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Abstract

Fish is without doubt the most important source of valuable omega-3 (n-3) fatty acids (FA), especially the long chain polyunsaturated fatty acids (LCPUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Aquaculture production of Atlantic salmon (*Salmo salar*) is one of the largest fish industries in the world and a dominant source of fish in Western diet. Fish meal and fish oil (FO) are important dietary components in fish feed and vital sources of nutrients and fatty acids in salmonid aquaculture. However, due to overfishing and high pressure on the aquatic ecosystem feeding the growing aquaculture industry have become a sustainability issue. To solve the issue, FO has increasingly been replaced with vegetable oils (VO). This has documented effects on the lipid profile of fish flesh e.g. by lowering the amount of the valuable omega-3 LCPUFAs.

Nutrigenomic approaches have been used to try to increase the levels of LCPUFAs in Atlantic salmon as well as other salmonids fed VO diets by the addition of bioactive compounds e.g. sesamin a bioactive compound extracted from sesame seeds. Sesamin has been shown to affect expression of genes involved in the lipid metabolism, but little is known about the underlying molecular regulation mechanism.

MicroRNAs (miRNAs) are small non-coding RNAs involved in post-transcriptional regulation and communication within as well as between cells. Increased understanding of how miRNAs effect the regulation of lipid metabolism, specifically the desaturation and elongation cascade of omega-3 (n-3) and omega-6 (n-6) LCPUFAs, can contribute in the search for a more sustainable aquaculture. Understanding the role of miRNA and other small non-coding RNAs as well as other post-transcriptional regulation mechanisms might help in finding a sustainable feeding method without losing the nutritional value of the fish filé.

The aim of this thesis is to investigate if genes involved in the lipid metabolism are under regulatory control of miRNAs in Atlantic salmon. Expression of 19 different miRNAs were compared in Atlantic salmon fed different feeding profiles of VO that differ in n-6 to n-3 FA ratio (low or high) and sesamin content (high, low or no sesamin).

The result indicates that miRNA most likely are a part of the feedback regulation mechanisms of lipid metabolism and that at least seven of the 18 miRNAt tested might regulate genes involved in the lipid homeostasis. Many mRNA showed a significant difference in expression levels in fish fed VO compared to the FO diet. Further, supplementation of sesamin to VO diet had a significant effect on the expression of several miRNAs. In many cases the addition of sesamin to the VO diet restored the miRNA expression to levels similar to that of fish fed the FO diet.

Sammanfattning

Fisk är en av de viktigaste källorna till värdefulla omega-3-fettsyror, speciellt långa fleromättade fettsyror (LCPUFA) så som eicosapentaenoic acid (EPA) och docosahexaenoic acid (DHA). Akvakultur med lax (*Salmo salar*) är en av de största fiskindustrierna i Världen och producerar en allt mer dominerande del av den fisk som konsumeras i västvärlden idag. Fiskfoder består till stor del av fiskmjöl och fiskolja (FO) som tillverkas av småfisk och fiskrens från fiskindustrin. Fiskmjöl och FO bidrar till en betydande del av fodrets näringsvärde. Utfiskning och förstörelse av havens ekosystem har skapat en ohållbar trend som medför att man på sikt inte kommer kunna producera tillräckligt med FO för att täcka behovet i den växande akvakulturindustrin. Därför har FO i fiskfodret successivt ersatt med vegetabiliska oljor (VO), som är ett billigare, mer hållbart samt ett mer miljövänligt alternativ. Förändringarna i fodersammansättningen där man går mot ett stigande innehåll av VO har en dokumenterad påverkan på lipidsammansättningen i fisken genom att sänka halten av de nyttiga omega-3 (n-3) fettsyrorna (FA) i synnerhet n-3 LCPUFA.

Inom nutrigenomikforskningen har man försökt öka fiskens egen produktion av de nyttiga n-3 LCPUFAs i lax genom att tillsätta olika bioaktiva ämnen, såsom sesamin till det VO baserade fodret. Sesamin är ett bioaktivt ämne utvunnet ur sesamfrön. Tillsatsen av sesamin har visats påverka uttrycket av flera gener involverade i lipidmetabolismen, men lite är ännu känt om vad som orsakar denna förändring på molekylär nivå. MikroRNA (miRNA) är små icke-kodande RNA som bidrar till reglering av cellernas proteinnivåer genom att binda till mRNA vilket i sin tur förhindrar translationen till protein. Kunskap om hur miRNA kan påverka lipidmetabolismen, kan vara en viktig del i förståelsen av hur man kan motverka hur en stigande inblandning av VO i fiskfodret förändrar fiskens lipidsammansättning. Detta skulle i sin tur kunna bidra till en hållbar fiskproduktion utan att göra avkall på fiskens höga näringsvärde.

Syftet med det här arbetet är att undersöka huruvida miRNA är en del av regleringen av gener involverade i lipidmetabolismen i lax. Uttrycket av 19 olika miRNA har undersökts i lever hos lax som fått antingen FO baserat foder eller olika typer av VO foder med varierande omega-6/omega3 (n-6/n-3) innehåll (hög eller låg) samt olika mängd sesamin (hög, låg eller utan sesamin). Resultaten tyder på att miRNA är en bidragande faktor i feedback regleringen av lipidmetabolismen. Av de 18 miRNA testade utgör 7 möjliga kandidater, vilka bör utvärderas vidare. Tillsatt sesamin hade en signifikant påverkan på uttrycket av flera miRNA. I flera fall där miRNA uttrycket signifikant skilt sig mellan en VO diet och FO diet har tillsatsen

av sesamin i en VO diet återställde uttrycket till nivåer liknande de uppmätta i fisk matad FO dieten.

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Abbreviations

cDNA	Complementary DNA
Δ5FAD	Delta 5 fatty acid desaturase
Δ6FAD	Delta 6 fatty acid desaturase
ACC	Acetyl-CoA carboxylase
DHA	Docosahexaenoic acid (22:6 n-3)
DPA	Docosapentaenoic acid (22:5n-3)
ELOVL	Elongase of very long chain fatty acids (four different transcripts)
EPA	Eicosapentaenoic acid (20:5n-3)
FA	Fatty acid
FO	Fish oil
LA	Linoleic acid (18:2n-6)
LCPUFA	Long chain polyunsaturated fatty acids
LDL	Low-density lipoprotein
miRNA	MicroRNA
n-3	Omega-3
n-6	Omega-6
n-6/n-3	n-6/n-3 PUFA
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
PUFA	Polyunsaturated fatty acid
SAFA	Saturated fatty acid
SNP	Single nucleotide polymorphism
SD	Standard deviation
TAG	Triacylglycerol
VO	Vegetable oil

1 Introduction

With the scientific development and the technological progress during the last two decades, a new and promising direction of nutritional science has developed that aims to describe how fundamental molecular processes are affected by diet. This direction has been named nutrigenomics and includes the use of biochemistry, physiology, nutrition, genomics, proteomics, metabolomics, transcriptomics and epigenomics to investigate how nutrients and bioactive dietary components affect gene expression and thereby the individual phenotype. By increasing knowledge of the underlying molecular processes of how diet induced gene regulations, scientists hope to clarify the relation between diet- and human health on one hand and diet and disease progression on the other (Ferguson *et al.*, 2016; Sales *et al.*, 2014; Ronteltap *et al.*, 2009).

Today, healthy diet recommendations are based on an approach where a few guidelines are assumed to fit the entire population. This population-based approach has its limitations since each person reacts differently to a particular food depending on gender, age, lifestyle, socioeconomic characteristics and genetic heritage (Allès *et al.*, 2016; Ferguson *et al.*, 2016; El Hajj *et al.*, 2014; Ronteltap *et al.*, 2009).

Nutrients in food are components that have been identified as necessary for life but along with the nutrients, food also consists of a wide variety of bioactive compounds (non-nutrients). How these nutrients and bioactive compound effect metabolic processes and gene regulation pathways has been intensively studied including long term effects of under- as well as overfeeding (Sales *et al.*, 2014; Tudose & Patras, 2013; Ronteltap *et al.*, 2009).

Several examples of nutrigenomic effects have been linked to historical cases of famine including the Dutch “hunger winter”, the Finnish famine, the Chinese Great famine and the siege of Leningrad. Exposure to famine during pregnancy

and early life development has been shown to affect health later in life by increased prevalence of cardiovascular diseases, diabetes, obesity, breast cancer and increased mortality (Ekamper *et al.*, 2015; Rotar *et al.*, 2015; El Hajj *et al.*, 2014; Veenendaal *et al.*, 2013; Painter *et al.*, 2008). Studies on individuals conceived during the Dutch "hunger winter" have been shown not only to exhibit these kinds of effects later in life but also pass parts of these effects forward to their children as well as to their grandchildren. These findings suggest that epigenetic changes in a parent caused by famine, can be passed down through generations (Veenendaal *et al.*, 2013; Painter *et al.*, 2008).

Today, obesity is increasing worldwide and overnutrition has been shown to have similar long-lasting effects as undernutrition. Epigenetic changes due to maternal obesity are introduced to the offspring at a sensitive window in the early development and may program long-lasting epigenetic alterations resulting in metabolic diseases later in life and thereby contributing to the worldwide metabolic disease epidemics (El Hajj *et al.*, 2014).

The supply of food is today very different from the food available to humans during the early stages of our evolution. These changes started first slowly with the beginning of the agriculture revolution, approximately 10 000 years ago, followed by a rapid shift during the industrial revolution. One of the big nutritional differences can be found in the food composition of dietary fatty acids (FA) and antioxidants. During human evolution, fat consumption was lower and consisted of higher level of polyunsaturated fatty acid (PUFA) with an equal intake of omega-3 (n-3) and omega-6 (n-6) FAs mainly originating from animal sources. Today the fat intake is higher with more saturated fatty acids and higher n-6/n-3 ratio as well as lower concentration of antioxidants. This change is mainly due to the increased use of grain and oil plants, both directly in human food but also in animal feed. Grain and oil crop are rich in n-6 FA whereas grasses that previously dominated animal feed, is high in n-3 FA. Today's diet is therefore lacking in n-3 PUFA in comparison to the diet humans have evolved from (Simopoulos, 1999).

A large body of evidence link a high dietary intake of n-3 PUFA, especially the long-chain polyunsaturated fatty acids (LCPUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), with improved cardiometabolic outcomes (Calder, 2013; Hull, 2011). Recent evidence show that the positive effect of n-3 PUFA might be mediated through several epigenetic mechanisms such as DNA methylation and microRNA (miRNA) interference (Lind *et al.*, 2015; Aslibekyan *et al.*, 2014; Gil-Zamorano *et al.*, 2014; Hoile *et al.*, 2014).

The n-6/n-3 ratio is increasing in our Western diet, as result of a more dominating terrestrial food origin, and a diminishing portion of aquatic species that are the biggest dietary source of n-3 LCPUFAs. Further today a large portion of fish for human consumption comes from aquaculture production. Atlantic salmon (*Salmo salar*) is one of the most commonly produced fish globally and salmonids are among the most frequently served fish in Western diet. Fish oil and fish meal have long been important pats in the feed of Atlantic salmon, but with an expanding aquaculture industry, the source of fish oil and fish meal has become limited and thereby created a sustainability issue. The most common way to handle this issue of sustainability is to partly replace FO with terrestrial VO, which effect the fatty acid composition by lowering the amount of n-3 LCPUFAs in fish flesh. By doing so we decrease or even risk eliminating the best source of n-3 LCPUFA for human consumption (Sprague *et al.*, 2016). A deeper knowledge of how dietary FAs effect lipid metabolism regulation would be of big use in the development of sustainable aquaculture and would open the way to increase the portion of VO in the feed to salmonids without lowering the amount of nutritionally valuable n-3 LCPUFAs in the fish flesh.

The main question of this thesis is if altered expression of genes involved in the homeostasis of LCPUFAs in Atlantic salmon is partly caused by changes in the composition of non-coding RNA transcripts induced by unbalanced nutrient uptake. To investigate this, expression of several miRNAs was measured in the white muscle and liver of Atlantic salmon given fish feed where FO fully or partly has been replaced by different compositions of VO with or without sesamin supplementation.

2 Literature review

2.1 Nutrigenomics

Food is essential for life and long-term health. Nutrients are the compounds in food that have been identified as necessary for life and they provide energy and building materials for the body. Food also contains a wide range of non-nutrient compounds (e.g. bioactive plant compounds, foodborne chemicals) that also can have both a positive and negative effect on health.

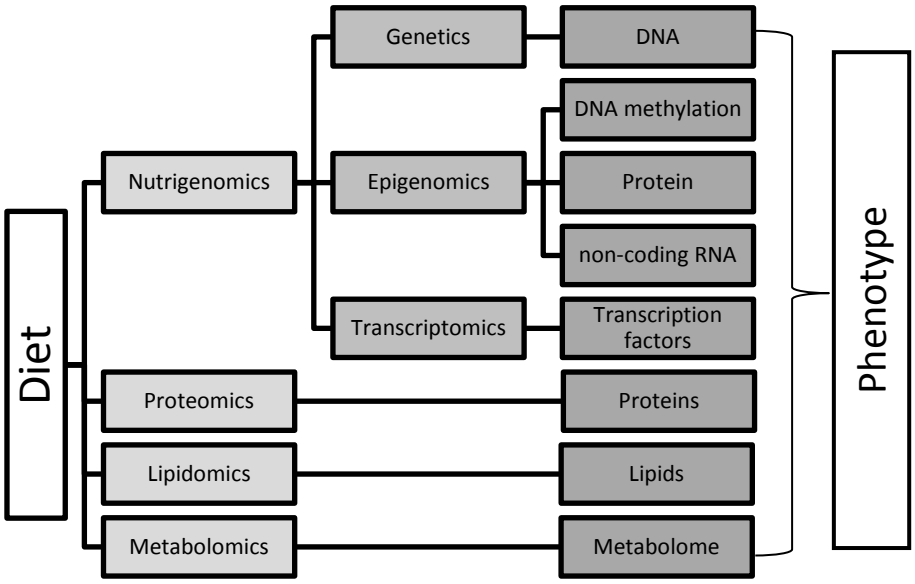
Genotypic variations are known to affect the enzyme activities in metabolic pathways, which in turn influences how dietary compounds are metabolized. This variation will influence these dietary compounds ability to keep a person health or their potency in causing diseases (Ferguson *et al.*, 2016; Sales *et al.*, 2014). The fact that nutrition and diet affect our overall health have increased the public interest in the beneficial effect that the diet can provide on our health. To be able to explore the relationship between diet and pathophysiological response it is necessary to understand the underlying physiological, biochemical and metabolic pathways (Ferguson *et al.*, 2016; Banerjee *et al.*, 2015).

Nutrigenomics studies how nutrients affect the genome, proteome and metabolome and uses molecular tools within these fields to gain a deeper understanding of how the diet can alter the expression of genes (Ferguson *et al.*, 2016; Ouml *et al.*, 2016; Sales *et al.*, 2014; Tudose & Patras, 2013). Since the diet consists of a multitude of nutrients and non-nutrient compounds, and each is involved in one or several biological processes, one need to take a multi-parametric approach in the diet related research field. However nutrigenomics is also a useful tool to focus on single specific biological events of effects elicited by a specific nutrient in a complex data-rich landscape, while reducing background noise (Tudose & Patras, 2013). Nutrigenomics is used in many different food production sectors e.g. pork,

poultry and fish industries. Nutrigenomics approaches are used to optimize feed efficiency by using different combinations of feed (Banerjee *et al.*, 2015).

2.1.1 The importance of combined "omics" science to reveal interactions between nutrition phenotype

To be able to understand how nutrients effect the body research including the genome, epigenome, transcriptome, proteome, lipidome, metabolome and microbiome needs to be included since they all effects the phenotype. This has given rise to several "omic" science fields including nutrigenomics (nutrigenetics, epigenimics and transcriptomics) proteomics, lipidomics, metabolomics (figure 1) (Ferguson *et al.*, 2016; Ouml *et al.*, 2016; Sales *et al.*, 2014; Tudose & Patras, 2013).



The different "omics" provides a wide range of techniques useful in the struggle to investigate the relationship between

Figure 1: How "Omics" science are used to understand the relationship between diet and Phenotype.

genetic variation, diet and several diseases e.g cardiovascular disease (Ferguson *et al.*, 2016; Banerjee *et al.*, 2015), cancer (Banerjee *et al.*, 2015; Sales *et al.*, 2014; Sharma *et al.*, 2010; Mulero-Navarro & Esteller, 2008), obesity (Banerjee *et al.*, 2015; Sales *et al.*, 2014; Milagro *et al.*, 2013) and diabetes (Sales *et al.*, 2014).

The importance of combining these different fields to strengthen the research of diet/health interaction have recently been highlighted (Ferguson *et al.*, 2016; Ouml *et al.*, 2016). One example are Ferguson *et al.* (2016) who reviewed the study of nutrition and cardiometabolic diseases from its beginning in the 1950s, progress made, and how the field of research has changed. They highly encourage combined research from the different "omics" and highlights that all fields provide a part of the solution of the understanding of the interaction between nutrition and cardiometabolic disease.

This thesis will focus on miRNA which are a part of the epigemone. The focus from now on will therefore focus on epigenomics and especially miRNAs.

2.1.2 Epigenomics and epigenetics

One of the factors involved in cell differentiation besides genes, are the epigenomic parameters. The epigenome refers to chemical compounds that are located on the genome (epigenetic markers), and regulates the expression of the genes and thereby marking what a cell can do and when to do it (Banerjee *et al.*, 2015; Sales *et al.*, 2014). Epigenomics is the research of the complete epigenetic modifications of a cell or tissue at a specific time. Like the genome, the epigenome is inherited as the cell duplicates (Sales *et al.*, 2014).

Epigenetics can be described as heritable changes in the gene expression that cannot be explained solely by changes in the DNA sequences (Milagro *et al.*, 2013; Sharma *et al.*, 2010; Kim *et al.*, 2008). There are six main epigenetic mechanisms that are involved in regulating the gene expression:

- DNA methylation
- Histone modifications
- Other nuclear proteins critical for epigenetic regulations (chromatin remodeling complex, effector proteins, insulator proteins)
- Genomic imprinting
- non-coding RNAs (including microRNAs)
- non-covalent mechanisms (physical alterations in nucleosomal positioning via nucleosome remodeler or replacement of canonical histone proteins with specialized histone variants)

Among these epigenetic modifications changes in DNA methylation, covalent histone modification and miRNA patterns are most extensively studied (Sales *et al.*, 2014; Milagro *et al.*, 2013; Sharma *et al.*, 2010; Kim *et al.*, 2008).

2.1.3 Epigenetic biomarkers

Epigenetic biomarkers are alterations in the epigenome that have been linked to a specific phenotype. Most commonly used epigenetic markers have been identified among the DNA methylation, post-translation histone modification and miRNAs (Chan & Baylin, 2012; Guil & Esteller, 2009).

DNA methylation is located on the 5' position of cytosine bases that are part of CpG dinucleotides. These dinucleotides are concentrated in specific regions called CpG islands in the 5' ends of many genes but can also occur in other positions in the genome. According to computational estimations, approximately 60% of all genes have a CpG island located on its 5' end. Methylation of these CpG island results in regulation of the gene (Chan & Baylin, 2012; Guil & Esteller, 2009).

Post-translational histone modifications control the functional state of the chromatin and thereby regulate whether the genes located in the chromatin are activated or repressed. Chromatin is composed of DNA, histones and other proteins. The histone tail is subject to a number of post-translational modifications which lead to remodeling of the chromatin and thereby which parts of the genome that are available for transcription (Chan & Baylin, 2012; Guil & Esteller, 2009).

MicroRNAs (miRNAs) are specific types of small RNAs that regulate gene expression of an mRNA which matches the complimentary strand of the miRNA (Guil & Esteller, 2009). Gene regulation by miRNA is the main objective of this thesis and the function of miRNA will be further described in sections 2.6.

2.2 Nutrigenomics within aquaculture

The national food administration of Sweden have recommendations that fish should be part of our diet for health reasons, mainly because of the content of n-3 LCPUFAs (SLV, 2007). The supply, as well as demand, for food fish is steadily increasing. The largest contribution to the annual increase in food fish comes from aquaculture production while the wild captured fish remains stable. In 2007, farmed fish constituted approximately 35% of the total food fish produced worldwide while that number has increased to 42% in 2012. The world fish aquaculture is estimated to increase at an average annual rate of 6.2% in a period of 2000-2012. Norway is the biggest producer of farmed fish in Europe and the sixth largest in the world (SOFIA, 2014).

Fish meal and FO are important parts of the feed in aquaculture when carnivore species such as Atlantic salmon are produced. This creates a sustainability issue in aquaculture production since even though the farmed fish production is increasing, the production of fish meal and FO is decreasing. Fish meal and FO can be pro-

duced from both whole fish or fish by-products such as heads, tails and bones. The amount of fish meal and FO produced from whole fish is decreasing and instead being replaced by fish remains and waste products to a higher extent. Even with a more efficient use of waste products in the production of fish meal and FO, other replacements need to be found to be able to provide a more sustainable aquaculture production (SOFIA, 2014).

2.2.1 Effects of replacing fish oil with vegetable oil in fish feed

The lack of FO has been compensated with oil from terrestrial plants, with rapeseed oil as the most common replacement in Europe. This is mainly due to its low price and ready availability (Sprague *et al.*, 2016).). Even though replacement of FO to VO show no major effect on growth, it affects the FA composition in the fish especially the amount of n-3 LCPUFAs (Sprague *et al.*, 2016; Leaver *et al.*, 2008c; Tocher *et al.*, 2003a; Tocher *et al.*, 2003b). Terrestrial plants have a very different lipid profile compared to FO, being rich in oleic (18:1n-9), linoleic (18:2n-6), to a lesser extent α-linolenic (18:3n-3) and completely lack the n-3 LCPUFAs such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). FO has a higher proportion of saturated fatty acids, long chain monoenes (20:1 and 22:1) and n-3 PUFA especially EPA and DHA as well as being low in the n-6 class of PUFA (Sprague *et al.*, 2016; Tocher *et al.*, 2003b). This change in the FA composition of the fish feed is highly reflected in the composition of the fish fatty acid and therefore affects the health value of fish as a nutrient source (Sprague *et al.*, 2016; Leaver *et al.*, 2008c; Tocher *et al.*, 2003a; Tocher *et al.*, 2003b). The proportion of the n-6 class of PUFA has doubled from 2010 to 2015 in Atlantic salmon and the level of EPA and DHA has been reduced by half. Even though the level of EPA and DHA is decreasing due to the replacement of FO by VO, farmed salmon still contains higher levels of EPA and DHA in weigh/volume than wild catch salmon or terrestrial livestock (Sprague *et al.*, 2016).). Even though Atlantic salmon, along with other farmed fish with high fat content, still is a superior source of n-3 LCPUFA, more fish need to be produced and consumed to get the same nutrition value as fish produced with fish oil as the main lipid source.

2.2.2 Epigenetic effects of exchanging fish oil with vegetable oil in fish feed

Replacement of FO to vegetable oil in fish feed does not only change the FA-composition by changing the basis of exogenous lipids, but is also altering processes involved in the synthesis of endogenous lipids (Schiller Vestergren, 2014; Trattner, 2009; Moya-Falcón *et al.*, 2005; Tocher *et al.*, 2003a; Tocher *et al.*,

2003b). Synthesis of highly unsaturated fatty acids (HUFA) has been shown to increase in fish fed a VO-rich diet compared to a FO-diet (Tocher *et al.*, 2003b). Moreover, there is a negative correlation between dietary EPA and DHA and HUPF synthesis (Tocher *et al.*, 2003a). Even with the increased HUFA synthesis, fish fed a VO diet are not able to produce the same level of EPA and DHA as fish fed a FO diet (Moya-Falcón *et al.*, 2005; Tocher *et al.*, 2003a; Tocher *et al.*, 2003b). A VO diet has been shown to affect several genes involved in lipid metabolism (Table 1) (Pan *et al.*, 2013; Schiller Vestergren *et al.*, 2012b; Morais *et al.*, 2011). When comparing expression outcome from several feeding trials, where the effect of a VO diet on the expression of lipid-related genes, the response in mRNA expression appears to be sensitive even to small changes in dietary composition (Bonacic *et al.*, 2016; Pan *et al.*, 2013; Schiller Vestergren *et al.*, 2012a; Morais *et al.*, 2011; Schiller Vestergren *et al.*, 2011). This indicates that fatty acids are involved in a complex regulatory pathway affecting lipid metabolism in Atlantic salmon that cannot be explained only by changes in mRNA expression levels.

Bonacic *et al.* (2016) revealed significant correlation not only between lipid source (FO or VO) and expression of lipid-related genes, but also a correlation between lipid level (% of lipids included in the feed) and expression of lipid-related genes in Senegalese sole (*Solea senegalensis*). It has been shown that n-3 PUFA level in the flesh of Atlantic salmon is a highly heritable trait (Leaver *et al.*, 2011). Fish families with higher level of n-3 PUFA had a clear increase in the expression of genes related to lipid transport, while desaturases and elongases were unchanged (Leaver *et al.*, 2011). Also Morais *et al.* (2011) demonstrated that two fish families bred towards either “lean” or “fat” lipid content reacted differently on VO feed depending on genotype. Similar to Leaver *et al.* (2011), Morais *et al.* (2011) found that genotype had a low impact on lipid synthesis-related genes but they also concluded a high impact on signaling pathways.

Table 1. *Genes effected by replacement of FO to a VO based diet*

Gene		Effect of VO diet	Reference
Desaturation	Δ6 fad, Δ5 fad,	Up-regulation (higher in lean fish than fat fish)	(Morais *et al.*, 2011)
	Δ6 fad	Up-regulated (TAG, MO, VO-1). Down regulated (VO-0,5)	(Pan *et al.*, 2013; Schiller Vestergren *et al.*, 2012a)
	Δ5 fad	Up-regulated (VO-1)	(Schiller Vestergren *et al.*, 2012a)
E 1	ELOVL2	Up-regulation in lean fish	(Morais *et al.*, 2011)

	ELOVL2	Up-regulated (VO-1)	(Schiller Vestergren *et al.*, 2012a)
	ELOVL5b	Up-regulated (VO-0.5)	(Schiller Vestergren *et al.*, 2012a)
	ELOVL4	Down-regulated	(Schiller Vestergren *et al.*, 2012a)
Fatty acid synthesis	FAS	Up-regulation	(Morais *et al.*, 2011)
Transcription factors	SREBP-1	Up-regulation in fat fish Down regulated (VO-0.5)	(Schiller Vestergren *et al.*, 2012a; Morais *et al.*, 2011)
	SREBP-2, LXR	Down-regulated (VO-0.5)	(Schiller Vestergren *et al.*, 2012a)
	PPARα	Down-regulation in lean fish	(Morais *et al.*, 2011)
	PPARα	Up-regulation (MO)	(Pan *et al.*, 2013)
	PPARβ1A, PPARγ	Up-regulated (LO, TAG, MO)	(Pan *et al.*, 2013)
	PGC-1	Up-regulated (VO-1)	(Schiller Vestergren *et al.*, 2012a)
β-oxidation	CPT-1	Up-regulated (TAG MO, VO-1, VO-0.5)	(Pan *et al.*, 2013; Schiller Vestergren *et al.*, 2012a)
	ACO	Up-regulated	(Pan *et al.*, 2013)
Xenobiotic metabolism	CRP	Down-regulated	(Morais *et al.*, 2011)
Sterol biosyn-thesis	SQLE	Up-regulation (higher in lean fish than fat fish)	(Morais *et al.*, 2011)
	PPARβ, PPARγ, CPT1, ACO	Unaffected in lean and fat fish	(Morais *et al.*, 2011)
	PPARα	Unaffected (VO-1, VO-0.5)	(Schiller Vestergren

	PPARβ1A, PPARγ, ELOVL5a	*et al.*, 2012a)

Δ6 fad Δ6 desaturase, Δ 5 fad Δ5 desaturase, ELOVL elongase (three different transcripts), FAS fatty acid synthase, LXR liver X receptor α, SREBP Sterol regulatory element binding protein, PPAR peroxisomal proliferator-activator receptor, PGC-1 proliferator-activated receptorgamma coactivator 1 alfa, CPT-1 carnitin palmitoyl transferase 1, ACO acyl-CoA oxidase, CRP cytochrome P450 reductase, SQLE squalene epoxidase,

2.3 Lipid metabolism

Lipids are along with proteins the largest organic components in fish and they provide an essential source of metabolic energy for the fish. Fish are rich in a wide range of PUFA, among them n-3 LCPUFAs which are vital for humans. Fish is the most important food source for these nutrients and since an increasing part of consumed fish comes from aquaculture it is vital that farmed fish nutritional values are kept at the same levels as wild caught fish. To be able to ensure this it is important to understand the basic biochemistry and genetics of lipid metabolism in fish (Tocher, 2003).

Lipids in fish as well as and mammals consist of structural lipids that compose the cell membrane (mainly phospholipids and free cholesterol), and storage lipids (mainly triacylglycerols) in body fat or as adipose lipids. Lipids circulate in the serum (blood) of the organism mainly as lipoproteins. In this mobile phase, lipoprotein function is mainly as transporter of hydrophobic lipids between the sites of synthesis (liver or intestine) and the target tissue. Lipids in the body have two sources of origin: synthesized within the cell, called endogenous lipids or derived from dietary fat, called exogenous lipids (Griffin, 2013; Leaver *et al.*, 2008a; Tocher, 2003).

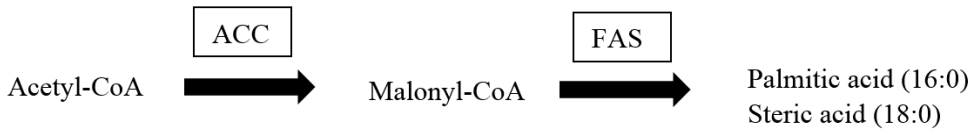
2.3.1 Synthesis of exogenous lipids

Digestion of lipids in fish as well as in mammals starts with hydrolysis of triacylglycerols (TAG), phospholipids and wax esters by the action of pancreas lipase into free FAs, acyl glycerols, 1-acyl-lysoglycerophospholipids, cholesterols and long chain alcohols. The products are then diffused into the intestinal mucosa and taken up into the enterocytes. The hydrolytic products are solubilized or emulsified in bile salt micelles before diffusion (Tocher, 2003). Once the FAs and other

products from lipid digestion have entered the enterocyte they are activated by acetyl-CoA and re-esterified into TAG and phospholipids via sequential enzymatic actions of CoA ligase, mono-glycerol acyltransferase, diacylglycerol acyltransferase and acyl-CoA:cholesterol acyltransferase (ACAT). The re-synthesized lipids are then transported from the cells in the gut to its functional place in the body through the lipoproteins in the serum(Griffin, 2013).

2.3.2 Lipogenesis

Lipogenesis is the biosynthesis of new endogenous lipids in the cell and is considered essentially identical within fish and humans (Leaver *et al.*, 2008a). Acetyl-CoA formed in the mitochondria is the primary carbon source in lipogenesis. This forms a "head", onto which multiple two-carbon acetyl units, depending on the length of the final fatty acid, are attached (Tocher, 2003). The two-carbon acetyl units are carboxylated by acetyl-CoA carboxylase (ACC) into malonyl-CoA followed by stepwise addition to the primary acetyl-CoA by the enzymatic activity from the cytosolic fatty acid synthetase (FAS) multienzyme complex (figure 2). The main products from the lipogenesis are saturated 16:0 palmitic acid and 18:0 steric acid (Leaver *et al.*, 2008a; Tocher, 2003).



Figur 2: Lipogenesis pathway

2.3.3 Unsaturated fatty acid biosynthesis

The 16:0 palmitic and 18:0 steric acids can de desaturated into 16:1n-7 palmitoleic acid and 18:1n-9 oleic acid by the activity of Δ9 fatty acid desaturase (Leaver *et al.*, 2008b; Tocher, 2003). Desaturation of FAs drastically lowers the melting point, which is of importance for fish to keep their movability in cold water (Tocher, 2003). All vertebrates, including fish, lack the Δ12- and Δ15-desaturase enzymes and are therefore not able to continue the desaturation of 18:1n to 18:2n-6 and 18:3n-3 which are the starting point for further elongation and desaturation of PUFA within the n-3 and n-6 families. The 18:2n-6 and 18:3n-3 FAs are therefore considered essential dietary FAs for vertebrates (Leaver *et al.*, 2008b; Tocher, 2003). Desaturation and elongation of the n-3 and n-6 lipid families compete for

the same enzymes where the n-3 family has a higher affinity than the n-6 family (Monroig *et al.*, 2010; Hastings *et al.*, 2005).

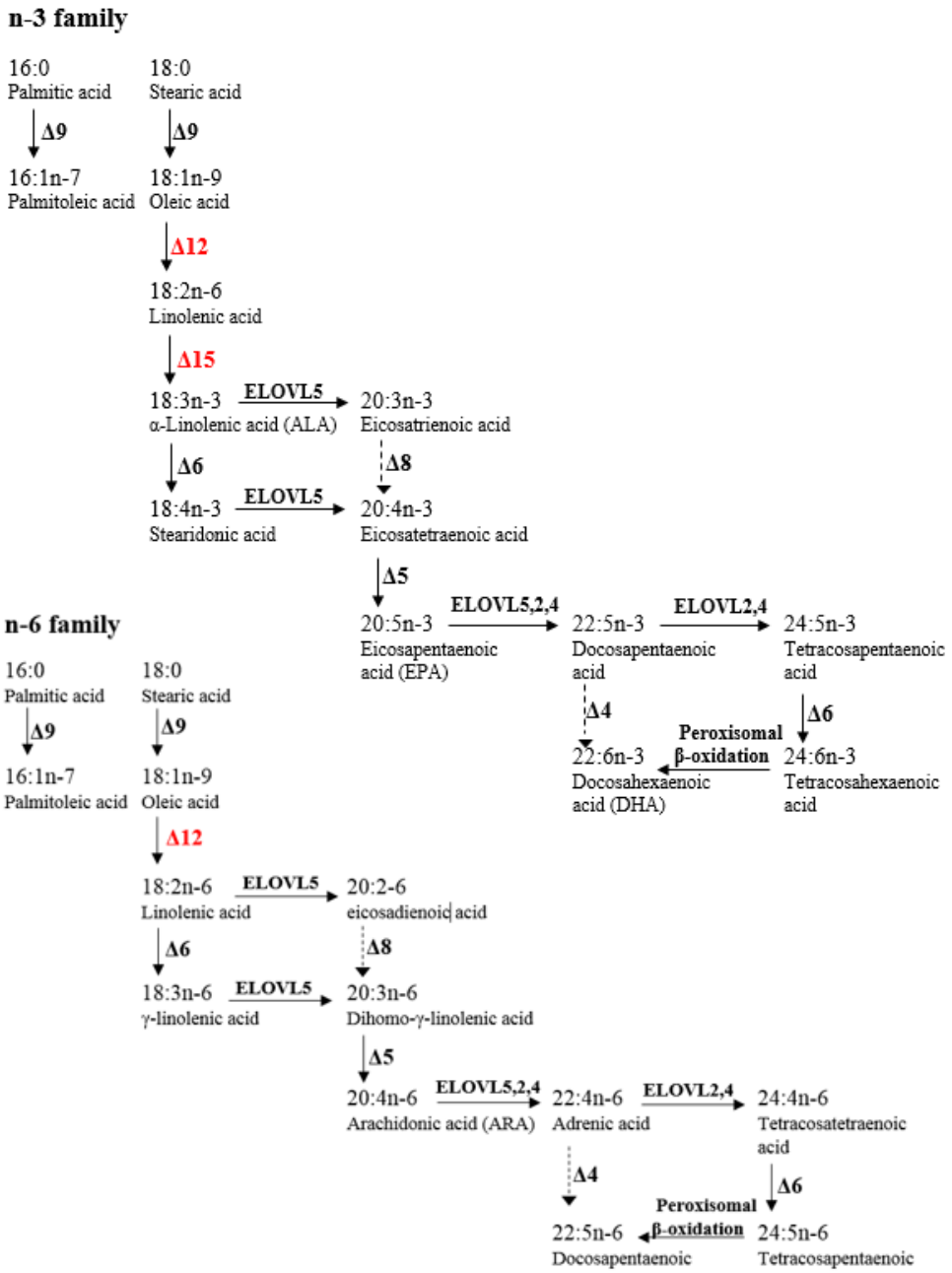


Figure 3: Elongation and desaturation of n-3, n-6 PUFA families.

2.3.4 Elongation and desaturation of fatty acids in Salmo salar

Desaturation and elongation of fatty acids in Atlantic salmon are dependent on a wide range of enzymes. Two desaturases (Δ5- and Δ6 fatty acid desaturase; FAD) and four elongases (ELOVL5a, ELOVL5b, ELOVL2 and ELOVL4) have been identified and characterized in Atlantic salmon (figure 3)(Carmona-Antoñanzas *et al.*, 2011; Monroig *et al.*, 2011a; Monroig *et al.*, 2011b; Monroig *et al.*, 2010; Morais *et al.*, 2009; Hastings *et al.*, 2005; Zheng *et al.*, 2005).

Desaturation of fatty acids

Atlantic salmon was the first fish shown to have two separate desaturases for Δ5 and Δ6 activity (Δ5FAD and Δ6FAD) (Hastings *et al.*, 2005; Zheng *et al.*, 2005). Three separate genes have been found to encode for three Δ6FADs (*Δ6fad_a*, *Δ6fad_b* and *Δ6fad_c*) and one for Δ5FAD (Δ5fad) (Monroig *et al.*, 2010). Both Δ6Fad_b, Δ6Fad_c and Δ5FAD have higher affinity to the n-3 family of the fatty acids than the n-6 family (Monroig *et al.*, 2010; Hastings *et al.*, 2005). Δ6Fad_b, Δ6Fad_c and Δ5Fad show limited Δ8 desaturation activity and Δ6Fad_b shows the highest conversion rate which allows an alternative pathway for the conversion of α-linolenic acid (18:3n-3, ALA) into eicosapentaenoic acid (20:5n-3) (figure 3)(Monroig *et al.*, 2011a).

Elongation of fatty acids

Elongase5a (Hastings *et al.*, 2005) and elongase5b have the highest activity towards C18 PUFA, decreasing with increasing fatty acid length ($C_{18\text{-}20} > C_{20\text{-}22} > C_{22\text{-}24}$). Elongase2 shows opposite activity preference ($C_{22\text{-}24} > C_{20\text{-}22} > C_{18\text{-}20}$). Elovl5b shows the same affinity for both the n-3 and the n-6 family of PUFA in contrast to ELOVL5a (Morais *et al.*, 2009).

Expression of transcription and elongation enzymes is affected by the lipid profile of the feed in Atlantic salmon which has been shown in farmed fish where fish oil has been replaced partly or fully by plant oil (Morais *et al.*, 2009; Zheng *et al.*, 2005).

EPA and DHA biosynthesis

DHA can either be exogenous from the feed or synthesized in the fish from EPA. Synthesis of DHA from EPA, called “Sprecher pathway”, that involves two elongation steps followed by Δ6 desaturation and peroxisomal β-oxidation was first shown in rats (Voss *et al.*, 1991). Atlantic salmon has since been shown to have all necessary enzymes for synthesis of DHA from EPA through the “Sprecher pathway” and therefore has a possible pathway of DHA synthesis (figure 3). An alter-

native to the “Sprecher pathway” has been shown in two different fish species: *Siganus canaliculatus* (herbivore, order Perciformes)(Li *et al.*, 2010) and *Solea senegalensis* (carnivorous, order Pleuronectiformes) (Morais *et al.*, 2012) involving an elongation step of EPA followed by Δ4 desaturation by Δ4FAD. *S. canaliculatus* was the first vertebrate shown to have activity of Δ4FAD. The Δ4FAD pathway requires only processes present in the endoplasmic reticulum, in contrast to the “Sprecher pathway” that requires additional enzymatic steps as well as additional transport to the peroxisome (figure 3). Δ4FAD has not yet been found in Atlantic salmon. But it has been found in distantly related species of both herbivore and carnivorous fish it is possibly a widespread pathway in all fish species (Morais *et al.*, 2012; Li *et al.*, 2010).

2.3.5 Fatty acid catabolism

The process of lipid metabolism is called β-oxidation and occurs in the cellular organelles mitochondria and peroxisomes. The importance of mitochondrial or peroxisomal β-oxidation is highly dependent on species, tissue and life stage (Leaver *et al.*, 2008a) . In Atlantic salmon peroxisomal β-oxidation compose a considerable part of the β-oxidation in liver and red muscle (100% and 70%, respectively) early in the development of the fish. These levels are significantly lowered after the seawater phase of the fish life which are decreased to 60% in the liver and 3% in the red muscle. In contrast, mitochondrial β-oxidation are the main β-oxidation within white muscle of Atlantic salmon both pre and post sweater transfer (Stubhaug *et al.*, 2007). The peroxisomal and mitochondrial β-oxidation are similar but the differ in some enzymatic activity (Poirier *et al.*, 2006). During β-oxidation in the mitochondria, acyl-CoA has to enter the inner compartment of the mitochondria. Acyl-CoAs are therefore converted into acyl-carnitine by carnitine palmitoyl transferase-1 (CPT-1) located on the outer membrane of the mitochondria. acyl-carnitine are then transported into the mitochondria matrix where it is converted back to acyl-CoA by carnitine palmitoyl transferase-2 (Leaver *et al.*, 2008a). Four enzymatic activities then compose the core of the β-oxidation cycle converting acyl-CoA esters into acyl-CoA and acetyl-CoA or propionyl-CoA which are described below (figure 4) (Poirier *et al.*, 2006).

1. Acyl-CoA is converted into 2E-enoyl-CoA by the function of acyl-CoA dehydrogenase in mitochondria and acyl-CoA oxidase in peroxisomes.
2. 2E-enoyl-CoA is converted into L-3-hydroxyacyl-CoA by the function of enoyl-CoA hydratase.

3. L-3-hydroxyacyl-CoA is converted into 3-ketoacyl-CoA by the function of 3-hydroxyacyl-CoA dehydrogenase
4. 3-ketoacyl-CoA is converted into Acyl-Coa and Acetyl-CoA or propionyl-CoA.

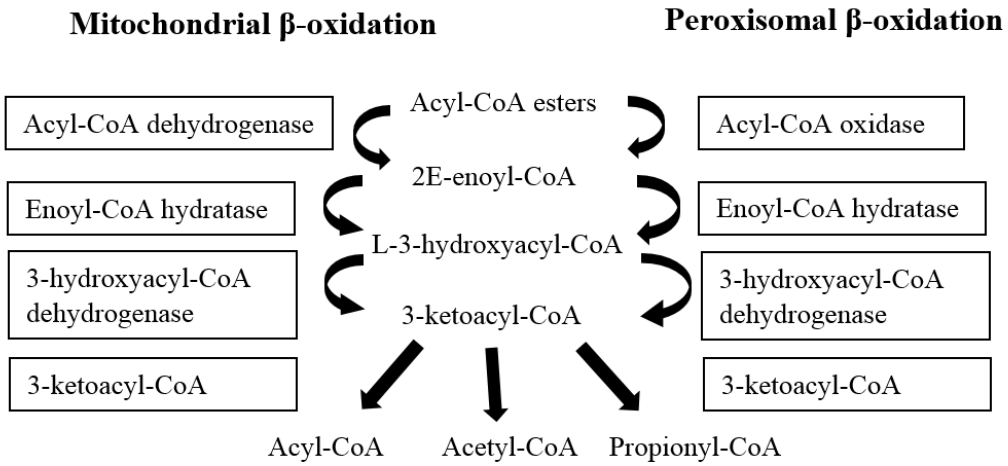


Figure 4: β-oxidation cycle within the mitochondria and the peroxisome. Further described in the text.

2.4 Feedback regulation

2.4.1 Possible feedback regulation pathways

Nutrigenomics feedback regulation can occur when a nutrient is either synthesized in the body or provided by the feed and thereby affecting the synthetization pathway of the specific nutrient in a positive or negative manner. There are several alternative pathways for feedback regulation (figure 5). The first and most commonly investigated is negative feedback regulation on a transcriptional level, i.e. inhibition of transcription of DNA into mRNA (step I in Figure 5) leading to a decrease in mRNA as a result. The second is post-transcriptional regulation which results in mRNA silencing (step II in figure 5). mRNA silencing leads to a decrease in expressed enzymes/proteins and might, but does not necessarily, also result in a decrease of mRNA. The third pathway is silencing of the expressed protein/enzyme, preventing the synthesis of the end product (step III in figure 5). The last pathway alternative (step IV in figure 5) is inhibition of genes that code

for transcription factors that affect the gene of interest resulting in a decrease of expressed mRNA.

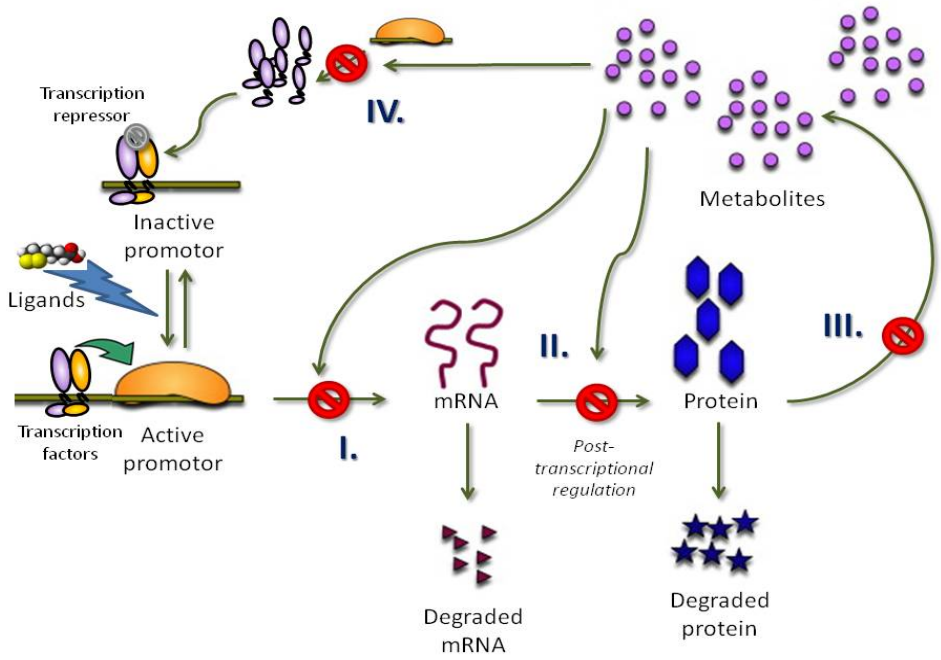


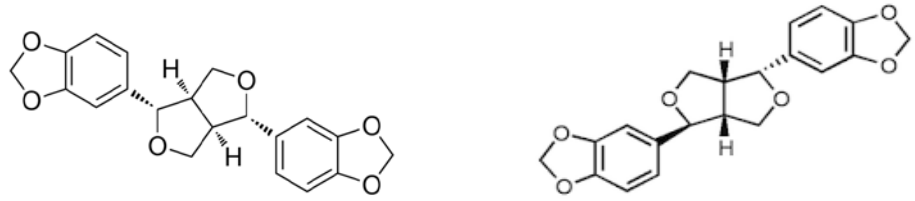
Figure 5: Schematic representation of four possible negative feedback regulation mechanisms

2.4.2 Feedback regulation on the synthesis of PUFA (EPA and DHA) in *Salmo salar*

Schiller Vestergren (2014) studied the effect of altering the lipid content, with or without supplemented bioactive compounds, in the feed of Atlantic salmon on the expression of genes involved in transcription, lipid uptake, desaturation, elongation and β-oxidation to detect possible metabolites involved in negative feedback regulation pathways. Schiller Vestergren (2014) displayed a sensitive mRNA relationship between mRNA expression and diet changes but could not generate equivalent variations in the FA composition and therefore concluded that mRNA variations (pathway I and IV in figure X) could not alone explain the interaction between diet and FA composition. Post-transcriptional regulation and/or inactivation of translated proteins/enzymes are therefore likely to be a part of PUFA synthesis.

2.5 Sesamin

Sesame oil contains oil soluble compounds known as ligands and one of the most thoroughly studied ones are sesamin. During the production of sesame oil approximately half for the sesamin are turned into its epimer episesamin (figure 6) (Jeng & Hou, 2005; Kushiro *et al.*, 2002).



Sesamin **Episesamin**

Figure 6: Chemical structure of sesamin and episesamin

2.5.1 Effect of sesamin on lipid metabolism

Sesamin and episesamin have been characterized to affect the lipid metabolism through many different pathways in rats. They have been shown to increase fatty acid β-oxidation by increased gene expression and activation of various enzymes in the mitochondrial and peroxisomal β-oxidation pathway as well as decrease the hepatic lipogenic activity by downregulation of enzymes of the lipogenesis (Jeng & Hou, 2005; Ide *et al.*, 2001; Ashakumary *et al.*, 1999; Fujiyama-Fujiwara *et al.*, 1995). The increase in β-oxidation enzymes induced by sesamin/episesamin are thought to be mainly due to activation of peroxisome proliferator activated receptor (PPAR) and the decrease in lipogenic enzyme is due to suppression of gene expression of sterol regulator element binding protein-1 (SREBP-1) (Ide *et al.*, 2001). Sesamin and episesamin have also been shown to alter the lipid profile both by affecting the desaturation and elongation through decreased Δ5desaturase activity, and lowering the n-6/n-3 ration (Jeng & Hou, 2005; Fujiyama-Fujiwara *et al.*, 1995).

Even though the effect of sesamin on lipid metabolism have been thoroughly characterized in rats it is not until recently that the effect of dietary sesamin have been studied in fish.

2.5.2 Effect of sesamin on lipid metabolism in Atlantic salmon

High inclusion of sesamin have been shown to affect several metabolites both in the liver as well as in white muscle of Atlantic salmon. Trattner *et al.* (2008a) showed that addition of sesamin in the feed increased the level of DHA in the white muscle of Rainbow trout. Trattner *et al.* (2008b) could also display a significant increase in elongation and desaturation of 18:3n-3 to DHA and an increased proportion of β-oxidation products. Along with these changes in FAs metabolism, sesamin has been shown to affect the expression of several lipid-related genes (down regulation of Δ6-, Δ5-desaturases, PPARα, PPARγ, cd36 and SRB-I, up-regulation of CTP1) (Trattner *et al.*, 2008b). However the metabolic alterations shown by Trattner *et al.* (2008a) was not supported by later publications, where sesamin did not significantly increase the amount of DHA or 18.3n-3 neither in rainbow trout nor in Atlantic salmon (Pan *et al.*, 2013; Schiller Vestergren *et al.*, 2012a; Schiller Vestergren *et al.*, 2011). However Schiller Vestergren *et al.* (2012a) concluded that FA involved in DHA synthesis were affected by the addition of sesamin in VO diet. Many studies have also shown that several genes involved in the lipid synthesis are affected by sesamin even though mRNA expression measurement are not enough to display any clear regulatory pathways (Pan *et al.*, 2013; Schiller Vestergren *et al.*, 2012a; Schiller Vestergren *et al.*, 2011).

Besides affecting lipid metabolism in Atlantic salmon sesamin has also been shown to alter several more metabolites (Wagner *et al.*, 2014). Wagner *et al.* (2014) displayed an increase in metabolites associated with energy metabolism (e.g. glucose, glycogen, leucine, valine, carnitine, lactate and nucleoside) suggesting that sesamin inclusion affects liver and white muscle metabolism.

Zajic *et al.* (2015) concluded that in the contrast to the carnivorous species. Atlantic salmon and Rainbow trout, elongation and desaturation cascade to produce n-3 LC-PUFA in omnivore species such as common carp (*Cyprinus carpio L.*) most likely are not effected by sesamin. This indicates that sesamin might only affect lipid metabolism in carnivorous species.

2.5.3 Effect of sesamin on detoxification processes in fish

Plant-based dietary components along with drugs or environmental contaminants might interfere with detoxification by altering the cytochrome P450 (CYP450) enzyme system and thereby decrease the organism ability to eliminate toxic substances. It is therefore important to evaluate dietary ingredients effect on detoxification processes (Zlabek *et al.*, 2015). CYP1A are the most commonly studied isoform in fish and its activity is usually measured as ethoxyresorufin-O-deethylase (EROD) activity. EROD along with other CYP450-mediated activities

are used as biomarkers to detect pollutants (Zlabek *et al.*, 2015; Wagner *et al.*, 2013; Trattner *et al.*, 2008a). Wagner *et al.* (2013) were the first to study the effect of sesamin on CYP450 activity in fish (Atlantic salmon, common carp) and suggested that sesamin can inhibit the activity of CYP1A- and CYP2E1-like isoforms in a mechanism-based manner. Also Zlabek *et al.* (2015) displayed a dose dependent correlation between sesamin and CYP450 activities in Atlantic salmon. The fact that sesamin effects the CYP450 enzyme system indicates that sesamin are recognized as a xenobiotic compound in Atlantic salmon which were also supported by the finding of (Trattner *et al.*, 2008a). Further studies are therefore needed to evaluate the effect of sesamin on the overall health of Atlantic salmon.

2.6 MicroRNA

MicroRNAs (miRNA) are endogenous ~22nt long non-coding RNAs that comprise one abundant class of gene regulator molecules by translation inhibition of mRNAs (Bartel, 2004). The translation inhibition can occur either by complete or incomplete complimentary base paring resulting in cleavage and degradation of the mRNA or blocking of the translation, respectively (He & Hannon, 2004). In human more than 60% of all protein coding genes have one or several conserved miRNA- binding sites (Friedman *et al.*, 2009) and miRNAs are involved in a variety of biological processes such as, cell differentiation (Zhu *et al.*, 2014; Kim *et al.*, 2010; Lin *et al.*, 2009) and metabolism (Zhang *et al.*, 2014; Mennigen *et al.*, 2013; Mennigen *et al.*, 2012). Dysregulations of miRNAs have also been associated with human diseases including cancer (Fabbri *et al.*, 2008).

2.6.1 MicroRNA synthesis

MiRNA can be expressed either alone as single miRNA or in bigger miRNA clusters were several miRNA are expressed as one bigger unit (Lau *et al.*, 2001). In both cases miRNA is first transcribed into a pri-miRNA by RNA polymerase II and then processed in two steps, the first one occurs inside the nucleus and the second one in the cytoplasm (Lee *et al.*, 2002)(figure 7). The pri-miRNa has one or more hairpin loops that are recognized by the double-stranded-RNA-binding protein, DGCR8 which together with of the RNase III family nuclease, Drosha (Lee *et al.*, 2003) consist a microprocessor complex which cleaves the pri-miRNA into a ~70 nt pre-miRNA (Denli *et al.*, 2004; Gregory *et al.*, 2004; Han *et al.*, 2004)(figure 7). The pre-miRNAs are then exported from the nucleus into the cy-

toplasm by the Ran-GTP dependant nucleo/cytoplasmic cargo transporter Exportin 5 (Exp5) (Yi *et al.*, 2003; Lund & Guttinger)(figure 7). In the cytoplasm, the pre-miRNA is cleaved by a complex composed by the RNase-III enzyme, Dicer and TRBP (human immunodeficiency virus transactivating response RNA-binding protein) into a ~22nt dsRNA duplex containing both the miRNA strand and its complimentary strand (miRNA*) (Chendrimada *et al.*, 2005; Haase *et al.*, 2005; Bernstein *et al.*, 2001; Ketting *et al.*, 2001; Knight & Bass, 2001)(figure 7). The argonaute protein (Ago) associates to the RNA duplex creating a pre-RNA-inducing silencing complex (pre-RISC) which removes the passenger strand generation the RNA-inducing silencing complex (RISC) (Ha & Kim, 2014). There are four different argonaute proteins (Ago 1-4) in humans that associates with almost identical sets of miRNAs (Ha & Kim, 2014). The RISC with the single stranded miRNA is capable of binding to the target mRNA by complementary base paring and induces translation inhibition (Bartel, 2004; He & Hannon, 2004)(figure 7).

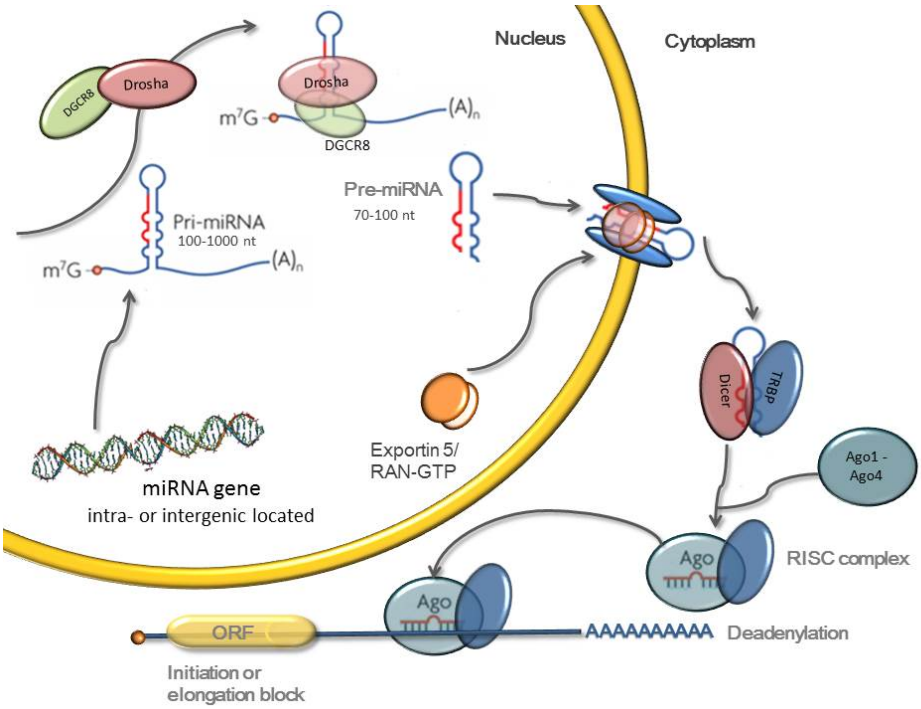


Figure 7. Biosynthesis of miRNA (Schiller Vestergren, 2014).

2.6.2 miRNA classification and naming

When a newly discovered miRNA is registered to the miRBase database the miRNA is provided sequential numerical identifiers that consist of three to four prefixes to specify the species, “miR” are added to mature sequences and “mir” to precursor sequences and last a number. miRNAs with the same number between several species are orthologous e.g. hsa-miR-122 (*Homo sapiens*) and ssa-miR-122 (*Salmo salar*). Paralogous sequences that differs in one or two positions in the mature sequence are given lettered suffixes (ssa-miR-122a and ssa-miR-122b) and precursor sequences that differs in a few positions but provides identical mature sequences re given numbered suffixes (ssa-miR-122a-1 and ssa-miR-122a-2)(Griffiths-Jones *et al.*, 2006).

2.6.3 miRNA in Atlantic salmon

Andreassen *et al.* (2009) found several 7-mers mofits in the 3’untranslated regions (3’UTRs) that were identical to highly conserved miRNA targets in Atlantic salmon *(Salmo salar)*. This indicated that miRNA is part of the regulation of protein expression in Atlantic salmon. It is not until recent years research about miRNA in Atlantic salmon *(Salmo salar)* have started to increase. Andreassen *et al.* (2013) discovered and characterized 180 conserved mature miRNA and 13 novel mature miRNA through deep sequencing. These miRNAs are currently the only *Salmo salar* miRNAs submitted to the miRNAbase even though it is not the first or last sequencing analysis done on *Salmo salar* miRNAs.

Arm domination of the hairpin are decided by comparing read counts and the one with the highest count are expected to be the dominant one. In *Salmo salar* miRNA the 5’ arm have been shown to be dominant further extant (60%) than the 3’arm (Andreassen *et al.*, 2013).

In this study 18 mature miRNA were selected from miRNA discovered in the sequencing of miRNA extracted from *Salmo salar* liver preformed of Schiller Vestergren (2014). Schiller Vestergren (2014) described 22 miRNA families to have high abundance (> 0,5%) in liver tissue of Atlantic salmon. 13 (mir-122, mir-21a, mir-21b, mir-722, mir-26a, mir-26b, mir-451a, mir-451b, mir-143, mir-194a, mir-17, mir-27a, mir-27b) out of the 18 miRNA tested in this thesis belonged to the miRNAs families with high abundance and 5 miRNA (mir-30c-3p, mir-30c-5p, mir-10a, mir140-3p and mir-140-5p) belonged to miRNAs families with low abundance (figure 8).

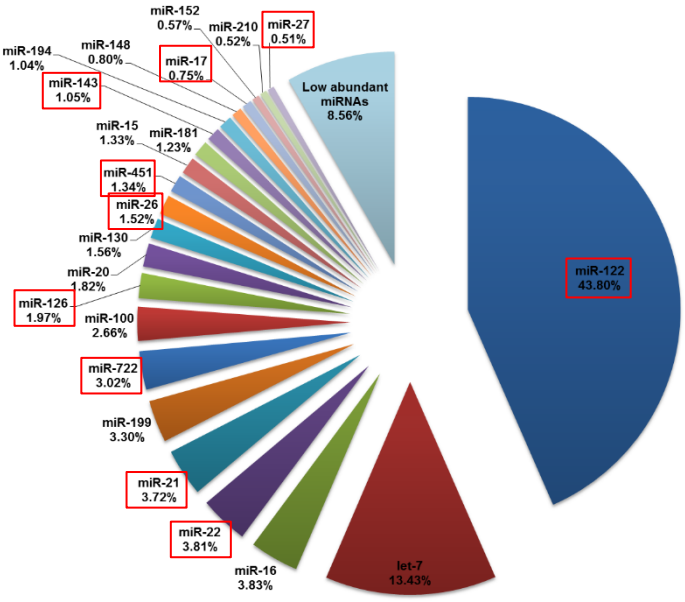
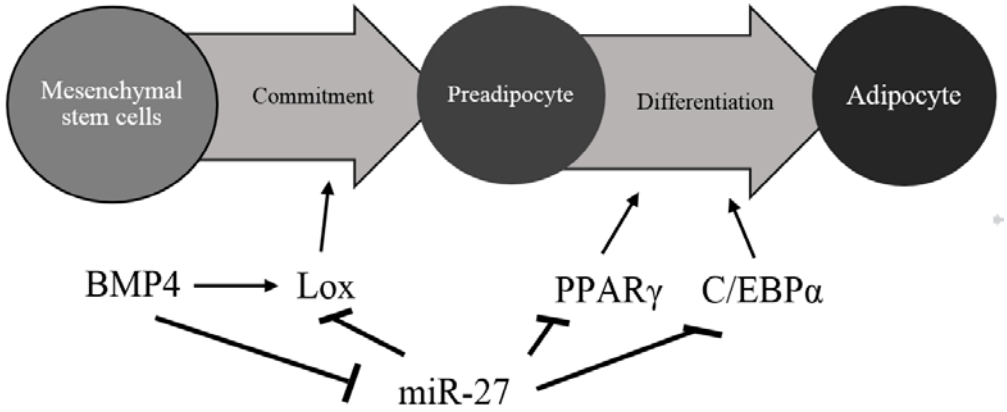


Figure 8: Relative presence of high abundant miRNAs in mature liver samples from Atlantic salmon received from Illumina sequencing performed by Schiller Vestergren (2014). The miRNA that were chosen for miRNA expression measurement in this thesis are marked with red. (Schiller Vestergren, 2014)

Many of these 18 miRNA are involved in the lipid metabolism by targeting various genes of the different processes in lipid metabolism and one example are miR27.

The miR27 family includes the isoform miR27a, miR27b which both have been shown to have inhibition function during adipogenesis by affect both the impaired adipocyte linage commitment from stem cells into preadipocyte and the differentiation of preadipocyte into adipocyte in both human and mice (Chen *et al.*, 2015; Zhu *et al.*, 2014; Kim *et al.*, 2010; Lin *et al.*, 2009). During BMP-induced adipocyte linage commitment mirR27a and miR27b have an antiadipogenic function by inhibiting lysyl oxidase (Lox) expression. miR27a and miR27b are in their turn inhibited by bone morphogenic protein 4 (BMP4) (Chen, Xu et al. 2015). At the

differentiation stage of preadipocyte, miR27a and miR27b have been shown to have inhibiting function by targeting peroxisome proliferator-activated receptor γ (PPARγ) (Kim *et al.*, 2010; Karbiener *et al.*, 2009), CCAAT-enhancer-binding protein α (C/EBPα) (Karbiener *et al.*, 2009; Lin *et al.*, 2009), cAMP response element binding protein (CREB) (Zhu *et al.*, 2014), which are all important regulators



in adipogenesis (figure 9.

Figure 9: Model of miR-27-mediated regulation of adipocyte commitment and differentiation. Alter from Chen *et al.* (2015)

3 Objectives

The main objective of this trial is to determine the effect of replacing fish oil with vegetable oil with and without supplemented sesamin in the diet of Atlantic salmon on the miRNA profile in the fish liver. The trial also includes investigation on the effect of different dietary ratios of n-6 to n-3 fatty acids and sesamin concentration. This is extending measurements using the same fish tissue as Schiller Vestergren (2014) where the mRNA expression of several genes involved in lipid metabolism was measured as well as fat content, fatty acid composition and fish performance.

Including a miRNA profile to the measurement previously conducted by Schiller Vestergren (2014) is done to provide more information about regulation of lipid related genes and if miRNAs are a part of this regulation. To do this, the expression of 18 miRNAs in Atlantic salmon are measured using qPCR.

4 Material and method

The fish trial and sampling had already been conducted (Schiller Vestergren, 2014) as described in the section below (4.1 Trail design).

Liver samples from each feeding profile (n=6) stored in RNAlater were used for the mRNA measurements. miRNA from the livers was extracted and the expression profile for 18 different mRNAs (mir-122, mir-21a, mir-21b, mir-722, mir-26a, mir-26b, mir-451a, mir-451b, mir-143, mir-194a, mir-17, mir-27a, mir-27b, mir-30c-3p, mir-30c-5p, mir-10a, mir140-3p and mir-140-5p) was measured using qPCR amplification. Gene expression of miR27d in the liver samples from Älvkarleby Research station was used as endogenous control. Extraction procedure, cDNA synthesis and qPCR amplification were performed simultaneously for the test samples as for the endogenous control and are described in section 4.2-4.3.

4.1 Trial design

Atlantic salmon were fed seven different diets produced by Skretting ARC Feed Technology Plant (Stavanger Norway). The feed varied between 3 different doses of sesamin, and 2 dietary n-6/n-3 ratios. A positive control with fish oil added to the feed was included.

4.1.1 Feed production

The basal diet was based on Skretting's commercial diet (Skretting Transfer 3.0; Skretting, Stavanger, Norway) for fish of size 100-300g but with reduced levels of fish meal and the fat level was maximized within present recommendations.

Experimental diet: fish meal (Scandinavian LT meal, Skretting, Stavanger), Wheat gluten (107,4 g/kg), soya concentrate (243,2 g/kg), wheat (89,5 g/kg), premixes (9 g/kg).

Oils included: a blend of plant oils, rapeseed (Skretting, Stavanger), palm (Fritex 24, Aarhuskarlshamn Sweden AB, Karlshamn, Sweden) and linseed oil (Elbe Fetthandel GmbH, Hamburg, Germany) was used as a starting point to design two oil mixes with different n-6/n-3 ratios (0.5, 1.0) for the present trial. V0.5 diets consisted of 53.5 g/kg rapeseed oil, 135 g/kg linseed oil and 50 g/kg palm oil and the V1 diets of 138,5 g/kg rapeseed oil, 40 g/kg linseed oil and 60 g/kg palm oil. The analyzed n-6/n-3 ration of V1 was 0.9 but still referred to as V1. A standard Scandinavian fish oil (Skretting, Stavanger) was used as additional control

Sesamin: Sesamin (98% purity) was obtained from KEB Biotech (Beijing, China). The sesamin was added as a dry powder accorded to ARC's calculations on required amounts. The powder was added to the feed mixed together with premixes pior to extrusion. Three different doses were included: 0 - no addition, 1.16 g/kg – low dose and 5.8 g/kg – high dose.

Feed was produced as 4 mm pellets including three different kernels with increasing sesamin content and were then coated with 3 different oils resulting in 7 different diets (Table 2). After production of the feed, it was analyzed for proximate composition and fatty acid content at ARC lab and the level of sesamin was measured (Uppsala) to make sure that the actual sesamin dose corresponded to the theoretical dose (Table 2).

Table 2: Experimental diets

Diet	Oil	Sesamin, g/kg (theory)	Sesamin, g/kg (actual)
A	1: n-6/n-3 = 0.5	0	n.d
B	1: n-6/n-3 = 0.5	1.16	2.19
C	1: n-6/n-3 = 0.5	5.80	5.68
D	2: n-6/n-3 = 1.0	0	1.41
E	2: n-6/n-3 = 1.0	1.16	2.37
F	2: n-6/n-3 = 1.0	5.80	6.18
G	Fish oil	0	1.34

4.1.2 Fish trial

The trial was conducted at Skretting ARC Fish Trial Station (Stavanger, Norway) and 7 circular tanks (1 m diameter) were used and all fish were individually tagged with micro-transponders (Trac ID System AS, Stavanger, Norway) well in ad-

vance of tail start up. The stock in each with 35 fish and the growth of each individual fish were followed. Atlantic salmon with an initial weight of 104.6g ± 9.9g and well adapted to sea water were kept at 12°C flow through for 4 moonths (November-March).

Automatic feeders were used to feed the fish in slight excess of their appetite and the excess was removed daily to calculate the feed consumption in each tank.

The following parameters were registered during the fish trial:

- Daily control of environmental parameters
- Daily feed and waste feed per tank
- Individual fish weight at start, after 1 month and at the end of trial. (after 2 months, the fish were bulk weighed and counted in each tank)
- The length of each fish was registered at the end of the trial

4.1.3 Samples and analyses

At slaughter (average fish weight 553.6 ± 88.1 g), 11 fish of average length per tank were included. 5 were sampled for proximate composition and fatty acid content at the ARC lab and the liver weights were recorded. Six were sampled for further studies and the liver and fillets were dissected out. Liver weights were recorded before they were frozen in liquid nitrogen and stored at -80°C. The filet was frozen (skin on) and both liver and muscle samples were sent to Uppsala for further analysis. Liver pieces used for the mRNA measurement were stored in RNAlater-ICE (AM7030, Applied Biosystems Part of Life Technologies, Carlsbad, CA US) directly before storage at -20°C.

4.1.4 Tissue sample used as positive control

The Atlantic salmon used for the positive control sample in the mRNA measurments came from Älvarleby Research station, Älkarleby, Sweden. The fishes were approximately 10g and 10 months of age and fed commercial diet (Alleraqua Preforma pellet size 2 and 9% fat content, Aller Aqua A/S, Christiansfeld, Danmark) prior to the sampling and kept in one tank with water from the river Dalälven at 10°C. Tissue samples from the liver were collected and stored in RNAlater (Life Technologies, Carlsbad, CA, USA) and sored at -80°C until further analysis.

4.2 Isolation of RNA

RNA was isolated from the liver samples by the use of miRVana miRNA isolation kit from Ambion (Life Technologies, Carlsbad, CA, US). Enrichment procedure for small RNAs was followed according to the manufacturer's instructions without

modifications. The optical density (NanoVue Spectrometer) was measured to ensure the purity and density of the extracted RNA.

4.3 cDNA synthesis and qPCR amplification

To synthesize cDNA and for the qPCR analysis a TaqMan® small RNA assay was used (table 3). The components were mixed according to manufacturer's instructions and the reverse transcription (RT) reaction was carried out in a Veriti® 96-well Fast Thermal Cycler (Applied Biosystems Part of Life Technologies, Carlsbad, CA, USA). The RT condition was set to one cycle: 16°C/30 mins, 42°C/30 mins, 85°C/5mins and hold at 4°C. 5µl of the extracted DNA were used for the RT-reaction. The purity and density were decided by measuring the optical density (NanoVue Spectrometer) of the synthesized cDNA.

The qPCR reactions were mixed after manufacturer's instructions for 20µl reactions with 1.33µl of the RT reaction used per reaction. The qPCR was performed using a StepOnePlus™ qPCR system (Applied Biosystems of Life Technologies, Foster City, CA, USA). The qPCR reaction was set to 41 cycles, cycle 1: 95°C/10min (enzyme activation), cycle 2-41: 95°C/15s and 60°C/60s (annealing and elongation). All samples (n=6 for 7 different feeding profiles) were run for each mRNA in triplicates. Non-template controls (singlet) and endogenous control (triplicates) were included on every plate. The data were collected and the expression of the different mRNAs were evaluated using the DataAssist software version 2.0 (Applied Biosystems of Life Technologies, Foster City).

Tabell 3. *miRNA primer used with miRBase accession number*

Assay Name (miRBase ID)	Mature miRNA Sequence	miRBase accession number
Has-miR-27b-3p (ssa-miR-27b-3p)	UUCACAGUGGCUAAGUUCUGC	MIMAT0000419
Ipu-miR-27c (ssa-miR-27d-3p)	UUCACAGUGGUUAAGUUCUG	MIMAT0029489
Has-miR-30a-3p (ssa-miR-30c-3p)	CUUUCAGUCGGAUGUUUGCAGC	MIMAT0000088
Bta-miR-30e-5p (ssa-miR-30c-5p)	UGUAAACAUCCUUGACUGGAAGCU	MIMAT0003799
Dre-miR-10c (ssa-miR-10a-5p)	UACCCUGUAGAUCCGGAUUUGU	MIMAT0003087
Has-miR-26a	UUCAAGUAAUCCAGGAUAGGCU	MIMAT0032563

(ssa-miR-26a-5p)		
Dre-miR-26b (ssa-miR-26b-5p)	UUCAAGUAAUCCAGGAUAGGUU	MIMAT0029486
Ssa-miR-451b		
Mmu-miR-451	AAACCGUUACCAUUACUGAGUU	MIMAT0001632
Has-miR-143 (ssa-miR-143-3p)	UGAGAUGAAGCACUGUAGCUCA	MIMAT0000849
Has-miR-194 (ssa-miR-194a-5p)	UGUAACAGCAACUCCAUGUGGA	MIMAT0000460
Has-miR-17 (ssa-miR-17-5p)	CAAAGUGCUUACAGUGCAGGUAG	MIMAT0000070
Dre-miR-27a-3p (ssa-miR-27a-3p)	UUCACAGUGGCUAAGUUCCGCU	MIMAT0029487
Has-miR-122 (ssa-miR-122-5p)	UGGAGUGUGACAAUGGUGUUUG	MIMAT0000421
Ssa-miR-21a-5p (ssa-miR-21a-5p)	UAGCUUAUCAGACUGGUGUUGACU	MIMAT0032533
Dre-miR-21 (ssa-miR-21b-5p)	UAGCUUAUCAGACUGGUGUUGGC	MIMAT0001787
ssa-miR-722-5p (ssa-miR-722-5p)	AUUUGAAACGUUUUAGCCAAA	MIMAT0032667
mmu-miR-140* (ssa-miR-140-3p)	UACCACAGGGUAGAACCACGGA	MIMAT0032360
Mmu-miR-140 (ssa-miR-140-5p)	CAGUGGUUUUACCCUAUGGUAG	MIMAT0000151

4.4 Statistical analysis

Relative expression of the different microRNAs, in relation to the reference miRNA (miR-27d-3p) were calculated according to the Livak method. The Livak method consists of three steps, (1) normalization of C_T (target gene) to C_T (reference gene) for all treatments to gain ΔC_T (test diet) and ΔC_T (reference diet), (2) normalization of ΔC_T (test diet) test diet to the ΔC_T (reference diet) to gain $\Delta\Delta C_T$ (3) calculation of the fold change between the test diet and the reference diet by the form $2^{-\Delta\Delta CT}$.

The data are presented as mean values ± standard deviation. Tukey's Kramer test was used to detect differences between the different diets and $p<0.05$ was regarded as significant.

5 Results

5.1 Gene expression of miRNA in liver of Atlantic salmon

Expression of miRNA was measured in liver of Atlantic salmon by real time q-PCR and miR-27d was used as endogenous control. Also the read count for the miRNAs from the Illumina sequencing performed by Schiller Vestergren (2014) was calculated as fold change compared to miR-27d. The same liver samples were used in both the qPCR measurement and the Illumina sequencing.

miR-122-5p showed the highest expression out of the 18 miRNAs tested in both the qPCR measurement and Illumina sequencing even though the read count showed a fourfold higher expression than the qPCR measurement in comparison to the reference miRNA (miR-27d-3p) (figure 10a). miR-21b-5p showed the second highest read count and a fold change of more than 30 in comparison to the reference gene while the gene expression measured by qPCR had a slightly lower gene expression than the reference gene (figure 10b). miR-451b-5p, miR-194a-5p, miR26b-5p, miR-26a-5p and miR-17-5p all had a higher (figure 10b) and miR-143-3p, miR-27a-3p, miR-27b-3p and miR-21a-5p a lower (figure 11a) gene expression (qPCR) and read count (Illumina sequencing) than the reference gene. miR-722-5p and miR-451a-5p both had a higher expression rate than the reference gene but a lower read count (figure 11a).

miR-30, miR-140 and miR-10 were described as low abundant miRNA by Schiller Vestergren (2014) due to low read count and are therefore presented with a combined read count for all mature sequence arms for the respective miRNA family while the qPCR data measured the specific mature sequence arms (miR-30c-3p, miR-30c-5p, miR-140-3p, miR-140-5p, miR-10a-5p)(figure 11b).

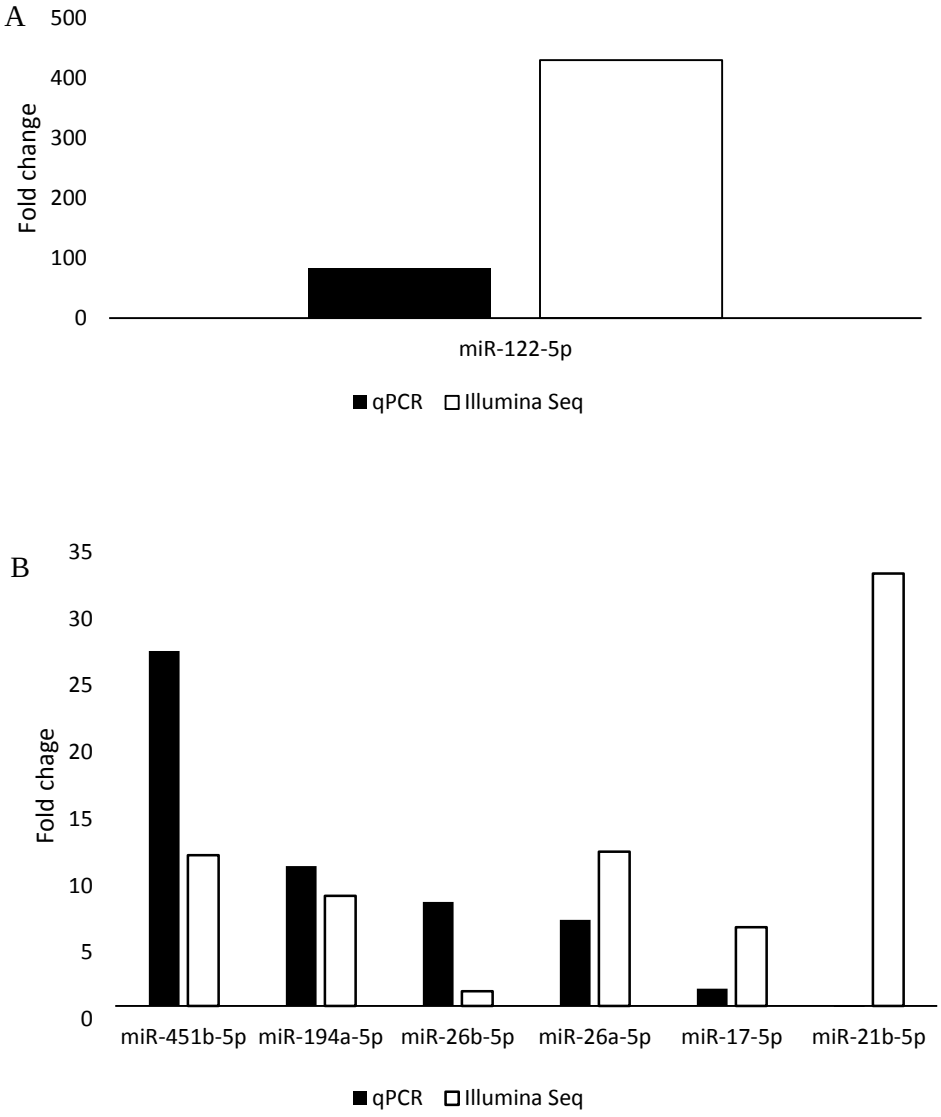


Figure 10. (A and B) Expression of miRNA measured by qPCR and read counts from Illumina sequencing presented as fold change in comparison to the reference miRNA (miR-27d-3p). Read counts data modified from (Schiller Vestergren, 2014).

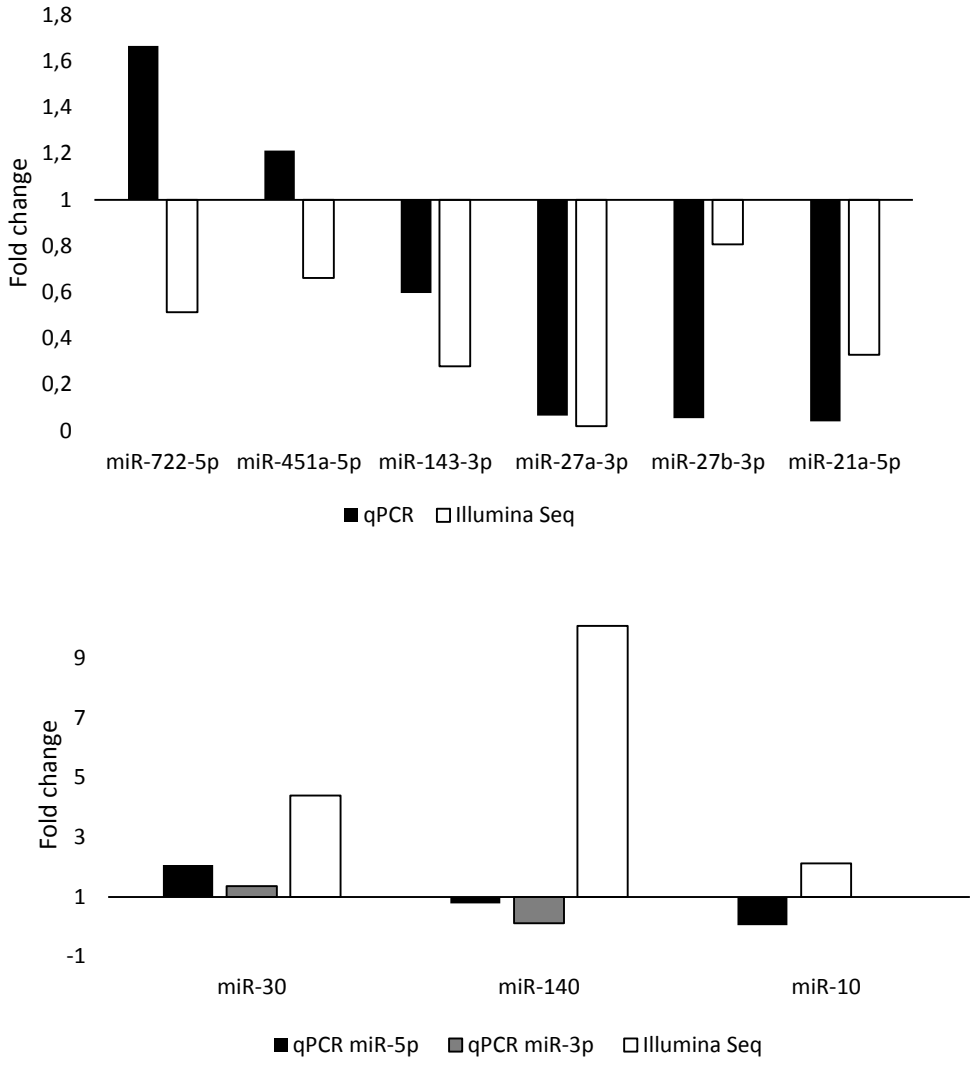


Figure 11. A) Expression of miRNA measured by qPCR and read counts from Illumina sequencing presented as fold change in comparison to the reference miRNA (miR-27d-3p). B) Expression of miRNA (miR-30c-3p, miR-30c-5p, miR-140-3p, miR-140-5p, miR-10a-5p) measured by qPCR and read count for all mature miRNA sequences in the miR-30, miR-140 and miR-10 family measured by Illumina sequencing presented as fold change in comparison to the reference miRNA (miR-27d-3p). Read counts data modified from (Schiller Vestergren, 2014)

5.2 Effect of vegetable oil replacement for fish oil on the miRNA expression

Effect of VO replacement compared to FO diet (figure 12 A-C) on the miRNA expression was measured and plotted as fold change to the reference diet (FO) (presented as a dashed line, figure 12 A-C). Expression of miRNA in the liver of Atlantic salmon feed diet were FO had been replaced with VO with either high or low n6/n3 ratio were compared with the reference diet containing FO. Among the 18 miRNAs used 11 (miR-140-5p, miR-194a-5p, miR-21a-5p, miR26a-5p, miR-143-3p, miR-17-5p, miR26b-5p, miR-27a-5p, miR27b-5p, miR-30c-3p, miR451b-5p) showed no difference in miRNA expression in fish fed VO diet with either high or low n6/n3 ratio without sesamin in compared to the reference (FO) diet ($p>0.05$, n=6) (figure 12a-b). The expression of 5 miRNAs (miR-10a-5p, miR140*-3p, miR-30c-5p, miR-122-5p and miR-21b-5p) had increased in fish fed VO diet with low (0.5) n6/n3 ration while the miRNA expression fish fed VO diet with high (1) n6/n3 ratio were unchanged compared to the reference (FO) diet ($p<0.05$, n=6) (figure 12a-c). miR-451a-5p were the only miRNA which showed an increase in mRNA expression in both fish fed VO diet with low and high n6/n3ration compared to the FO diet (figure 12b) and miR-722-5p the only one that sowed an increase expression in fish fed VO diet with high n6/n3 ratio (1) but no difference in fish fed VO diet with low n3/n6 ratio (0.5) (figure 12c) compared to the reference diet (FO) ($p<0.05$, n=6).

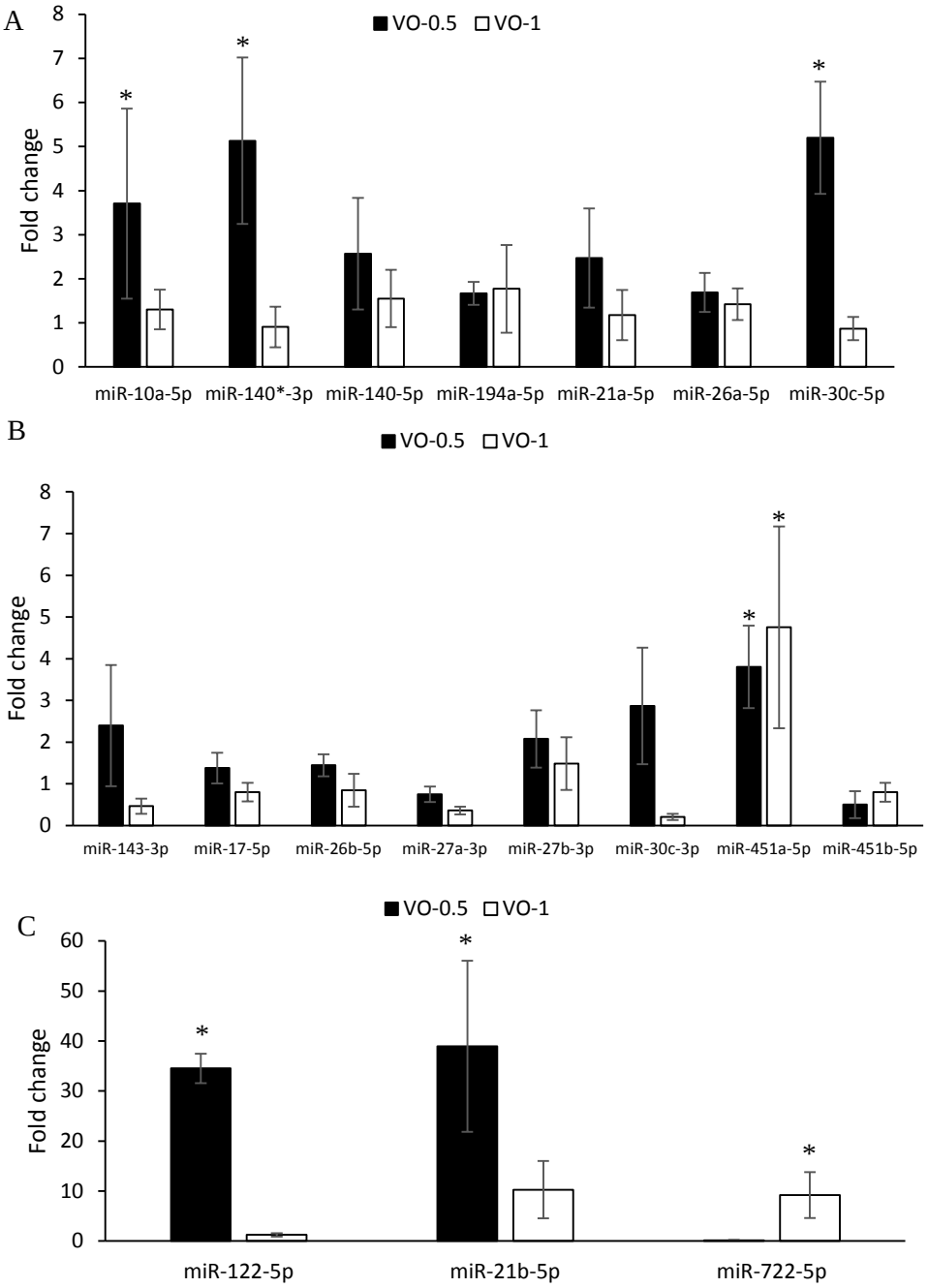


Figure 12. Expression of miRNA in fish feed VO diet either with low (0.5) or high (1)

n6/n3 ratio shown as fold change difference in comparison to the reference diet FO (dashed line) (A-C). * Denotes significant difference from fish fed the FO diet ($p < 0.05$), n = 6. miR-27d were used as endogenous control.

5.3 Effect of sesamin supplementation to the vegetable oil diet on the miRNA expression

Effect of sesamin addition in either high (SH), low (SL) or no amount (S0) to the VO diet with low (0.5) (figure 13 A-C, figure 14A-C) or high (1) (figure 13 D-F, figure 14 D-F) n-6/n-3 ratio on the miRNA expression was measured and plotted as fold change to the reference diet (FO) (presented as a dashed line, figure 13 A-F, figure 14A-F). Out of 18 miRNAs tested there were no measurable changes in fish fed either of the different fed profiles in 6, regarding miRNA expression (miR-140-5p, miR-26a-5p, miR-17-5p, miR-27b-3p, miR-451b-5p and miR-30c-3p) (Figure 14 and 13).

Of the five miRNAs with increased expression in fish fed a VO diet with low (0.5) n-6/n-3 ratio (miR-10a-5p, miR140*-3p, miR-30c-5p, miR-122-5p and miR-21b-5p), four showed a significantly ($p<0.05$ n=6) lower miRNA expression in fish fed the VO-0.5 diet with sesamin of either high or low concentration added (miR140-3p, miR-30c-5p, miR-122-5p and miR-21b-5p), one only with the high sesamin concentration (miR-10a-5p) ($p<0.05$, n=6) (figure 13a and 14b-c).

miR-451a-5p that showed significantly increased miRNA expression in fish fed a VO diet with low or high n-6/n-3 ratio compared to the reference diet (FO) displayed a significant ($p<0.05$, n=6) decrease in fish fed a VO-0.5+SL, VO-1+SL and VO+SH diet back to levels not different from fish fed the reference diet (figure 4b and 4e).

miR-26b-5p, miR-27a-3p, miR-122-5p all display a miRNA expression significantly ($p<0.05$, n=6) higher in VO with low and/or high n-6/n-3 ratio that includes either high and/or low sesamin supplementation (figure 13b, 13e, 14a and 14d)

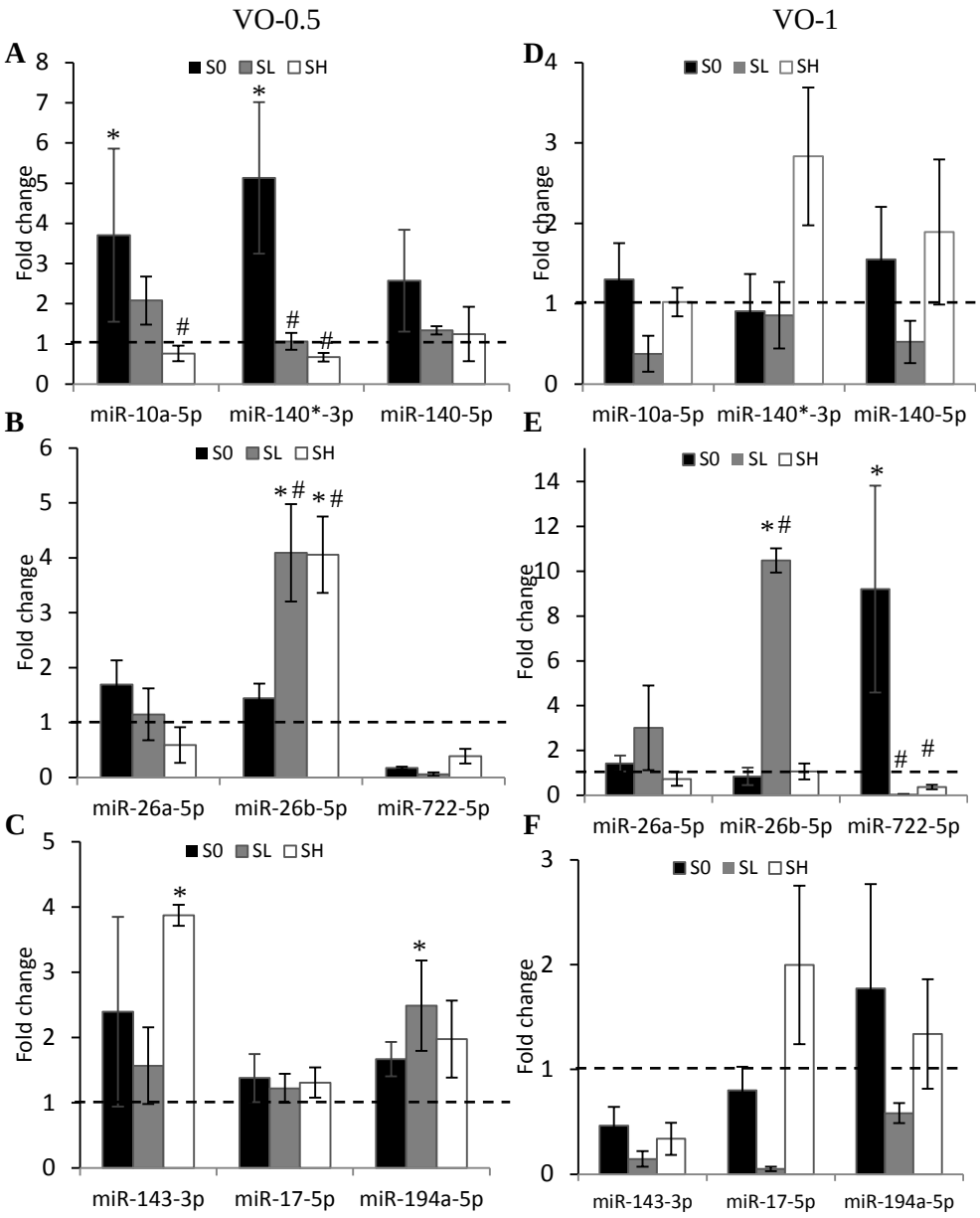


Figure 13. (A-C) Expression of miRNA in fish feed VO diet with low (0.5) n6/n3 ratio supplemented with no (S0), low (SL) or high (SH) level of sesamin compared to the reference diet FO (dashed line). (D-F) Expression of miRNA in fish feed VO diet with high (1) n6/n3 ratio supplemented with no (S0), low (SL) or high (SH) level of sesamin compared to the reference diet FO (dashed line). * Denotes significant difference from reference diet ($p < 0.05$), n = 6. # Denotes significant difference from VO diet without sesamin ($p < 0.05$), n = 6. MiR-27d was used as endogenous control.

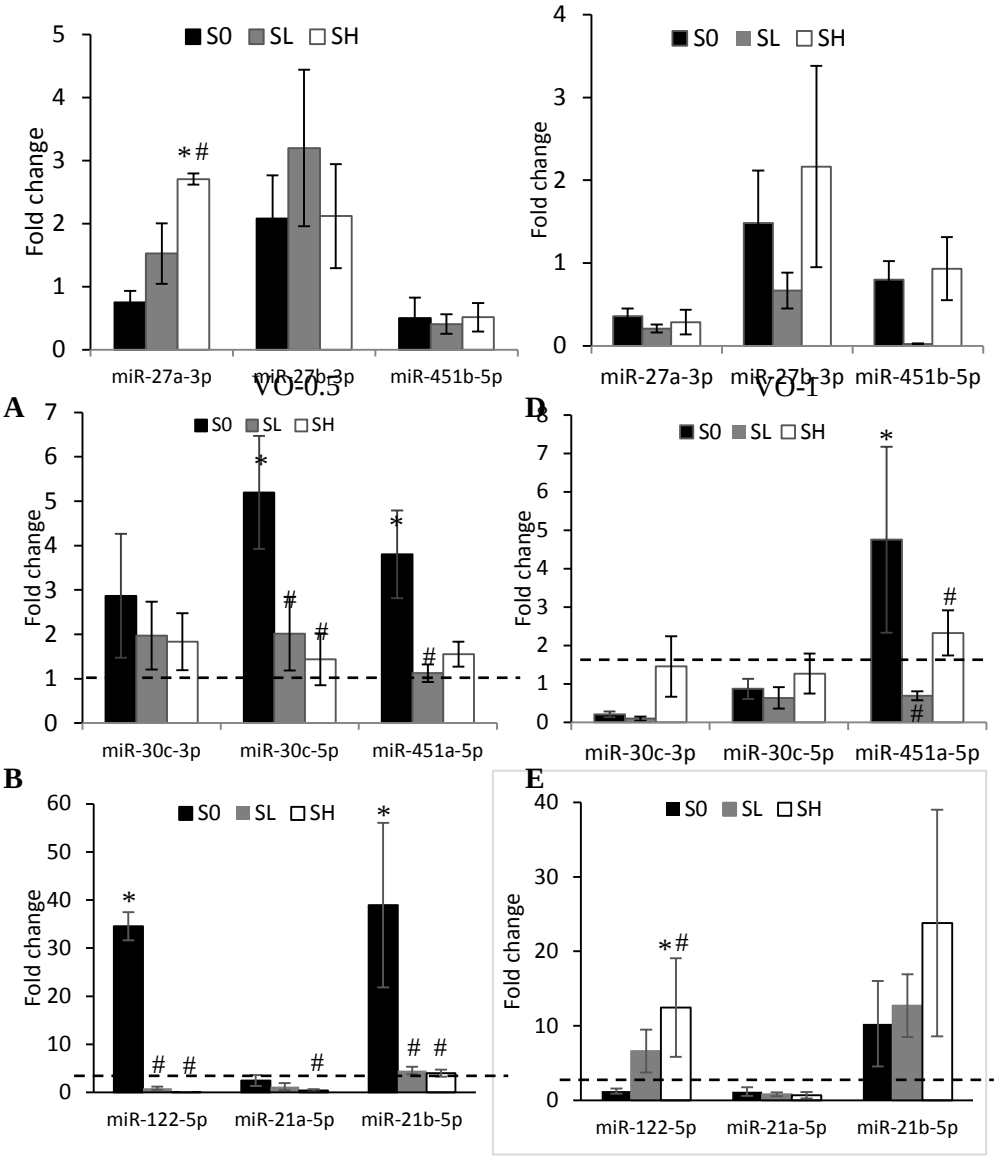


Figure14. (A-C) Expression of miRNA in fish feed VO diet with low (0.5) n6/n3 ratio supplemented with no (S0), low (SL) or high (SH) level of sesamin compared to the reference diet FO (dashed line). (D-F) Expression of miRNA in fish feed VO diet with high (1) n6/n3 ratio supplemented with no (S0), low (SL) or high (SH) level of sesamin compared to the reference diet FO (dashed line). * Denotes significant difference from fish fed the FO diet ($p < 0.05$), n = 6. # Denotes significant difference from fed FO diet without sesamin ($p < 0.05$), n = 6. MiR-27d was used as endogenous control.

6 Discussion

Due to draining of the valuable marine resources and decrease in availability of fish meal and fish oil a more sustainable aquaculture of carnivore fish species as Atlantic salmon is needed without altering the nutrition value of the fish. Fish lipid content are reflected by the lipid content of the feed by several manners. The dietary lipids compose a big part of the base of exogenous lipids that are then synthesized further to PUFA among many other lipids (Griffin, 2013). Dietary lipids are also involved in the regulation pathway if lipid synthesis and therefore affects the lipid content in the body of the organism through several ways (Bonacic *et al.*, 2016; Pan *et al.*, 2013; Schiller Vestergren *et al.*, 2012a; Morais *et al.*, 2011; Tocher *et al.*, 2003a; Tocher *et al.*, 2003b). Altering lipid synthesis by the addition of bioactive compounds have been widely studied (Zajic *et al.*, 2015; Schiller Vestergren, 2014; Wagner *et al.*, 2014; Trattner *et al.*, 2008a; Trattner *et al.*, 2008b). mRNA measurement cannot alone explain the alteration in expression of lipid-related genes and post transcriptional regulation pathways need to be taken into consideration (Schiller Vestergren, 2014). The most commonly studied epigenetic alteration are DNA methylation, post-translation histone modification and miRNAs and may all be involved in the lipid metabolism regulation pathway(Chan & Baylin, 2012; Guil & Esteller, 2009). This thesis has focus on the possible relation between 18 of the most abundant miRNAs in Atlantic salmon (Schiller Vestergren, 2014) were fish oil is replaced with vegetable oil with and without supplemented sesamin.

6.1 miRNA expression

Schiller Vestergren (2014) recently characterized the expression profile of hepatic miRNAs in Atlantic salmon by Illumina Genome Analyzer. A comparison be-

tween the qPCR data from this study and the Illumina sequencing data from Schiller Vestergren (2014) were compared to validate the qPCR technique used. Schiller Vestergren (2014) annotated 202 different miRNA families were miRNA from 22 of the miRNA families accounted for 91% of the miRNA expressed in the liver of Atlantic Salmon. Of the 18 miRNA included in this study 14 belonged to 9 of the 22 families that showed the highest abundance in study from Schiller Vestergren (2014) were miR-122 showed the highest followed by, miR-21, miR722, miR-26, miR451, miR-143, miR194, miR-17 and mir-27. The five remaining miRNA are included in 3 miRNA families that were classified as low abundant miRNA (miR-30, miR-10 and miR140) by Schiller Vestergren (2014).

According to the qPCR conducted in this thesis miR-122-5p were the miRNA with the highest miRNA expression which are supported by the illumine sequencing of Schiller Vestergren (2014) Except for miR-21b-5p that displayed the second highest abundancy according to the Illumina data by Schiller Vestergren (2014) one of the lowest expression rate in the qPCR measurement. Otherwise the qPCR expressions were quite similar to the illumine data form Schiller Vestergren (2014).

6.2 Effect of vegetable oil replacement for fish oil on the miRNA expression

In this thesis 7 miRNA candidates possible involved in regulation of lipid metabolism (miR-10a-5p, miR140*-3p, miR-30c-5p, miR-122-5p and miR-21b-5p, miR-451a-5p, 722-5p) has been identified. The VO-0.5 diet had a much greater impact on the miRNA expression in Atlantic salmon then the VO-1 diet. Lipid metabolism regulation appears to be sensitive for even small changes in the dietary lipid both to lipid composition, origin and level (Bonacic *et al.*, 2016; Pan *et al.*, 2013; Schiller Vestergren *et al.*, 2012a; Morais *et al.*, 2011; Tocher *et al.*, 2003a; Tocher *et al.*, 2003b). miR-10a-5p, miR140*-3p, miR-30c-5p, miR-122-5p and miR-21b-5p were all significant upregulated ($p < 0.05$) in fish fed VO-0.5 diet compared to the FO diet were no effect could be seen in the VO-1 and the FO diet. This indicates that these miRNAs are involved in a regulatory pathway sensitive to high dietary inclusion of ALA (18:3n-3). Schiller Vestergren *et al.* (2012a) showed that the three transcription factors (SREBP-1, SREBP-2 and LXR) involved in the lipid homeostasis that were significantly down regulated ($p < 0.05$) in fish fed the VO-0.5 diet compared to the FO diet but with no difference between the VO-1 and the FO diet. It is possible that the upregulated miRNAs are involved in the regulatory

system of the downregulated transcription factors but this need further investigations.

miR451a were the only miRNA that were significant upregulated ($p < 0.05$) in both the VO-0.5 diet an VO-1 diet compared to the FO diet. Several lipid synthesis-related genes have been affected by both the VO-0.5 and the VO-1 diet. PGC-1α and ELOVL4 were significant downregulated ($p < 0.05$) in both VO diets, CPT1 and ELOVL5b were significant up regulated ($p < 0.05$) in both VO diets and Δ5 fad were significant ($p < 0.05$) downregulated in the VO-0.5 diet but significant upregulated ($p < 0.05$) in the VO-1 diet (Schiller Vestergren *et al.*, 2012a).

miR-722-5p had a significant increase in expression in the VO-1 diet but no difference in the VO-0.5 diet in comparison to the FO diet. Also the elongation factor ELOVE 2 and desaturase Δ6 fad were significant upregulated by the VO-1 diet but not in the VO-0.5 diet compared to the FO diet (Schiller Vestergren *et al.*, 2012a). Further studies are needed to conclude any regulation of the lipid synthesis-related genes by the tested miRNAs.

It has earlier been shown that the miR-27 family are involved in the regulatory system of PPARα transcription factor but since neither PPARα or the miR-27 tested were affected by replacement of FO to VO this could not be supported or argued with (Pan *et al.*, 2013; Schiller Vestergren *et al.*, 2012a).

Leaver *et al.* (2011) showed that n-3 PUFA are a highly heritable trait and Morais *et al.* (2011) showed that fish families with different genotype react differently to a VO based diet. Variation in genotype between fishes should therefore be taken in consideration. The fishes in the trial of this study was not related, which could therefore cause difference in genotype which could have contributed to fluctuation in the dataset. However desaturases, elongases and cholesterol biosynthetic enzymes did not differ between families with different n-3 rations in their flesh instead were the biggest difference among genes involved in the lipid transport genes (Leaver *et al.*, 2011). Also Morais *et al.* (2011) found that genotype had a low impact on lipid synthesis-related genes but they also concluded a high impact on signaling pathways. Genotype might therefore not be a big factor on studies conducted regarding genes involved in the lipid synthesis. However, the effect on genotype on miRNA involved in the lipid synthesis pathway are yet to be investigated.

6.3 Effect of sesamin addition to fish feed a Vegetable oil based diet

The inclusion of sesamin had a dose-dependent negative effect on growth (Schiller Vestergren *et al.*, 2012a). This is consistent with the results shown by Wagner *et al.* (2014) which indicated that sesamin inclusion affects the energy metabolism by increasing many metabolites associated with energy metabolism in Atlantic salmon. Sesamin display a ability to reduce miRNA expression increased by VO diet back to expression rates similar to the FO diets. In five (miR-140-3p, 722-5p, miR-30c-5p, miR-122-5p and miR-21b-5p) of the seven miRNAs upregulated by either of the VO diets were the gene expression restored in VO supplemented with either high or low sesamin to expression reduced back to the expression rates of the FO diet. miR-10a-5p displayed a lower expression in the VO-0.5SL diet but not significant different form the VO-0.5S0 diet and a significant decrease back to the levels provided by the FO diet by the VO-0.5SH diet. miR-451a-5p displayed a decrease in the VO-0.5SL and VO-0.5SH compared to the VO-0.5S0 diet but only the VO-0.5SL were significant lower than the VO-0.5S0 diet. The increase in the VO-1S0 diet of the miR-451a-5p were significant reduced back to the expression rate of the FO diet in the VO diet with either of the sesamin concentrations. Five miRNA (miR-26b-5p, miR-143-3p, miR-194a-5p, miR27a-3p and miR21a-5p) that showed not difference between fish fed the VOS0 diet and the FO diet displayed a significant upregulation of one or several VO diets supplemented with either high or low sesamin. These results indicate that at sesamin supplementation reduces changes in the miRNA expression induced by replacement of FO diet to a VO diet. This is novel data since to my knowledge no studies have been conducted on the effect of sesamin supplemented VO diet on the miRNA expression in Atlantic salmon before.

Several studies have indicated that sesamin are recognized as xenobiotic compound in Atlantic salmon (Zlabek *et al.*, 2015; Wagner *et al.*, 2013; Trattner *et al.*, 2008a). Therefore, it necessary to further investigate the long-term health effect of sesamin supplemented in the feed in Atlantic salmon.

7 Conclusion

Nutrigenomics approaches provide tools that can help to increase the understanding of interaction between diet and fatty acid metabolism and thereby taking us one step closer to a more sustainable aquaculture industry. Epigenetic regulation of lipid metabolism is complex and not yet fully understood. This thesis concludes that miRNA most likely are a part of the feedback regulation of lipid metabolism and 7 possible miRNA candidates were identified.

The results from this study supports the claim that sesamin effects the lipid metabolism not only by effecting several lipid synthesis related genes but also by altering miRNA expression. Several studies have indicated that sesamin are recognized as xenobiotic compound in Atlantic salmon and sesamin might also enhance energy metabolism. Therefore, it necessary to further investigate the long-term health effect of sesamin supplemented in the feed in Atlantic salmon.

The role of miRNA along with other post transcriptional regulation pathways and effect of genotype need to be further investigated to fully understand how lipid synthesis works and are affected by dietary alterations and compounds.

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8 Supplementary material

miRNA	Mature sequence
miR-21a	**ssa-miR-21a-5p** UAGCUUAUCAGACUGGUGUUGACU **bta-miR-21-5p** UAGCUUAUCAGACUGAUGUUGACU **sha-miR-21** UAGCUUAUCAGACUGAUGUUGACUG **hsa-miR-21-5p** UAGCUUAUCAGACUGAUGUUGA
miR21b	**ssa-miR21b-5p** UAGCUUAUCAGACUGGUGUUGGC **dre-miR-21** UAGCUUAUCAGACUGGUGUUGGC **fru-miR-21** UAGCUUAUCAGACUGGUGUUGGC **tni-miR-21** UAGCUUAUCAGACUGGUGUUGGC **ccr-miR-21** UAGCUUAUCAGACUGGUGUUGGC **hsa-miR-21-5p** UAGCUUAUCAGACUGAUGUUGA
miR-772	**Ssa-miR-772-5p** AUUUGAAACGUUUUAGCCAAA **ccr-miR-722** UUUUUUGCAGAAACGUUUCAG **nve-miR-2041a-5p** AUUUUGCUCUUACGUUUCACU **dre-miR-722** UUUUUUGCAGAAACGUUUCAGAUU
miR-26a	**ssa-miR-26a-5p** UUCAAGUAAUCCAGGAUAGGCU **hsa-miR-26a-5p** UUCAAGUAAUCCAGGAUAGGCU **mmu-miR-26a-5p** UUCAAGUAAUCCAGGAUAGGCU **rno-miR-26a-5p** UUCAAGUAAUCCAGGAUAGGCU **dre-miR-26a-5p** UUCAAGUAAUCCAGGAUAGGCU **ssc-miR-26a** UUCAAGUAAUCCAGGAUAGGCU **ccr-miR-26a** UUCAAGUAAUCCAGGAUAGGCU
miR-26b	**ssa-miR-26b-5p** UUCAAGUAAUCCAGGAUAGGUU **dre-miR-26b** UUCAAGUAAUCCAGGAUAGGUU **pma-miR-26b-5p** UUCAAGUAAUCCAGGAUAGGUU **ipu-miR-26b** UUCAAGUAAUCCAGGAUAGGUU **hsa-miR-26a-5p** UUCAAGUAAUCCAGGAUAGG**C**U
miR-451a	**Ssa-miR-451a**

miR-451b	**Ssa-miR-451b**
miR-143	**ssa-miR-143-3p** UGAGAUGAAGCACUGUAGCUC **hsa-miR-143-5p** UGAGAUGAAGCACUGUAGCUC **mmu-miR-143-5p** UGAGAUGAAGCACUGUAGCUC **rno-miR-143-5p** UGAGAUGAAGCACUGUAGCUCA **dre-miR-143** UGAGAUGAAGCACUGUAGCUC **ccr-miR-143** UGAGAUGAAGCACUGUAGCU
miR-194a	**ssa-miR-194a-5p** UGUAACAGCAACUCCAUGUGGA **hsa-miR-194-5p** UGUAACAGCAACUCCAUGUGGA **mmu-miR-194-5p** UGUAACAGCAACUCCAUGUGGA **rno-miR-194-5p** UGUAACAGCAACUCCAUGUGGA **gga-miR-194** UGUAACAGCAACUCCAUGUGGA **ccr-miR-194** UGUAACAGCAACUCCAUGUGGA
miR-17a	**Ssa-miR-17a**
miR-27a	**ssa-miR-27a-3p** UUCACAGUGGCUAAGUUCCGCU **dre-miR-27a-3p** UUCACAGUGGCUAAGUUCCGCU **ipu-miR-27a** UUCACAGUGGCUAAGUUCCGCU **hsa-miR-27a-3p** UUCACAGUGGCUAAGUUCCGC **mmu-miR-27a-3p** UUCACAGUGGCUAAGUUCCGC **ssc-miR-27a** UUCACAGUGGCUAAGUUCCGC
miR-27b	**Ssa-miR-27b-3p** UUCACAGUGGCUAAGUUCUGC **hsa-miR-27b-3p** UUCACAGUGGCUAAGUUCUGC **mmu-miR-27b-3p** UUCACAGUGGCUAAGUUCUGC **rno-miR-27b-3p** UUCACAGUGGCUAAGUUCUGC **gga-miR-27b-3p** UUCACAGUGGCUAAGUUCUGC **dre-miR-27b-3p** UUCACAGUGGCUAAGUUCUGC**A**
miR-27d	**Ssa-miR-27d-3p** UUCACAGUGGUUAAGUUCUG **ola-miR-27c-3p** UUCACAGUGGUUAAGUUCUG **dre-miR-27c-3p** UUCACAGUGGUUAAGUUCUG**C** **fru-miR-27c** UUCACAGUGGUUAAGUUCUG**C** **ccr-miR-27c-3p** UUCACAGUGGUUAAGUUCUG**CC** **hsa-miR-27b-3p** UUCACAGUGG**C**UAAGUUCUG**C**
miR-30c	**ssa-miR-30c-5p** UGUAAACAUCCUUGACUGGAAGCU **bta-miR-30e-5p** UGUAAACAUCCUUGACUGGAAGCU **ssc-miR-30e-5p** UGUAAACAUCCUUGACUGGAAGCU **tgu-miR-30a-5p** UGUAAACAUCCUUGACUGGAAGCU **hsa-miR-30e-5p** UGUAAACAUCCUUGACUGGAAG **mmu-miR-30e-5p** UGUAAACAUCCUUGACUGGAAG
miR-30c	**ssa-miR-30c-3p** CUUUCAGUCGGAUGUUUGCAGC **hsa-miR-30a-3p** CUUUCAGUCGGAUGUUUGCAGC **mmu-miR-30a-3p** CUUUCAGUCGGAUGUUUGCAGC **rno-miR-30a-3p** CUUUCAGUCGGAUGUUUGCAGC **gga-miR-30a-3p** CUUUCAGUCGGAUGUUUGCAGC
miR-10a	**ssa-miR-10a-5p** UACCCUGUAGAUCCGGAUUUGU **dre-miR-10c-5p** UACCCUGUAGAUCCGGAUUUGU **fru-miR-10c** UACCCUGUAGAUCCGGAUUUGU **tni-miR-10c** UACCCUGUAGAUCCGGAUUUGU **ccr-miR-10c** UACCCUGUAGAUCCGGAUUUGUG

	hsa-miR-10a-5p	UACCCUGUAGAUCCGAAUUUGUG
	mmu-miR-10a-5p	UACCCUGUAGAUCCGAAUUUGUG
miR-140	**ssa-miR-140-5p**	CAGUGGUUUUACCCUAUGGUAG
	hsa-miR-140-5p	CAGUGGUUUUACCCUAUGGUAG
	mmu-miR-140-5p	CAGUGGUUUUACCCUAUGGUAG
	rno-miR-140-5p	CAGUGGUUUUACCCUAUGGUAG
	dre-miR-140-5p	CAGUGGUUUUACCCUAUGGUAG
	ccr-miR-140-5p	CAGUGGUUUUACCCUAUGGUAG
miR-140	**ssa-miR-140-3p**	UACCACAGGGUAGAACCACGGA
	bta-miR-140	UACCACAGGGUAGAACCACGGA
	oan-miR-140-3p	UACCACAGGGUAGAACCACGGA
	ccr-miR-140-3p	UACCACAGGGUAGAACCACGGA
	dre-miR-140-3p	UACCACAGGGUAGAACCACGGAC
	ssc-miR-140-3p	UACCACAGGGUAGAACCACGGAC

Ssa – *Salmo salar* (Atlantic salmon), Bta – *Bos Taurus* (Taurine cattle), Sha - *Sarcophilus harrisii* (Tasmanian devil), Ccr – *Cyprinus carpio* (Common carp), Fru – *Fugu rubripes* (Japanese puffer), Dre – *Danio rerio* (Zebrafish), Hsa - *Homo sapiens* (Human), Mmu – *Mus musculus* (House mouse), Rno – *Rattus norvegicus* (Brown rat), Ssc – *Sus scrofa* (Wild boar), Ccr – *Cyprinus carpio* (Common carp), Pma – *Petromyzon marinus* (sea lamprey), Ipu – *Ictalurus punctatus* (Channel catfish), Gga – *gallus gallus* (red junglefowl), Ola – *Oryzias latipes* (Japanese rice fish), Tgu – *Taeniopygia guttat* (Zebra finch), Oan – *Ornitorhynchus anatinus* (Platypus)