc}	5.3^{b}				
Paper III	-	-	Culture media	-	0.0	-	0.03	0.15	-0.03	0.05

1) Vegetable oil control diet without supplementation of bioactive compounds; 2) Rainbow trout; 3) Atlantic salmon

Table 6f. *Effect of bioactive compounds on docosahexaenoic acid (DHA, 22:6n-3) in the triacylglycerol and phospholipid fractions (difference in percentage units of total identified, mean value) of white muscle (Papers I & II) and liver from fish fed a vegetable oil diet (Paper II only) compared with fish fed a fish oil-based diet and in hepatocytes after incubation with the respective treatment (Paper III)*

	Feed	Tissue	Fraction	Bioactive compound supplementation						
				VO control[1)]	Sesamin	High sesamin	Episesamin	Lipoic acid	Low genistein	High genistein
Paper I[2)]	Linseed oil (LO)	White muscle	Triacylglycerols	-4.9^{b}	-4.5^{b}					
			Phospholipids	-18.9^{b}	-18.2^{b}					
	Purified Linseed oil (TAG)	White muscle	Triacylglycerols	-4.8^{b}	-4.7^{b}					
			Phospholipids	-17.7^{b}	-16.9^{b}					
	Mixed oil (MO)	White muscle	Triacylglycerols	-5.0^{b}	-4.3^{b}					
			Phospholipids	-18.8^{b}	-16.3^{b}					
Paper II[3)]	Low n-6/n-3	White muscle	Triacylglycerols	-8.3^{b}	-8.2^{b}	-8.4^{b}				
			Phospholipids	-14.7^{b}	-14.1^{b}	-13.1^{b}				
		Liver	Triacylglycerols	-9.4^{bc}	9.1^{b}	-10.1^{c}				
			Phospholipids	-11.4^{d}	-10.4^{cd}	-12.2^{d}				
	High n-6/n-3	White muscle	Triacylglycerols	-8.6^{b}	-8.5^{b}	-8.4^{b}				
			Phospholipids	-15.7^{b}	-15.1^{b}	-14.2^{b}				
		Liver	Triacylglycerols	-9.3^{bc}	-9.8^{bc}	-9.9^{bc}				
			Phospholipids	-8.6^{bc}	-9.3^{bc}	-7.9^{b}				
Paper III	-	-	Culture media	-	-1.3	-	-1.0	0.7	-0.1	-0.5

1) Vegetable oil control diet without supplementation of bioactive compounds; 2) Rainbow trout; 3) Atlantic salmon

The decrease was independent of the n-6/n-3 value in the diet fed to the fish. Of the individual SAFA, 16:0 was most clearly affected by the addition of sesamin to the feed, followed by 18:0. The addition of bioactive compounds to the hepatocyte cell media did not affect the content of SAFA in Paper III.

The amount of MUFA (*Table 6b*) in the triacylglycerol and PL fractions from rainbow trout white muscle samples (Paper I) fed the FO diet was 27.9% and 8.0%, respectively. A significant increase in the amount of MUFA was detected in fish fed all the different VO diets with the exception for LO (non-significant). When sesamin was added to the feed, there tended to be an additional increase in MUFA content, but this effect was not significant.

In Paper II, the MUFA content in the triacylglycerol and PL fractions for Atlantic salmon white muscle were 48% and 14.5%, respectively. In the liver the corresponding triacylglycerol and PL fractions were much higher, 51.4% and 32.3%, respectively. The level of MUFA in the PL fraction of the liver was significantly decreased regardless of n-6/n-3 ratio and sesamin supplementation. In contrast, the MUFA content in the PL fraction of the white muscle was significantly increased regardless of n-6/n-3 ratio and sesamin supplementation. In the triacylglycerol fractions of both white muscle and liver in fish fed the diet with the higher n-6/n-3 ratio, the amount of MUFA was at a significantly higher level than in the control fish fed FO diets. Sesamin, when added to the feed, significantly decreased the level of MUFA in the triacylglycerol fractions for the higher n-6/n-3 ratio group in both white muscle and liver. The results were quite different in the triacylglycerol fraction for fish fed the low n-6/n-3 diet, where the amount of MUFA in the liver was the same as in fish fed the FO diet and no effect was seen with the addition of sesamin. This can be compared with the significant increase in MUFA in the corresponding triacylglycerol fraction in fish fed the high n-6/n-3 VO feed, followed by a significant decrease on addition of sesamin to the diet.

In white muscle samples from fish fed the low n-6/n-3 diet, the amount of MUFA in the triacylglycerol fraction was significantly decreased compared with fish fed the FO diet, but no increase or additional decrease was seen when sesamin was added.

In Paper III, no effect was observed in the MUFA content after bioactive compound supplementation.

In Paper I, the amount of LA increased in both the triacylglycerol and PL fractions, but the increase in the triacylglycerol fraction was twice that in the PL fraction. There were no significant differences in how LA was incorporated from the two linseed oils, but twice the amount of LA was detected in white muscle from rainbow trout given MO.

In Paper II, the amount of LA increased in triacylglycerol and PL fractions in both white muscle and liver samples from fish fed the VO diets (*Table 6c*), regardless of n-6/n-3 ratio, compared with fish fed FO diet. No significant effects were seen on LA content after incubation with sesamin, regardless of concentration. However, there was a trend towards high incorporation of LA into both white muscle and liver after diet supplementation with sesamin.

In Paper II, the amount of LA in the triacylglycerol and PL fractions of white muscle from fish fed FO was 5.05% and 1.9%, respectively. The corresponding values in the liver were 5.71% and 2.1%.

When FO was exchanged for VO (Paper I), the amount of ALA increased significantly in both the triacylglycerol and PL fractions. The amount of ALA measured in the triacylglycerol and PL fractions from fish fed FO was 1.37% and 0.61%, respectively. There were significant differences in the amount of ALA incorporated into white muscle of rainbow trout depending on the type of VO used as feed. When purified TAG was used instead of crude LO, the incorporation of ALA declined, and when mixed oils (MO) were used the incorporation of ALA into white muscle declined even further.

The amount of ALA in the triacylglycerol and PL fractions increased in both white muscle and liver samples from fish fed the VO diets in all three studies (*Table 6d*). The increase was dependent on the n-6/n-3 ratio fed to the fish, with the percentage increase in ALA being twice as high in fish fed the low n-6/n-3 ratio compared with the high n-6/n-3 ratio. This was not observed for LA (*Table 6c*). In Paper II, no effects were seen on ALA composition after incubation of any of the bioactive compounds used.

No effects of bioactive compound supplementation on either LA (*Table 6c*) or ALA (*Table 6d*) were observed in any of Papers I-III.

On comparing the changes in EPA and DPA in *Table 6e* and *Table 6f*, it can be seen the amount of EPA increased in both the triacylglycerol and PL fractions in the liver of fish fed the VO diet, regardless of n-6/n-3 ratio (Paper II). When sesamin was added to the feed the amount of EPA increased significantly further. In contrast, EPA decreased in the white muscle samples in all fish fed VO and no effect of addition of sesamin was observed. Similarly, the amount of EPA decreased in white muscle of all fish fed VO diets in Paper I, regardless of VO composition.

The DHA content decreased in all fish fed VO diets, regardless of treatment. There was a tendency for a greater increase in the PL fraction than in the triacylglycerol fraction in the white muscle of fish fed all VO diets in Paper I and fish fed the lower n-3/n-6 ratio in Paper II. However, this tendency was not statistically significant.

7.2 Gene expression

An overview of the relative changes in gene expression reported as fold change in Papers I, II and III is given in *Table 7a* and *7b*.

In Paper II and III, the results were normalized against a housekeeping gene selected on the basis that this gene was unaffected by any of the experimental parameters tested. A second round of normalization was then performed against the control treatment (FO). The final results are presented as relative fold change compared with the control. In Paper I, the fold change calculations were similar for the liver samples tested, but for the white muscle samples no data were available for the fish fed FO, so the fold change data show the effect of sesamin supplementation on fish fed the VO diet, not the total effect of VO compared with the FO diet.

In Paper I (*Table 7a*), different combinations of LO-based VO in combination with sesamin supplementation were examined. In fish fed LO or TAG, the effect on all genes tested was similar in white muscle. In general, the expression of *PPAR*s, *Δ6FAD* and *ACO* decreased in white muscle when sesamin was added to the VO diet, except for the gene coding for the rate limiting CPT1 enzyme involved in mitochondrial β-oxidation, where the gene expression increased significantly compared with fish fed a strict VO diet.

In the liver samples, changing to a VO diet in general, regardless of the type of VO used, resulted in increased expression of all genes tested except *PPARα*, where no effect or a small decrease was observed. On adding sesamin to the diet, gene expression was unaffected for *PPARα* and *PPARγ*, but increased for the other genes tested, in fish fed the LO diet. For *Δ6FAD* and *ACO*, the increase in expression after sesamin supplementation was consistent regardless of the type of linseed oil used, in comparison to fish fed FO and fish fed the VO diet. For fish fed either the TAG or MO diet, the effect of sesamin supplementation was limited for *CPT1* compared with a strict VO diet, but there was still a significant increase compared with liver samples from fish fed FO. For *PPARα*, *PPARβ1* and *PPARγ*, addition of sesamin to the diet resulted in a slight to significant decrease in expression for both the TAG and MO diets.

In the experiment described in Paper II (*Table 7a*), no clear significant picture emerged for any of the transcription factors tested following sesamin addition. There was a general downregulating tendency among the transcription factors. In the case of *PPARγ* in Paper II, significant upregulation was seen when the highest concentration of sesamin was added to the high n-6/n-3 ratio diets, while for *PPARβ1A* high concentrations of sesamin decreased the mRNA expression level, regardless of n-6/n-3 level. The exceptions were *SREBP-1*, *SREPB-2* and *LXR*, where clear downregulation was evident in fish fed the low n-6/n-3 VO diet and where there was an increase in expression to

equal or above the levels detected in fish fed the FO diet after addition of the lower amount of sesamin. For fish fed the high n-6/n-3 VO diet, no clear effect was seen on *SREBP-1* and *SREPB-2* and *LXR* compared with fish fed the FO diet. Moreover, sesamin supplementation did not have any distinct effect except for significant downregulation of *SREBP-1* when the lower concentration of sesamin was added to the high n-6/n-3 VO diet.

In general, in fish fed the low n-6/n-3 ratio VO diet, genes coding for enzymes in the elongation and desaturation cascade showed lower expression than in fish fed the FO diet, except for *ELOVL2* and *ELOVL5b*, which were higher in expression rate in fish fed VO. On the other hand, in fish fed the high n-6/n-3 diet, the same set of genes was generally significantly upregulated compared with fish fed the FO diet. In most cases the lower concentration of sesamin increased the expression of genes coding for enzymes in the elongation and desaturation cascade. The exceptions were *ELOVL5a*, *ELOVL5b* and *ELOVL4*, which showed no effect or significant downregulation when sesamin was added to the high n-6/n-3 VO diet.

The effects of the different bioactive compounds (Paper III) were more apparent after 48 hours of incubation (*Table 7b*). In general, sesamin, episesamin and LPA increased the expression of *Δ5FAD*, *Δ6FAD*, *ELOLV2*, *ELOLV5a*, *CD36*, *CPT-1* and *ACO*. Sesamin also increased *PPAR1β* and *PPARγ* and episesamin increased *PPARα* and *PPAR1β*. Incubation with LPA for 12 hours increased the expression of all *PPAR*, but further incubation for 48 hours decreased the expression of *PPARα* and *PPAR1β*. During the same incubation period, LPA significantly increased *PPARγ* transcription.

Table 7a. *Effect of sesamin (S) on relative gene expression (fold change) in white muscle and liver from rainbow trout (Paper I) and liver from Atlantic salmon (Paper II) fed vegetable oil diets (VO). Increase in fold change are all values >1.00 and all data <1.00 express a decrease compared with the control fish oil (FO) diet. Data with values close to 1.00 indicate no change, a"*" indicate significantly different from FO and "#" significantly different from VO.*

		Paper I									Paper II					
		Linseed oil (LO)			Purified Linseed oil (TAG)			Mixed oil (MO)			Low n-6/n-3			High n-6/n-3		
		White muscle	Liver		White muscle	Liver		White muscle	Liver		VO	Sesamin	High sesamin	VO	Sesamin	High sesamin
		VO+S	VO	VO+S	VO+S	VO	VO+S	VO+S	VO	VO+S						
Transcription factors	PPARα	0.59*	1.27	1.20	0.64*	1.15	0.86	1.36	1.53*	0.93#	0.88	0.90	0.61	0.93	0.92	0.90
	PPARβ1A	0.81.	3.41*	4.44*#	0.35*	3.48*	2.06*#	1.66*	4.77*	4.16*	0.82	0.78	0.57*	0.77	0.74	0.51*#
	PPARγ (long/short)	0.65*	2.14*	2.13*	0.09*	1.50*	01.57*	1.10	2.21*	0.88*	1.05	1.17	1.00	0.99	0.99	1.64*#
	PCG-1										0.41	0.60*	0.40*	0.66*	0.83	1.12
	SREBP-1										0.53	1.93*#	1.05#	0.73	0.46*#	0.74
	SREBP-2										0.57*	1.40*#	0.71	0.93	0.99	0.91
	LXR										0.56*	1.10#	1.46*#	1.03	1.03	1.20
Uptake	CD36										0.87	1.06	0.73	0.89*	0.69*#	0.63
	SP-B1										1.20	1.29	0.91	1.12	0.84	1.29
Elongation & Desaturation	Δ5FAD										0.45	2.34*#	2.87*#	2.59*	1.86*#	1.27
	Δ6FAD	0.70*	0.74	1.63*#	0.22*	1.82*	3.17*#	0.79	2 .23*	6.91*#	0.65*	2.70*#	3.36*#	4.38*	1.61*#	0.94#
	ELOVL2										1.47	2.13*#	0.87#	2.38*	1.85	1.50#
	ELOVL5a										0.78	1.18	1.80*#	0.99	1.11	1.15
	ELOVL5b										2.04*	2.34*	1.69*#	1.55	0.89#	1.09
	ELOVL4										0.44*	2.19*#	0.42*	0.27*	0.16*	1.43#
β-oxidation	CPT1	2.06*	1.00	2.84*#	1.50*	1.99*	1.55*	1.14	3.12*	3.40*	1.51*	1.35*	0.82#	1.64*	2.05*#	0.97#
	ACO	0.70*	39.5*	63.6*#	0.57*	52.0*#	58.9*	1.21	76.9*#	61.4*#	0.54	0.93	2.69*#	0.68	0.77	0.55*

Table 7b. E*ffect of bioactive compounds on relative gene expression (fold change) in hepatocytes (Paper III) after incubation with the respective treatment after 12 hr or 48 hr respectively. Increase in fold change are all values >1.00 and all data <1.00 express a decrease in gene expression compared with the control fish oil (FO) diet. Data with values close to 1.00 indicate no change and a"∗" indicate significantly different from FO.*

		Paper III									
		Sesamin		Episesamin		Lipoic acid		Low genistein		High genistein	
		12 hr	48 hr	12 hr	48 hr	12 hr	48 hr	12 hr	48 hr	12 hr	48 hr
Transcription factors	PPARα	1.54	0.79	1.67∗	1.30	2.89∗	0.78∗	3.77∗	0.62∗	0.76	0.70
	PPARβ1A	2.39∗	1.51∗	1.01	1.77∗	3.22∗	0.43∗	2.14∗	0.99	0.81	1.36∗
	PPARγ (long/short)	1.99∗	2.57∗	1.17	1.27	3.22∗	4.70∗	1.82∗	0.49∗	0.74	0.81
	PCG-1										
	SREBP-1										
	SREBP-2										
	LXR										
Uptake	CD36	1.11	1.66∗	1.11	2.99∗	1.57∗	2.09∗	1.06	0.74∗	1.37∗	1.06
	SP-B1										
Elongation & Desaturation	Δ5FAD	0.38∗	1.90∗	1.18	1.98∗	0.07∗	1.86∗	0.09∗	0.86	1.02	1.03
	Δ6FAD	1.02	1.71∗	1.02	1.89∗	0.79	1.77∗	1.04	0.87	0.98	1.12
	ELOVL2	12.2∗	2.61∗	0.81	1.96∗	10.9∗	3.01∗	10.7∗	1.15	0.91	1.11
	ELOVL5a	0.93	2.00∗	1.20	2.10∗	1.33	3.44∗	1.39	0.93	1.30	1.00
	ELOVL5b										
	ELOVL4										
β-oxidation	CPT1	1.55	1.91∗	1.01	2.46∗	2.25∗	1.88∗	0.80	1.10	1.87	0.89
	ACO	2.19∗	2.51∗	0.80	1.88∗	2.98∗	3.00∗	2.74∗	1.07	1.08	0.94

7.3 miRNome analysis of liver in mature Atlantic salmon

Total RNA from the liver from six mature individuals at the post-smolt developmental stage was extracted, size-separated and successfully subjected to NGS using the Illumina® HiSeq™ 2000 Sequencing System. Roughly 45 million reads were obtained and of these, 41 million or 90.2% were annotated against the *S. salar* miRBase, roughly 1.5% were annotated against zebrafish (*Danio rerio*) and 8.29% remained unannotated (*Figure 7*).

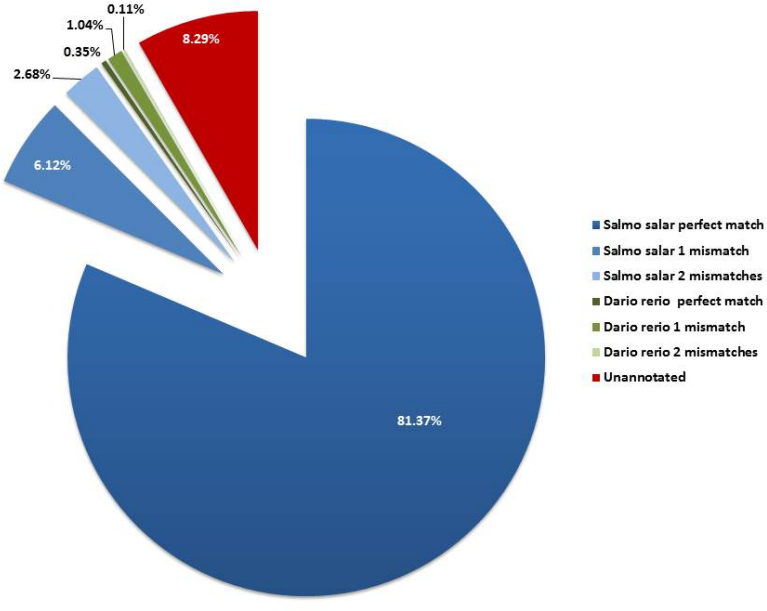


Figure 7. Percentage distribution of the 45 million expression reads generated from the liver of fully grown Atlantic salmon (*Salmon salar*) post-smoltification, divided into reads with a perfect match, reads with one mismatch and reads with two mismatches against annotated miRNAs in *S. salar* and homologues miRNAs in zebrafish (*Dario rerio*), as well as remaining annotated reads.

In total, 229 distinct conserved miRNAs (or 159 miRNA families) were identified (Paper V), not counting the isomiRs. According to recommendations by Ambros *et al.* (2003) and Griffith-Jones *et al.* (2006), the dominant strand must have 10 or more reads to ensure it is not a transcription error. In the material tested, 92 sequences had an abundance below 10. Thus in total, in Paper V we identified 22 conserved miRNAs with high abundance and 137 low abundance conserved miRNAs (Supplementary Table S3 in Paper V), as well as a considerable amount of new isomiRs (Supplementary Table S4 in Paper V). Of the 159 conserved miRNAs identified, 30 were novel conserved miRNAs previously annotated for other vertebrates in miRBase (*Table 8*).

A total of 779 471 small distinct RNA tags were found and of these 4.6% were annotated against *S. salar* entries corresponding to 369 of the 371 existing entries (*Figure 8A*). The two miRNAs not represented were *ssa-miR-219c* and *ssa-miR-7552a*. Of the 369 entries annotated against *S. salar*, 316 (red bars) were also found in *D. rerio*, 244 in *Ictalurus punctatus* (channel catfish) and so on. Of the 743 615 unannotated RNA tags that remained after screening against the *S. salar* miRNA library, an additional 293 were identified as putative orthologues miRNAs annotated against *D. rerio*, 244 against *I. punctatus etc*. (yellow bars in *Figure 8*).

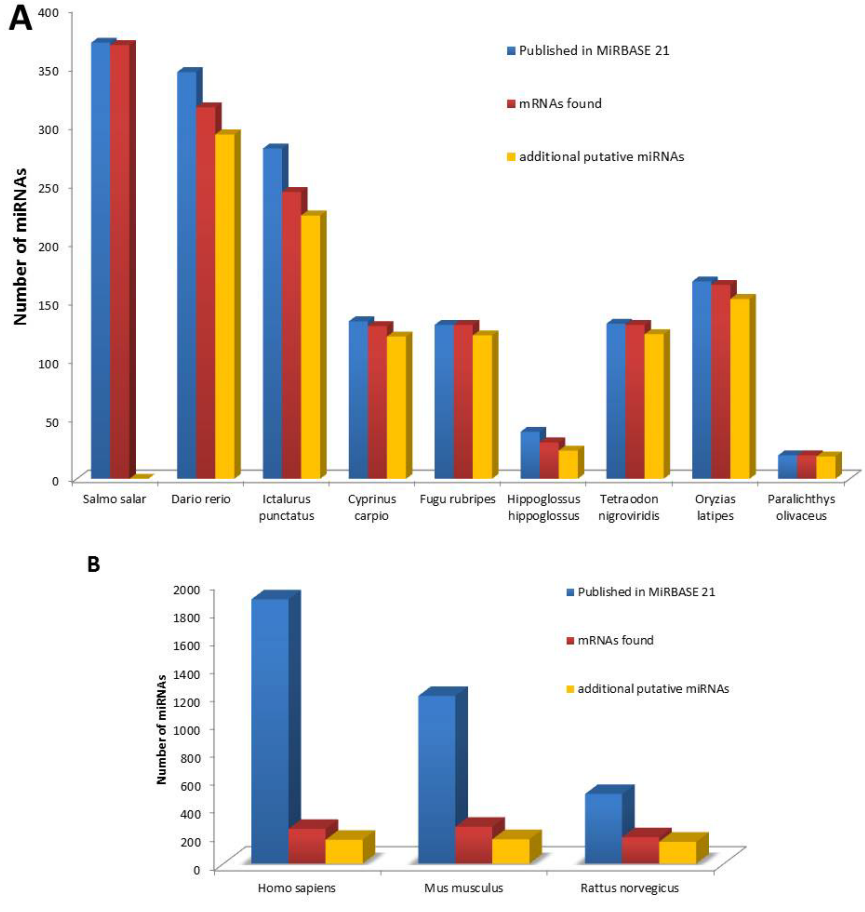


Figure 8. Number of conserved miRNAs and isomiRs identified (red bars) corresponding to published *S. salar* miRNA entries in MiRBase version 21 (blue bars), and the additional number of putative miRNAs identified in the unannotated set after screening against *S. salar* (green bars) in: A) orthologues from other teleost species and B) orthologues from humans, mice and rats.

The residual 95.4% or 743 622 tags remained unannotated. The unannotated tags were once more BLAST-searched and mapped against the published miRNAs in miRBase. The identified miRNAs cover a large proportion of reported miRNAs in

Pisces, including zebrafish, common carp (*Cyprinus carpio*), Atlantic halibut (*Hippoglossus hippoglossus*), torafugu/Japanese pufferfish (*Fugu rubripes*), channel catfish, medaka/Japanese rice fish (*Oryzias latipes*), olive flounder (*Paralichthys olivaceus*) and green spotted puffer fish (*Tetraodon nigroviridis*) (*Figure 8A*), compared with a rather low representation of the published miRNAs belonging to humans (*Homo sapiens*), mice (*Mus musculus*) and rats (*Rattus norvegicus*) (*Figure 8B*). In Paper V the overlap of the miRNA sequences identified was compared with those identified or predicted in earlier studies of Atlantic salmon and rainbow trout, since not all miRNA sequences have been entered into MiRBase ver. 21 (Andreassen *et al.*, 2013; Bekaert *et al.*, 2013; Barozai, 2012; Ma *et al.*, 2012; Salem *et al.*, 2010; Ramachandra *et al.*, 2008). The data proved to be consistent with previous miRNA annotation for *S. salar*.

All miRNAs with an individual abundance above 0.50% (*Figure 9*) and with over 1000 copies of each isomiR are listed in Supplementary Table 4 in Paper V. Star In total, 22 miRNAs had an abundance above 0.5%. Moreover, 43 new isomiRs were identified, of which two were isomiRs of the sequence (*ssa-miR-100-3p* and *ssa-miR-130-5p*). Two new precursors (*ssa-miR-126* and *ssa-miR-130*) were also identified. Three miRNAs (*ssa-miR-722*, *ssa-miR-126* and *ssa-miR-143*) did not have any variants in the material tested.

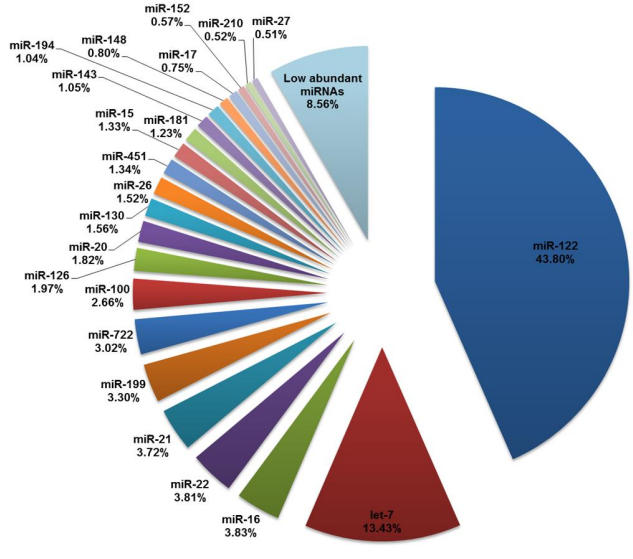
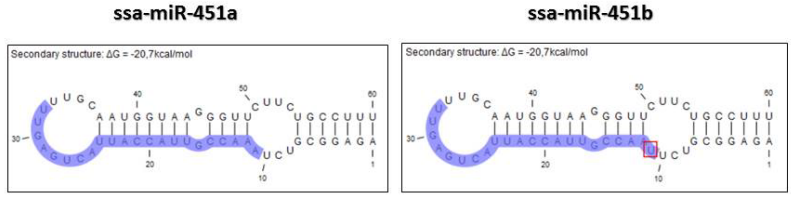


Figure 9. Relative presence of the most common miRNAs in mature liver samples, as a function of expression values.

The relative abundance of miRNAs varied greatly among the conserved miRNAs (*Figure 9*). The most abundant miRNAs included *ssa-miR-122*, *ssa-let-7*, *ssa-miR-16*, *ssa-miR-22*, *ssa-miR-21*, *ssa-miR-199*, *ssa-miR-722*, *ssa-miR-100*, *ssa-miR-126*, *ssa-miR-20*, *ssa-miR-130*, *ssa-miR-26*, *ssa-miR-451*, *ssa-miR-15*, *ssa-miR-181*, *ssa-*

mir-143 and *ssa-miR-194*, which accounted for ~91% of the 41 682 086 reads mapped to miRBase. Of the *ssa-let-7* family, *ssa-let7a*, *ssa-let7e* and *ssa-let7j* were the most abundant.

We also identified 30 novel conserved miRNAs which were homologues to miRNA annotated in MiRBase but not previously described in Atlantic salmon (*Table* 8). Of these novel conserved miRNAs identified, the majority were low abundant (below 0.5%) except for *ssa-miR-451*, which was highly abundant with a presence of 1.43%. Two isoforms of *ssa-miR-451* were identified, where *omy-miR-451a* has been annotated previously in rainbow trout. However, the isomiR *ssa-miR-451b* was by far the most dominantly expressed, with three-fold higher expression than *ssa-miR-451a*. *Ssa-miR-451a* and *ssa-miR-451b* were found to be highly conserved when aligned against previously annotated *miR-451* in miRBase (*Figure 10*).



Salmo salar liver	ssa-mir-451b	AGAGGCGUCUUAACCGUUACCAUUACUGAGUUUUGCAAUGGUAAGGGUUCUUCUGCCUUU
Salmo salar liver	ssa-mir-451a	AGAGGCGUCUAAACCGUUACCAUUACUGAGUUUUGCAAUGGUAAGGGUUCUUCUGCCUUU
Danio rerio	dre-mir-451	AGAGGCGGCGAAACCGUUACCAUUACUGAGUUUAGUAAUGGUAAGGGUUCUGCUGCCUUU
Homo sapiens	hsa-mir-451a	AAUGGCAAGGAAACCGUUACCAUUACUGAGUUUAGUAAUGGUAAUGGUUCUCUUGCUAUACC
Sus scrofa	ssc-miR-451	GGUGGCGAGCAAACCGUUACCAUUACUGAGUUUAGUAAUGGUAACGGUUCUCUUGCUGCACC
Pongo pygmaeus	ppy-mir-451	AAUGGCAAGGAAACCGUUACCAUUACUGAGUUUAGUAAUGGUAAUGGUUCUCUUGCUAUACCCAGA
Equus caballus	eca-miR-451	AAUGUCGAGGAAACCGUUACCAUUACUGUGUUUAGUAAUGGUAACGGUUCUCUUGCUGUACCCAGA
Canis familiaris	cfa-miR-451	GAUGGCGAGGAAACCGUUACCAUUACUGAGUUUAGUAAUGGUAAUGGUUCUCUUGCUGUACCCA
Bos taurus	bta-miR-451	GGUGGCGAGGAAACCGUUACCAUUACUGAGUUUAGUAAUGGUAACGGUUCUCUUGCUGCACCCAGA
Pan troglodytes	ptr-mir-451	AAUGGCAAGGAAACCGUUACCAUUACUGAGUUUAGUAAUGGUAAUGGUUCUCUUGCUAUACCCAGA
Macaca mulatta	mml-mir-451	AAUGGCAAGGAAACCGUUACCAUUACUGAGUUUAGUAAUGGUAAGGGUUCUCUUGCUAUAUCCAGA
Gallus gallus	gga-mir-451	GCUGGCAGGGAAACCGUUACCAUUACUGAGUUUAGUAAUGGUAACGGUUCUGCUGACAGCCA
Xenopus tropicalis	xtr-mir-451	AGUGGCAAUGAAACCGUUACCAUUACUGAGUUUAGUAAUGGUAAGGGUUCUGUUGCUGCUC
Anolis carolinensis	aca-mir-451	UGUGUUGGCGAAACCGUUACCAUUACUGAGUUUAGUAAUGGUAAGGGUUCUUCUAUCACGCCGACU
Rattus norvegicus	rno-mir-451	AAUGGCGAGGAAACCGUUACCAUUACUGAGUUUAGUAAUGGUAAUGGUUCUCUUGCUGCUCCCACA
Mus musculus	mmu-mir-451a	AAUGGCGAGGAAACCGUUACCAUUACUGAGUUUAGUAAUGGUAACGGUUCUCUUGCUGCUCCCACA
Ophiophagus hannah	oha-mir-451	GUCAGCGAAACCGUUACCAUUACUGAGUUUAGUAAUGGUAAGGGUUCUUCUGCCCCGCC
Capra hircus	chi-mir-451	GGUGGCGAGGAAACCGUUACCAUUACUGAGUUUAGUAAUGGUAACGGUUCUCUUGCUGCACCCAGA
Petromyzon marinus	pma-mir-451	AGUGGCGACAAAACCGUUACCAUUAUUGUAACCAAUAAUGGUAAGGGUUCUCUUGCUGCUCCCCGCU
Taeniopygia guttata	tgu-mir-451	GCCGGCGGGGAAACCGUUACCAUUACUGAGUUUAGUAAUGGUAACGGUUCUGCCGACGGCCGGCAAGGCGGCA

Figure 10. Alignment of the novel conserved miRNA *miR-451* against orthologous entries in MiRBase version 21. Comparison of two novel conserved isomiRs (*ssa-miR-451a* and *ssa-miR-451b*) differing only in one single nucleotide in the mature miRNA (marked with a red box). No difference can be seen in hairpin loop structure.

Of all conserved miRNAs found in the mature liver samples, 79.91% were located on 5′stem and 18.16% on 3′stem when the evaluation was based on individual transcription rate data for the whole population. However, of the 22 highest expressed mature miRNA, 12 were located on the 5′strand and 10 on the 3′strand. Both *ssa-miR-122* and *ssa-let-7* had a mature 5′strand and these two miRNAs covered more than 57% of all transcribed miRNAs in mature liver of Atlantic salmon.

Table 8. *Novel conserved miRNAs identified in liver of mature Atlantic salmon post-smoltification*

miRNA name	Homologue species	Orthologues miRNA	Dominant strand	5′mature	3′mature	Expression value
ssa-miR-451b	*Danio rerio*	*dre-miR-451*	5’	UAACCGUUACCAUUACUGAGUU		449 355
ssa-miR-451a	*Danio rerio*	*dre-miR-451*	5’	AAACCGUUACCAUUACUGAGUU		108 803
ssa-miR-6240	*Mus musculus*	*mmu-miR-6240*	5’	ACAAAGCATCGCGAAGGCCCAAGGTG		3 161
ssa-miR-7641	*Hippoglossus hippoglossus*	*hhi-miR-7641*	5’	CTGAATACGCCCGATCTCGT		2 467
ssa-miR-7550	*Ictalurus punctatus*	*ipu-miR-7550*	5’	ATCCGGCTCGAAGGACCA		1 805
ssa-miR-3963	*Mus musculus*	*mmu-miR-3963*	5’	TGTATCCCACTTCTGACAC		1 392
ssa-miR-1957	*Mus musculus*	*mmu-miR-1957*	5’	CAGTGGTAGAGCATTTGAC		686
ssa-miR-2189	*Danio rerio*	*dre-miR-2189*	3’	TGATTATTTGAATCAGCTGTGT		497
ssa-miR-7977	*Homo sapiens*	*hsa-miR-7977*	5’	TTCCCGGCCAACGCACCA		402
ssa-miR-1260a	*Homo sapiens*	*hsa-miR-1260a*	5’	ATCCCACCGCTGCCACCA		385
ssa-miR-4454	*Homo sapiens*	*hsa-miR-4454*	5’	GGATCCGGGTCACGGCACCA		369
ssa-miR-3618	*Ictalurus punctatus*	*ipu-miR-3618*	5’	GATTTCCAATAATTGAGACAGT		329
ssa-miR-217	*Danio rerio*	*dre-miR-217*	5’	TACTGCATCAGGAACTGATTGG		315
ssa-miR-141	*Ictalurus punctatus*	*ipu-miR-141*	3’	CATCTTACCTGACAGTGCTCGG	TAACACTGTCTGGTAACGATGC	309
ssa-miR-1983	*Mus musculus*	*mmu-miR-1983*	3’	CTCACCTGGAGCACCTTTTCT		276
ssa-miR-457	*Cyprinus carpio*	*cca-miR-457*	5’	AGCAGCACGTAAATACTGGAG		185
ssa-miR-215	*Homo sapiens*	*hsa-miR-215*	5’	ATGACCTATGAATTGACAGAC		184
ssa-miR-3966	*Mus musculus*	*mmu-miR-3966*	5’	AGCTGCCAGCTGAAGAACTGT		144
ssa-miR-187	*Cyprinus carpio*	*cca-miR-187*	3’		TCGTGTCTTGTGTTGCAGCCAGT	104
ssa-miR-733	*Danio rerio*	*dre-miR-733*	5’	GCGCTGGTGTAGCTCAGTGGTT		86
ssa-miR-3120	*Homo sapiens*	*hsa-miR-3120*	3’	CCTGTCTGTGCCTGCTGTACA	TGCACAGCAAGTGTAGACAGGC	77
ssa-miR-7558	*Ictalurus punctatus*	*ipu-miR-7558*	5’	AGCTGAGATTAGGAGCACACTC		57
ssa-miR-98	*Homo sapiens*	*hsa-miR-98*	5’	TGAGGTAGTAAGTTGTATTGTT		54
ssa-miR-4443	*Homo sapiens*	*hsa-miR-4443*	5’	TTGGAGGCGTGGGTTTT		39
ssa-miR-6239	*Mus musculus*	*mmu-miR-6239*	5’	TTAGCGGTGGATCACTCA	TAGCGGTGGATCACTCGG	36
ssa-miR-7147	*Danio rerio*	*dre-miR-7147*	5’	TGTACCATGCTGGTAGCCGGT		33
ssa-miR-184	*Danio rerio*	*dre-miR-184a/b*	3’		TGGACGGAGAACTGATAAGGGC	24
ssa-miR-3964	*Mus musculus*	*mmu-miR-3964*	3’		ATAAAGTAGAAAGCACTAAA	22
ssa-miR-3591	*Homo sapiens*	*hsa-miR-3591*	5’	TTTAGTGTGATAATGGCGTTTG		17
ssa-miR-6412	*Mus musculus*	*mmu-miR-6412*	3’		TCGAAACCATCCTCTGCTACCA	11

7.4 Evaluation of miRNA endogenous controls

Similarly to mRNA expression analysis, miRNA qPCR analysis requires thoroughly investigated endogenous controls. In Paper IV the expression of *ssa-let-7a*, *ssa-miR-16a*, *ssa-miR-16b*, *ssa-miR-194a*, *ssa-miR-22a*, *ssa-miR-22b*, and *ssa-miR-27c* was examined with regard to their use as endogenous controls in microRNA expression studies. The ideal endogenous control genes should be expressed on a constant level in different tissues, regardless of developmental stage and unaffected by experimental treatment. The most suitable endogenous control was *ssa-miR-27c*.

7.5 Tissue distribution of selected miRNAs

In Paper IV, the distribution of selected miRNAs in nine somatic tissues (liver, heart, brain, kidney, spleen, intestine, gill, white, and red muscle) was evaluated. The majority of the miRNAs tested were highly tissue-specific. Some miRNAs were only expressed in one tissue. For example, *ssa-miR-122*, *ssa-miR-722* and *ssa-miR-92a* were almost exclusively expressed in the liver and *ssa-miR-16a* and *ssa-miR-21* were only expressed in the brain. Both *ssa-miR-143* and *ssa-let-7a* were expressed in the kidney, but showed preferential expression in gills and red muscle, respectively.

The different isomiRs showed distinct differences in transcription levels and tissue specificity. As mentioned previously, *ssa-miR-16a* was brain-specific but *ssa-miR-16b* showed high levels of expression in the brain but also in the liver, red muscle and kidney. The expression of *ssa-miR-16a* tended to be higher than that of *ssa-miR-16b*, but the difference was not significant. Similarly, *ssa-miR-22a* was predominantly expressed in liver, red muscle and stomach, whereas *ssa-miR-22b* showed a much lower expression rate overall and was more evenly expressed between tissues except for no detectable levels in gills and white muscle.

8 General discussion

The objective of this thesis was to determine the effect of adding bioactive compounds both *in vivo* and *in vitro* on the expression of genes related to lipid homeostasis in salmonids.

The main aim was to investigate the underlying molecular mechanisms of n-3 LCPUFA biosynthesis and regulation in salmonids. The overall objective was to deliver a comprehensive map of miRNAs expressed in the liver of mature Atlantic salmon and identify novel liver-predominant miRNAs. The intention was for the knowledge acquired to be used in future guidance on how to optimize the content of n-3 LCPUFA in salmonids through enhanced activity of the desaturation and elongation pathway, thus enabling efficient and effective use of VO in aquaculture while maintaining the high nutritional quality of wild catch fish.

8.1 Effects on growth performance

Replacement of FO with VO did not affect growth performance in Papers I and II, which confirms previous findings for Atlantic salmon (Torstensen *et al.*, 2005a; Bell *et al.*, 2001; Torstensen *et al.*, 2000) and rainbow trout (Trattner *et al.*, 2008a).

Addition of sesamin had a dose-dependent negative effect on growth in Paper II, but only the highest inclusion rate resulted in significantly reduced final weight. This is contradictory to growth performance results in studies resting sesamin inclusion in the feed of Atlantic salmon (Trattner *et al.*, 2011), rainbow trout (Trattner *et al.*, 2008a) and carp (Mraz *et al.*, 2010). However, similar results have been obtained in trials on juvenile barramundi (*Lates calcarifer*), where the growth rate was reduced in fish receiving sesamin, regardless of the dietary oil used (Alhazzaa *et al.*, 2012). The reduction in growth observed in this thesis with the addition of a high sesamin content is in

agreement with studies of other bioactive compounds, *e.g.* dodecylthioacetic acid and tetradecylthioacetic acid (TTA) (Kleveland *et al.*, 2006a) Moya-Falcón *et al*., (2004), where these substances significantly lowered the body weight of Atlantic salmon.

8.2 Effects on lipid content

8.2.1 Total lipid content

In the *in vivo* study in Papers I and II, the white muscle lipid content was not affected by sesamin supplementation. However, in Paper II the liver fat content was significantly increased by addition of the high level of sesamin, confirming previous findings in rats (Ashakumary *et al.*, 1999). This is contrary to results obtained by Moya-Falcón *et al.* (2004), where the liver lipid content was reported to be unaffected by the addition of TTA to Atlantic salmon diets. In another study, addition of conjugated linoleic acid or TTA did not affect the total amount of lipids in liver of Atlantic cod (*Gadus morhua*) (Kennedy *et al.*, 2007b).

8.2.2 Fatty acid composition

As expected, the composition of FAs in white muscle and liver strongly reflected the FA composition in the different diets in both rainbow trout and Atlantic salmon. For example, in Paper II the amount of MUFA in the TAG fraction was significantly higher in both white muscle and liver from fish fed the VO diet with the high n-6/n-3 compared with the control fish fed FO diets, as a consequence of the significantly higher amount of MUFA in the VO feed. Increased levels of LA and ALA were also observed as an effect of VO inclusion compared with FO in both rainbow trout and Atlantic salmon. In addition, the ALA content was lower in white muscle of rainbow trout fed the MO diet compared with the LO and TAG diets, reflecting the composition of the different diets in Paper I. Similarly, replacement of FO with VO reduced the proportion of DHA in both white muscle (Paper I) and white muscle/liver (Paper II). In Paper II, the two different n-6/n-3 ratios had an impact on n-3 FA content in fish, with the higher ratio mainly resulting in lower amounts of ALA. This decrease was not reflected in higher percentages of DHA and EPA. These results are in agreement with several studies where complete or partial replacement of FO with VO, such as rapeseed oil, palm oil, LO and/or soy oil, had a significant effect on lipid composition in salmonids (Pettersson *et al.*, 2009; Turchini & Francis, 2009; Jordal *et al.*, 2007; Torstensen *et al.*, 2005b; Bell *et al.*, 2001; Thomassen & Røsjø, 1989), lowering the proportion of DHA in both liver and muscle.

In Paper I, fish fed the TAG diet, where the polar phase had been removed, had a higher proportion of EPA and lower proportion of ALA in triacylglycerol fraction and an increased level of DPA in the PL fraction compared with the LO diet. These results could indicate that the polar fraction removed from the LO had some effect on the metabolism of PUFA. No additional effect of sesamin supplementation on FA composition was observed. However, in the triacylglycerol fraction of the LO+sesamin group, ALA was significantly decreased compared with the LO group, but no corresponding increase was seen in DHA content.

No effect of sesamin supplementation was seen in the amount of ALA or DHA in Paper II or Paper III. This contradicts previous studies, where a significant increase in DHA has been observed on addition of a dietary sesamin/episesamin mixture in both white muscle of rainbow trout (Trattner *et al.*, 2008a) and in Atlantic salmon hepatocytes (Trattner *et al.*, 2008b). However, sesamin addition affected the FA involved in the synthesis of DHA, *e.g*. 20:3n-3 increased and EPA and DPA decreased in white muscle in Paper II. In contrast, an increase in EPA and a very limited effect to slight decrease in DPA were seen in the liver after sesamin supplementation.

The difference in response to bioactive compounds compared with previously published results may be explained by several factors. First of all, the physiological response to bioactive compounds may vary depending on fish species. Seawater fish have a lower capacity to convert ALA to DHA than freshwater fish (Sales, 2010; Sargent & Tacon, 1999). However, in Papers I-III no distinction in response to sesamin was apparent between rainbow trout (Paper I) and Atlantic salmon (Papers II and III).

Within-species variations in the response to bioactive compounds may depend on age, gender, size and possibly environmental conditions such as temperature and feed composition. It has been shown that the synthesis of DHA decreases with size/age in Atlantic salmon (Zheng *et al.*, 2004). In Paper III, the fish were fed a commercial FO-based diet before sacrification and hepatocyte preparation. This diet is particularly high in n-3 LCPUFAs. The individual fish used in the experiment were therefore well nourished and had a high fat content. There were clear differences in age and size between fish in Papers I, II and III compared with the studies performed by Trattner *et al.* (2008a); (Zheng *et al.*, 2004) (final weight; ~88 g, ~105 g and ~1300 g, respectively, compared with ~35 g), which probably had an effect on the final results.

The feeding period can also affect physiological response to sesamin. In Paper II, the experimental diets were fed to fish for four months, which is

longer than in previous studies (5-11 weeks) (Trattner *et al.*, 2011; Mraz *et al.*, 2010; Trattner *et al.*, 2008a; Trattner *et al.*, 2008b).

Another possible reason for the lower effect on DHA in Papers I and II is that only sesamin was used, whereas an equimixture of sesamin and episesamin was used by Trattner *et al.* (2011; 2008a). Episesamin is reported to be a stronger lipid modulator of enzyme activity than sesamin in mammals (Kiso *et al.*, 2005; Kushiro *et al.*, 2002). Therefore, the presence of episesamin in the diet of fish may be important for modulation of FA composition.

8.3 Effects on lipid-related gene expression

As reported in previous studies, inclusion of VO as a natural consequence affected the expression of lipid-related genes (Torstensen *et al.*, 2009; Trattner, 2009; Leaver *et al.*, 2008a).

In Papers I and II, the reduction in dietary n-3 LCPUFA in favor of increased levels of n-3 MUFA and ALA resulted in alteration in expression of genes related to FA β-oxidation (*CPT1* and *ACO*) and *PPAR*, but the data were not consistent. The increased levels of FAs involved in PUFA synthesis and the increased expression of the genes involved observed in Papers I and II support the claim that sesamin has an impact on lipid metabolism.

In Paper III, where salmon hepatocytes were incubated with different bioactive compounds, only small effects were seen in the FA profile. The effect on the gene expression profile was more pronounced. After 48 hours of incubation with LPA, episesamin or sesamin, upregulation of all the genes chosen as markers (*ELOVL5a*, Δ*5FAD*, *ELOVL2* and Δ*6FAD*) for the biosynthesis of PUFAs was observed.

As previously mentioned, episesamin has been shown to be more potent than sesamin. In Paper III, there were differences in *CD36* and *CPT1*, where the expression was more upregulated with episesamin than sesamin. In contrast, the expression of *PPARγ* was higher in the sesamin-treated than episesamin-treated cells. In general, the results in Paper III suggest a time-dependent response regardless of the bioactive compound added to the medium, with more pronounced effects after 48 hours. Individual bioactive compounds generated different effects.

In view of the whole genome duplication event that occurred in the evolution of salmonids (Allendorf, 1978), gene expression studies are complex due to the presence of duplicate genes that may be differently regulated. In addition, the individual fish tested in Papers I-V were not genetically homogeneous or offspring from one single genetically identical family. It can be assumed that there was genetic variation between individual fish both within

trials and between replicates from each tank, which may have affected the experimental outcome. One solution to decrease this variation would be to increase the number of individuals. A greater number of replicates would influence the statistic reliability, the correlation between FA composition and gene expression results in these supplementation studies. This is exemplified by the work done on SNP allele frequency studies using pyrosequencing (Wasson *et al.*, 2002), which shows that doubling the sample size drastically improves the detection accuracy.

8.3.1 Uptake of fatty acids

Markers for FA uptake (*CD36* and *SR-B1*) were only tested in Papers II and III. Both *PPARα* and *PPARγ* have been shown to induce the transcription of *CD36* and *SR-B1* (Torstensen *et al.*, 2009; Malerød *et al.*, 2005; Malerød *et al.*, 2003; Poirier *et al.*, 2001; Motojima *et al.*, 1998), so it was not surprising that the effects on these genes were limited. No effects were seen on *SR-B1* in Paper II and *CD36* only showed significant downregulation in tissues from fish fed the higher dietary ratio of n-6/n-3 with sesamin added. No effects were seen on either *PPARα* or *PPARγ* in Paper II.

In Paper III, the expression of *CD36* followed the same expression pattern as *PPARγ* in all treatments after 48 hours, but the trend was not as clear for *PPARα*. After 48 hours, expression of *CD36* was upregulated following incubation with episesamin, sesamin and LPA for 12 and 48 hours. Similarly, in Paper III expression of *PPARα* was related to expression of the long chain FA transporter, *CD36* in LPA (12 h), genistein 0.005mM (48 h) and episesamin (48 h). The effect of episesamin was twice as high as that of sesamin. This finding is in agreement with Kushiro *et al.* (2002), who reported more potent effects of episesamin than sesamin.

8.3.2 Elongation and desaturation

The transcription rate of all elongases except *ELOVL5a* was increased in the liver of salmon fed VO, irrespective of n-6/n-3 ratio, compared with that of fish fed the FO diet. This is in line with Morais *et al.* (2009), who reported that expression of *ELOVL5b* and *ELOVL2*, but not of *ELOVL5a*, was significantly increased in both liver and intestine when Atlantic salmon were fed VO instead of FO. *ELOVL5b* codes for genes involved in the elongation of C18 to C20 PUFA and *ELOVL2* for genes involved in the elongation of C20 to C22 (Morais *et al.*, 2009; Hastings *et al.*, 2004). *SREBP-1* and *SREBP-2* were only evaluated in Paper II, but expression was significantly increased by the lower addition rate of sesamin to the low n-6/n-3 ratio diets. In fish fed these diets, the *SREBP* target genes, desaturases and elongases were also all significantly

upregulated. This is in agreement with hepatocyte studies in rodents, where the expression of *ELOVL5*, Δ*6FAD* and Δ*5FAD* has been shown to be regulated by both *PPARα* and *SREBP-1c* (Qin *et al.*, 2009; Matsuzaka *et al.*, 2002). Paper II showed increased expression of *SREBP*, elongases and desaturases, but not of *PPARα*. The increase in *ELOVL5b* and *ELOVL2* in fish fed the VO diet (V0.5) with low sesamin addition was accompanied by a non-significant increase in the amount of DHA in both the TAG and PL fractions. Expression of desaturation and elongation genes increased significantly with addition of sesamin. The effect of sesamin seemed to be influenced by the FA composition and n-6/n-3 ratio in the VO formulation (Papers I and II). For the lower n-6/n-3 ratio, sesamin increased desaturation and elongation, while for the higher n-6/n-3 ratio, sesamin decreased both desaturation and elongation. This is similar to results reported by Trattner *et al.* (2008b). In Paper II, fish fed the V0.5SL and V0.5SH diets showed increased expression of LXR compared with fish fed the V0.5S0 diet (see *Table 1* for diet abbreviations). This could indicate that sesamin acts on *SREBP-1c* directly, or indirectly by activation of *LXR*. The increased expression of *LXR* and *SREBP* can also be related, since *LXRα* and *LXRβ* have been shown to activate *SREBP-1c* in rodents (Cruz-Garcia *et al.*, 2009; Zhou *et al.*, 2008).

After 12 hours of incubation, *Δ5FAD* showed significant downregulation in cells incubated with genistein 0.005mM, LPA and sesamin. However, the expression of both *Δ6FAD* and *Δ5FAD* was upregulated by episesamin, sesamin and LPA after 48 hours. There were striking and rapid changes in *Δ5FAD* gene expression, from pronounced downregulation to upregulation, with genistein 0.005mM, LPA and sesamin. The upregulation of desaturases after 48 hours of incubation with sesamin contradicts results obtained by Trattner *et al.* (2008a), where downregulation of both *Δ6FAD* and *Δ5FAD* was detected after incubation with sesamin. However, the downregulation of the desaturases was seen in combination with an increased amount of radio-labelled DHA synthesised from 14C 18:3n-3 (Trattner *et al.,* 2008a). In Paper III, no significant effects were observed on DHA levels, but there were effects on stearidonic acid (18:4n-3) and 20:4n-3, possibly due to differences in fish size.

Desaturase and elongase activities, gene expression and, consequently, the response to treatment may depend on how fatty the individual fish was at the time of sampling. The fish used in Paper III were fatter than those in the study by Trattner *et al* (2008a). For further studies, determining the weight and length relationship could be interesting and helpful in understanding the metabolism of lipids. Furthermore, in the previous study radio-labelled FA was used, whereas Paper III total FA were analyzed and some changes could have

been masked by the endogenous pool of FA. In addition, consideration should be given to the feed the fish were fed prior to hepatocyte isolation, since the fish in Paper III were fed a commercial diet high in n-3 LCPUFA, which is known to inhibit enzymes involved in the elongation and desaturation cascade (Tocher *et al.*, 2003a). In contrast, a rapeseed oil-based diet prior to hepatocyte isolation increases enzyme activity of *Δ5FAD*, *Δ6FAD* and elongases (Moya-Falcón *et al.*, 2005). A possible interaction between feed FA composition and content of bioactive compound could explain the different results between the groups in present studies. The lack of correlation between desaturase expression and LCPUFA biosynthesis may also indicate that the effect of sesamin was mediated through other mechanisms not yet understood.

8.3.3 β-oxidation

In Paper I, the reduction in dietary n-3 LCPUFA in favor of increased levels of n-3 MUFA and ALA, resulted in alteration in expression of genes related to FA β-oxidation and *PPAR*. Sesamin had a weak to clear downregulating effect on *PPARα* expression in liver of fish fed any of the three VO tested, which confirms findings by Trattner *et al.* (2008a) that expression of *PPARα* is significantly downregulated in the liver of rainbow trout fed sesamin/episesamin as a supplement to a mixed oil diet. The same downregulation was seen in white muscle for fish fed the LO and TAG diets.

No significant effect was seen on *PPARα* after sesamin addition to the diet in Paper II or to the media in Paper III. Since *PPARα* is considered to be the main inducer of β-oxidation (Leaver *et al.*, 2006), a corresponding downregulation in the β-oxidation markers could be expected. However, the β-oxidation markers tested, *CPT1* and *ACO*, were significantly upregulated in both fish fed VO diets and fish given sesamin supplementation in Paper I. *PPARβ/δ* and *PPARγ* have also been shown to target the genes coding for the β-oxidation enzymes, *CPT1* and *ACO*, in liver and white muscle of rainbow trout and Atlantic salmon (Torstensen *et al.*, 2009; Du *et al.*, 2004; Ruyter *et al.*, 1997). In Papers I and III, both *PPARβ/δ* and *PPARγ* followed the same expression pattern as *CPT1* and *ACO*. One could speculate that the effect of sesamin is not caused by ligand binding to the *PPARα*. Cloning analysis of the ligand-binding regions of *PPARα* and *PPARγ* genes in Atlantic salmon have revealed that they contain additional amino acid residuals, which could suggest that the ligand-binding properties in salmon *PPAR*s differ from those seen in rodents (Andersen *et al*., 2000). This could explain the deviation from findings in rodents (Ashakumary *et al*., 1999).

The upregulation of β-oxidation markers is in agreement with a previous study by our research group, which showed increased levels of β-oxidation

products after addition of sesamin to Atlantic salmon hepatocytes (Trattner *et al.,* 2008a) and in studies on rodents (Jeng & Hou, 2005; Ashakumary *et al.,* 1999). In the low n-6/n-3 ratio groups in Paper II, similar results were found for *ACO*, but not for *CPT1*. However, in that study both *ACO* and *CPT1* were significantly downregulated after high sesamin addition in the high n-6/n-3 ratio diet. This might indicate that the n-6/n-3 ratio influenced the response of β-oxidation genes to sesamin.

After 12 hours of incubation in Paper III, *PPARγ* was upregulated in the genistein 0.005mM treatment and increased mRNA levels for β-oxidation markers were seen. These findings agree with previous reports that genistein is effective in increasing the activity of enzymes involved in β-oxidation in rodents (Takahashi *et al.*, 2009) and that it acts in the same manner as fibrates, known agonists of *PPARα*, in both *in vivo* and *in vitro* studies in rodents (Ricketts *et al.*, 2005) and as a ligand for *PPARγ* (Dang *et al.*, 2003).

All three *PPAR*s and markers for β-oxidation were upregulated in response to 12 hours of incubation with LPA addition to the hepatocyte media. Similarly, in a study on rodents LPA also increased β-oxidation (Huong & Ide, 2008). This indicates that LPA can activate the different *PPAR*s and thereby trigger the expression of target genes for mitochondrial β-oxidation.

8.4 Feedback regulation

A surplus of n-3 LCPUFA in salmonid feed prior to a shift in dietary oils has been shown to reduce the capacity of salmonids to swiftly increase the synthesis of n-3 LCPUFA when the oil in the aquafeed is changed from FO to VO (Moya-Falcón *et al.*, 2005; Tocher *et al.*, 2003a). Furthermore, Tocher *et al.* (2003b) demonstrated a significant correlation between the activity of the LCPUFA biosynthetic pathway and dietary n-3 LCPUFA levels. PUFAs suppress expression of lipogenic genes and induce expression of *PPAR*s, which simultaneously induce the transcription of genes encoding proteins of lipid oxidation and thermogenesis (Price *et al.*, 2000).

In Paper III, the fish were fed commercial FO based diet prior to dissection and hepatocytes preparation. Commercial FO based is high in n-3 LCPUFAs. The individual fish used in the experiment had equal condition factor and high fat content. Even though the amount of EPA and DHA was low in the hepatocyte culture media, it is likely that the feeding conditions prior to slaughter, with high levels of both EPA and DHA, could have influenced the potential positive effect of bioactive compounds on the process of desaturation, elongation and β-oxidation. This suggestion is supported by data presented by Henderson & Sargent (1984), which show that peroxisomal β-oxidation is

increased only when there is an imbalance between the amount of MUFA 22:1 and PUFAs, a situation which is very unlikely to occur in natural fish diets or in standard FO diets.

Negative feedback regulation at the transcriptional level (*Figure 11*) is one of the most common motifs in gene regulatory networks (Zeron & Santillán, 2010). Tocher *et al.* (2003a) and Nakamura & Nara (2004), among others, have suggested that Δ*5FAD* and Δ*6FAD* are regulated by a negative feedback loop and that excessive intake of LA, ALA or any other type of PUFA can be a problem, leading to suppression of the PUFA metabolic pathway.

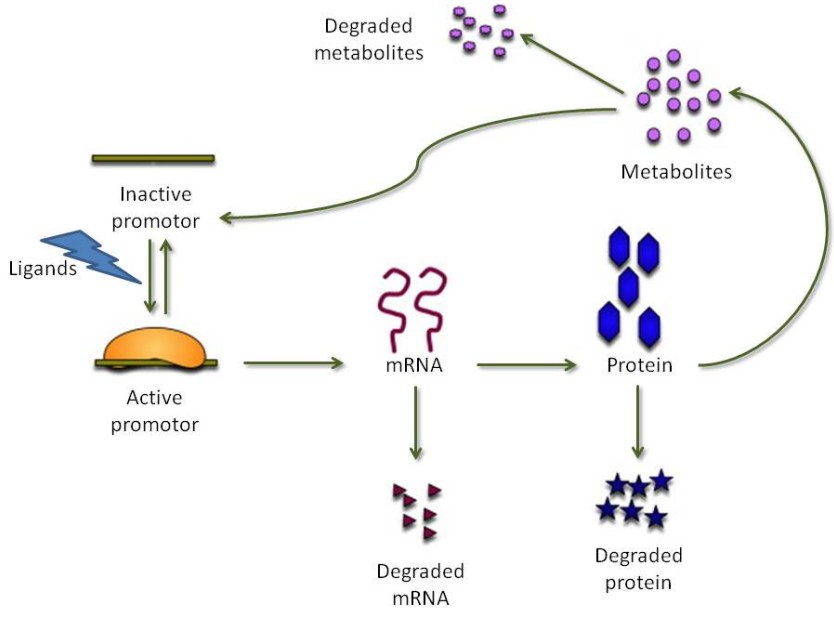


Figure 11. Schematic representation of a gene expression system subject to negative feedback regulation, where a decrease in amount of metabolite below a certain level sends a signal back to the promoter region of the gene in question, resulting in triggering of mRNA transcription. (Modified after Zeron & Santillán, 2010).

Thomassen *et al.* (2012) showed that when EPA and DHA were added to a rapeseed oil diet, the total process of desaturation, elongation and β-oxidation to DHA was significantly reduced (by about 50%) in Atlantic salmon. *In vitro* studies indicate that the inhibition is triggered by DHA and not by EPA accumulation. The inhibition occurred mainly at the Δ6 desaturation step from 24:5n-3 to 24:6n-3, and at the second elongation step (*ELOVL2*) from DPA to

24:5n-3. From this, it can be postulated that FO actually suppresses the desaturation and elongation of LCPUFAs.

Figure 12 summarizes the different regulation alternatives in the desaturation and elongation process of LCPUFAs. It is likely that not one but several regulation pathways are involved in the desaturation and elongation cascade. The first and conventional alternative for feedback inhibition (step I in *Figure 12*) is a high level of the metabolite (in this case DHA) directly inhibiting the transcription of DNA to mRNA, with an associated decrease in mRNA expression as the end result. If this mode of action dominated, in the current situation we would expect a decrease in gene expression of Δ*5FAD*, Δ*6FAD*, *ELOVL5* or *ELOVL2*, followed by a decrease in enzyme activity and finally a decrease in amount of DHA.

The second alternative is post-transcriptional regulation or RNA silencing (step II in *Figure 12*). Here, no inhibition needs to be seen on the mRNA level, but ultimately a decrease in the amount of expressed protein/enzyme. In some cases it may even be possible to detect an increase in mRNA expression. In step III in *Figure 12*, the feedback mechanism inactivates the Δ*5FAD* or Δ*6FAD* desaturase and/or *ELOVL5* or *ELOVL2* elongase, generating no end product (DHA).

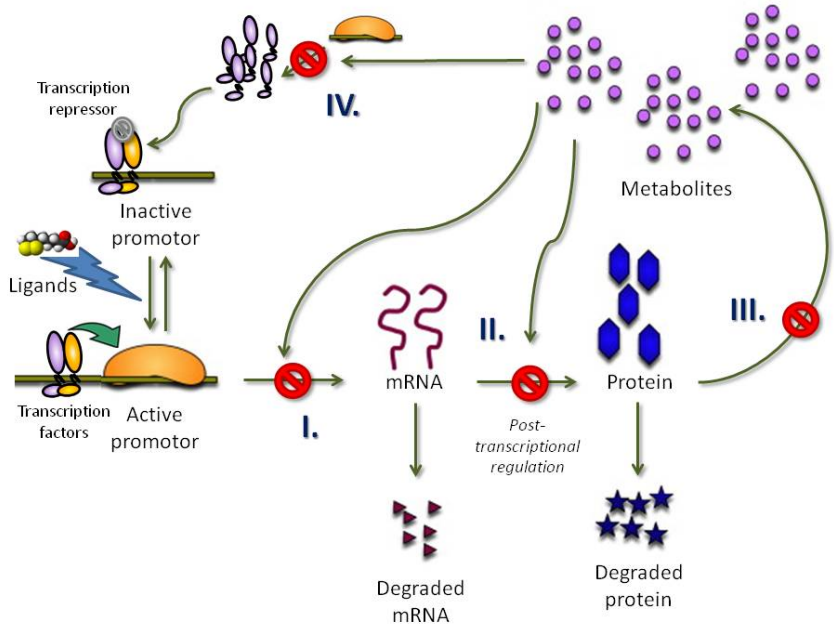


Figure 12. Schematic representation of four possible negative feedback regulation mechanisms in fish rich in EPA and/or DHA.

In a final alternative, the feedback mechanism could target other target genes or genes coding for transcription factors with effects on that particular gene expression as the primary effect, *e.g.* decreased expression of *PPAR* or *SREBP* (step IV in *Figure 12*).

The turnover of mRNA is extremely sensitive to dietary changes, resulting in fluctuations in mRNA levels, which in Papers I-III did not generate equivalent variations in the FA composition. Such fluctuations could indicate that using mRNA analysis as the only measurement, without considering potential post-transcriptional regulation mechanisms, protein levels or enzyme activity measurements, is a limited tool to explain biochemical results.

8.5 Epigenetic regulation

It is possible that the unlimited excess of n-3 LCPUFA in salmonid feed for generations of both wild fish and fish in aquaculture can be under epigenetic regulation and is the cause of the reduced capacity in salmonids to swiftly increase synthesis of n-3 LCPUFA when the oil in the aquafeed is changed from FO to VO.

During recent decades the picture of gene regulation has become still more complex with the discovery of epigenetic regulation. The four major components of epigenetic regulation are promoter methylation, histone modification, chromatin conformation changes and altered expression of non-coding RNAs, especially miRNAs.

8.6 MicroRNA regulation in liver of Atlantic salmon

The discrepancy between increased expression of target genes in the desaturation and elongation cascade and the lack of an actual response in the FA content of EPA and DHA is most likely the result of a combination of feedback regulation and post-transcriptional regulation such as RNA silencing. However, this needs to be verified and one step towards this is mapping of the expression of different miRNAs in the liver of Atlantic salmon.

8.6.1 Identification of hepatic miRNA

At present, more than 28 645 entries have been made in miRBase version 21 (June 2014) representing hairpin precursor miRNAs, expressing 35,828 mature miRNA products annotated in 223 different species (Griffiths-Jones *et al.*, 2006). The number of known fish miRNAs is not comparable to those for human and mouse, considering the conserved nature of miRNAs among different species. For a long time, the miRNAs identified were limited to

model species such as zebrafish (Mishima, 2012; Kloosterman *et al.*, 2006; Schier & Giraldez, 2006), tiger blowfish, Medaka (Li *et al.*, 2010a) and green spotted puffer. The miRNAs identified in non-model species were limited to rainbow trout (Salem *et al.*, 2010; Ramachandra *et al.*, 2008), Atlantic halibut (Bizuayehu *et al.*, 2012) and Atlantic cod (Johansen *et al.*, 2011; Johansen *et al.*, 2009), and were identified using more or less extensive direct cloning, sequencing and northern blot analyzes. However, there are some restrictions to these methods, such as their limited capability to detect low abundant miRNAs and therefore mainly uncovering conserved miRNAs (Ruby *et al.*, 2007; Ruby *et al.*, 2006). This can partly explain the limited number of miRNAs detected in teleosts.

The introduction of the NGS platforms has made it possible to precisely identify and characterize non-conserved or low expressed miRNAs in Atlantic salmon and rainbow trout (Andreassen *et al.*, 2013; Bekaert *et al.*, 2013; Ma *et al.*, 2012). Depending on the experimental set-up, NGS technologies can provide more complete view of the miRNA transcriptome (miRNome), including identification and quantification of both highly abundant conserved miRNA and non-conserved or low abundance miRNA, spanning nearly six orders of magnitude of expression (Morin *et al.*, 2008).

While the interaction of the miRNA and liver transcriptome had been well established in mammals (Nan *et al.*, 2013; Gatfield *et al.*, 2009b; Girard *et al.*, 2008; Varnholt, 2008), the importance of post-transcriptional regulation through miRNA intervention on diverse metabolic processes in salmonids remains to be determined. This thesis presents the first deep sequencing results for the miRNome in liver of Atlantic salmon, and provides a basis for further studies on post-transcriptional regulation of hepatic lipogenesis and homeostasis.

In this thesis, 159 conserved previously described miRNA families (Andreassen *et al.*, 2013; Bekaert *et al.*, 2013; Ma *et al.*, 2012) were annotated in Atlantic salmon liver, of which ssa-miR-122 was by far the most abundant miRNA, with a presence of 48%. It has been reported that *miR-122* is a highly abundant conserved liver-specific miRNA, with *e.g.* over 72% of all miRNA molecules present in mouse hepatocytes being variants of *miR-122* (Lagos-Quintana *et al.*, 2002). However, this is probably somewhat of an overestimate, since the results were achieved using tissue-specific cloning, which generates far more limited statistical material than NGS, although *miR-122* is clearly highly abundant. Studies in mammals have shown that *miRNA-122* is implicated together with transcription factor HNF6 in the differentiation and maintenance of the hepatic phenotype (Laudadio *et al.*, 2012; Jung *et al.*, 2011; Xu *et al.*, 2010) and the regulation of lipid metabolism (Girard *et al.*, 2008;

Esau *et al.*, 2006a; Krützfeldt *et al.*, 2005). Gatfield *et al.* (2009) also showed that REV-ERBα, the major circadian regulator in mammals, regulates the expression of miR-122. In turn, miR-122 participates in the circadian control of genes in hepatic metabolism through the *miR-122* targets *PPARβ/γ* and *SMARCD1/BAF60a*, which is a coactivator of *PPARα*. In rainbow trout, *omy-miR-122* has also been linked to the regulation of cholesterol and lipid metabolism, but the results indicate that the metabolic consequences of *omy-miRNA-122* inhibition differ quantitatively between vertebrate species and that distinct direct molecular targets of *miRNA-122* may mediate different metabolic effects depending on vertebrate species (Mennigen *et al.*, 2014a; Mennigen *et al.*, 2014b).

The second most abundant miRNA family found in Atlantic salmon in this thesis was *ssa-let-7*, which is probably the best-known highly conserved miRNA and functions as the key regulator of developmental timing in almost all animal species (Bartel, 2009; Ambros, 2001).

All of the 13 distinct novel miRNAs discovered by Andreassen *et al*. (2013) were identified. However, none of these 13 novel miRNAs had a presence above 0.3‰. Even the novel ssa-miR-8163 was present with a low number of reads (7 425). This was somewhat unexpected, since differential expression data from Andreassen *et al*. (2013) suggested that this miRNA should be enriched in liver tissue. A reason for this could be that *ssa-miR-8163* has a different role in the post-smolt Atlantic salmon mature liver than in the pre-smolt and one-day-old individuals tested by Andreassen and co-workers.

In this thesis, 30 novel conserved miRNAs were identified in the liver of Atlantic salmon, of which *ssa-miR-451* was the most abundant (Table S2 in Paper V). To the best of my knowledge, *ssa-miR-451b* has not been identified previously and to date only *omy-miR-451a* has been reported in rainbow trout eggs (Ma *et al.*, 2012).

With NGS, a new magnitude of variability in mature miRNA sequences has been observed. These sequence variants are referred to as isomiR. An isomiR is a multiple mature sequence that shows variations in one or several nucleotides compared with the reference miRNA sequence annotated in miRBase. In many cases, isomiRs of the miRNA* sequence (passenger strand) are also observed (Morin *et al.*, 2008). In this thesis, a total of 43 new isomiRs not yet described were identified among the 22 miRNAs with the highest transcription rate in mature liver of Atlantic salmon, including two 3′isomiRs (Table S4 in Paper V). These isomiR variations might have a profound impact on target recognition and post-transcriptional mRNA degradation, especially when they are located in the seed region. For the other variations the effect

might not be as pronounced, but they are probably part of the fine-tuning of expression of the target miRNA (Brennecke *et al.*, 2005).

8.6.2 Evaluation for endogenous controls in miRNA qPCR

Before the effect of miRNAs on gene regulation can be widely studied, a robust method for profiling the expression level of each miRNA in a sample is required. Ribosomal RNAs, small nuclear RNA (snRNA), small nucleolar RNAs and U6snRNA (RNU6B) have been frequently used as endogenous controls in qPCR studies of miRNA distribution. Criticisms have been expressed about these approaches (Li *et al.*, 2012a). Identifying and using miRNAs as endogenous controls would be preferable, since they are exposed to the same treatment, are of similar size and exist in approximately similar quantities as target miRNAs. Identification of one or more specific endogenous control miRNAs is crucial for accurate comparison of miRNA expression.

The expression of seven putative endogenous control genes (*ssa-let-7a*, *ssa-miR-16a*, *ssa-miR-16b*, *ssa-miR-194a*, *ssa-miR-22a*, *ssa-miR-22b*, and *ssa-miR-27c*) was evaluated as regards their tissue distribution and use as endogenous controls in microRNA expression studies. The increased expression of *ssa-miR-16a* in brain made it less suitable for tissue distribution studies and therefore only *ssa-miR-27c* was chosen as an endogenous control for the tissue studies in this thesis. However, *ssa-miR-16a* would work acceptably as an endogenous control in studies of liver and white muscle, among others. It has previously been used as an endogenous control in porcine blastocytes (Li *et al.*, 2012a).

Both *ssa-miR-16a* and *ssa-miR-16b* (*Figure 13*), as well as *ssa-miR-22a* and *ssa-miR-22b*, are isomiRs. As can be seen from the diagram, *ssa-miR-16a* and *ssa-miR-16b* only differ in one nucleotide in the second last position after 3′end. However, this affects the size and position of the central loop, as well as minimum free energy (ΔG). Even if they have identical seed regions, Ye *et al.* (2008) showed that the location of the central loop is another important factor affecting the repression efficiency of gene regulation mediated by miRNAs. In Paper V, the transcription rate differed considerably between isomiRs in mature liver of Atlantic salmon, with 995 507 reads for *ssa-miR-16a* compared with 238 475 for *ssa-miR-16b* (Supplementary Table S4 in Paper V). The differences in transcription rate between the isomiRs *ssa-mir-16a* and *ssa-miR-16b*, and between *ssa-miR-22b* and *ssa-miR-22b*, demonstrated using qPCR analysis in Paper IV agreed with the results from NGS data in Paper V.

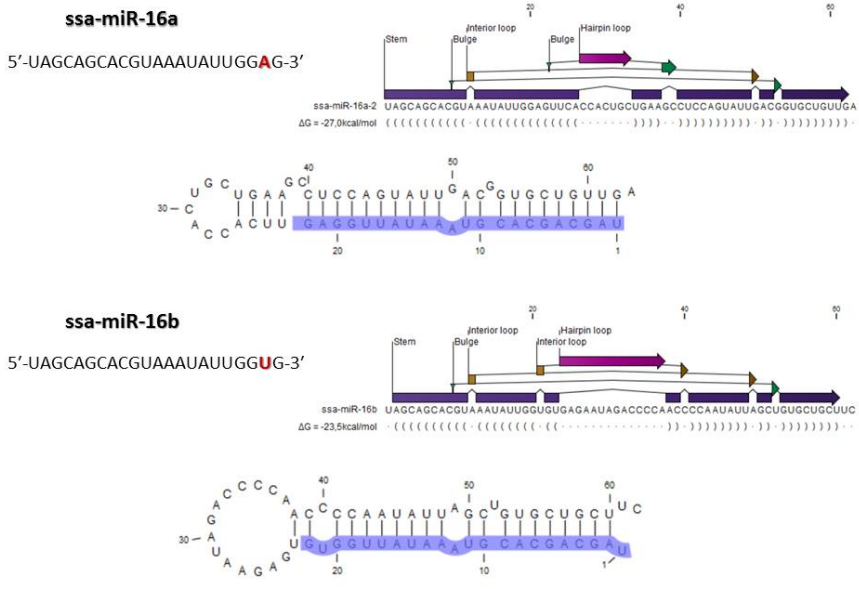


Figure 13. Sequence of the mature isomiRs, loop structures and minimum free energy (ΔG) of the putative endogenous control genes *ssa-miR-16a* and *ssa-miR-16b*.

8.6.3 Tissue distribution of conserved miRNA

Quantifying miRNAs in different tissues is an important initial step to investigate functions of miRNAs. The tissue distribution of miRNAs provides baseline references to analyze variations in miRNA expression under various environmental and physiological conditions.

A few of the miRNAs tested in this thesis, such as *ssa-miR-22b*, *ssa-miR-26b* and *ssa-miR-27c*, showed ubiquitous expression in all tissues, which indicates that they can be linked to basic fundamental functions. Ramachandra *et al.* (2008) found that *omy-miR-26a* was evenly represented in all tissues. This agrees with the findings for *ssa-miR-26b* in Paper V, since *ssa-miR-26b* only differed in one base in the second last position from the 3′end compared with *omy-miR-26a* and otherwise they have identical seed regions. However, the difference observed in expression pattern between the isomiRs *ssa-miR-16a* and *ssa-miR-16b* in both Papers IV and V suggests this cannot be taken for granted.

Most of the miRNAs tested in Paper IV were expressed in a tissue-specific manner, although some overlap in expression was seen. This agrees with findings in rodents, fruit fly (*Drosophila* sp.) and zebrafish (Mishima, 2012; Kloosterman & Plasterk, 2006).

9 Main findings and conclusions

The main conclusion reached in this thesis is that it is possible to interact with the metabolism of lipids in farmed fish by addition of bioactive compounds. Lipoic acid, sesamin and episesamin showed more potent effects on gene expression than genistein. Lipoic acid, sesamin and episesamin also caused some changes in FAs, whereas genistein did not.

This thesis presents an in-depth analysis of the expression of miRNAs in the liver of Atlantic salmon. Some of these miRNAs have been shown to be regulating transcription factors and genes involved in cholesterol and lipid homeostasis in mammals. These findings provide a basis for understanding the role of miRNAs in the regulation of lipid metabolism in teleost fish and for devising novel approaches to improve aquaculture production of healthy fish. Furthermore, miRNAs can be used as biomarkers or as tools in molecular manipulation.

10 Future perspectives

Future studies should continue research within the new fields of transcriptional regulation, use of biomarkers and investigating the mechanisms of specific pathways in fatty acid biosynthesis. Such studies should include:

- Verification of the most abundant miRNAs and isomiRs identified in liver from mature Atlantic salmon post-smoltification using quantitative RT-PCR.
- Since miRNAs exert their function by regulating target mRNAs, the identification of miRNA targets involved in lipid homeostasis is critical to understanding their role. Future experiments should focus on identification of miRNA targets and functional characterization of their response *in vitro* and *in vivo* using synthetic premiRs and antimiRs.
- Deep sequencing miRNA profiling of different somatic tissues in fish post-smoltification and exposed to changes in feed formulation from fish oil to vegetable oils.

Acknowledgements

First of all, I would like to thank my supervisors. A special warm thank you to my main supervisor, Professor **Jana Pickova**, for your valuable guidance and your appreciation for new approaches and testing new technology platforms. I am grateful to you for giving me the privilege of working with fish, fish physiology and the wonderful world of PUFAs, as well as opening the door to your home, your friendship and all your good cooking – and for introducing me to Ben & Jerry's "Cherry Garcia" ice cream before it was too late.

Dr. **Sofia Trattner**, my wonderful co-supervisor, I would like to thank you for your never-ending encouragement, constructive suggestions, long nice "future dream" talks and for charging my love of molecular biology. Wish that Nyköping was closer to Uppsala!!

Particular thanks to all the people at the Department of Food Science and fellow PhD students, who gave me the privilege of being part of their community during these past years. Sincere thanks for your warm welcome and for becoming my friends.

Special thanks go to **Honza** for all good discussions and nice dinners with you and your family, as well as to my co-author and sweet roommate **Liane.**

To **Galia Zamaratskaia**, I will not thank you for all the extra kilos you helped putting around my waist with your fantastic Russian patisseries and yams, but I will thank you for introducing me to the challenging world of HPLC and for being such a warm and generous person.

To **Monika** and **Bosse** for always contributing to make everything more enjoyable, to all the laughter and late hour beers

I would not have started on this journey if it had not have been for some remarkable men and women in companies with extraordinary technologies crossing my path:

- For opening the doors to Pharmacia Biotech and for the introduction to the wonderful world of multivariate analysis, I would like to send a lot of good thoughts to **Per Kårsnäs**, wherever you may be in the world right now.
- To the ALFexpress team, especially **Tomas Hultman**, **Anna Edman Örlefors**, **Peter Hagerlid** and **Gunilla Jakobsson,** and the best automated sequencing instrument ever to see the light of day.
- Special thanks to **Sol** for sharing long hours both in the lab and at "News" – for being a true friend through ups and downs in life.
- To **Björn Ekström**, **Marie Ellow, Lisa Westin, John Kleine**, **Mats Bergström** and the rest of the fireflies at Pyrosequencing AB. Pyro took my biotech heart and never gave it back!
- To **Björn Ingemarsson**, for being my mentor and role model in the intriguing world of science and biotechnology.
- To the Nordic commercial team at Applied Biosystems giving me RT-PCR, RNA interference, microRNA and Next Generation Sequencing. Special warm thoughts to 2x**Jeanette**, **Brigitte Fortnagle** and **Nick Jordan**.
- To **Helena Nilshans** for bringing me into her fantastic Biomolecular Imaging team – **Frida**, **Helena**, **Karola**, **Joe** and **Mats** as well as introducing me to the fabulous world of Product Management at GE Healthcare.
- To **Anna Sylvan** for sharing this journey with me, being such a good friend and for reminding me where I truly belong!

Most of all, I would like to thank those closest to my heart. Without you, none of this would have been possible or meaningful.

To **Peter**, the love of my life, my husband and life companion – I would like to thank you for being there, supporting me and cheering me on, telling me that I am intelligent, beautiful and definitely am going to make it during the very darkest stretches of this journey.

To **Mark**, for being the meaning of my life and the glitter in my eyes. Thanks for being such a fantastic son! To **Franz** for giving you to me.

Sara “Sötnos” and **Carl Magnus** “Pannkaka”, thank you for welcoming me into your family and letting me be a part of your life. You symbolize the very true meaning of what a “bonus” can be. To **Susanne** for being my friend even if we had all odds against us.

To my sweet new-comers and family members - **Johanna**, **Carro**, **William** and **Åke**. Special thanks for taking so much of the workload and making this day a very special day.

To my brother, **Karl** for never letting me feel sorry for myself and for always giving me wonderful stories and laughs to carry with me. To his wonderful **Veronica** and their sons, **Viktor** and **Jonathan**.

My Father, **Bernt Schiller**, for never ending encouragement and long breakfast discussions and to **Gill** for being my friend and my father’s beloved companion.

Sussie - my oldest friend and the sister of my heart. I cannot with words describe my gratitude and love for you and your family.

To **Lina** and her daughter **Cornelia** for putting Uppsala on the map and becoming my friend after long hours in the fields of Scania.

To my lovely and beautiful goddaughters – **Hanna**, **Saga** and **Linn** and their mothers and fathers, the best of friends – **Sussie** & **Bosse**, **Anna** & **Henrik** and **Solan** & **Håkan.**

In memory of my friends who should have been here today – **Helle**, **Eva** and **Janne**, the world is a much colder place without you.

To **Xano** for keeping me sane during hours of stress by forcing me to go for good long walks.

To **AKKA V** for symbolizing everything good in life

December 2014

References

Ackman, R.G. (1996). DHA: can it benefit salmon marketing? *Journal of Aquatic Food Product Technology* 5(4), 7-26.

Ae Park, S., Choi, M.S., Cho, S.Y., Seo, J.S., Jung, U.J., Kim, M.J., Sung, M.K., Park, Y.B. & Lee, M.K. (2006). Genistein and daidzein modulate hepatic glucose and lipid regulating enzyme activities in C57BL/KsJ-db/db mice. *Life Sciences* 79(12), 1207-1213.

Alhazzaa, R., Bridle, A.R., Carter, C.G. & Nichols, P.D. (2012). Sesamin modulation of lipid class and fatty acid profile in early juvenile teleost, Lates calcarifer, fed different dietary oils. *Food Chemistry* 134(4), 2057-2065.

Allendorf, F.W. (1978). Protein polymorphism and the rate of loss of duplicate gene expression. *Nature* 272(5648), 76-78.

Allendorf, F.W. & Utter, F.M. (1976). Gene duplication in the family Salmonidae. III. Linkage between two duplicated loci coding for aspartate aminotransferase in the cutthroat trout (Salmo clarki). *Hereditas* 82(1), 19-24.

Ambros, V. (2001). microRNAs:Tiny regulators with great potential. *Cell* 107, 823-826.

Ambros, V. (2004). The functions of animal microRNAs. *Nature* 431, 350-355.

Ambros, V., Bartel, B., Bartel, D.P., Burge, C.B., Carrington, J.C., Chen, X., Dreyfuss, G., Eddy, S.R., Griffiths-Jones, S., Marshall, M., Matzke, M., Ruvkun, G. & Tuschl, T. (2003). A uniform system for microRNA annotation. *RNA* 9(3), 277-279.

Andersen, Ø., Eijsink, V.G.H. & Thomassen, M. (2000). Multiple variants of the peroxisome proliferator-activated receptor (PPAR) [gamma] are expressed in the liver of Atlantic salmon (Salmo salar). *Gene* 255(2), 411-418.

Andreassen, R., Lunner, S. & Høyheim, B. (2009). Characterization of full-length sequenced cDNA inserts (FLIcs) from Atlantic salmon (Salmo salar). *BMC Genomics* 10(502).

Andreassen, R., Worren, M.M. & Høyheim, B. (2013). Discovery and characterization of miRNA genes in atlantic salmon (Salmo salar) by use of a deep sequencing approach. *BMC Genomics* 14(1).

Aoi, W., Naito, Y., Mizushima, K., Takanami, Y., Kawai, Y., Ichikawa, H. & Yoshikawa, T. (2010). The microRNA miR-696 regulates PGC-1α in mouse skeletal muscle in response to physical activity. *American Journal of Physiology - Endocrinology and Metabolism* 298(4), E799-E806.

Appelqvist, L.-Å. (1968). Rapid methods of lipid extraction and fatty acid methyl ester preparation for seed and leaf tissue with special remarks on preventing the accumulation of lipid contaminants. *Arkiv För Kemi* 28, 551-570.

Aranda, A. & Pascual, A. (2001). Nuclear hormone receptors and gene expression. *Physiological Reviews* 81(3), 1269-1304.

Ashakumary, L., Rouyer, I., Takahashi, Y., Ide, T., Fukuda, N., Aoyama, T., Hashimoto, T., Mizugaki, M. & Sugano, M. (1999). Sesamin, a sesame lignan, is a potent inducer of hepatic fatty acid oxidation in the rat. *Metabolism-Clinical and Experimental* 48(10), 1303-1313.

Ason, B., Darnell, D.K., Wittbrodt, B., Berezikov, E., Kloosterman, W.P., Wittbrodt, J., Antin, P.B. & Plasterk, R.H.A. (2006). Differences in vertebrate microRNA expression. *Proceedings of the National Academy of Sciences of the United States of America* 103(39), 14385-14389.

Bahuaud, D., Mørkøre, T., Østbye, T.K., Veiseth-Kent, E., Thomassen, M.S. & Ofstad, R. (2010). Muscle structure responses and lysosomal cathepsins B and L in farmed Atlantic salmon (Salmo salar L.) pre- and post-rigor fillets exposed to short and long-term crowding stress. *Food Chem* 118(3), 602-615.

Bailey, G.S., Poulter, R.T.M. & Stockwell, P.A. (1978). Gene duplication in tetraploid fish: model for gene silencing at unlinked duplicated loci. *Proceedings of the National Academy of Sciences of the United States of America* 75(11), 5575-5579.

Barozai, M.Y.K. (2012). Identification and characterization of the microRNAs and their targets in Salmo salar. *Gene* 499(1), 163-168.

Bartel, D.P. (2004). MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell* 116(2), 281-297.

Bartel, D.P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* 136(2), 215-33.

Bartz, M., Kociucka, B., Mankowska, M., Switonski, M. & Szydlowski, M. (2014). Transcript level of the porcine ME1 gene is affected by SNP in its 3′UTR, which is also associated with subcutaneous fat thickness. *Journal of Animal Breeding and Genetics* 131(4), 271-278.

Bast, A. & Haenen, G. (2003). Lipoic acid: A multifunctional antioxidant (Reprinted from Thiol Metabolism and Redox Regulation of Cellular Functions). *BioFactors* 17(1-4), 207-213.

Bekaert, M., Lowe, N.R., Bishop, S.C., Bron, J.E., Taggart, J.B. & Houston, R.D. (2013). Sequencing and Characterisation of an Extensive Atlantic Salmon (Salmo salar L.) MicroRNA Repertoire. *PLoS ONE* 8(7).

Bell, J.G., McEvoy, J., Tocher, D.R., McGhee, F., Campbell, P.J. & Sargent, J.R. (2001). Replacement of fish oil with rapeseed oil in diets of atlantic salmon (Salmo salar) affects tissue lipid compositions and hepatocyte fatty acid metabolism. *Journal of Nutrition* 131(5), 1535-1543.

Bell, J.G., McGhee, F., Campbell, P.J. & Sargent, J.R. (2003a). Rapeseed oil as an alternative to marine fish oil in diets of post-smolt Atlantic salmon (*Salmo salar*): changes in flesh fatty acid composition and effectiveness of subsequent fish oil ‘‘wash out’’. *Aquaculture* 218, 515–528.

Bell, J.G., Tocher, D.R., Henderson, R.J., Dick, J.R. & Crampton, V.O. (2003b). Altered fatty acid compositions in Atlantic salmon (Salmo salar) fed diets containing linseed and rapeseed oils can be partially restored by a subsequent fish oil finishing diet. *Journal of Nutrition* 133(9), 2793-2801.

Berge, G.M., Bakke, H., Baranski, M., Kjær, M.A., Moghadam, H., Østbye, T.-K., Ruyter, B., Sigholt, T., Sonesson, A., Thomassen, M. & Zamaratskaia, G. (2014). Potential for production of Atlantic salmon families with improved capacity for EPA and DHA production. *ISSFAL 11th Congress of the International Society for the Study of Fatty Acids and Lipids. Stockholm Sweden; 2014-06-28 - 2014-07-02*.

Berge, G.M., Østbye, T.-K.K., Sonesson, A.K., Kjær, M.A., Baranski, M., Krasnov, A., Moghadam, H., Bakke, H., Thomassen, M.S. & Sigholt, T. (2013). The capacity of Atlantic salmon to produce EPA and DHA can be improved by genetic selection and nutrition. *European Aquaculture Conference (EAS); 2013-08-10 - 2013-08-12 NOFIMA UMB*.

Berthelot, C., Brunet, F., Chalopin, D., Juanchich, A., Bernard, M., Noël, B., Bento, P., Da Silva, C., Labadie, K., Alberti, A., Aury, J.M., Louis, A., Dehais, P., Bardou, P., Montfort, J., Klopp, C., Cabau, C., Gaspin, C., Thorgaard, G.H., Boussaha, M., Quillet, E., Guyomard, R., Galiana, D., Bobe, J., Volff, J.N., Genêt, C., Wincker, P., Jaillon, O., Crollius, H.R. & Guiguen, Y. (2014). The rainbow trout genome provides novel insights into evolution after whole-genome duplication in vertebrates. *Nature Communications* 5.

Betancor, M.B., McStay, E., Minghetti, M., Migaud, H., Tocher, D.R. & Davie, A. (2014). Daily rhythms in expression of genes of hepatic lipid metabolism in Atlantic salmon (Salmo salar L.). *PLoS ONE* 9(9).

Betel, D., Koppal, A., Agius, P., Sander, C. & Leslie, C. (2010). Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. *Genome Biology* 11(8).

Bizuayehu, T.T., Lanes, C.F.C., Furmanek, T., Karlsen, B.O., Fernandes, J.M.O., Johansen, S.D. & Babiak, I. (2012). Differential expression patterns of conserved miRNAs and isomiRs during Atlantic halibut development. *BMC Genomics* 13(1).

Brennecke, J., Stark, A., Russell, R.B. & Cohen, S.M. (2005). Principles of microRNA-target recognition. *PLoS Biol* 3(3), e85.

Bushati, N. & Cohen, S.M. (2007). MicroRNA functions. In. pp. 175-205.

Buzzi, M., Henderson, R.J. & Sargent, J.R. (1996). The desaturation and elongation of linolenic acid and eicosapentaenoic acid by hepatocytes and liver microsomes from rainbow trout (Oncorhynchus mykiss) fed diets containing fish oil or olive oil. *Biochimica et Biophysica Acta - Lipids and Lipid Metabolism* 1299(2), 235-244.

Buzzi, M., Henderson, R.J. & Sargent, J.R. (1997). Biosynthesis of docosahexaenoic acid in trout hepatocytes proceeds via 24-carbon

intermediates. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* 116(2), 263-267.

Campos, C., Sundaram, A.Y.M., Valente, L.M.P., Conceição, L.E.C., Engrola, S. & Fernandes, J.M.O. (2014). Thermal plasticity of the miRNA transcriptome during Senegalese sole development. *BMC Genomics* 15(1).

Carmona-Antoñanzas, G., Monroig, Ó., Dick, J.R., Davie, A. & Tocher, D.R. (2011). Biosynthesis of very long-chain fatty acids (C>24) in Atlantic salmon: Cloning, functional characterisation, and tissue distribution of an Elovl4 elongase. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* 159(2), 122-129.

Carrington, J.C. & Ambros, V. (2003). Role of microRNAs in plant and animal development. *Science* 301(5631), 336-8.

Castro, V., Grisdale-Helland, B., Helland, S.J., Kristensen, T., Jørgensen, S.M., Helgerud, J., Claireaux, G., Farrell, A.P., Krasnov, A. & Takle, H. (2011). Aerobic training stimulates growth and promotes disease resistance in Atlantic salmon (Salmo salar). *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* 160(2), 278-290.

Chen, R., D'Alessandro, M. & Lee, C. (2013). MiRNAs are required for generating a time delay critical for the circadian oscillator. *Current Biology* 23(20), 1959-1968.

Clop, A., Carre, W. & Amills, M. (2011). Polymorphisms in the microRNA pathway as a source of phenotypic variation in animals. In: *MicroRNA: Expression, Detection and Therapeutic Strategies*. pp. 165-188.

Clop, A., Marcq, F., Takeda, H., Pirottin, D., Tordoir, X., Bibé, B., Bouix, J., Caiment, F., Elsen, J.M., Eychenne, F., Larzul, C., Laville, E., Meish, F., Milenkovic, D., Tobin, J., Charlier, C. & Georges, M. (2006). A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. *Nature Genetics* 38(7), 813-818.

Coccia, E., Varricchio, E., Vito, P., Turchini, G.M., Francis, D.S. & Paolucci, M. (2014). Fatty Acid-Specific Alterations in Leptin, PPARα, and CPT-1 Gene Expression in the Rainbow Trout. *Lipids*.

Cook, H.W. & McMaster, C.R. (2002). Fatty acid desaturation and chain elongation in eukaryotes. In. New Comprehensive Biochemistry pp. 181-204.

Cruz-Garcia, L., Minghetti, M., Navarro, I. & Tocher, D.R. (2009). Molecular cloning, tissue expression and regulation of liver X Receptor (LXR) transcription factors of Atlantic salmon (Salmo salar) and rainbow trout (Oncorhynchus mykiss). *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* 153(1), 81-88.

Cruz-Garcia, L., Sánchez-Gurmaches, J., Gutiérrez, J. & Navarro, I. (2011). Regulation of LXR by fatty acids, insulin, growth hormone and tumor necrosis factor-α in rainbow trout myocytes. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* 160(2), 125-136.

Dang, Z.C., Audinot, V., Papapoulos, S.E., Boutin, J.A. & Löwik, C.W.G.M. (2003). Peroxisome proliferator-activated receptor γ (PPARγ) as a

molecular target for the soy phytoestrogen genistein. *Journal of Biological Chemistry* 278(2), 962-967.

Dannevig, B.H. & Berg, T. (1985). Endocytosis of galactose-terminated glycoproteins by isolated liver cells of the rainbow trout (Salmo gairdneri). *Comparative Biochemistry and Physiology -- Part B: Biochemistry and and Molecular Biology* 82(4), 683-688.

Dávalos, A., Goedeke, L., Smibert, P., Ramírez, C.M., Warrier, N.P., Andreo, U., Cirera-Salinas, D., Rayner, K., Suresh, U., Pastor-Pareja, J.C., Esplugues, E., Fisher, E.A., Penalva, L.O.F., Moore, K.J., Suárez, Y., Lai, E.C. & Fernández-Hernando, C. (2011). miR-33a/b contribute to the regulation of fatty acid metabolism and insulin signaling. *Proceedings of the National Academy of Sciences of the United States of America* 108(22), 9232-9237.

Du, N.H., Arpat, A.B., De Matos, M. & Gatfield, D. (2014). MicroRNAs shape circadian hepatic gene expression on a transcriptome-wide scale. *eLife* 2014(3).

Du, Z.Y., Demizieux, L., Degrace, P., Gresti, J., Moindrot, B., Liu, Y.J., Tian, L.X., Cao, J.M. & Clouet, P. (2004). Alteration of 20:5n-3 and 22:6n-3 fat contents and liver peroxisomal activities in fenofibrate-treated rainbow trout. *Lipids* 39(9), 849-855.

Dweep, H., Sticht, C., Pandey, P. & Gretz, N. (2011). MiRWalk - Database: Prediction of possible miRNA binding sites by " walking" the genes of three genomes. *Journal of Biomedical Informatics* 44(5), 839-847.

Esau, C., Davis, S., Murray, S.F., Yu, X.X., Pandey, S.K., Pear, M., Watts, L., Booten, S.L., Graham, M., McKay, R., Subramaniam, A., Propp, S., Lollo, B.A., Freier, S., Bennett, C.F., Bhanot, S. & Monia, B.P. (2006a). miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab* 3(2), 87-98.

Esau, C., Kang, X., Peralta, E., Hanson, E., Marcusson, E.G., Ravichandran, L.V., Sun, Y., Koo, S., Perera, R.J., Jain, R., Dean, N.M., Freier, S.M., Bennett, C.F., Lollo, B. & Griffey, R. (2004). MicroRNA-143 regulates adipocyte differentiation. *Journal of Biological Chemistry* 279(50), 52361-52365.

Esau, C.C., Davis, S. & Murray, S.F. (2006b). miRNA-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab.* 3, 87–98.

Esau, C.C. & Monia, B.P. (2007). Therapeutic potential for microRNAs. *Advanced Drug Delivery Reviews* 59(2-3), 101-114.

Evans, H., De Tomaso, T., Quail, M., Rogers, J., Gracey, A.Y., Cossins, A.R. & Berenbrink, M. (2008). Ancient and modern duplication events and the evolution of stearoyl-CoA desaturases in teleost fishes. *Physiological Genomics* 35(1), 18-29.

FAO (2006). Use of Fishary Resources as feed inputs to aquaculture development: Trends and Policy implications. *FAO Fisheries Circular* 1018.

FAO (2010). World aquaculture 2010. *FAO Fisheries and Aquaculture Technical Paper* 500(1).

FAO (2012). The state of world fisheries and aquaculture. *FAO Fisheries and Aquaculture Technical Paper*.

Feillet-Coudray, C., Aoun, M., Fouret, G., Bonafos, B., Ramos, J., Casas, F., Cristol, J.P. & Coudray, C. (2013). Effects of long-term administration of

saturated and n-3 fatty acid-rich diets on lipid utilisation and oxidative stress in rat liver and muscle tissues. *British Journal of Nutrition* 110(10), 1789-1802.

Fernández-Hernando, C., Suárez, Y., Rayner, K.J. & Moore, K.J. (2011). MicroRNAs in lipid metabolism. *Current Opinion in Lipidology* 22(2), 86-92.

Fujiyama-Fujiwara, Y., Umeda-Sawada, R., Kuzuyama, M. & Igarashi, O. (1995). Effects of Sesamin on the Fatty Acid Composition of the Liver of Rats Fed N-6 and N-3 Fatty Acids-Rich Diet. *j.Nutr.Sci.Vitaminol.* 41, 217-225.

Gatfield, D., Le Martelot, G., Vejnar, C.E., Gerlach, D., Schaad, O., Fleury-Olela, F., Ruskeepaa, A.L., Oresic, M., Esau, C.C., Zdobnov, E.M. & Schibler, U. (2009). Integration of microRNA miR-122 in hepatic circadian gene expression. *Genes Dev* 23(11), 1313-26.

Girard, M., Jacquemin, E., Munnich, A., Lyonnet, S. & Henrion-Caude, A. (2008). miR-122, a paradigm for the role of microRNAs in the liver. *Journal of Hepatology* 48(4), 648-656.

Gjedrem, T. (1997). Flesh quality improvement in fish through breeding. *Aquaculture International* 5(3), 197-206.

Griffiths-Jones, S., Grocock, R.J., van Dongen, S., Bateman, A. & Enright, A.J. (2006). miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic acids research.* 34(Database issue), D140-144.

Griffiths-Jones, S., Saini, H.K., Van Dongen, S. & Enright, A.J. (2008). miRBase: Tools for microRNA genomics. *Nucleic Acids Research* 36(SUPPL. 1), D154-D158.

Gräff, J., Kim, D., Dobbin, M.M. & Li-Huei, T. (2011). Epigenetic Regulation of Gene Expression in Physiological and Pathological Brain Processes. *Physiological Reviews* 91(2), 603-649.

Hara, A. & Radin, N.S. (1978). Lipid extraction of tissue with low toxicity solvent. *Analytic Biochemistry* 90, 420-426.

Hastings, N., Agaba, M.K., Tocher, D.R., Zheng, X., Dickson, C.A., Dick, J.R. & Teale, A.J. (2004). Molecular cloning and functional characterization of fatty acyl desaturase and elongase cDNAs involved in the production of eicosapentaenoic and docosahexaenoic acids from α-linolenic acid in Atlantic salmon (Salmo salar). *Marine Biotechnology* 6(5), 463-474.

Henderson, R.J. (1996). Fatty acid metabolism in freshwater fish with particular reference to polyunsaturated fatty acids. *Archives of Animal Nutrition/Archiv fur Tierernahrung* 49(1), 5-22.

Henderson, R.J. & Sargent, J.R. (1984). Lipid metabolism in rainbow trout (Salmo gairdnerii) fed diets containing partially hydrogenated fish oil. *Comparative Biochemistry and Physiology -- Part B: Biochemistry and* 78(3), 557-564.

Henderson, R.J. & Sargent, J.R. (1985). Chain-length specificities of mitochondrial and peroxisimal β-oxidation of fatty acids in livers of rainbow trout (Salmo gairdneri). *Comparative Biochemistry and Physiology -- Part B: Biochemistry and* 82(1), 79-85.

Hesketh, J.E., Vasconcelos, M.H. & Bermano, G. (1998). Regulatory signals in messenger RNA: Determinants of nutrient-gene interaction and metabolic compartmentation. *British Journal of Nutrition* 80(4), 307-321.

Hiltunen, J.K., Chen, Z., Haapalainen, A.M., Wierenga, R.K. & Kastaniotis, A.J. (2010). Mitochondrial fatty acid synthesis - An adopted set of enzymes making a pathway of major importance for the cellular metabolism. *Progress in Lipid Research* 49(1), 27-45.

Hordvik, I. (1998). The impact of ancestral tetraploidy on antibody heterogeneity in salmonid fishes. *Immunological Reviews* 166, 153-157.

Horton, J.D., Goldstein, J.L. & Brown, M.S. (2002). SREBPs: Activators of the complete program of cholesterol and fatty acid synthesis in the liver. *Journal of Clinical Investigation* 109(9), 1125-1131.

Huong, D.T.T. & Ide, T. (2008). Dietary lipoic acid-dependent changes in the activity and mRNA levels of hepatic lipogenic enzymes in rats. *British Journal of Nutrition* 100(1), 79-87.

Ide, T., Ashakumary, L., Takahashi, Y., Kushiro, M., Fukuda, N. & Sugano, M. (2001). Sesamin, a sesame lignan, decreases fatty acid synthesis in rat liver accompanying the down-regulation of sterol regulatory element binding protein-1. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids* 1534(1), 1-13.

Ide, T., Azechi, A., Kitade, S., Kunimatsu, Y., Suzuki, N. & Nakajima, C. (2012). Combined effect of sesamin and α-lipoic acid on hepatic fatty acid metabolism in rats. *European Journal of Nutrition*, 1-13.

Ide, T., Hong, D.D., Ranasinghe, P., Takahashi, Y., Kushiro, M. & Sugano, M. (2004). Interaction of dietary fat types and sesamin on hepatic fatty acid oxidation in rats. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids* 1682(1-3), 80-91.

Ide, T., Kushiro, M., Takahashi, Y., Shinohara, K., Fukuda, N. & Sirato-Yasumoto, S. (2003). Sesamin, a Sesame Lignan, as a Potent Serum Lipid-Lowering Food Component. *Japan Agricultural Research Quarterly (JARQ)* 37(3), 151 – 158.

Inui, M., Martello, G. & Piccolo, S. (2010). MicroRNA control of signal transduction. *Nat Rev Mol Cell Biol* 11(4), 252-263.

Issemann, I. & Green, S. (1990). Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* 347(6294), 645-50.

Jeng, K.C.G. & Hou, R.C.W. (2005). Sesamin and sesamolin: Nature's Therapeutic Lignans. *Current enzyme Inhibition* 1(1), 11-20.

Johansen, S.D., Coucheron, D.H., Andreassen, M., Karlsen, B.O., Furmanek, T., Jørgensen, T.E., Emblem, A., Breines, R., Nordeide, J.T., Moum, T., Nederbragt, A.J., Stenseth, N.C. & Jakobsen, K.S. (2009). Large-scale sequence analyses of Atlantic cod. *New Biotechnology* 25(5), 263-271.

Johansen, S.D., Karlsen, B.O., Furmanek, T., Andreassen, M., Jørgensen, T.E., Bizuayehu, T.T., Breines, R., Emblem, A., Kettunen, P., Luukko, K., Edvardsen, R.B., Nordeide, J.T., Coucheron, D.H. & Moum, T. (2011). RNA deep sequencing of the Atlantic cod transcriptome. *Comparative*

Biochemistry and Physiology - Part D: Genomics and Proteomics 6(1), 18-22.

Jordal, A.-E.O., Lie, Ø. & Torstensen, B.E. (2007). 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* 13, 114–130.

Jorgensen, S.M., Kleveland, E.J., Grimholt, U. & Gjoen, T. (2006). Validation of reference genes for real-time polymerase chain reaction studies in Atlantic salmon. *Marine Biotechnology* 8(4), 398-408.

Jump, D.B., Botolin, D., Wang, Y., Xu, J., Christian, B. & Demeure, O. (2005). Fatty Acid Regulation of Hepatic Gene Transcription. *J. Nutr.* 135, 2503–2506.

Jump, D.B. & Clarke, S.D. (1999). Regulation of gene expression by dietary fat. *Annu. Rev. Nutr.* 19, 63-90.

Jung, C.J., Iyengar, S., Blahnik, K.R., Ajuha, T.P., Jiang, J.X., Farnham, P.J. & Zern, M. (2011). Epigenetic modulation of miR-122 facilitates human embryonic stem cell self-renewal and hepatocellular carcinoma proliferation. *PLoS One* 6(11).

Kamal-Eldin, A., Frank, J., Razdan, A., Tengblad, S., Basu, S. & Vessby, B. (2000). Effects of dietary phenolic compounds on tocopherol, cholesterol, and fatty acids in rats. *Lipids* 35(4), 427-435.

Kavsan, V., Koval, A., Petrenko, O., Roberts, C.T. & Leroith, D. (1993). Two Insulin Genes Are Present in the Salmon Genome. *Biochemical and Biophysical Research Communications* 191(3), 1373-1378.

Kennedy, S.R., Bickerdike, R., Berge, R.K., Dick, J.R. & Tocher, D.R. (2007a). Influence of conjugated linoleic acid (CLA) or tetradecylthioacetic acid (TTA) on growth, lipid composition, fatty acid metabolism and lipid gene expression of rainbow trout (Oncorhynchus mykiss L.). *Aquaculture* 272(1-4), 489-501.

Kennedy, S.R., Bickerdike, R., Berge, R.K., Porter, A.R. & Tocher, D.R. (2007b). Influence of dietary conjugated linoleic acid (CLA) and tetradecylthioacetic acid (TTA) on growth, lipid composition and key enzymes of fatty acid oxidation in liver and muscle of Atlantic cod (*Gadus morhua* L.). *Aquaculture* 264(1-4), 372-382.

Kersten, S. (2008). Peroxisome proliferator activated receptors and lipoprotein metabolism. *PPAR Research*.

Kim, D.J., Seok, S.H., Baek, M.W., Lee, H.Y., Na, Y.R., Park, S.H., Lee, H.K., Dutta, N.K., Kawakami, K. & Park, J.H. (2009). Developmental toxicity and brain aromatase induction by high genistein concentrations in zebrafish embryos. *Toxicology Mechanisms and Methods* 19(3), 251-256.

Kim, M.S., Park, J.Y., Namkoong, C., Jang, P.G., Ryu, J.W., Song, H.S., Yun, J.Y., Namgoong, I.S., Ha, J., Park, I.S., Lee, I.K., Viollet, B., Youn, J.H., Lee, H.K. & Lee, K.U. (2004). Anti-obesity effects of alpha-lipoic acid mediated by suppression of hypothalamic AMP-activated protein kinase. *Nature Medicine* 10(7), 727-733.

Kim, V.N. (2005). MicroRNA biogenesis: Coordinated cropping and dicing. *Nature Reviews Molecular Cell Biology* 6(5), 376-385.

Kiso, Y., Tsuruoka, N., Kidokoro, A., Matsumoto, I. & Abe, K. (2005). Sesamin ingestion regulates the transcription levels of hepatic metabolizing enzymes for alcohol and lipids in rats. *Alcoholism: Clinical and Experimental Research* 29(11 SUPPL.).

Kjær, M.A., Vegusdal, A., Gjøen, T., Rustan, A.C., Todorčević, M. & Ruyter, B. (2008). Effect of rapeseed oil and dietary n-3 fatty acids on triacylglycerol synthesis and secretion in Atlantic salmon hepatocytes. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* 1781(3), 112-122.

Kleveland, E.J., Ruyter, B., Vegusdal, A., Sundvold, H., Berge, R.K. & Gjøen, T. (2006a). Effects of 3-thia fatty acids on expression of some lipid related genes in Atlantic salmon (Salmo salar L.). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 145(2), 239-248.

Kleveland, E.J., Syvertsen, B.L., Ruyter, B., Vegusdal, A., Jørgensen, S.M. & Gjøen, T. (2006b). Characterization of Scavenger Receptor Class B, Type I in Atlantic Salmon (*Salmo salar* L.). *Lipids* 71, 1017–1027.

Kloosterman, W.P. & Plasterk, R.H.A. (2006). The Diverse Functions of MicroRNAs in Animal Development and Disease. *Developmental Cell* 11(4), 441-450.

Kloosterman, W.P., Steiner, F.A., Berezikov, E., de Bruijn, E., van de Belt, J., Verheul, M., Cuppen, E. & Plasterk, R.H.A. (2006). Cloning and expression of new microRNAs from zebra fish. *Nucleic Acids Res.* 34, 2558.

Kozlov, A.V., Gille, L., Staniek, K. & Nohl, H. (1999). Dihydrolipoic acid maintains ubiquinone in the antioxidant active form by two-electron reduction of ubiquinone and one-electron reduction of ubisemiquinone. *Archives of Biochemistry and Biophysics* 363(1), 148-154.

Krek, A., Grün, D., Poy, M.N., Wolf, R., Rosenberg, L., Epstein, E.J., MacMenamin, P., Da Piedade, I., Gunsalus, K.C., Stoffel, M. & Rajewsky, N. (2005). Combinatorial microRNA target predictions. *Nature Genetics* 37(5), 495-500.

Kris-Etherton, P.M., Hecker, K.D., Bonanome, A., Coval, S.M., Binkoski, A.E., Hilpert, K.F., Griel, A.E. & Etherton, T.D. (2002). Bioactive compounds in foods: Their role in the prevention of cardiovascular disease and cancer. *American Journal of Medicine* 113, PII S0002-9343(01)00995-0.

Krützfeldt, J., Rajewsky, N., Braich, R., Rajeev, K.G., Tuschl, T., Manoharan, M. & Stoffel, M. (2005). Silencing of microRNAs in vivo with 'antagomirs'. *Nature* 438(7068), 685-689.

Krützfeldt, J. & Stoffel, M. (2006). MicroRNAs: A new class of regulatory genes affecting metabolism. *Cell Metab* 4(1), 9-12.

Kushiro, M., Masaoka, T., Hageshita, S., Takahashi, Y., Ide, T. & Sugano, M. (2002). Comparative effect of sesamin and episesamin on the activity and gene expression of enzymes in fatty acid oxidation and synthesis in rat liver. *Journal of Nutritional Biochemistry* 13(5), 289-295.

Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W. & Tuschl, T. (2002). Identification of Tissue-Specific MicroRNAs from Mouse. *Current Biology* 12(9), 735-739.

Lau, N.C., Lim, L.P., Weinstein, E.G. & Bartel, D.P. (2001). An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science* 294(5543), 858-862.

Laudadio, I., Manfroid, I., Achouri, Y., Schmidt, D., Wilson, M.D., Cordi, S., Thorrez, L., Knoops, L., Jacquemin, P., Schuit, F., Pierreux, C.E., Odom, D.T., Peers, B. & Lemaigre, F.P. (2012). A feedback loop between the liver-enriched transcription factor network and miR-122 controls hepatocyte differentiation. *Gastroenterology* 142(1), 119-129.

Leaver, M.J., Bautista, J.M., Björnsson, B.T., Jönsson, E., Krey, G., Tocher, D.R. & Torstensen, B.E. (2008a). Towards fish lipid nutrigenomics: Current state and prospects for fin-fish aquaculture. *Reviews in Fisheries Science* 16(SUPPL.1), 71-92.

Leaver, M.J., Ezaz, M.T., Fontagne, S., Tocher, D.R., Boukouvala, E. & Krey, G. (2007). Multiple peroxisome proliferator-activated receptor β subtypes from Atlantic salmon (Salmo salar). *Journal of Molecular Endocrinology* 38(3-4), 391-400.

Leaver, M.J., Taggart, J.B., Villeneuve, L., Bron, J.E., Guy, D.R., Bishop, S.C., Houston, R.D., Matika, O. & Tocher, D.R. (2011). Heritability and mechanisms of n- 3 long chain polyunsaturated fatty acid deposition in the flesh of Atlantic salmon. *Comparative Biochemistry and Physiology - Part D: Genomics and Proteomics* 6(1), 62-69.

Leaver, M.J., Tocher, D.R., Obach, A., Jensen, L., Henderson, R.J., Porter, A.R. & Krey, G. (2006). Effect of dietary conjugated linoleic acid (CLA) on lipid composition, metabolism and gene expression in Atlantic salmon (Salmo salar) tissues. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* 145(2), 258-267.

Leaver, M.J., Villeneuve, L.A.N., Obach, A., Jensen, L., Bron, J.E., Tocher, D.R. & Taggart, J.B. (2008b). Functional genomics reveals increases in cholesterol biosynthetic genes and highly unsaturated fatty acid biosynthesis after dietary substitution of fish oil with vegetable oils in Atlantic salmon (*Salmo salar*). *BMC Genomics* 9(299).

Lee, R.C., Feinbaum, R.L. & Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 75(5), 843-854.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Rådmark, O., Kim, S. & Kim, V.N. (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425(6956), 415-419.

Lefaucheur, L., Milan, D., Ecolan, P. & Le Callennec, C. (2004). Myosin heavy chain composition of different skeletal muscles in Large White and Meishan pigs. *Journal of Animal Science* 82(7), 1931-1941.

Lemoine, C.M.R., Lougheed, S.C. & Moyes, C.D. (2010). Modular evolution of PGC-1α in vertebrates. *Journal of Molecular Evolution* 70(5), 492-505.

Lewis, B.P., Burge, C.B. & Bartel, D.P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120(1), 15-20.

Li, J., Wei, H.X., Li, Y., Li, Q.Y. & Li, N. (2012a). Identification of a suitable endogenous control gene in porcine blastocysts for use in quantitative PCR analysis of microRNAs. *Science China Life Sciences* 55(2), 126-131.

Li, R.W., Lin, Y.Q., Zheng, Y.C., Lu, B.M., Lin, J.C., Huang, L. & Liu, Z.X. (2012b). Cloning, expression and polymorphism analyses of PGC-lα gene of Schizothorax prenanti. *Asian Journal of Animal and Veterinary Advances* 7(10), 928-939.

Li, S.C., Chan, W.C., Ho, M.R., Tsai, K.W., Hu, L.Y., Lai, C.H., Hsu, C.N., Hwang, P.P. & Lin, W.C. (2010a). Discovery and characterization of medaka miRNA genes by next generation sequencing platform. *BMC Genomics* 11(SUPPL. 4).

Li, Y., Monroig, O., Zhang, L., Wang, S., Zheng, X., Dick, J.R., You, C. & Tocher, D.R. (2010b). Vertebrate fatty acyl desaturase with Δ4 activity. *Proceedings of the National Academy of Sciences of the United States of America* 107(39), 16840-16845.

Lie, Ø., Waagbø, R. & Sandnes, K. (1988). Growth and chemical composition of adult Atlantic salmon (Salmo salar) fed dry and silage-based diets. *Aquaculture* 69(3-4), 343-353.

Lin, J., Liu, F. & Jiang, Y. (2012). Antisense technologies targeting fatty acid synthetic enzymes. *Recent Patents on Anti-Cancer Drug Discovery* 7(2), 198-206.

Livak, K.J. & Schmittgen, T.D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* 25(4), 402-408.

Lykkesfeldt, J., Hagen, T.M., Vinarsky, V. & Ames, B.N. (1998). Age-associated decline in ascorbic acid concentration, recycling, and biosyntheis in rat hepatocytes - Reversal with (R)-α-lipoic acid supplementation. *FASEB Journal* 12(12), 1183-1189.

Ma, H., Hostuttler, M., Wei, H., Rexroad Iii, C.E. & Yao, J. (2012). Characterization of the rainbow trout egg microRNA transcriptome. *PLoS ONE* 7(6).

Malerød, L., Juvet, L., Gjøen, T. & Berg, T. (2002). The expression of scavenger receptor class B, type I (SR-BI) and caveolin-1 in parenchymal and nonparenchymal liver cells. *Cell and Tissue Research* 307(2), 173-180.

Malerød, L., Sporstøl, M., Juvet, L.K., Mousavi, A., Gjøen, T. & Berg, T. (2003). Hepatic scavenger receptor class B, type I is stimulated by peroxisome proliferator-activated receptor γ and hepatocyte nuclear factor 4α. *Biochemical and Biophysical Research Communications* 305(3), 557-565.

Malerød, L., Sporstøl, M., Juvet, L.K., Mousavi, S.A., Gjøen, T., Berg, T., Roos, N. & Eskild, W. (2005). Bile acids reduce SR-BI expression in hepatocytes by a pathway involving FXR/RXR, SHP, and LRH-1. *Biochemical and Biophysical Research Communications* 336(4), 1096-1105.

Matsuzaka, T., Shimano, H., Yahagi, N., Amemiya-Kudo, M., Yoshikawa, T., Hasty, A.H., Tamura, Y., Osuga, J.I., Okazaki, H., Iizuka, Y., Takahashi, A., Sone, H., Gotoda, T., Ishibashi, S. & Yamada, N. (2002). Dual

regulation of mouse Δ5- and Δ6-desaturase gene expression by SREBP-1 and PPARα. *Journal of Lipid Research* 43(1), 107-114.

McFarlane, C., Vajjala, A., Arigela, H., Lokireddy, S., Ge, X., Bonala, S., Manickam, R., Kambadur, R. & Sharma, M. (2014). Negative auto-regulation of myostatin expression is mediated by Smad3 and MicroRNA-27. *PLoS ONE* 9(1).

McKay, S.J., Trautner, J., Smith, M.J., Koop, B.F. & Devlin, R.H. (2004). Evolution of duplicated growth hormone genes in autotetraploid salmonid fishes. *Genome* 47(4), 714-23.

Mennigen, J.A., Martyniuk, C.J., Seiliez, I., Panserat, S. & Skiba-Cassy, S. (2014a). Metabolic consequences of microRNA-122 inhibition in rainbow trout, Oncorhynchus mykiss. *BMC Genomics* 15, 70.

Mennigen, J.A., Panserat, S., Larquier, M., Plagnes-Juan, E., Medale, F., Seiliez, I. & Skiba-Cassy, S. (2012). Postprandial regulation of hepatic microRNAs predicted to target the insulin pathway in rainbow trout. *PLoS One* 7(6).

Mennigen, J.A., Plagnes-Juan, E., Figueredo-Silva, C.A., Seiliez, I., Panserat, S. & Skiba-Cassy, S. (2014b). Acute endocrine and nutritional co-regulation of the hepatic omy-miRNA-122b and the lipogenic gene fas in rainbow trout, Oncorhynchus mykiss. *Comp Biochem Physiol B Biochem Mol Biol* 169, 16-24.

Mennigen, J.A., Skiba-Cassy, S. & Panserat, S. (2013). Ontogenetic expression of metabolic genes and microRNAs in rainbow trout alevins during the transition from the endogenous to the exogenous feeding period. *Journal of Experimental Biology* 216(9), 1597-1608.

Minghetti, M., Leaver, M.J. & Tocher, D.R. (2011). Transcriptional control mechanisms of genes of lipid and fatty acid metabolism in the Atlantic salmon (Salmo salar L.) established cell line, SHK-1. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* 1811(3), 194-202.

Mishima, Y. (2012). Widespread roles of microRNAs during zebrafish development and beyond. *Development Growth and Differentiation* 54(1), 55-65.

Moazzami, A.A. & Kamal-Eldin, A. (2006). Sesame seed is a rich source of dietary lignans. *Journal of the American Oil Chemists Society* 83(8), 719-723.

Monroig, Ó., Li, Y. & Tocher, D.R. (2011). Delta-8 desaturation activity varies among fatty acyl desaturases of teleost fish: High activity in delta-6 desaturases of marine species. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* 159(4), 206-213.

Monroig, Ó., Tocher, D.R. & Navarro, J.C. (2013). Biosynthesis of polyunsaturated fatty acids in marine invertebrates: Recent advances in molecular mechanisms. *Marine Drugs* 11(10), 3998-4018.

Monroig, Ó., Zheng, X., Morais, S., Leaver, M.J., Taggart, J.B. & Tocher, D.R. (2010). Multiple genes for functional 6 fatty acyl desaturases (Fad) in Atlantic salmon (Salmo salar L.): Gene and cDNA characterization, functional expression, tissue distribution and nutritional regulation. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* 1801(9), 1072-1081.

Moore, M.J. (2005). From birth to death: the complex lives of eukaryotic mRNAs. *Science* 309(5740), 1514-8.

Morais, S., Castanheira, F., Martinez-Rubio, L., Conceição, L.E.C. & Tocher, D.R. (2012a). Long chain polyunsaturated fatty acid synthesis in a marine vertebrate: Ontogenetic and nutritional regulation of a fatty acyl desaturase with Δ4 activity. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* 1821(4), 660-671.

Morais, S., Monroig, O., Xiaozhong, Z., Leaver, M.J. & Tocher, D.R. (2009). Highly Unsaturated Fatty Acid Synthesis in Atlantic Salmon: Characterization of ELOVL5-and ELOVL2-like Elongases. *Marine Biotechnology* 11(5), 627-639.

Morais, S., Pratoomyot, J., Taggart, J.B., Bron, J.E., Guy, D.R., Bell, J.G. & Tocher, D.R. (2011). Genotype-specific responses in Atlantic salmon (Salmo salar) subject to dietary fish oil replacement by vegetable oil: A liver transcriptomic analysis. *BMC Genomics* 12.

Morais, S., Silva, T., Cordeiro, O., Rodrigues, P., Guy, D.R., Bron, J.E., Taggart, J.B., Bell, J.G. & Tocher, D.R. (2012b). Effects of genotype and dietary fish oil replacement with vegetable oil on the intestinal transcriptome and proteome of Atlantic salmon (Salmo salar). *BMC Genomics* 13(1).

Morash, A.J., Le Moine, C.M.R. & McClelland, G.B. (2010). Genome duplication events have led to a diversification in the CPT I gene family in fish. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* 299(2), R579-R589.

Morash, A.J. & McClelland, G.B. (2011). Regulation of carnitine palmitoyltransferase (CPT) I during Fasting in rainbow trout (Oncorhynchus mykiss) promotes increased mitochondrial fatty acid oxidation. *Physiological and Biochemical Zoology* 84(6), 625-633.

Morikawa, T., Yasuno, R. & Wada, H. (2001). Do mammalian cells synthesize lipoic acid? Identification of a mouse cDNA encoding a lipoic acid synthase located in mitochondria. *FEBS Letters* 498(1), 16-21.

Morin, R.D., O'Connor, M.D., Griffith, M., Kuchenbauer, F., Delaney, A., Prabhu, A.L., Zhao, Y., McDonald, H., Zeng, T., Hirst, M., Eaves, C.J. & Marra, M.A. (2008). Application of massively parallel sequencing to microRNA profiling and discovery in human embryonic stem cells. *Genome Research* 18(4), 610-621.

Motojima, K., Passilly, P., Peters, J.M., Gonzalez, F.J. & Latruffe, N. (1998). Expression of putative fatty acid transporter genes are regulated by peroxisome proliferator-activated receptor α and γ activators in a tissue- and inducer-specific manner. *Journal of Biological Chemistry* 273(27), 16710-16714.

Moya-Falcón, C., Hvattum, E., Dyrøy, E., Skorve, J., Stefansson, S.O., Thomassen, M.S., Jakobsen, J.V., Berge, R.K. & Ruyter, B. (2004). Effects of 3-thia fatty acids on feed intake, growth, tissue fatty acid composition, β-oxidation and Na+,K+-ATPase activity in Atlantic salmon. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* 139(4), 657-668.

Moya-Falcón, C., Thomassen, M.S., Jakobsen, J.V. & Ruyter, B. (2005). Effects of dietary supplementation of rapeseed oil on metabolism of [1-^{14}C]18:1n-9, [1-^{14}C]20:3n-6, and [1-^{14}C]20:4n-3 in Atlantic salmon hepatocytes. *Lipids* 40(7), 709-717.

Mráz, J. (2012). Lipids in common carp (*Cyprinus carpio*) and effects on human health. *Acta Universitatis agriculturae Sueciae* 2012:75.

Mraz, J., Schlechtriem, C., Olohan, L.A., Fang, Y.X., Cossins, A.R., Zlabek, V., Samuelsen, T. & Pickova, J. (2010). Sesamin as a potential modulator of fatty acid composition in common carp (Cyprinus carpio). *Aquaculture Research* 41(11), e851-e861.

Mráz, J., Zájic, T. & Pickova, J. (2012). Culture of common carp (Cyprinus carpio) with defined flesh quality for prevention of cardiovascular diseases using finishing feeding strategy. *Neuroendocrinology Letters* 33(SUPPL. 2), 60-67.

Nakamura, M.T. & Nara, T.Y. (2004). Structure, function, and dietary regulation of Δ6, Δ5, and Δ9 desaturases. In. pp. 345-376.

Nan, X.M., Wang, J.Q., Chen, H.Y. & Hu, H. (2013). MicroRNAs and lipids metabolism. *Progress in Biochemistry and Biophysics* 40(2), 118-129.

Nasopoulou, C. & Zabetakis, I. (2012). Benefits of fish oil replacement by plant originated oils in compounded fish feeds. A review. *LWT - Food Science and Technology* 47(2), 217-224.

Ohno, S. (1999). Gene duplication and the uniqueness of vertebrate genomes circa 1970-1999. *Seminars in Cell and Developmental Biology* 10(5), 517-522.

Ohno, S., Wolf, U. & Atkin, N.B. (1968). Evolution from fish to mammals by gene duplication. *Hereditas* 59(1), 169-187.

Olesen, I., Gjedrem, T., Bentsen, H.B., Gjerde, B. & Rye, M. (2003). Breeding Programs for Sustainable Aquaculture. *Journal of Applied Aquaculture* 13(3-4), 179-204.

Osborne, T.F. & Espenshade, P.J. (2009). Evolutionary conservation and adaptation in the mechanism that regulates SREBP action: What a long, strange tRIP it's been. *Genes and Development* 23(22), 2578-2591.

Pagot, Y. & Belin, J.M. (1996). Involvement of carnitine acyltransferases in peroxisomal fatty acid metabolism by the yeast Pichia guilliermondii. *Applied and Environmental Microbiology* 62(10), 3864-3867.

Panda, S., Antoch, M.P., Miller, B.H., Su, A.I., Schook, A.B., Straume, M., Schultz, P.G., Kay, S.A., Takahashi, J.S. & Hogenesch, J.B. (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* 109(3), 307-320.

Pasquinelli, A.E., Reinhart, B.J., Slack, F., Martindale, M.Q., Kuroda, M.I., Maller, B., Hayward, D.C., Ball, E.E., Degnan, B., Müller, P., Spring, J., Srinivasan, A., Fishman, M., Finnerty, J., Corbo, J., Levine, M., Leahy, P., Davidson, E. & Ruvkun, G. (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* 408(6808), 86-89.

Peñaloza, C., Hamilton, A., Guy, D.R., Bishop, S.C. & Houston, R.D. (2013). A SNP in the 5′ flanking region of the myostatin-1b gene is associated with harvest traits in Atlantic salmon (Salmo salar). *BMC Genetics* 14.

Pettersson, A., Johnsson, L., BrÄNnÄS, E. & Pickova, J. (2009). Effects of rapeseed oil replacement in fish feed on lipid composition and self-selection by rainbow trout (Oncorhynchus mykiss). *Aquaculture Nutrition* 15(6), 577-586.

Pickova, J., Dutta, P.C., Larsson, P.O. & Kiessling, A. (1997). Early embryonic cleavage pattern, hatching success, and egg-lipid fatty acid composition: comparison between two cod (Gadus morhua) stocks. *Canadian Journal of Fisheries and Aquatic Sciences* 54(10), 2410-2416.

Pickova, J. & Mørkøre, T. (2007). Alternate oils in fish feeds. *European Journal of Lipid Science and Technology* 109(3), 256-263.

Plat, J., Nichols, J.A. & Mensink, R.P. (2005). Plant sterols and stanols: effects on mixed micellar composition and LXR (target gene) activation. *J. Lipid Res.* 46(11), 2468-2476.

Poirier, H., Niot, I., Monnot, M.C., Braissant, O., Meunier-Durmort, C., Costet, P., Pineau, T., Wahli, W., Willson, T.M. & Besnard, P. (2001). Differential involvement of peroxisome-proliferator-activated receptors α and δ in fibrate and fatty-acid-mediated inductions of the gene encoding liver fatty-acid-binding protein in the liver and the small intestine. *Biochemical Journal* 355(2), 481-488.

Powell, K. (2003). Fish farming: Eat your veg. *Nature* 426(6965), 378-379.

Price, P.T., Nelson, C.M. & Clarke, S.D. (2000). Omega-3 polyunsaturated fatty acid regulation of gene expression. *Current Opinion in Lipidology* 11(1), 3-7.

Qin, Y.F., Dalen, K.T., Gustafsson, J.-Å. & Nebb, H.I. (2009). Regulation of hepatic fatty acid elongase 5 by LXRα-SREBP-1c. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* 1791(2), 140-147.

Ramachandra, R.K., Salem, M., Gahr, S., Rexroad Iii, C.E. & Yao, J. (2008). Cloning and characterization of microRNAs from rainbow trout (Oncorhynchus mykiss): Their expression during early embryonic development. *BMC Developmental Biology* 8.

Regost, C., Jakobsen, J.V. & Rørå, A.M.B. (2004). Flesh quality of raw and smoked fillets of Atlantic salmon as influenced by dietary oil sources and frozen storage. *Food Research International* 37(3), 259-271.

Rehmsmeier, M., Steffen, P., Hochsmann, M. & Giegerich, R. (2004). Fast and effective prediction of microRNA/target duplexes. *Rna* 10(10), 1507-17.

Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelll, A.E., Bettlnger, J.C., Rougvle, A.E., Horvitz, H.R. & Ruvkun, G. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature* 403(6772), 901-906.

Reppert, S.M. & Weaver, D.R. (2002). Coordination of circadian timing in mammals. *Nature* 418(6901), 935-941.

Reyes, D., Cepeda, V., Gonzalez, R. & Vidal, R. (2012). Ssa miRNAs DB: Online repository of in silico predicted miRNAs in Salmo salar. *Bioinformation* 8, 284–286.

Rhainds, D. & Brissette, L. (2004). The role of scavenger receptor class B type I (SR-BI) in lipid trafficking: Defining the rules for lipid traders. *The International Journal of Biochemistry & Cell Biology* 36(1), 39-77.

Ricketts, M.L., Moore, D.D., Banz, W.J., Mezei, O. & Shay, N.F. (2005). Molecular mechanisms of action of the soy isoflavones includes activation of promiscuous nuclear receptors. A review. *Journal of Nutritional Biochemistry* 16(6), 321-330.

Robin, J.H., Regost, C., Arzel, J. & Kaushik, S.J. (2003). Fatty acid profile of fish following a change in dietary fatty acid source: model of fatty acid composition with a dilution hypothesis. *Aquaculture* 225(1-4), 283-293.

Rosenlund, G. (2001). Effect of alternative lipid sources on long-term growth performance and quality of Atlantic salmon (Salmo salar L.). *Aquaculture Research* 32(SUPPL. 1), 323-328.

Ruby, J.G., Jan, C., Player, C., Axtell, M.J., Lee, W., Nusbaum, C., Ge, H. & Bartel, D.P. (2006). Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in C. elegans. *Cell* 127(6), 1193-207.

Ruby, J.G., Stark, A., Johnston, W.K., Kellis, M., Bartel, D.P. & Lai, E.C. (2007). Evolution, biogenesis, expression, and target predictions of a substantially expanded set of Drosophila microRNAs. *Genome Research* 17(12), 1850-1864.

Ruxton, C.H.S., Calder, P.C., Reed, S.C. & Simpson, M.J.A. (2005). The impact of long-chain n-3 polyunsaturated fatty acids on human health. *Nutrition Research Reviews* 18(1), 113-129.

Ruyter, B., Andersen, Ø., Dehli, A., Östlund Farrants, A.-K., Gjøen, T. & Thomassen, M.S. (1997). Peroxisome proliferator activated receptors in Atlantic salmon (*Salmo salar*): Effects on PPAR transcription and acyl-CoA oxidase activity in hepatocytes by peroxisome proliferators and fatty acids. *Biochimica et Biophysica Acta - Lipids and Lipid Metabolism* 1348(3), 331-338.

Ruyter, B., Røsjø, C., Einen, O. & Thomassen, M.S. (2000). Essential fatty acids in Atlantic salmon: Time course of changes in fatty acid composition of liver, blood and carcass induced by a diet deficient in n-3 and n-6 fatty acids. *Aquaculture Nutrition* 6(2), 109-117.

Ruyter, B. & Thomassen, M.S. (1999). Metabolism of n-3 and n-6 fatty acids in Atlantic salmon liver: Stimulation by essential fatty acid deficiency. *Lipids* 34(11), 1167-1176.

Sacco, J. & Adeli, K. (2012). MicroRNAs: Emerging roles in lipid and lipoprotein metabolism. *Current Opinion in Lipidology* 23(3), 220-225.

Safari, M.-R. & Sheikh, N. (2003). Effects of flavonoids on the susceptibility of low-density lipoprotein to oxidative modification. *Prostaglandins, Leukotrienes and Essential Fatty Acids* 69, 73–77.

Safdar, A., Abadi, A., Akhtar, M., Hettinga, B.P. & Tarnopolsky, M.A. (2009). miRNA in the regulation of skeletal muscle adaptation to acute endurance exercise in C57BI/6J male mice. *PLoS ONE* 4(5).

Salem, M., Xiao, C., Womack, J., Rexroad Iii, C.E. & Yao, J. (2010). A microRNA repertoire for functional genome research in rainbow trout (Oncorhynchus mykiss). *Marine Biotechnology* 12(4), 410-429.

Sales, J. (2010). Quantification of the Differences in Flesh Fatty Acid Components between Farmed and Wild Fish. *Journal of Aquatic Food Product Technology* 19(3), 298-309.

Sanden, M., Stubhaug, I., Berntssen, M.H.G., Lie, Ø. & Torstensen, B.E. (2011). Atlantic salmon (Salmo salar L.) as a net producer of long-chain marine ω-3 fatty acids. *Journal of Agricultural and Food Chemistry* 59(23), 12697-12706.

Sargent, J.R. & Tacon, A.G.J. (1999). Development of farmed fish: a nutritionally necessary alternative to meat. *Proceedings of the Nutrition Society* 58(2), 377-383.

Sassi-Messai, S., Gibert, Y., Bernard, L., Nishio, S.I., Ferri Lagneau, K.F., Molina, J., Andersson-Lendahl, M., Benoit, G., Balaguer, P. & Laudet, V. (2009). The phytoestrogen genistein affects zebrafish development through two different pathways. *PLoS ONE* 4(3).

Schier, A.F. & Giraldez, A.J. (2006). MicroRNA Function and Mechanism: Insights from Zebra Fish. *Cold Spring Harbor Symposia on Quantitative Biology*, 195-203.

Schneider, R. & Grosschedl, R. (2007). Dynamics and interplay of nuclear architecture, genome organization, and gene expression. *Genes and Development* 21(23), 3027-3043.

Schoonjans, K., Staels, B. & Auwerx, J. (1996). The peroxisome proliferator activated receptors (PPARs) and their effects on lipid metabolism and adipocyte differentiation. *Biochimica et Biophysica Acta - Lipids and Lipid Metabolism* 1302(2), 93-109.

Seglen, P.O. (1976). Preparation of isolated rat liver cells. *Methods in Cell Biology* Vol. 13, 29-83.

Shahi, P., Loukianiouk, S., Bohne-Lang, A., Kenzelmann, M., Küffer, S., Maertens, S., Eils, R., Gröne, H.J., Gretz, N. & Brors, B. (2006). Argonaute--a database for gene regulation by mammalian microRNAs. *Nucleic acids research.* 34(Database issue), D115-118.

Shende, V.R., Goldrick, M.M., Ramani, S. & Earnest, D.J. (2011). Expression and rhythmic modulation of circulating micrornas targeting the clock gene Bmal1 in mice. *PLoS ONE* 6(7).

Shende, V.R., Kim, S.M., Neuendorff, N. & Earnest, D.J. (2014). MicroRNAs function as cis- and trans-acting modulators of peripheral circadian clocks. *FEBS Letters* 588(17), 3015-3022.

Sprecher, H. (2000). Metabolism of highly unsaturated n-3 and n-6 fatty acids. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* 1486(2-3), 219-231.

Stachowiak, M., Szydlowski, M., Flisikowski, K., Flisikowska, T., Bartz, M., Schnieke, A. & Switonski, M. (2014). Polymorphism in 3′ untranslated region of the pig PPARA gene influences its transcript level and is associated with adipose tissue accumulation. *Journal of Animal Science* 92(6), 2363-2371.

Stubhaug, I., Tocher, D.R., Bell, J.G., Dick, J.R. & Torstensen, B.E. (2005a). Fatty acid metabolism in Atlantic salmon (Salmo salar L.) hepatocytes and

influence of dietary vegetable oil. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* 1734(3), 277-288.
Tacon, A.G.J. (2005). State of information on salmon aquaculture feed and the environment *Report prepared for the WWF US Initiated Salmon Aquaculture Dialogue*.
Tacon, A.G.J. & Metian, M. (2008). Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture* 285(1-4), 146-158.
Taggart, J.B., Bron, J.E., Martin, S.A.M., Seear, P.J., Høyhein, B., Talbot, R., Carmichael, S.N., Villeneuve, L.A.N., Sweeney, G.E., Houlihan, D.F., Secombes, C.J., Tocher, D.R. & Teale, A.J. (2008). A description of the origins, design and performance of the TRAITS–SGPAtlantic salmon *Salmo salar* L. cDNA microarray. *Journal of Fish Biology* 72, 2071–2094.
Takahashi, Y., Odbayar, T.-O. & Ide, T. (2009). A comparative analysis of genistein and daidzein in affecting lipid metabolism in rat liver. *Journal of Clinical Biochemistry and Nutrition* 44(3), 223-230.
Thanuthong, T., Francis, D.S., Manickam, E., Senadheera, S.D., Cameron-Smith, D. & Turchini, G.M. (2011). Fish oil replacement in rainbow trout diets and total dietary PUFA content: II) Effects on fatty acid metabolism and in vivo fatty acid bioconversion. *Aquaculture* 322-323, 99-108.
Thomassen, M.S., Rein, D., Berge, G.M., Østbye, T.-K. & Ruyter, B. (2012). High dietary EPA does not inhibit Δ5 and Δ6 desaturases in Atlantic salmon (Salmo salar L.) fed rapeseed oil diets. *Aquaculture* 360-361, 78-85.
Thomassen, M.S. & Røsjø, C. (1989). Different fats in feed for salmon: Influence on sensory parameters, growth rate and fatty acids in muscle and heart. *Aquaculture* 79(1-4), 129-135.
Tocher, D.R. (1990). Incorporation and metabolism of (n-3) and (n-6) polyunsaturated fatty acids in phospholipid classes in cultured rainbow trout (Salmo gairdneri) cells. *Fish Physiology and Biochemistry* 8(3), 239-249.
Tocher, D.R. (2003). Metabolism and functions of lipids and fatty acids in teleost fish. *Reviews in Fisheries Science* 11(2), 107-184.
Tocher, D.R. (2010). Fatty acid requirements in ontogeny of marine and freshwater fish. *Aquaculture Research* 41(5), 717-732.
Tocher, D.R., Bell, J.G., Dick, J.R. & Crampton, V.O. (2003a). Effects of dietary vegetable oil on Atlantic salmon hepatocyte fatty acid desaturation and liver fatty acid compositions. *Lipids* 38(7), 723-732.
Tocher, D.R., Bell, J.G., MacGlaughlin, P., McGhee, F. & Dick, J.R. (2001). Hepatocyte fatty acid desaturation and polyunsaturated fatty acid composition of liver in salmonids: Effects of dietary vegetable oil. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* 130(2), 257-270.
Tocher, D.R., Bell, J.G., McGhee, F., Dick, J.R. & Fonseca-Madrigal, J. (2003b). Effects of dietary lipid level and vegetable oil on fatty acid metabolism in Atlantic salmon (Salmo salar L.) over the whole production cycle. *Fish Physiology and Biochemistry* 29(3), 193-209.

Tocher, D.R., Carr, J. & Sargent, J.R. (1989). Polyunsaturated fatty acid metabolism in fish cells: differential metabolism of (n-3) and (n-6) series acids by cultured cells orginating from a freshwater teleost fish and from a marine teleost fish. *Comparative Biochemistry and Physiology -- Part B: Biochemistry and* 94(2), 367-374.

Tocher, D.R. & Dick, J.R. (1990a). Incorporation and metabolism of (n-3) and (n-6) polyunsaturated fatty acids in phospholipid classes in cultured atlantic salmon (Salmo salar) cells. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* 96(1), 73-79.

Tocher, D.R. & Dick, J.R. (1990b). Polyunsaturated fatty acid metabolism in cultured fish cells: Incorporation and metabolism of (n-3) and (n-6) series acids by Atlantic salmon (Salmo salar) cells. *Fish Physiology and Biochemistry* 8(4), 311-319.

Tocher, D.R. & Sargent, J.R. (1990). Incorporation into phospholipid classes and metabolism via desaturation and elongation of various14C-labelled (n-3) and (n-6) polyunsaturated fatty acids in trout astrocytes in primary culture. *Journal of Neurochemistry* 54(6), 2118-2124.

Todorčević, M., Vegusdal, A., Gjøen, T., Sundvold, H., Torstensen, B.E., Kjær, M.A. & Ruyter, B. (2008). Changes in fatty acids metabolism during differentiation of Atlantic salmon preadipocytes; Effects of n-3 and n-9 fatty acids. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* 1781(6-7), 326-335.

Torra, I.P., Ismaili, N., Feig, J.E., Xu, C.F., Cavasotto, C., Pancratov, R., Rogatsky, I., Neubert, T.A., Fisher, E.A. & Garabedian, M.J. (2008). Phosphorylation of liver X receptor α selectively regulates target gene expression in macrophages. *Molecular and Cellular Biology* 28(8), 2626-2636.

Torstensen, B.E., Bell, J.G., Rosenlund, G., Henderson, R.J., Graff, I.E., Tocher, D.R., Lie, O. & Sargent, J.R. (2005a). Tailoring of Atlantic salmon (Salmo salar L.) flesh lipid composition and sensory quality by replacing fish oil with a vegetable oil blend. *Journal of Agricultural and Food Chemistry* 53(26), 10166-10178.

Torstensen, B.E., Bell, J.G., Rosenlund, G., Henderson, R.J., Graff, I.E., Tocher, D.R., Lie, Ø. & Sargent, J.R. (2005b). Tailoring of Atlantic salmon (Salmo salar L.) flesh lipid composition and sensory quality by replacing fish oil with a vegetable oil blend. *Journal of Agricultural and Food Chemistry* 53(26), 10166-10178.

Torstensen, B.E., Frøyland, L. & Lie, Ø. (2004). Replacing dietary fish oil with increasing levels of rapeseed oil and olive oil - Effects on Atlantic salmon (*Salmo salar* L.) tissue and lipoprotein lipid composition and lipogenic enzyme activities. *Aquaculture Nutrition* 10(3), 175-192.

Torstensen, B.E., Lie, Ø. & Hamre, K. (2001). A factorial experimental design for investigation of effects of dietary lipid content and pro- and antioxidants on lipid composition in Atlantic salmon (Salmo salar) tissues and lipoproteins. *Aquaculture Nutrition* 7(4), 265-276.

Torstensen, B.E., Nanton, D.A., Olsvik, P.A., Sundvold, H. & Stubhaug, I. (2009). Gene expression of fatty acid-binding proteins, fatty acid transport

proteins (cd36 and FATP) and β-oxidation-related genes in Atlantic salmon (Salmo salar L.) fed fish oil or vegetable oil. *Aquaculture Nutrition* 15(4), 440-451.

Torstensen, B.E., Øyvind, L. & Frøyland, L. (2000). Lipid metabolism and tissue composition in Atlantic salmon (Salmo salar L.) - Effects of capelin oil, palm oil, and oleic acid-enriched sunflower oil as dietary lipid sources. *Lipids* 35(6), 653-664.

Trattner, S. (2009). Quality of lipids in fish fed vegetable oils. *Diss. (sammanfattning/summary) Uppsala : Sveriges lantbruksuniv., Acta Universitatis agriculturae Sueciae, 1652-6880 ; 2009:31*

Trattner, S., Kamal-Eldin, A., Brännäs, E., Moazzami, A.A., Žlábek, V., Larsson, P., Ruyter, B., Gjøen, T. & Pickova, J. (2008a). Sesamin Supplementation Increases White Muscle Docosahexaenoic Acid (DHA) Levels in Rainbow Trout (Oncorhynchus mykiss) Fed High Alpha-Linolenic Acid (ALA) Containing Vegetable Oil: Metabolic Actions. *Lipids* 43(11), 989-997.

Trattner, S., Pickova, J., Park, K.H., Rinchard, J. & Dabrowski, K. (2007). Effects of alpha-lipoic and ascorbic acid on the muscle and brain fatty acids and antioxidant profile of the South American pacu *Piaractus mesopotamicus*. *Aquaculture* 273(1), 158-164.

Trattner, S., Ruyter, B., Ostbye, T.K., Gjøen, T., Zlabek, V., Kamal-Eldin, A. & Pickova, J. (2008b). Sesamin Increases Alpha-Linolenic Acid Conversion to Docosahexaenoic Acid in Atlantic Salmon (Salmo salar L.) Hepatocytes: Role of Altered Gene Expression. *Lipids* 43(11), 999-1008.

Trattner, S., Ruyter, B., Østbye, T.K., Kamal-Eldin, A., Moazzami, A., Pan, J., Gjøen, T., Brännäs, E., Zlabek, V. & Pickova, J. (2011). Influence of dietary sesamin, a bioactive compound on fatty acids and expression of some lipid regulating genes in Baltic Atlantic salmon (Salmo salar L.) juveniles. *Physiological Research* 60(1), 125-137.

Treiber, T., Treiber, N. & Meister, G. (2012). Regulation of microRNA biogenesis and function. *Thrombosis and Haemostasis* 107(4), 605-610.

Tu, W.C., Mühlhäusler, B.S., James, M.J., Stone, D.A.J. & Gibson, R.A. (2012). An alternative n-3 fatty acid elongation pathway utilising 18:3n-3 in barramundi (Lates calcarifer). *Biochemical and Biophysical Research Communications* 423(1), 176-182.

Turchini, G.M. & Francis, D.S. (2009). Fatty acid metabolism (desaturation, elongation and β-oxidation) in rainbow trout fed fish oil- or linseed oil-based diets. *British Journal of Nutrition* 102(1), 69-81.

Turchini, G.M., Francis, D.S. & De Silva, S.S. (2007a). Finishing diets stimulate compensatory growth: Results of a study on Murray cod, Maccullochella peelii peelii. *Aquaculture Nutrition* 13(5), 351-360.

Turchini, G.M., Francis, D.S. & De Silva, S.S. (2007b). A whole body, in vivo, fatty acid balance method to quantify PUFA metabolism (desaturation, elongation and beta-oxidation). *Lipids* 42(11), 1065-71.

Turchini, G.M., Hermon, K., Cleveland, B.J., Emery, J.A., Rankin, T. & Francis, D.S. (2013a). Seven fish oil substitutes over a rainbow trout grow-out

cycle: I) Effects on performance and fatty acid metabolism. *Aquaculture Nutrition* 19, 82-94.

Turchini, G.M., Hermon, K., Moretti, V.M., Caprino, F., Busetto, M.L., Bellagamba, F., Rankin, T. & Francis, D.S. (2013b). Seven fish oil substitutes over a rainbow trout grow-out cycle: II) Effects on final eating quality and a tentative estimation of feed-related production costs. *Aquaculture Nutrition* 19, 95-109.

Wada, H., Shintani, D. & Ohlrogge, J. (1997). Why do mitochondria synthesize fatty acids? Evidence for involvement in lipoic acid production. *Proceedings of the National Academy of Sciences of the United States of America* 94(4), 1591-1596.

Wang, T., Li, M., Guan, J., Li, P., Wang, H., Guo, Y., Shuai, S. & Li, X. (2011). MicroRNAs miR-27a and miR-143 regulate porcine adipocyte lipid metabolism. *International Journal of Molecular Sciences* 12(11), 7950-7959.

Waples, R.S. (1988). Estimation of allele frequencies at isoloci. *Genetics* 118(2), 371-384.

Varanasi, U., Chu, R., Huang, Q., Castellon, R., Yeldandi, A.V. & Reddy, J.K. (1996). Identification of a peroxisome proliferator-responsive element upstream of the human peroxisomal fatty acyl coenzyme A oxidase gene. *Journal of Biological Chemistry* 271(4), 2147-2155.

Varnholt, H. (2008). The role of microRNAs in primary liver cancer. *Annals of Hepatology* 7(2), 104-113.

Wasson, J., Skolnick, G., Love-Gregory, L. & Permutt, M.A. (2002). Assessing allele frequencies of single nucleotide polymorphisms in DNA pools by Pyrosequencing™ technology. *BioTechniques* 32(5), 1144-1152.

Vegusdal, A., Sundvold, H., Gjøen, T. & Ruyter, B. (2003). An in vitro method for studying the proliferation and differentiation of Atlantic salmon preadipocytes. *Lipids* 38(3), 289-296.

Wienholds, E., Koudijs, M.J., Van Eeden, F.J.M., Cuppen, E. & Plasterk, R.H.A. (2003). The microRNA-producing enzyme Dicer1 is essential for zebrafish development. *Nature Genetics* 35(3), 217-218.

Wightman, B., Ha, I. & Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell* 75(5), 855-862.

Voss, A., Reinhart, M., Sankarappa, S. & Sprecher, H. (1991). The metabolism of 7,10,13,16,19-docosapentaenoic acid to 4,7,10,13,16,19-docosahexaenoic acid in rat liver is independent of a 4-desaturase. *Journal of Biological Chemistry* 266(30), 19995-20000.

Xiao, F., Zuo, Z., Cai, G., Kang, S., Gao, X. & Li, T. (2009). miRecords: An integrated resource for microRNA-target interactions. *Nucleic Acids Research* 37(SUPPL. 1), D105-D110.

Xu, H., He, J.H., Xiao, Z.D., Zhang, Q.Q., Chen, Y.Q., Zhou, H. & Qu, L.H. (2010). Liver-enriched transcription factors regulate microRNA-122 that targets CUTL1 during liver development. *Hepatology (Baltimore, Md.)* 52(4), 1431-1442.

Yasuda, K., Ikushiro, S., Wakayama, S., Itoh, T., Yamamoto, K., Kamakura, M., Munetsuna, E., Ohta, M. & Sakaki, T. (2012). Comparison of metabolism of sesamin and episesamin by drug-metabolizing enzymes in human liver. *Drug Metabolism and Disposition* 40(10), 1917-1926.

Ye, W., Lv, Q., Wong, C.-K.A., Hu, S., Fu, C., Hua, Z., Cai, G., Li, G., Yang, B.B. & Zhang, Y. (2008). The Effect of Central Loops in miRNA:MRE Duplexes on the Efficiency of miRNA-Mediated Gene Regulation. *PLoS ONE* 3(3), e1719.

Yi, X. & Maeda, N. (2006). α-Lipoic acid prevents the increase in atherosclerosis induced by diabetes in apolipoprotein E-deficient mice fed high-fat/low-cholesterol diet. *Diabetes* 55(8), 2238-2244.

Young, W.P., Wheeler, P.A., Coryell, V.H., Keim, P. & Thorgaard, G.H. (1998). A detailed linkage map of rainbow trout produced using doubled haploids. *Genetics* 148(2), 839-850.

Yuan, J.P., Wang, J.H. & Liu, X. (2007). Metabolism of dietary soy isoflavones to equol by human intestinal microflora - Implications for health. *Molecular Nutrition and Food Research* 51(7), 765-781.

Zeron, E.S. & Santillán, M. (2010). Distributions for negative-feedback-regulated stochastic gene expression: Dimension reduction and numerical solution of the chemical master equation. *Journal of Theoretical Biology* 264(2), 377-385.

Zheng, X., Seiliez, I., Hastings, N., Tocher, D.R., Panserat, S., Dickson, C.A., Bergot, P. & Teale, A.J. (2004). Characterization and comparison of fatty acyl Δ6 desaturase cDNAs from freshwater and marine teleost fish species. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* 139(2), 269-279.

Zheng, X., Tocher, D.R., Dickson, C.A., Bell, J.G. & Teale, A.J. (2005a). Highly unsaturated fatty acid synthesis in vertebrates: New insights with the cloning and characterization of a Δ6 desaturase of atlantic salmon. *Lipids* 40(1), 13-24.

Zheng, X., Torstensen, B.E., Tocher, D.R., Dick, J.R., Henderson, R.J. & Bell, J.G. (2005b). Environmental and dietary influences on highly unsaturated fatty acid biosynthesis and expression of fatty acyl desaturase and elongase genes in liver of Atlantic salmon (*Salmo salar*). *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids* 1734(1), 13-24.

Zheng, X.Z., Leaver, M.J. & Tocher, D.R. (2009). Long-chain polyunsaturated fatty acid synthesis in fish: Comparative analysis of Atlantic salmon (Salmo salar L.) and Atlantic cod (Gadus morhua L.) Delta 6 fatty acyl desaturase gene promoters. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* 154(3), 255-263.

Zhong, D., Huang, G., Zhang, Y., Zeng, Y., Xu, Z., Zhao, Y., He, X. & He, F. (2013). MicroRNA-1 and microRNA-206 suppress LXRα-induced lipogenesis in hepatocytes. *Cellular Signalling* 25(6), 1429-1437.

Zhou, J., Febbraio, M., Wada, T., Zhai, Y., Kuruba, R., He, J., Lee, J.H., Khadem, S., Ren, S., Li, S., Silverstein, R.L. & Xie, W. (2008). Hepatic Fatty Acid Transporter Cd36 Is a Common Target of LXR, PXR, and PPAR[gamma] in Promoting Steatosis. *Gastroenterology* 134(2), 556-567.e1.

Zhou, J., Zhai, Y., Mu, Y., Gong, H., Uppal, H., Toma, D., Ren, S., Evans, R.M. & Xie, W. (2006). A novel pregnane X receptor-mediated and sterol regulatory element-binding protein-independent lipogenic pathway. *Journal of Biological Chemistry* 281(21), 15013-15020.