Atlantic salmon (*Salmo salar L.*) sterol metabolism and metabolic health - impact of dietary lipids

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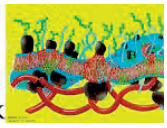

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Nina

Contents

Abbreviations

ARA arachidonic acid

DHA docosahexaenoic acid

EPA eicosapentaenoic acid

ER endoplasmic reticulum

FA fatty acid

FFA free fatty acid

FO fish oil

LAP land-animal by-products

LXR liver x receptor

MAG monoacylglycerol

MUFA monounsaturated fatty acids

PAH polyaromatic hydrocarbons

PG prostaglandin

PUFA polyunsaturated fatty acids

SFA saturated fatty acids

SREBP sterol responsive element binding protein

TAG triacylglycerol

VLC very long chained

VO vegetable oil

Summary

Marine resources are today limited, and due to this there is an increasing inclusion of non-marine lipids in the diet of farmed salmonids. An optimal diet for farmed Atlantic salmon (*Salmo salar* L.) should not only promote fast growth, but also keep the fish at good health, making it robust to face changes and stressors from the surrounding environment. There is, however, still knowledge lacking about the nutritional needs of Atlantic salmon. Lipid sources vary in their fatty acid (FA) composition, as well as in content of other lipid soluble compounds, such as sterols and environmental contaminants. The focus of this thesis has been to study the effect of FAs and lipid soluble compounds on Atlantic salmon lipid metabolism and metabolic health.

Very long-chained n-3 polyunsaturated fatty acids (VLC n-3 PUFAs), typically found in the marine environment, have on several occasions shown to have lipid-lowering properties. The lower dietary and tissue concentrations of these FAs in Atlantic salmon due to the substitution of marine oils with terrestrial oils, may thus have effects on the lipid metabolism. This was studied by performing four dietary trials using feeds with low concentrations of marine oils (0 – 86 g marine oils kg^{-1} feed), and thus also low dietary concentrations of VLC n-3 PUFAs (1.5 - 8.5 % eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) of total FAs and 0.5 – 6.6 % of diet). Lower dietary concentrations of VLC n-3 PUFAs tended to increase liver lipids in all the dietary trials. However, the observed changes in liver lipid content could not solely be explained by dietary concentrations of VLC n-3 PUFAs. Thus, dietary concentrations of saturated fats or plant sterols, commonly known as phytosterols, are hypothesised to affect liver lipid stores. Phytosterols are well-known to lower the uptake and synthesis of cholesterol, and might therefore have caused a cholesterol deficiency in the fish fed the low-marine diets containing low cholesterol levels. The transcription factors affected by phytosterols in the tissues are also involved in processes of lipogenesis, and disturbances in sterol metabolism could therefore affect the lipid storage in the liver. The effects of SFAs could not be separated from the effects of phytosterols with the current experimental designs. Thus, low dietary EPA and DHA concentrations (<2 % of diet) increased the risk of higher lipid depositions in the liver and changes in lipid

metabolism. The magnitude of liver lipid levels were further elevated when low dietary concentrations of EPA and DHA were combined with high dietary phytosterols (1100 mg kg^{-1}) and low saturated fat (<5 % of diet).

The VLC n-3 PUFAs are also potent inhibitors of inflammation, and thus help maintaining a balanced function of the inflammatory system. Decreasing the concentrations of dietary VLC n-3 PUFAs and simultaneously increasing dietary concentrations of the pro-inflammatory n-6 FAs, common in vegetable oils, might skew the balance of the inflammatory system leading to a chronic low-grade inflammation. Temperature-dependant changes in inflammatory status were seen in Atlantic salmon fed very low dietary concentrations of VLC n-3 PUFAs (0.5 % EPA + DHA of diet), indicating dietary EPA and DHA below the minimum requirement for the fish to maintain an optimal inflammatory status. However, no effects on the response to a bacterial infection *in vitro* or on adiposity were seen due to the low dietary concentrations of VLC n-3 PUFAs or n-3/n-6 ratios as low as 1 to 0.3.

Concomitant with changes in lipid composition, are the changes in environmental contaminants delivered via the diet when fish oil is replaced by vegetable oils. Many of these contaminants are highly lipid soluble, enabling them to bioaccumulate in the lipid portion of organisms. The same physical properties enable them to be embedded in biological membranes, where they can perturb the structure, and thus also the function, of the membranes. An increased fluidity of model membranes was observed when adding two different types of typical vegetable oil contaminants, polyaromatic hydrocarbons (PAHs). Some of the observed toxic effect of these compounds on fish could therefore be partially due to this membrane perturbing effect of the PAHs.

The combined effect of non-optimal concentrations of various lipids and lipid soluble compounds might thus negatively affect the health of Atlantic salmon by affecting the liver lipid metabolism as well as inflammation and the physico-chemical properties of the lipid bilayer.

List of Figures

List of Tables

List of publications

Paper I: Liland, N.S., Rosenlund, G., Berntssen, M.H.G, Brattelid, T., Madsen, L. & Torstensen, B.E. (2013a): "Net production of Atlantic salmon (FIFO, Fish in Fish out < 1) with dietary plant proteins and vegetable oils", Aquaculture Nutrition, Vol. 19: 289-300.

Paper II: Liland, N.S., Espe, M., Rosenlund, G., Waagbø, R., Hjelle, J. I., Lie, Ø., Fontanillas, R. & Torstensen, B. E. (2013b): "Diets high in plant protein and phytosterols affect cholesterol metabolism in Atlantic salmon, *Salmo salar* L.", British Journal of Nutrition, Vol. 10: 258-272.

Paper III: Liland, N.S., Hatlen, B., Takle, H., Venegas, C., Espe, M., Torstensen, B. E. & Waagbø, R. (2014): "Including processed poultry and porcine by-products in diets high in plant ingredients reduced liver TAG in Atlantic salmon, *Salmo salar* L.", Aquaculture Nutrition, Available online from 17th of October 2014, DOI: 10.1111/anu.12190.

Paper IV: Liland, N.S., Rocha, S., Pittman, K., Campo, A., Holen, E., Torstensen, B. E., Rosenlund, G. & Sissener, N. H.: "Dietary EPA and DHA and water temperature affect inflammation but not visceral adipose tissue in Atlantic salmon (*Salmo salar* L.)", Manuscript.

Paper V: Liland, N. S., Simonsen, A. C., Duelund, L., Torstensen, B. E., Berntssen, M. G. & Mouritsen, O. G. (2014): "Polyaromatic hydrocarbons do not disturb liquid-liquid phase coexistence, but increase the fluidity of model membranes", Chemistry and Physics of Lipids, Vol. 184: 18-24.

Papers I, II, III and V have been granted reprint permissions from journals.

1. Introduction

The amount of fish produced from aquaculture has had a rapid increase from 14 million tons in 1990 to 60 million tons in 2010, while fish catches from marine capture fisheries have been stable at approximately 80 million tons (FAO 2012, 1994). Fish meal and fish oil (FO) have long been the most important ingredients in fish feed, and a growing aquaculture industry has thus lead to an increased demand of these marine products. A limited production of fish meal and FO and an increased demand of these products due to growing global aquaculture, have led to an extensive use of alternative feed ingredients, mainly of terrestrial origin (Figure 1). Fish are, however, not in need of specific raw materials, but have nutrient requirements they need to cover through the feed. General consensus has been that the fish meal and FO provide nutrients in concentrations which cover the dietary requirements for several nutrients for the fish and by increasing the use of non-marine feed ingredients, some negative effects have been detected, especially in modern intensive culture. Some of the observed effects of diets low in marine ingredients on Atlantic salmon (*Salmo salar* L.) are accumulation of fat in the whole fish, liver and plasma, as well as changes in the lipid metabolism (Jordal *et al.*, 2007, Leaver *et al.*, 2008, Torstensen *et al.*, 2011). These changes can potentially affect both the growth and the welfare of the fish and it is therefore important that the nutrient requirements for Atlantic salmon are decided to allow for formulation of an optimal feed composition, regardless of feed ingredients utilised. Currently, there is an approximate 20 % loss in the seawater stage of cultured salmonids (Hjeltnes, 2014), which could possibly be partially counteracted by optimal formulation of the diets.

Diets low in marine ingredients and high in vegetable oils may be deficient in several lipids, like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), saturated fatty acids (SFAs), and cholesterol, and can also contain high concentrations of typical plant fatty acids (FAs) like 18:1n-9 (oleic acid), 18:2n-6 (linoleic acid) and 18:3n-3 (α-linolenic acid). A large number of studies have been performed to study the effect of non-marine feed ingredients on Atlantic salmon, showing that partially replacing marine lipid sources with vegetable- (Bell *et al.*, 2002, 2001, 1993, 1991, Nanton *et*

al., 2007, Regost *et al.*, 2004, Rosenlund *et al.*, 2001, Stubhaug *et al.*, 2007, Torstensen *et al.*, 2008, 2005, Waagbø *et al.*, 2013) or land-animal by-product (LAP) ingredients (Emery *et al.*, 2014, Hatlen *et al.*, 2013, Higgs *et al.*, 2006, Rosenlund *et al.*, 2001, Turchini *et al.*, 2009) is possible without reducing growth. There are, however, some uncertainties as to which components of the oils are causing the reported negative health effects, and the nutrient requirements are yet to be decided for many essential compounds (NRC, 2011, Torstensen *et al.*, 2013). The aim of this thesis was therefore to investigate the effects of specific nutrients that change in concentrations when switching from a high- to a low-marine diet on the Atlantic salmon, using lipid metabolism and metabolic health as endpoints.

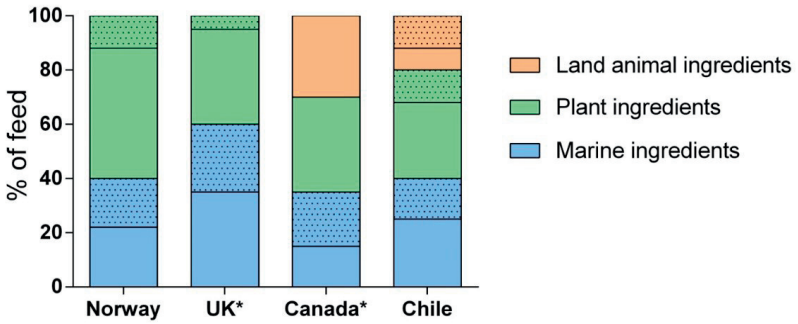

Figure 1: Origin of ingredients used in typical diets for cultured Atlantic salmon in different countries per 2010. Bars marked with * are 2008 values. Lipid ingredients within each class are marked with dots. Source: (Tacon *et al.*, 2011).

2. Aims

The aim of this PhD thesis was to determine the effects on Atlantic salmon lipid metabolism of specific lipids and lipid soluble compounds in the diet. Molecular, chemical, histological and physical methods were used to do a thorough evaluation of how specific nutrients from different dietary oil sources can affect Atlantic salmon performance and health. The PhD thesis will cover the following aims:

- Describe the impact of diets low in EPA and DHA and high in 18 carbon fatty acids on lipid metabolism and lipid accumulation in the liver of Atlantic salmon (Papers I, II and III).

- Elucidate effects of diets low in cholesterol and high in phytosterols on sterol metabolism of Atlantic salmon (Papers II and III).

- Evaluate inflammation status in Atlantic salmon fed diets with low concentrations of EPA and DHA and varying n-3/n-6 ratios (Paper IV).

- Understand the effects of selected contaminants from vegetable oil sources (polyaromatic hydrocarbons) on physico-chemical properties of model membranes (Paper V).

3. Background

Modern diets for farmed Atlantic salmon are high in fat (~40 % of diet), and the composition of the oils used to formulate their diets has a potentially great impact on the fish. Dietary FAs are not only substrates for energy and growth; they also have several roles as bioactive components (Vance and Vance, 2008). This biologically active role of FAs means that they can affect many aspects of health and welfare. There are also other components in the oil sources with potential to affect health and welfare of the fish, such as sterols and contaminants. The effect of specific lipid-soluble compounds from the diet on Atlantic salmon lipid metabolism was the focus of this thesis.

3.1 Fish oil vs. terrestrial lipids

3.1.1 Lipid composition of oils

Marine oils differ from terrestrial oils in their high concentrations of very long-chained n-3 polyunsaturated fatty acids (VLC n-3 PUFA) like 20:5n-3 (EPA) and 22:6n-3 (DHA). Fish oil (FOs) will also vary in their fatty acid (FA) composition depending on the species and also the time of year the fish is caught. Such variations will also be found in VOs, which may contain high levels of shorter n-3 FAs like 18:3n-3 (α-linolenic acid) as well as being rich in n-6 FAs like 18:2n-6 (linoleic acid) and/or n-9 FAs such as 18:1n-9 (oleic acid). Lipids from terrestrial animals, such as poultry, will vary according to their dietary lipid source (Kaul *et al.*, 2008). In addition, FO contains high concentrations of SFAs, and most VOs (except for e.g. palm oil) have low concentrations of SFAs. Poultry oil, however, is an excellent source of SFAs. Lipids from terrestrial animals are also good sources of cholesterol, which VOs do not contain. VOs, on the other hand, contain plant sterols, i.e. phytosterols (Figure 2), which are the plant equivalents of the animal cholesterol. The composition of the sterols in the oils will also vary, with β-sitosterol and campesterol usually being the most abundant in commercially available VOs (Harwood *et al.*, 1994, Phillips *et al.*, 2002). A

presentation of typical FA composition and sterol concentration of different oils is presented in Table 1.

Table 1: Typical fatty acid compositions (weight %) and sterol concentrations (g kg^{-1} diet) of various oils.

	Sardine[1]	Capelin[1]	Rapeseed	Soybean	Linseed	Palm	Sunflower	Poultry[2]
Total SFA	27	18.9	8.0	15.7	10.0	50.4	12.8	23-33
18:1n-9	9.3	6.7	60.1	23.9	18.4	39.1	22.1	24-38
20:1n-9	1.5	15.9	1.4	0.1	-	0.1	0.2	0.4-0.5
22:1n-11	0.9	20.2	0.4	-	-	-	-	<0.1
Total MUFA	25.4	56.2	62.4	24.2	18.5	39.4	22.4	26-46
18:2n-6	1.1	1.3	21.5	52.1	16.8	10.2	65.6	19-42
18:3n-3	0.6	0.7	9.9	7.8	55.0	0.3	0.5	1-27
Total PUFA	39.6	21.1	31.5	59.8	71.8	10.5	66.0	21-49
EPA + DHA	30.7	13.2	-	-	-	-	-	≤0.04
Total n-6	1.9	1.3	21.6	52.1	16.8	10.2	65.6	20-43
Total n-3	37.7	19.8	9.9	7.8	55.0	0.3	0.5	1-27
Ratio n-3/n-6	19.5	15.2	0.46	0.15	3.27	0.03	0.007	0.05-1.2
Phytosterols[3]	-	-	6.1	3.6	4.2	2.5	2.7	-
Cholesterol[4]	7.7	7.1	-	-	-	-	-	8.5

Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Sources: Fatty acids of vegetable oils: Dubois *et al.* (2007); [1] Torstensen *et al.* (2013), [2] Kaul *et al.* (2008), Fatty acid composition of chicken fat pad lipids [3] Harwood *et al. (1994)*; sunflower oil: Phillips *et al. (2002)*, [4] USDA food list, Nutrient data 04542 Fat, chicken, 04590 Fish oil, herring and 04594 Fish oil, sardine.

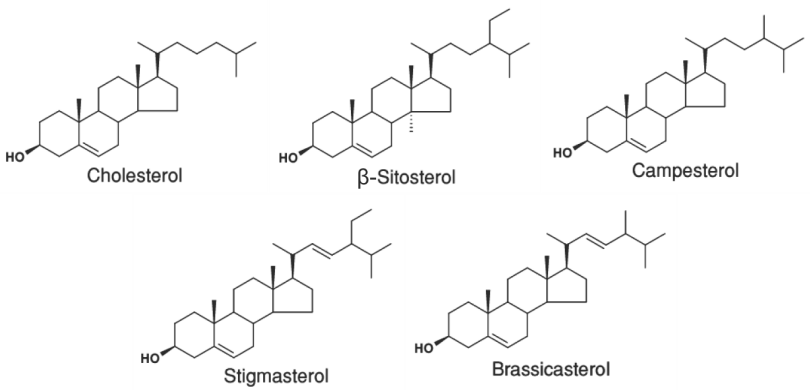

Figure 2: Molecular structures of cholesterol and the most common phytosterols.

3.1.2 Contaminants

All feed ingredients contribute with undesirable substances. However, concentrations and types of undesirable components vary according to origin of the ingredient. Marine lipid sources contain contaminants that bioaccumulate through the trophic levels, like dioxins and PCB. Concentrations of contaminants are higher in organisms at high trophic levels. Due to this, fatty fish and FOs are sources of these typical marine contaminants (Berntssen *et al.*, 2010, 2005). VOs do not contain high levels of marine contaminants, but may, on the other hand, contain other contaminants like pesticides, herbicides and products related to the production of VOs (e.g. polyaromatic hydrocarbons, PAH). A decrease in marine contaminants and an increase in plant contaminants has been reported both in the fish feeds as well as in the fillet of Atlantic salmon during the last decade (Berntssen *et al.*, 2010, 2005, Sanden *et al.*, 2013, Sissener *et al.*, 2013). These plant contaminants are relatively new to the aquaculture industry and the investigation of the mechanics of accumulation and absorption of them is of importance to assess the possible negative implications of using these feed ingredients.

3.2 Lipid metabolism in Atlantic salmon

3.2.1 Lipid absorption

Atlantic salmon are carnivorous and their optimal diet is therefore rich in proteins and fat. Fat concentrations have increased in the diet of cultured salmonids, from ~10 % of feed in the 1970's to the current levels of ~30-40 % (percent of total weight) (Tacon *et al.*, 2011). Most of the dietary lipids are in the form of triacylglycerols (TAG) and the remaining fat will vary according to lipid source, but may also contain sterols, phospholipids, lipid-soluble vitamins and other lipids such as wax-esters (Waagbø *et al.*, 2001). The pyloric caeca and the duodenum are the main sites for lipid absorption in salmonids, where a reduced pH increases the secretion of cholecystokinin which induces the release of bile acids into the intestine. The bile acid increases the surface of the lipids by forming micelles which enhances the uptake through the enterocytes.

In addition to containing neutral lipids, such as TAG, the micelles will also contain other lipid soluble compounds like sterols and vitamins D and A. Inside the micelles, lipases and colipases break the FAs free from the glycerol backbone of the TAG. In humans this results in two free FAs (FFAs) and one monoacylglycerol (MAG), but fish do not have colipase, and thus all three FAs are released from the glycerol due to lack of specificity (Lie and Lambertsen, 1985, Saele *et al.*, 2011, 2010). Once absorbed by the enterocytes, the FFAs will be re-esterified with glycerol to form TAG or with lysophospholipids to form phospholipids. Native chylomicrons, consisting of lipids and enterocyte-produced apolipoprotein B will then transport the lipids to the tissues. Once in the blood stream they take up additional apolipoproteins and are now called mature chylomicrons. There is no defined lymphatic system in teleosts, as there is in mammals (Waagbø *et al.*, 2001). After delivering lipids to the tissues, they return to the liver as chylomicron remnants. Humans produce a shorter intestine-specific version of the apolipoprotein B, called ApoB48, but it is suspected that fish only have the full-length version of this apolipoprotein, called ApoB100 (Conticello *et al.*, 2005). The liver of Atlantic salmon is the main organ for metabolism and further transport of the lipids and is not a lipid-storing organ, as it is in lean fish species, like Atlantic cod (*Gadus morhua* L.) (Bratberg *et al.*, 2013). Once absorbed, the lipids can either be stored, the majority in adipocytes or as intracellular lipid droplets in the muscle and visceral cavity (Nanton *et al.*, 2007), or they can be β-oxidised and converted into energy (Stubhaug *et al.* 2007). In addition, FAs have several other important roles in the organism, such as making up the FA portion of phospholipids in cell membranes, being used as a substrate for eicosanoid production as well as regulating gene expression (Vance and Vance, 2008).

Uptake of sterols from the intestinal lumen to the enterocytes is mediated by Niemann-Pick C1-like 1 receptors (NPC1L1, Figure 3) (Davis *et al.*, 2004, Davis Jr and Altmann, 2009, Othman *et al.*, 2013). Once inside the enterocytes, their uptake into chylomicrons, and thus further transport into the body, is dependent on esterification by Acyl-CoA cholesterol acyltransferases 1 and 2 (ACAT1 and -2). In humans, ACAT1 esterifies phytosterols more efficiently than ACAT2, but the esterification of

phytosterols is still much less efficient than for cholesterol, leading to a lower absorption of phytosterols compared to cholesterol (Temel *et al.*, 2003). The uptake mechanisms of sterols is not yet described in Atlantic salmon. The amount of sterol absorbed will also depend on the speed at which unesterified sterols are pumped back into the intestinal lumen by a dimer of ATP-binding cassette sub-family G member-transporter proteins called ABCG5/8. Much cholesterol is excreted as bile acids, and most of this is reabsorbed from the intestine, making this enterohepatic circulation a crucial step for maintaining cholesterol homeostasis (Redinger, 2003). In humans, ~0.4 - 3.5 % of the phytosterols are absorbed, compared to 35 - 70% of cholesterol (Temel *et al.*, 2003). There is limited knowledge regarding the digestibility of phytosterols in Atlantic salmon, but one trial showed a higher digestibility of phytosterols in Atlantic salmon than in humans, ranging from 2 – 15 % depending on the dietary sterol composition (Miller *et al.*, 2008).

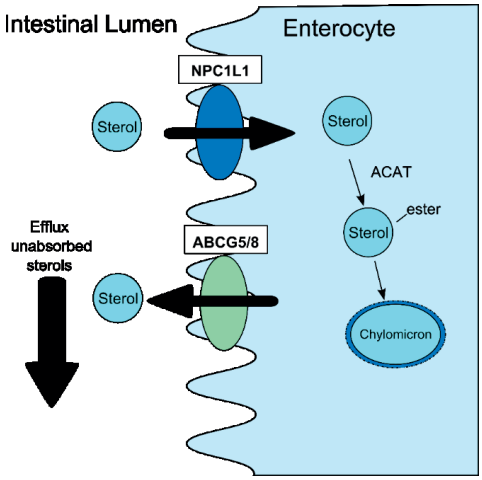

Figure 3: Schematic view of the intestinal absorption of sterols. Adapted from: (Agellon, 2008, Voelker, 2008). Acyl-CoA cholesterol acyltransferases (ACAT), ATP-binding cassette sub-family G 5 and 8 (ABCG5/8); Niemann-Pick C1-like 1 receptor (NPC1L1).

3.2.2 Lipid transport

Apolipoproteins produced in the liver, such as ApoB100, are packed together with lipids into very low density lipoprotein (VLDL) particles, which transport the endogenously produced lipids from the liver to the peripheral tissues. As the lipids from VLDL are absorbed by the tissues, the density of the particle increases, and the particle turns into low density lipoprotein (LDL), which main function is to transport cholesterol to the tissues. Native high density lipoproteins (HDL) particles are also produced in the liver and return lipids and cholesterol from the peripheral tissues to the liver. An increased ratio of LDL- to HDL-cholesterol in plasma is often associated with an increased risk of cardiovascular disease, since less HDL-particles able to bind cholesterol increases the risk of cholesterol accumulation on the arterial walls (Castelli *et al.*, 1992). The American Heart Association recommends that a level of total cholesterol level of 5 mmol L^{-1} or a LDL-C under 3 mmol L^{-1} is an optimal level in humans, protecting from development of atherosclerosis. Atlantic salmon, however, have much higher normal levels of total plasma cholesterol, often higher than 10 mmol L^{-1} (Sandnes *et al.*, 1988), with approximately 80% of the plasma cholesterol as HDL-cholesterol (Farrell and Munt, 1983).

3.3 Dietary lipids and lipid metabolism

As described in section 3.1, multiple factors in the diet change when the FO is replaced with oils of terrestrial origin. Many studies have been performed with Atlantic salmon to elucidate the effects of such diets on the fish health and performance; the effect of VOs on the fish has been an especially investigated topic. A great effort has also been put forth to decide the nutrient requirements in Atlantic salmon for specific FAs and to investigate the effects of single dietary components on the metabolism of the fish. The current status of knowledge in the field is summarized in the coming sections.

3.3.1 Dietary requirements of EPA and DHA

The minimum dietary requirements for EPA and DHA of Atlantic salmon are not yet quantified (Torstensen *et al.*, 2013). Several feeding trials have demonstrated good growth and performance in the seawater stage when EPA + DHA concentration is below 2 % of the diet (Bell *et al.*, 2002, 2001, 1993, Bell *et al.*, 2010, 1991, Nanton *et al.*, 2007, Regost *et al.*, 2004, Stubhaug *et al.*, 2007, Tocher *et al.*, 2003b, Torstensen *et al.*, 2005, 2000). This has also been verified in an industry-scaled trial, with no reduction in any growth or feeding parameter after reducing EPA + DHA from 2.6 % of diet to 1.6 % of diet (Rosenlund *et al.*, 2014). To assess the requirements of marine FAs in seawater stage Atlantic salmon, studies running over longer periods are needed. The fish will have a gradual and slow decrease in tissue concentrations of marine FAs, and several months of feeding a diet low in EPA and DHA is therefore required for the first signs of a potential deficiency to be seen (Unpublished results, Dr. G. Rosenlund). Long-term studies on an industrial scale are therefore needed to evaluate the effects of low-marine diets in a realistic scenario.

Several dietary trials have, however, seen negative health effects due to feeding low-marine diets, such as increased liver lipids (Table 2). An increase in liver lipid is in humans regarded as a negative development in health status, and can lead to reduction- and ultimately loss of function of the organ (Eckel *et al.*, 2005). Table 2 presents the published studies that have reported increased liver lipids in Atlantic salmon when fed low-marine diets. As seen in Table 2, many different lipid sources are reported to increase liver lipids, and there are thus several nutritional factors being the cause leading to increased liver lipid stores.

Table 2: Overview of dietary trials reporting increased liver lipids in Atlantic salmon fed diets low in EPA and DHA.

EPA and DHA [a]	Lipid source	Dietary factors that could have affected lipid accumulation	Duration of trial	Final weight	Reference
4.2 (1.2)	VO blend [b]	Phytosterols and 18:1n-9	42 weeks	2000 g	Petropoulos *et al.* 2009
4.9 (1.6)	Linseed oil or soybean oil	18:3n-3 or 18:2n-6	16 weeks	400 g	Leaver *et al.* 2008
2.2 (0.7)	RAFOA-mix [c]	Phytosterols and 18:1n-9	2 years	4000 g	Jordal *et al*. 2007
4.9 (1.5)	Soybean oil	18:2n-6	26 weeks	3000 g	Ruyter *et al.* 2006
4.5 (1.4)	Linseed oil or rapeseed oil	18:3n-3 or phytosterols and 18:1n-9	50 weeks	2500 g	Tocher *et al.*2003a
3.7 (1.4)	Linseed oil and rapeseed oil (1:1)	18:3n-3, phytosterols and 18:1n-9	2 years	2000 g	Tocher *et al.*2003b
2.5 (0.8)	Linseed oil	18:3n-3	40 weeks	1900 g	Tocher *et al.*2002
5.4 (1.4)	Rapeseed oil	Phytosterols and 18:1n-9	17 weeks	350 g	Bell *et al.* 2001
8.6 (2.8)	RAFOA-mix [c]	18:3n-3, phytosterols and 18:1n-9	1 year	4000 g	Torstensen *et al.* 2011

[a] % of total FA (% of total diet), [b] Rapeseed, palm and camelina oil (50:30:20, by weight), [c] Rapeseed, palm and linseed oils (55:30:15, by vol.).

3.3.2 EPA and DHA as regulators of lipid metabolism

It is known from rodent studies that both EPA and DHA inhibit TAG excretion in hepatocytes (Berge *et al.*, 1999, Ikeda *et al.*, 1998, Nossen *et al.*, 1986, Rustan *et al.*, 1988). Studies on humans with non-alcoholic liver disease (NAFLD) report low EPA and DHA in both liver and other tissues of those who suffer from this disease compared to healthy subjects (Araya *et al.*, 2004). The authors suggests that this could be due to the ability of the VLC n-3 PUFAs to suppress lipogenesis via a transcription factor called sterol responsive element binding protein 1 (SREBP1) (Lee *et al.*, 2008). Also shorter FAs can inhibit SREBP expression, but the inhibiting effect diminishes with shorter chain length and increasing saturation (Worgall *et al.*, 1998). Mammalian SREBP1 exists in two isoforms, namely 1a and 1c (Sul and Smith, 2008). SREBP1c is mainly involved in FA synthesis, whilst SREBP1a can activate both cholesterogenesis and lipogenesis (Amemiya-Kudo *et al.*, 2002). Atlantic salmon SREBP1 has been described, and its functions are similar to those in humans and mice (Minghetti *et al.*, 2011). There is, however, no proof of different isoforms of SREBP1 existing in Atlantic salmon (Kortner *et al.*, 2014, Morais *et al.*, 2011), and the SREBP1 homologue used in this thesis is a sequence shared by both SREBP1a and -1c of mammals. The inhibiting effect of EPA and DHA on lipogenesis has also been shown in Atlantic salmon hepatocytes (Kjær *et al.*, 2008, Leaver *et al.*, 2008, Vegusdal *et al.*, 2005) and in the SHK-1 cell line from Atlantic salmon (Minghetti *et al.*, 2011). Also, a link has been reported between high dietary inclusion of VOs, SREBP expression and increased liver lipids in Atlantic salmon (Leaver *et al.*, 2008). Furthermore, low inclusion of FO and thus low concentrations of dietary EPA and DHA, have also been reported to increased lipid concentrations in liver (Bell *et al.*, 2001, Jordal *et al.*, 2007, Leaver *et al.*, 2008, Petropoulos *et al.*, 2009, Ruyter *et al.*, 2006, Tocher *et al.*, 2003a, 2003b, 2002). By using a triangular design with feeds containing FO, linseed oil or rapeseed oil, Tocher and colleagues (2003a) showed a negative correlation between EPA and DHA in diet and liver lipid concentration. Additionally, VLC n-3 PUFAS regulate the expression of liver x-receptor (LXR), a transcription factor that also regulates several lipogenic processes (Sul and Smith, 2008) (Figure 4). It is therefore clear that by replacing large proportions of the dietary FO with terrestrial lipid sources,

accumulation of lipids in the liver is one of the unwanted consequences, and that this could occur due to low dietary concentrations of EPA and DHA. The studies that have shown increase in liver lipids in Atlantic salmon due to FO replacement are listed in Table 2.

VLC-PUFAs are not efficiently produced in most vertebrates, and an insufficient intake of such FAs will lower their concentrations in the membrane phospholipids (Waagbø *et al.*, 1993). The FA composition of the membrane phospholipids will affect the physical aspects (thickness, fluidity) of the membrane (Murphy, 1990); with VLC-PUFAs rendering the membrane more fluid than SFAs. In addition, membrane 20 carbon FAs are substrates for other physiological processes in an organism and can affect the functioning of membrane-embedded proteins (Hulbert *et al.*, 2005). Eicosanoids, such as prostaglandins and leukotrienes, are important signalling molecules in inflammatory reactions and are produced from VLC-PUFAs released from the cell membranes by lipases (Calder, 2001). Inflammation is an organisms' defence system, and consists of reactions within an organism to protect against harmful stimuli like pathogens and injuries by removing the objects causing the harm followed by repair of the tissue. A well-functioning inflammatory system will be adaptable; increasing its activity when stressors are present and decreasing when the threat is over to avoid unnecessary damage to the tissues. The VLC n-3 PUFAs are precursors for a group of eicosanoids, commonly given anti-inflammatory properties due to their lower ability to initiate inflammatory reactions than their n-6 counterparts. The n-6 derived eicosanoids are the most abundant in most animals, but a lower synthesis of the n-3 derived eicosanoids can skew the balance between the anti- and pro-inflammatory eicosanoids and result in a chronic low-grade inflammation (Calder, 2009, 2006). A chronic low-grade inflammation is, together with increased liver lipids, some of the symptoms associated with the lifestyle disease termed 'metabolic syndrome' in humans (Grundy *et al.*, 2004).

Tissue concentrations of VLC n-3 PUFAs are naturally higher in Atlantic salmon than in humans, and they therefore have a higher production of the anti-inflammatory n-3 derived eicosanoids (Araujo *et al.*, In Press). ARA, is, however, still the most important eicosanoids precursor in Atlantic salmon (Araujo *et al.*, In Press, Bell *et al.*, 1996, Holen *et al.*, In Press). Also the immune system of the fish can be affected by changes in the diet, and Atlantic salmon fed diets low in both n-3 and n-6 VLC-PUFA had lower production of prostaglandins in gills (Tocher *et al.*, 2003b), in stimulated blood cells (Bell *et al.*, 1993), and kidney macrophage-enriched leucocytes (Bell *et al.*, 1996). This could be associated with a lower ability to respond to infections. With the decreasing concentrations of VLC n-3 PUFAs in the diet of Atlantic salmon, it is important to assess the effects on the inflammatory system, and the influences of the changes in eicosanoid production on the robustness of the fish.

3.3.3 18 carbon fatty acids

Increased Atlantic salmon liver lipids is also positively correlated with high inclusions of VOs with high concentrations of 18 carbon FAs (Table 2). One study showed a correlation with dietary 18:2 n-6, but not 18:3n-3, and hepatic lipid concentrations (Tocher *et al.*, 2003a). As mentioned in the previous chapter, VLC n-3 PUFAs inhibit the lipogenic transcription factor SREBP1, and results from mice have shown an inhibiting effect on SREBP also by shorter FAs like 18:1n-9, 18:2n-6 and 18:3n-3 (Thewke *et al.*, 1998). It is, however, not certain if the 18 carbon FAs can function as independent inhibitors, or if they only have an enhancing effect of other SREBP agonists, like sterols (Worgall *et al.*, 1998) (section 3.3.5).

Higher concentrations of 18:2n-6 in the Atlantic salmon diet have shown to increase the production of n-6 derived eicosanoids *in vivo* (Bell *et al.*, 1996,1993, 1992, Gil Martens *et al.* 2010) as well as *in vitro* (Araujo *et al.*, In Press, Holen *et al.*, 2011). The 18 carbon FAs can, however, only affect the eicosanoid production after having been elongated to FAs with 20 carbons or more, and are therefore not as efficient as the VLC PUFAs at changing the eicosanoids production (Calder, 2006, 2001). Thompson and colleagues (1996) reported increased mortality in Atlantic salmon fed sunflower oil

after a challenge test with *Aeromonas salmonicida* and *Vibrio anguillarum* compared to fish fed FO. This was thought to be due to the very low n-3/n-6 ratio (0.30) in the fish fed the n-6 rich sunflower oil diet. A similar trial was repeated by Gjøen and colleagues (2004), using soybean oil as lipid source (n-3/n-6 = 0.24), reporting no difference in mortality between the soybean oil fed fish and the FO control after an *A. salmonicida* challenge test. A study by Waagbø *et al.* (1993) showed that dietary lipids affected disease resistance (*Vibrio salmonicida*) differently and temperature dependently. The current studies on immune response and effect of dietary lipids are showing varying results, and more studies on this topic are therefore needed to understand the complex mechanisms involved.

3.3.4 Saturated fats

In addition to a decreased dietary concentration of VLC n-3 PUFAs, all the diets used in the feeding trials presented in Table 2 (except for the trials using palm oil in the VO blend) had a decrease in SFAs. Lowering dietary SFAs increases feed utilisation in Atlantic salmon, especially at low temperatures (Emery *et al.*, 2014, Menoyo *et al.*, 2003, Ng and Gibon, 2011, Ng *et al.*, 2004, Torstensen *et al.*, 2000), but it is not known how much SFA the Atlantic salmon require for maintaining optimal function (Torstensen *et al.*, 2013). Using rapeseed oil as only lipid source led to increased liver lipids in Atlantic salmon (Bell *et al.*, 2001) and this effect was not present in a similar feeding trial using palm oil as lipid source (Bell *et al.*, 2002), indicating dietary fatty acids provided by the vegetable oil affected the liver lipid levels to a higher extent than the decrease in marine lipids.

Membrane content of SFAs have been shown in humans and rodents to largely unaffected due to diet composition (Hulbert *et al.*, 2005), which also seem to be the case for Atlantic salmon (Bell *et al.*, 1996, Ruyter *et al.*, 2006). There is, however, a strong effect of temperature on the FA composition of the membrane phospholipids in fish (homeoviscous adaptation) (Sinensky, 1974), and an increased temperature will decrease the PUFA concentrations and increase concentrations of SFAs (Hazel, 1979). Due to the current low dietary concentrations of SFAs in the typical diet for cultured

salmonids, it is of importance to determine the optimal dietary concentration of SFAs in the Atlantic salmon diet.

3.3.5 Sterols

Phytosterols are proven to lower circulating LDL-cholesterol in humans (Jones *et al.*, 1997). They are also efficient at lowering plasma lipids and increasing the beneficial HDL-cholesterol when administered together with VLC n-3 PUFAs (Micallef and Garg, 2008). It is thought that phytosterols compete with cholesterol for a space in the micelles, leading to a lower uptake of cholesterol when phytosterols are introduced to the diet (Ikeda *et al.*, 1989). More recent work, however, indicate that this is only part of the mechanism, and that the effect of phytosterols might be caused by the phytosterols directly affecting the proteins involved in sterol uptake, like NPC1L1 (Brown *et al.*, 2010, Calpe-Berdiel *et al.*, 2009) (Figure 3).

There are only a few studies on phytosterols in fish, and these have reported reduced plasma cholesterol concentrations and changes in the endocrine system when providing phytosterols to fish via the water (Tremblay and Kraak, 1999, Tremblay and Van Der Kraak, 1998) or with implants in the visceral cavity (Gilman *et al.*, 2003). Diets high in phytosterols have shown to reduce cholesterol digestibility also in Atlantic salmon (Miller *et al.*, 2008), which is suspected to cause a cholesterol deficiency in fish fed high-plant diets due to the already low dietary cholesterol concentrations in such diets (Carmona-Antoñanzas *et al.*, 2014, Leaver *et al.*, 2008, Torstensen *et al.*, 2013). Low cellular levels of cholesterol releases the transcription factor SREBP2 from its membrane-bound form, which then enter the nucleus and initiate transcription of mRNA translating into proteins responsible for the production of cholesterol (e.g. mevalonate kinase (MVK) and 7-dehydroxycholesterolsynthase (DHCR7) (Figure 4).

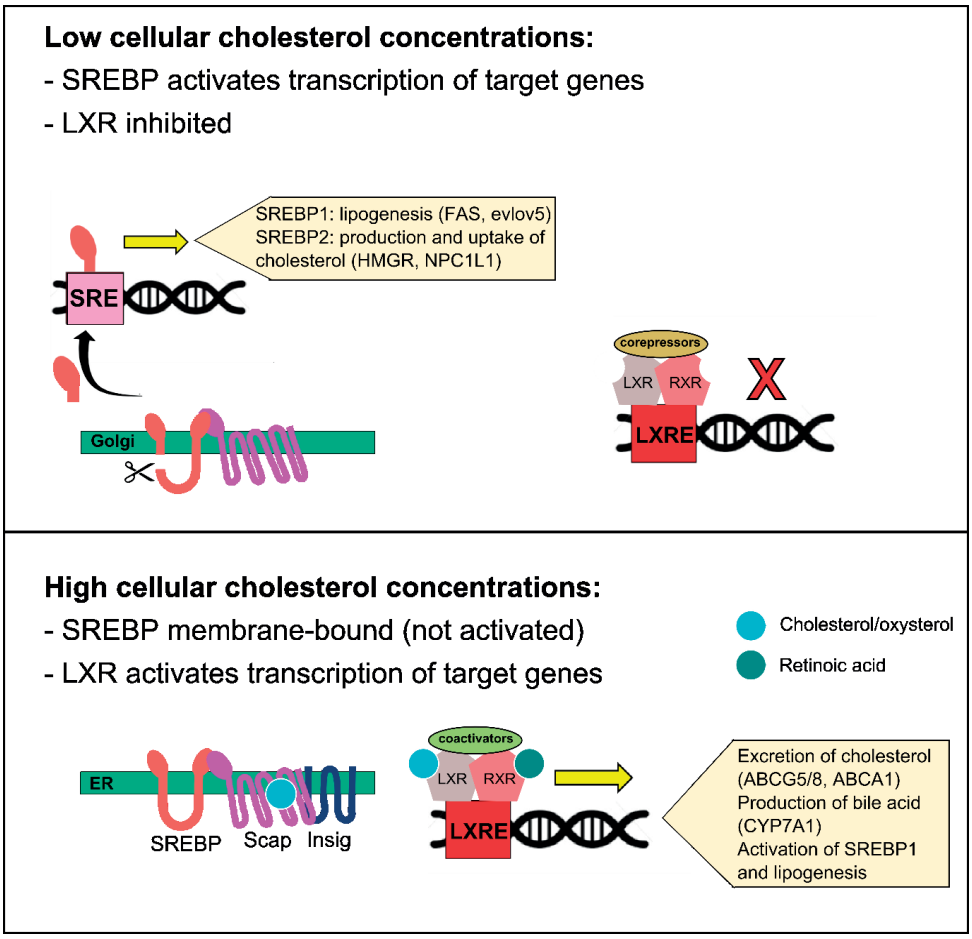

Figure 4: Illustration of LXR and SREBP activation and expression. In the presence of cholesterol, SREBPs are bound to the endoplasmic reticulum (ER) membrane in a complex with Insig and Scap. Low cholesterol will release Insig from the ER and Scap will chaperone SREBP to golgi where it is processed into mature SREBP. SREBP then enter the nucleus to activate transcription of genes involved in cholesterol and lipid synthesis by binding to the sterol responsive element (SRE). LXR is, in the case of low cholesterol, inactive and bound to the LXR response element (LXRE) in a heterodimer with RXR, bound to corepressors and repress the expression of target genes. In the presence of ligands (e.g. oxysterols), the corepressors will be released and replaced with coactivators, for subsequent transcription of genes involved in cholesterol excretion and lipogenesis. Sources: (Alrefai *et al.*, 2007, Chiang *et al.*, 2001, Ducheix *et al.*, 2013, Horton *et al.*, 2002).

Oxidised cholesterol, i.e. oxysterols, is a sign of high cellular cholesterol levels and will activate LXR to initiate processes involved in the excretion of cholesterol (Figure 4). Phytosterols can act as cholesterol 'mimics' if tissue concentrations are sufficiently high, disrupting the cholesterol homeostasis by inhibiting the processing of SREBP2 and activating LXR in mice (Kaneko *et al.*, 2003, Yang *et al.*, 2004). Since both LXR and SREBPs are known to activate processes of lipogenesis it is possible that the influence of phytosterols could increase lipid production in the liver (Arts and Kohler, 2008). This is supported by a study where mice over-expressing SREBP had an enlarged fatty liver (Shimano *et al.*, 1996). Since several feeding trials with typical high-phytosterol lipids (like rapeseed oil) have reported increased liver lipids, the link between this dietary component and lipid metabolism needs to be elucidated.

3.3.6 Contaminants

One type of contaminant found in VOs are polyaromatic hydrocarbons (PAHs). PAHs are compounds produced under incomplete burning processes and are introduced to the VOs by direct-fire drying of the grains or oilseeds (Larsson *et al.*, 1987, SCF, 2002, Speer *et al.*, 1990). In this group of compounds, most attention has been focused on benzo[a]pyrene, which is officially labelled as carcinogenic (IARC, 2010, 1983, 1973). Many other PAHs also have well-documented toxic effects (NLM, 2014) which are hypothesised to be caused partially by their ability to insert into biological membranes due to their highly lipophilic properties (Mackay *et al.*, 2006, Schirmer *et al.*, 1998, Sikkema *et al.*, 1995). Because they are also slightly soluble in water they may be presented to aquatic organisms and have been shown to be very toxic in aquatic environments (Schirmer *et al.*, 1998, Tuvikene, 1995). However, when PAH are presented to the fish via feed, they are in a lipophilic medium, which might increase the uptake, especially of the larger and more hydrophobic PAHs like benzo[a]pyrene (Castelli *et al.*, 2002, 2001). With increasing concentrations of plant ingredients in the feeds, it is of importance to investigate the toxic mechanisms of such typical VO contaminants. The focus in this thesis was the incorporation of PAHs into synthetic model membranes, to assess possible physico-chemical changes in the membrane.

4. Methodological Considerations

4.1 Experimental design

The research within salmonid aquaculture nutrition has during the last decade evolved from focusing on the suitability of various sources of ingredients, to focusing on separate nutrients and their requirements. The dietary trials included in this thesis had experimental designs focusing on specific nutrients, to be able to detect differences due to changes in lipid composition, such as n-3/n-6 ratio (Paper I and II) and EPA and DHA (Paper IV). Crystalized amino acids were added in all the dietary trials, to ensure that the studies could focus on the effect of the lipids, and not limitations of essential amino acids. The dietary trial presented in Paper III was designed to look at the suitability of LAP ingredients in combination with plant ingredients and was thus more an ingredient trial than focusing on specific nutrients. However, in the latter trial (Paper III), the diets were balanced in EPA and DHA, so that the dietary effects would be due to the other lipids varying between the diets, e.g., SFAs. Moving from the more basic research of suitability of various sources of ingredients to more nutrient-specific needs, is an important step towards a more flexible use of ingredients. Dietary requirements have been determined for many dietary components such as vitamins and amino acids (NRC, 2011), but much work is still lacking in the area of lipids (Torstensen *et al.*, 2013). Simple endpoints such as growth or feed efficiency are important to have an idea of the basic functionality of the feeds and rearing conditions, but in modern intensive aquaculture the fish are exposed to a variety of stressors and are selected for a faster growth than what they would experience in the wild. The diets should also fulfil other aims, like helping the fish to be as robust as possible to tolerate stress and infections.

All the selected endpoints used to measure effects of diets in this thesis were chosen due to their relevance towards the general robustness of the fish, and their potential as sensitive markers for diet-induced changes in metabolism. Both *in vivo* and *in vitro* measurements were made, and chemical, molecular and histological examinations were performed to look for effects of the diets, as well as for increasing the knowledge on

uptake and metabolism of lipid nutrients. One reoccurring effect on the fish were increased lipid concentrations in liver. This has also been seen by others before, but it has been difficult to pinpoint the cause for the increase in liver lipid concentrations (Table 2). This is partially because a change in liver lipid concentrations can be induced by several different factors independently. However, it is also partially because any feeding trial where you want to study the effect of replacing a given ingredient or reducing the concentrations of a lipid, you have to add something else in its place. This means that there will be an effect of adding components to the diets, as well as the effect of reducing other factors in the diet. For example, when you reduce the dietary FO inclusion and replace it with soybean oil, you increase the n-6 FA concentrations, on the expense of VLC n-3 PUFAs and SFAs. The observed effects on fish lipid metabolism and health could thus be caused by any of lipids, or a combination. Due to this complicating factor, several feeding trials with different setup and design made to isolate the effects of single nutrients are needed to be able to investigate the effects of specific nutrients on the metabolism of the fish. In this thesis the designs were made to be able to see the effects of varying dietary n-3/n-6 ratios and phytosterol concentrations (Paper II), as well as being able to separate the effect of phytosterol concentrations from phytosterol/cholesterol ratios (Papers II and III).

4.2 Assessing steatosis in Atlantic salmon

Diagnosing fatty liver (steatosis) is well-described for humans, defined as when >5 % of the hepatocytes have visible lipid droplets at 10× magnification (Sanyal *et al.*, 2011). Similar methods have also been used in this thesis (Paper III), using earlier described criteria for Atlantic salmon (Martinez-Rubio *et al.*, 2013). The criteria for categorizing liver lipids (level 0: no steatosis - level 5: severe steatosis) are slightly different, e.g. level 1 steatosis when >10 % of hepatocytes have lipid vacuoles at 20× magnification. However, in this thesis, liver TAG concentrations has been the main measure for assessing steatosis. Also liver enzymes associated with liver damage (aspartate transaminase, AST, and alanine transaminase, ALT) (Wu, 2002) were measured in plasma in all the dietary trials. The HPTLC method (Bell *et al.* 2003) used for analysis

of lipid classes, and thus TAG in this thesis, uses chemical visualization of double bonds as detection principle. A confounding factor of this method is that a sample containing more unsaturated FAs will give higher signal than samples with more SFAs. This can, to some extent, be corrected by calculating the number of double bonds per gram sample with results from an analysis of FA composition from the same tissue to calculate a correction factor for each sample. In all the results presented from this method in this thesis, the amount of double bonds in the same sample has been calculated and, based on those numbers, a correction factor for each sample was calculated, followed by normalisation of the data. This gives a more reliable comparison of liver lipid levels between the dietary trials where different FA compositions have been used in the diets, and ensures that the differences seen in liver TAG are reflecting the true differences in lipid levels. In all cases, the adjusted values showed that the differences between the groups were not overestimated by the HPTLC method, and that the method rather underestimated the differences between the dietary groups. As an example, the mean liver TAG concentrations of the PP-RO fed fish were 2.6x higher than the PP-FO fed fish (Paper I), and the adjusted values showed a mean difference of 3.4x. The same trend was also seen for the other dietary trials.

It is also relevant to determine which concentrations of liver lipids are within a normal range and when an increase in liver lipids evolve into a pathogenic state in Atlantic salmon. As mentioned above, histological methods are used for this in human diagnosis of fatty liver and has also been used in this thesis (Paper III) (Martinez-Rubio *et al.*, 2013). Recent unpublished results from Atlantic salmon are showing that histological methods are more sensitive to changes in lipid accumulation, since they can detect differences in size and number of lipid droplets without the total amount of lipids in liver differing (Dr. M. Sanden). This emphasizes the need for developing and testing new methods for early detection of metabolic imbalances. However, more information is needed on when the Atlantic salmon liver loses or changes its functions as a result of lipid accumulation. An increase in liver lipids could potentially be used as an early marker for nutritional imbalances, as it in humans is associated with other typical signs of sub-optimal diets, such as diabetes and hyperlipidemia (Poynard *et al.*, 2005). The

sensitivity of the various methods for measuring liver lipids should be investigated, to be able to choose the method giving the most accurate results. Standardised methodology for assessing the potential negative fish health effects of increased liver lipids in Atlantic salmon is required.

4.3 Solid-supported membranes as models for biological systems

Amphipathic substances like phospholipids have the ability to form vesicles in a charged environment, and this is the basis of the formation of all living cells. Eukaryotic cell membranes consist of a phospholipid bilayer, embedded with proteins and other functional components such as glycoproteins (Figure 5a) (Nicolson, 2013, Singer and Nicolson, 1972). The lipids of the cell membranes were earlier thought to be only a carrier medium for the other components in the membrane, but newer discoveries have unveiled their great importance in membrane homeostasis and function (Bagatolli *et al.*, 2010). Although there are many other components in a cell membrane, it is the physical properties of the lipids that it is built up from that lay the principal rules for membrane behaviour. Solid-supported lipid bilayers have gained popularity as a model for cell membranes, due to their simple design, fast preparation and the possibility to combine them with high-resolution imaging tools, like atomic force microscopy (AFM) and fluorescence microscopy (Veatch and Keller, 2005). These model membranes usually have an extremely simplified composition, commonly containing only two or three types of phospholipids and often no proteins at all. Compared to cell membranes, which are complex mixtures of lipids and with a great array of proteins embedded into them (Figure 5a), the model membranes might seem overly simplified (Figure 5b). The advantage of such a simplified system is that it will represent the pure effect of lipids on the membrane properties, excluding possible protein-effects. Changes in lipid behaviour will also be easier detected than when using cell membranes or more complex lipid bilayers (Korchowiec *et al.*, 2008). One method used to detect influences by contaminants in the lipid bilayer is differential scanning calorimetry (DSC), which detects the changes in enthalpy and temperature when a lipid

bilayer goes from a solid gel-phase to a more liquid phase when heated (Figure 6). Lipophilic compounds inside the membranes can change both the enthalpy and temperature change between these two phases compared to a control membrane without any additional compounds, and give indications on where these compounds position and how they influence the bilayer properties by either fluidizing it or making it more rigid (Castelli *et al.*, 2002). It is also possible to use this technique on cell membranes, but due to its complex composition, the transition temperature will be much broader and small effects will be difficult to detect. Model membranes are therefore more sensitive to small changes in lipid behaviour.

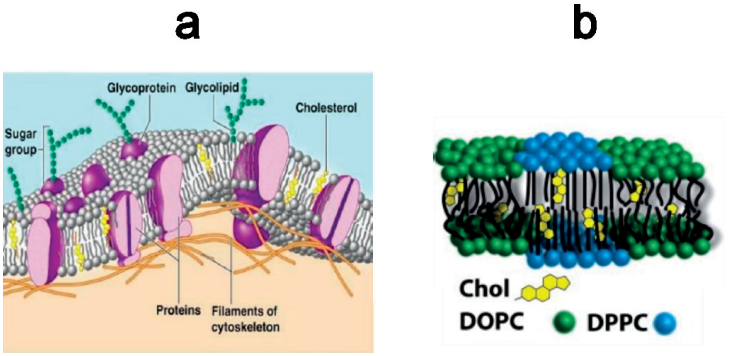

Figure 5: (a) Illustration of a cell membrane according to the fluid mosaic model. According to this model, the liquid lipid bilayer contains proteins and glycolipids that move freely around. Some structures are less mobile and are connected to the cytoskeleton. Source: www.sd84.k12.id.us/shs/departments/science/yost/Biology/ (b) Structure of a tri-lipid bilayer with less liquid regions (blue) consisting of saturated fat and higher concentrations of cholesterol. Dioleoylphosphocholine: DOPC; Dipalmitoylphosphatidylcholine: DPPC. Source: (Fritzsching *et al.*, 2013).

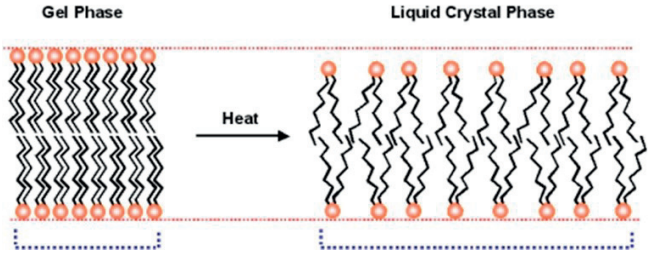

Figure 6: A lipid bilayer exposed to heat will undergo structural changes and have an increased disorder as well as increased area.

5. Discussion

Dietary lipids can affect a whole range of metabolic systems in the salmon, including the hepatic lipid metabolism, the inflammatory response and possibly also altering the physical properties of the cell membranes (Figure 7, Papers I, II, III, IV and V). Comparing liver TAG, measured in the dietary trials presented in Papers II and III, show that elevated concentrations in Paper II, were much lower (~2×) than the highest liver TAG concentrations measured in Paper III (Figure 8). When considering the dietary compositions of the trials, it is clear that no single factor, such as EPA and DHA, phytosterols or SFAs (Figures 9 - 11), can explain the variations in liver lipids independently. Low dietary EPA + DHA concentrations (< 2 % of diet), however, seem to have a negative effect on the metabolic health and increased lipid accumulation in the liver of Atlantic salmon. High dietary phytosterol/low cholesterol and/or low dietary concentrations of SFAs further increased liver TAG concentrations, possibly leading to additional changes in metabolic health. The possible mechanisms behind the observed effects of the low-marine diets are discussed in the following chapters.

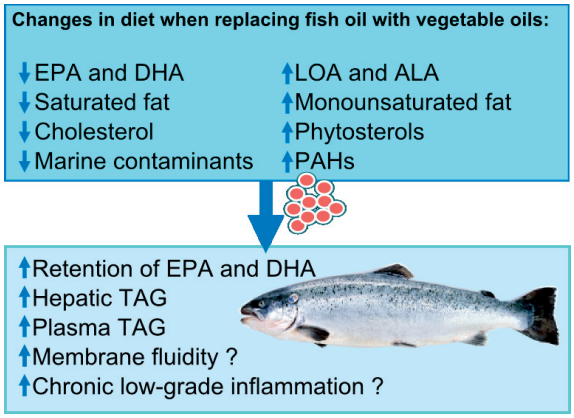

Figure 7: Summary of some dietary components changing when fish oil is replaced with vegetable oils and the effects seen in on Atlantic salmon eating feeds containing vegetable oils in this thesis. ALA, 18:3*n*-3 α-linolenic acid; DHA: 22:6n-3 docosahexaenoic acid; EPA, 20:5*n*-3 eicosapenteanoic acid; LOA, 18:2*n*-6 linoleic acid; PAH, polyaromatic hydrocarbons; TAG, triacylglycerol.

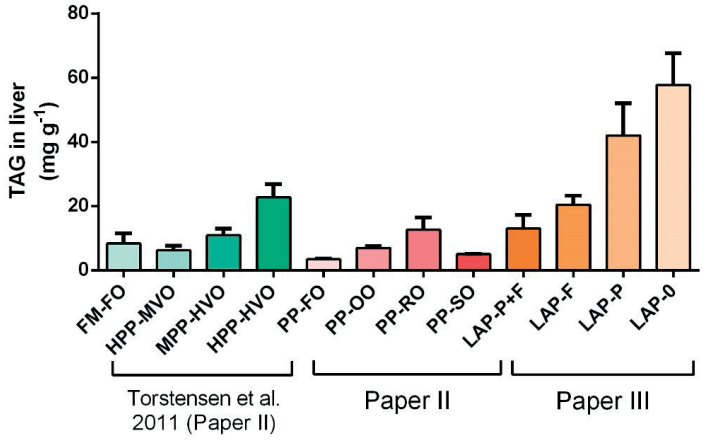

Figure 8: Liver TAG concentrations from trials presented in Papers II and III.

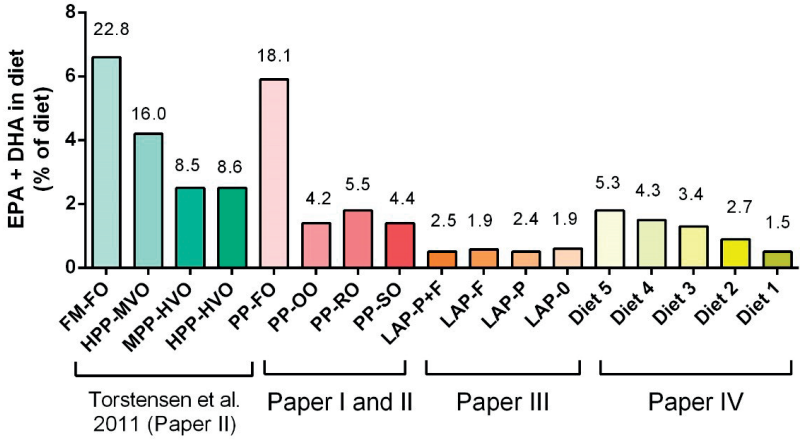

Figure 9: Dietary concentrations of EPA + DHA of the diets of all trials presented in thesis. Values for EPA + DHA concentrations as percent of total FAs are marked with numbers over the bars.

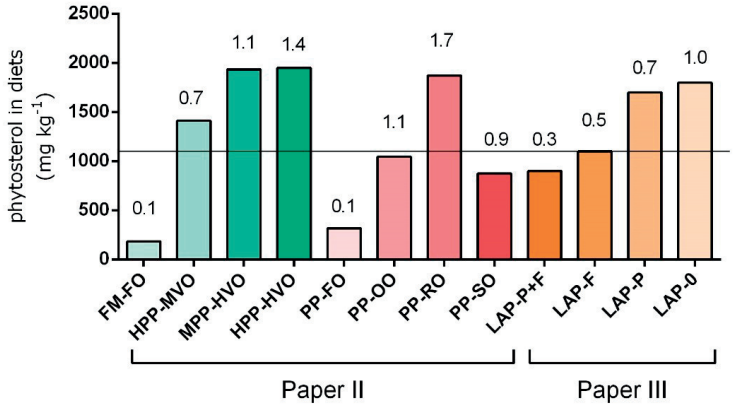

Figure 10: Dietary concentrations of phytosterols in the trials presented in this thesis where liver lipids have been measured. The ratios of phytosterol / cholesterol are denoted as numbers over each bar. The horizontal line is denoting the suggested limit of phytosterols where accumulation of dietary phytosterols in liver increases (1100 mg kg^{-1})(see section 5.4).

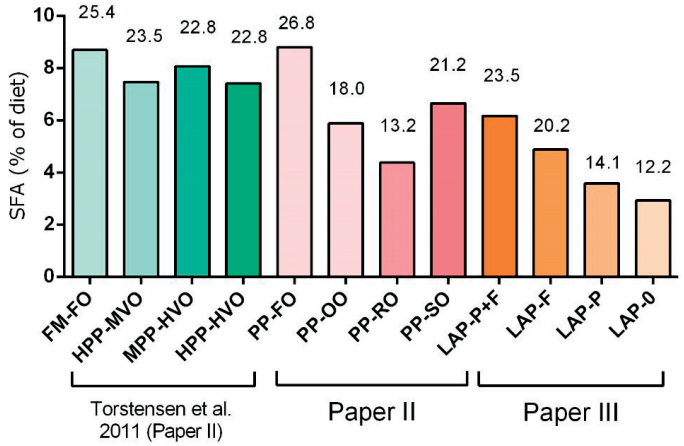

Figure 11: Dietary concentration of saturated fats (SFAs) in the trials presented in this thesis where liver lipids have been measured. Values for SFA concentrations as percent of total FAs are given as numbers above the bars.

5.1 Growth and survival on low-marine diets

There was low mortality, good growth and no external signs of poor health in any of the dietary trials of this thesis, indicating well-performed trials with diets fulfilling the basic nutrient requirements for growth and survival (Papers I, II, III and IV). Reduced growth due to low dietary concentrations of low FO inclusion has been reported earlier (Bell *et al.*, 2010, Grisdale-Helland *et al.*, 2002, Torstensen *et al.*, 2004) (0.4 % to 3.0 % EPA + DHA of diet). But, by using balanced mixtures of VOs there are no reductions in growth or feed efficiency, even in long-term feeding trials with EPA and DHA concentrations as low as 0.5 % of the diet (Papers I, III, IV, Torstensen *et al.*, 2005, Turchini *et al.*, 2009). The only increase in mortality seen in the trials of this thesis was registered in the fish fed a high n-6 diet (PP-SO: 70% of protein sources were plant protein and 70% of lipid sources were soybean oil) after an intermediate weighing (unpublished results from the feeding trial presented in Paper I, N. S. Liland). This was hypothesised to be related to the handling stress experienced by the fish when weighed, and could mean that diets with high concentrations of 18:2n-6 (39 % of dietary FAs) give a sub-optimal stress-response. This has also been noted earlier, where a greater transport-induced mortality was observed in Atlantic salmon fed sunflower-oil diets, also with high concentrations of 18:2n-6 (44 % of dietary FAs)(Bell *et al.*, 1991). Such observations emphasise the importance of finding early markers to monitor the health of the fish; growth may not deviate from the predicted, and a lower robustness might thus not be detected until the fish is subjected to stress or disease.

5.2 Dietary lipids and liver lipid metabolism

The liver is one of the main organs for lipid metabolism in Atlantic salmon, being involved in lipid β-oxidation (Stubhaug *et al.*, 2007, 2005), TAG- and lipoprotein synthesis (Kjær *et al.*, 2008, Moya-Falcón *et al.*, 2006, Vegusdal *et al.*, 2005) as well as being an important organ for uptake of lipids through the synthesis of cholesterol and bile (Cruz-Garcia, 2010, Kortner *et al.*, 2014, 2012, Leaver *et al.*, 2008). Any imbalance in the uptake-, production- or β-oxidation of FAs can lead to accumulation of lipid droplets in the hepatocytes, which is one of the symptoms of metabolic

syndrome in humans (Grundy *et al.*, 2004, Nguyen *et al.*, 2008). In mammals, an increase in liver lipids, also called non-alcoholic fatty liver disease (NAFLD), may lead to dyslipidemia and type 2 diabetes through important transcription factors like SREBP and PPAR (Malaguarnera *et al.*, 2009). Changes in the hepatic metabolism may also affect the plasma composition of Atlantic salmon, as the liver secretes both cholesterol and TAG in VLDL particles (Lie *et al.*, 1993). Changes in the metabolic processes of the liver therefore have the potential to affect metabolism and health of the whole body. In the trials presented in this thesis, the general trend was that the lower the dietary EPA and DHA concentrations (Figure 9), the more liver TAG was seen (Figure 8). The differences in EPA and DHA concentrations in the diets could, however, not explain the differences within each trial. The liver TAG concentrations obtained from high-performance thin layer chromatography (HPTLC) and histological analysis of lipid infiltration in liver showed the same trends of increasing liver lipids with higher plant ingredient inclusion (Paper III). Both methods therefore proved valid for determining liver lipid accumulation in that trial (Paper III).

Which levels of steatosis that could be considered pathogenic are not yet known. Total liver lipids in the current trials ranged from ~5 - 6 g 100 g^{-1} (Paper II) and ~6 - 11 g 100 g^{-1} (Paper III). Other published values on total liver lipids in Atlantic salmon have ranged from 3.3 - 7.3 g 100 g^{-1} (Bell *et al.*, 2002, 2001, Jordal *et al.*, 2007, Leaver *et al.*, 2008, Petropoulos *et al.*, 2009, Ruyter *et al.*, 2006). The liver lipid concentrations in Paper III are thus higher than most earlier reported concentrations. In the case of liver damage, however, human plasma concentrations of liver enzymes (AST and ALT) increase up to 20-fold compared to in subjects with healthy livers (Wu, 2002). No changes in Atlantic salmon plasma concentration of these liver enzymes (Paper I and unpublished results) indicate that no severe liver damage occurred in any of the trials included in this thesis. Fatty liver can be a symptom of intake of oxidized oils, partly due to the lowered levels of the potent antioxidant Vitamin E in oxidized oils (Mourente *et al.*, 2002, Tacon, 1992, Waagbø, 2006). There were, however, no signs of increased oxidation in the liver (TBARS) of the fish in the current feeding trials (unpublished results from trials in Paper II, III and IV).

Both the VLC n-3 PUFAs as well as phytosterols are known to affect transcription factors important for lipid homeostasis, like SREBP and LXR (sections 3.3.2 and 3.3.5). It has been suggested that Atlantic salmon diets high in VO might be too low in cholesterol (Torstensen *et al.*, 2013), although the optimal levels of dietary cholesterol for Atlantic salmon requirement is not yet determined (NRC, 2011). Since tissue phytosterols can increase cholesterol efflux and reduce cholesterol synthesis in mice (Yang *et al.*, 2004) (section 3.3.5), increased dietary phytosterols could elevate the risk of a cholesterol deficiency in Atlantic salmon. A phytosterol-induced increase in the cholesterol excretion combined with a reduced cholesterol synthesis could increase the risk of cholesterol deficiency at already low dietary concentrations of cholesterol.

5.3 Fatty acids as inhibitors of lipogenic processes

Atlantic salmon preferentially store VLC n-3 PUFAs in the tissues and also produce them from shorter and less unsaturated FAs through the action of desaturases and elongases, when supplied in low concentrations in the diet (Ruyter *et al.*, 2003, Sanden *et al.*, 2011, Stubhaug *et al.*, 2007, Tocher *et al.*, 2003a). This was also seen in the trials of this thesis, where the dietary EPA + DHA concentrations were lowered from ~5 % of total FAs (Paper I) to ~1 % of FAs (Paper III) (Figure 9), with no changes in the concentrations of EPA + DHA in the lipids of the whole fish (~6-7 % of total FAs, Figure 12). The EPA and DHA concentrations in whole fish expressed as g kg^{-1} were slightly lower in Paper III than in Paper I and II, but this was due to the smaller size and lower total lipid content of the fish in Paper III.

Currently, there is a need for determining the requirement for dietary EPA and DHA for commercially reared Atlantic salmon (Torstensen *et al.*, 2013). The assumed safe lower limit of dietary EPA + DHA based on current knowledge is ~5 % of total FAs, or 2 % of diet, although it has also been reported earlier that ~1 % EPA + DHA of diet is enough for salmon fry (Ruyter *et al.*, 2000a, b). A trial was performed feeding Atlantic salmon diets with constant EPA + DHA concentrations (~0.5 % of diet) in a two-way ANOVA design using a high-plant diet (LAP-0) and then partially replacing either the lipid and/or protein with LAP ingredients (Paper III). These diets (Paper III) provoked

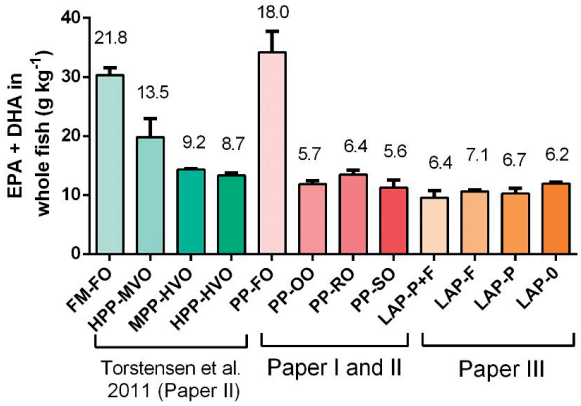

Figure 12: Whole fish concentrations of EPA and DHA from trials presented in thesis. Values for EPA and DHA concentrations as percent of total FAs are given as numbers above the bars.

the highest liver TAG concentrations of this thesis (Figure 8). This same dietary trial also had the lowest dietary concentrations of EPA + DHA of all the trials in this thesis where liver lipids were measured (Figure 9). SREBP activity is inhibited by VLC n-3 PUFAs in mice (Xu *et al.*, 1999a, 1999b, Yahagi *et al.*, 1999), so it is possible that the constant and low dietary concentrations of VLC n-3 FAs in Paper III led to an increased activity of SREBP1 and the lipogenic pathways that this transcription factor activates, ultimately resulting in increased liver TAG. The fact that no difference in the expression of *srebp1* was seen, could be indicating that the SREBP1 protein activity is regulated post-transcriptionally in Atlantic salmon, as it is in mice (Hoang *et al.*, 2012), or simply that all the dietary groups had an increased expression of this gene. Since there are several steps in the activation of SREBP, involving intracellular transport, post-translational modifications of protein structure, and binding to other proteins (Figure 4), it is likely that the regulation of SREBP1 activity is not solely on transcription level. Unpublished results suggest that VLC n-3 PUFAs could be regulating SREBP activity post-transcriptionally also in Atlantic salmon (Dr. M. Sanden). Due to the balanced concentrations of dietary VLC n-3 PUFAs (Paper III), the differences between the dietary groups in liver TAG of this feeding trial could not be attributed to differences in dietary VLC n-3 PUFAs. The increased expression of a FA desaturase, *fads1*, in the fish fed highest dietary inclusion of VO (LAP-0), however, indicates that some lipogenic pathways were activated. The lack of a direct relation within each dietary

trial between dietary EPA and DHA concentrations, gene expression and liver TAG, was also confirmed in the two feeding trials presented in Paper II, where Atlantic salmon were fed diets with large variations in EPA + DHA concentrations (1.4 - 6.6 % of diet) by replacing marine ingredients with plant protein and different types of VOs. Hence, there were no indications that the measured differences in gene expression or liver TAG were directly related to the EPA and DHA concentrations in the diets (Papers II and III).

The current results indicate that dietary EPA + DHA concentrations under ~2 % of diet increase the risk of increased liver TAG deposition and altered lipid metabolism (Papers II and III). The effects seen in the fish cannot, however, be explained solely by dietary EPA + DHA and other dietary components must also interact, affecting the lipid depositions in the liver of Atlantic salmon fed low-marine diets.

5.4 Imbalances in sterol metabolism leading to lipogenesis

Accumulated tissue phytosterols disrupt cholesterol homeostasis in mice, assumed to be due to the phytosterols acting as LXR agonists and inhibiting the cleavage of SREBP (Hoang *et al.*, 2012, Yang *et al.*, 2004). The uptake of phytosterols from the diet is, however, negligible compared to cholesterol (section 3.2.1) and only around 1 % of dietary phytosterols will be stored in the tissues (Sanders *et al.*, 2000, Yang *et al.*, 2004). Atlantic salmon liver phytosterol concentrations ranged from ~1% of total sterols to ~6 % of total sterols, depending on the dietary phytosterol concentrations (Paper II). Phytosterol concentrations in tissues and plasma of humans will, however, not only depend on the phytosterol concentrations in the diet, but also on the metabolic processes regulating uptake and excretion of sterols (Othman *et al.*, 2013) (section 3.2.1). This seems to be the case also for Atlantic salmon, where the dietary phytosterol concentrations (Figure 10) did not give a dose-dependent increase in the tissue concentrations of phytosterols (Figure 13) (Papers II and III). The current results suggest a threshold for the accumulation of phytosterols in the liver of Atlantic salmon, where dietary concentrations over 1100 mg kg^{-1} gave an approximate two-fold increase

in liver phytosterol concentrations compared to dietary phytosterol concentrations below 1100 mg kg^{-1}. Dietary and tissue phytosterols in mice affect sterol metabolism through interactions with sterol-regulated transcription factors, such as SREBP and LXR (Hoang *et al.*, 2012, Yang *et al.*, 2004). It is thus possible that feeding high dietary phytosterol concentrations to Atlantic salmon (>1100 mg kg^{-1}) will affect the same systems in Atlantic salmon.

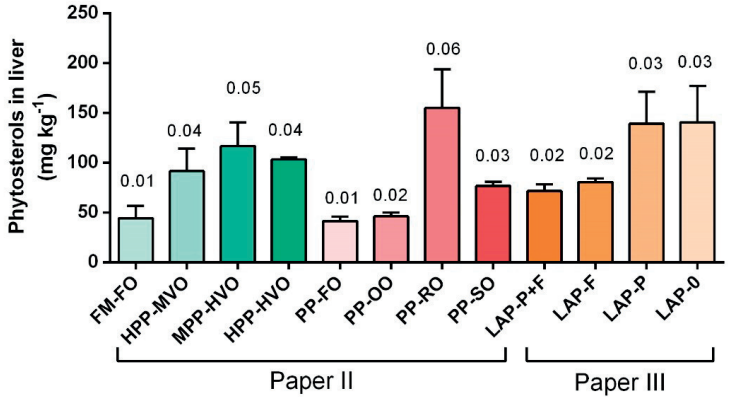

Figure 13: Comparison of liver phytosterol concentrations of the fish presented in Papers II and III. The ratios of phytosterol / cholesterol are given as numbers above each bar.

Vertebrates have the ability to produce cholesterol from acetate, and do therefore not have a requirement for cholesterol (Hellman *et al.*, 1954). Phytosterols, however, affect the cholesterol homeostasis by reducing the uptake of cholesterol as well as lowering cholesterol production and increasing cholesterol efflux (Calpe-Berdiel *et al.*, 2009). Atlantic salmon fed a high-phytosterol diet (PP-RO, 1872 g phytosterols kg^{-1} diet) during 6 months did not increase its cholesterol uptake or production when fed low dietary cholesterol concentrations. Fish fed VO diets with similar cholesterol concentrations and lower phytosterol concentrations, however, did upregulate the cholesterol production pathways (PP-OO and PP-SO, 1044 and 874 g phytosterols kg^{-1}, respectively) (Paper II). The PP-RO fed fish also had a highly fluctuating expression of *lxr* and one of its target genes (*cyp7a1*, rate-determining protein for bile acid production). The tissue phytosterol/cholesterol ratios in the PP-RO fed fish were ~2-3

times higher than in the other dietary groups (Paper II) and the phytosterols could therefore have been acting as cholesterol agonists; inhibiting the expression of SREBP and activating LXR and their respective target genes (Hoang *et al.*, 2012, Laraki *et al.*, 1993, Yang *et al.*, 2004). Since both SREBP and LXR are known to affect processes of lipogenesis (Figure 4), also in Atlantic salmon (Cruz-Garcia *et al.*, 2009, Kortner *et al.*, 2014, Minghetti *et al.*, 2011), the reported disturbances in the sterol metabolism (Paper II) might also affect the lipid metabolism. The liver TAG concentrations were higher in the PP-RO fed fish compared to the other dietary groups in that feeding trial and mirrored the liver phytosterol concentrations, supporting the hypothesised connection between the lipid- and sterol metabolism (Paper II). Phytosterols have shown to affect LXR and SREBP activity without promoting lipid production in mice (Hoang *et al.*, 2012, Yang *et al.*, 2004). Mammals, however, have two isotypes of SREBP, SREBP1a and -1c, each with a slightly different effect on the cholesterol and lipid metabolism (Amemiya-Kudo *et al.*, 2002), whilst only one version of SREBP1 has been detected in Atlantic salmon (Minghetti *et al.*, 2011). There could therefore be less plasticity in the salmon's response to increased phytosterol concentrations in the tissues, ultimately resulting in unintended changes in the liver lipid homeostasis, here manifested as increased liver and plasma TAG.

Two different designs regarding dietary phytosterol concentrations (Paper II and III) enabled us to determine whether it was the dietary phytosterol concentrations or the phytosterol/cholesterol ratios that decide the magnitude of the metabolic response to phytosterols. The most pronounced effects on the expression of *srebp*, *lxr* and the genes activated by these transcription factors (*npc1l1*, *dhcr7*, *cyp7a1*), were seen due to large differences in liver phytosterol/cholesterol ratio (0.01 – 0.06, Paper II). When liver phytosterol concentrations increased two-fold, but with only small differences in the liver phytosterol/cholesterol ratio (0.02 – 0.03, Paper III), there were no detectable differences in the liver expression of the above mentioned genes. However, in spite of little difference in liver ratios of phytosterols/cholesterol (Paper III), the liver lipids increased with higher dietary and liver phytosterol concentrations, indicating that the lipid metabolism of the fish was affected by the phytosterol in the diets (Paper III).

Both SREBP and LXR are known to be strongly post-transcriptionally regulated (Knight *et al.*, 2005, Xu *et al.*, 1999a), and unpublished results indicate that the same might be true for Atlantic salmon SREBP (assessed by a gel-shift assay) (Sanden *et al.*, 2014). However, in order to have a realistic assessment of the activity and diet-effects of LXR and SREBP in Atlantic salmon, more knowledge need to be gained on the mechanisms regulating their activity and the methods for detecting the amount of active transcription factors need to be further developed.

5.5 Role of saturated fat in lipid metabolism

In humans, diets high in saturated fat have long been associated with negative health effects such as hepatic steatosis and cardiovascular disease (Hu and Willett, 2002, Riccardi *et al.*, 2004). More recent work, however, indicate that the connection between SFAs and such negative health effects is not as clear after all (Siri-Tarino *et al.*, 2010). SFAs, once assumed less metabolically active than unsaturated fats, are now identified as having important roles in regulation of metabolism and gene expression (Legrand and Rioux, 2010). Atlantic salmon is a species possibly adapted to quite high concentrations of SFAs, since the marine food chain often contain significant amounts of SFAs (reflected in the high-marine diets FMFO and PPFO, Figure 11). It is also suggested that there is an optimal dietary level of SFAs for Atlantic salmon, and that some VO diets based on rapeseed oil might be too low in SFAs (Torstensen *et al.*, 2013). Some of the diets with high concentrations of VOs presented in this thesis were not particularly low in SFAs, due to the inclusion of palm oil. However, the PP-RO diet (Papers I and II), and the LAP-0 diet (Paper III), both had the lowest concentrations of dietary SFAs in their respective trials (< 5 % of diet). The fish fed these diets also had the highest concentrations of liver TAG in their respective trials (Figure 8). By using poultry oil as a lipid source (Paper III), SFAs were included in the diet and the liver lipids were lowered. An increased concentration of SFAs thus decreased the lipid accumulation in liver, but the simultaneous decrease in dietary phytosterols makes it impossible to separate the effects of SFAs from the effects of phytosterols in the current results. As mentioned in section 3.3.4, two separate, but similar, trials showed that by

using palm oil instead of rapeseed oil in diets for Atlantic salmon, an increase in liver lipids was prevented (Bell *et al.*, 2002, 2001). Whether this was due to the reduction in phytosterols or the increased SFAs could, however, not be deduced from these two studies.

SFAs are also important components of the cell membranes, and their concentration in fish membranes will vary according to water temperature to maintain optimal membrane fluidity (homeoviscous adaptation) (Hazel, 1979) (section 3.3.4). Low dietary concentrations of SFAs could therefore affect the ability of the fish to maintain optimal membrane fluidity at different water temperatures, especially at high temperatures. The current results thus indicate that dietary concentrations of SFAs below 5 % of the diet could be a risk factor for metabolic stress and increased lipid depositions in the liver. It remains to be elucidated whether there is an optimal level of SFAs for Atlantic salmon and which metabolic pathways are affected by the SFAs.

5.6 Lipid interactions on metabolic health

Based on the current results, only minor changes in liver TAG will develop due to influence of dietary phytosterols and or/SFAs as long as EPA + DHA concentrations are >2 % diet (Papers II and III). Dietary phytosterols is the most likely factor to explain many of the differences within each trial in both gene expression as well as liver TAG (section 5.4). In Paper III the highest liver TAG concentrations of this thesis were seen, in spite of the small size of the fish in Paper III (~800 g, compared to ~2500 g in Paper II) and the shorter feeding trial (4 months, compared to the 12 and 6 months in Paper II). Lipid storage in Atlantic salmon fillet and visceral fat stores typically increase with the size of the fish (Olsen and Skjervold, 1995), however this is not the case for liver lipid stores which is generally low and below 4 % of the liver weight (Bell *et al.*, 2002, 2001, Jordal *et al.*, 2007, Leaver *et al.*, 2008, Petropoulos *et al.*, 2009, Ruyter *et al.*, 2006) (section 5.2). Earlier studies have also shown dietary-induced changes in liver TAG concentrations of Atlantic salmon after only 3 months of feeding (Torstensen *et al.*, 2011). This makes liver TAG a good marker for changes in lipid metabolism, even

in shorter feeding trials. Nevertheless, longer trials are needed to assess long-term effects on lipid metabolism, since increases in liver TAG have been shown to revert over time in some VO fed fish (Torstensen *et al.*, 2011).

The current results thus indicate that feeding Atlantic salmon diets with dietary phytosterol concentrations above 1100 mg kg^{-1} lead to fish that do not absorb or produce more cholesterol as a response to lowered dietary cholesterol concentrations (Papers II and III). Cholesterol is crucial for many processes in the body, such as for maintaining membrane fluidity as well as for digestion of lipids as bile acids, and cellular levels of cholesterol are therefore tightly controlled by efficient systems present in all cells (Goedeke and Fernandez-Hernando, 2012). As discussed in chapter 5.3, low dietary concentrations of EPA + DHA (<2 % of diet) are likely affecting the same metabolic pathways as the phytosterols (SREBP and LXR). When low dietary EPA and DHA is combined with high phytosterol/low cholesterol, the result is an even higher liver lipid accumulation than when only one of these factors are present (Papers II and III). Thus, in diets with low dietary concentrations of cholesterol combined with low dietary VLC n-3 PUFA concentrations, commonly seen in low-marine/high-plant diets, attention should be paid to the phytosterol concentrations in the diets to maintain the cholesterol and lipid homeostasis of the fish. In contrast to what is seen in mice (Alvheim, 2012), there was no effect of changes in dietary n-3/n-6 ratio on liver lipid levels (Paper II). How SFAs act on the lipid metabolic health of Atlantic salmon is not clear, and the effects of using low dietary SFA concentration combined with low EPA and DHA and high phytosterol/low cholesterol in the diets should be investigated further.

5.7 Lipid-induced inflammation

Inflammation is a set of crucial reactions to external injuries and infections, aiming to accelerate healing and remove the components leading to infection (Calder, 2001, Waagbø, 2006). To prevent that the inflammatory system attacks healthy tissue, the inflammation needs to be activated only by injury or infection and should cease as soon as the threat is over (Calder, 2009). The VLC PUFAs of the membrane phospholipids,

are important for regulating the inflammatory mechanisms, as they are used for the production of inflammatory eicosanoids (section 3.3.3). One common symptom of metabolic syndrome in humans is a chronic low-grade inflammation, associated with increased plasma concentrations of pro-inflammatory cytokines and inflammation in the visceral fat (Grundy *et al.*, 2004). In humans, the VLC n-3 PUFAs reduce inflammation (Calder, 2006, Klein-Platat *et al.*, 2005). Fish have a "simpler" immune system than mammals, relying on more non-specific defence mechanisms, but involve many of the same systems (Trichet, 2010), and it is thus expected that lowered dietary and tissue concentrations of VLC n-3 PUFAs compared to the n-6 FAs could lead to a higher inflammation also in Atlantic salmon.

The effect on the Atlantic salmon inflammatory status of low dietary EPA + DHA (starting at 1.8 % of diet and reduced down to 0.5 % of diet in a regression design) at two different water temperatures (6 °C and 12 °C) was investigated (Paper IV). After twelve months on the low EPA and DHA diets at 6 °C, there was an increased expression of several genes related to inflammatory response (*cox2*, *alox5* and *nosip*) in the visceral fat of the fish fed the lowest dietary concentrations of EPA + DHA (0.5 % of diet). There were no differences in gene expression in the fish kept at 12 °C, but plasma concentrations of a potent pro-inflammatory eicosanoid produced from VLC n-6 PUFAs (PGE_2) were ~9× higher in the fish fed the lowest dietary EPA + DHA (0.5 % of diet) compared to the fish fed the highest dietary EPA + DHA (1.8 % of diet). The fish reared at 6 °C showed no differences in plasma PGE_2 concentrations between the groups fed the highest and lowest dietary concentrations of EPA and DHA. Thus, the dietary effects on plasma PGE_2 concentrations appear to be dependent on temperature; low EPA + DHA concentrations (0.5 % of diet) at 12 °C led to large increases in plasma PGE_2 in all fish, whilst this at 6 °C only changes in gene expression of inflammation markers was seen. Dietary EPA + DHA concentrations at 0.5 % of the diet might thus be too low for an optimal inflammatory status in Atlantic salmon. There was, however, no effect on the inflammation in visceral fat or head kidney of varying the dietary n-3/n-6 ratio (Paper IV).

In mammals, obesity results in more adipocytes bursting due to their large size (Murano *et al.*, 2008). This cell-rupturing is associated with inflammation in the visceral fat, visible in the tissue as immune cells surrounding the dead adipocytes, called crown-like structures (CLS) (Hertzel *et al.*, 2008) (Figure 2 in Paper IV). Mice fed diets high in n-6 FAs are more prone to develop both obesity and obesity-related disorders like chronic low-grade inflammation (Alvheim *et al.*, 2013, Midtbo *et al.*, 2013). There was no effect of lowering the dietary EPA and DHA concentrations (1.8 to 0.5 % of diet) or changing the dietary n-3/n-6 ratio on the density of CLS in visceral fat of Atlantic salmon (Paper IV). There were also no signs that low dietary EPA + DHA (0.5 % of diet) or changes in concentrations of dietary n-6 FAs caused any change in adiposity (Paper IV). Atlantic salmon is a species naturally adapted to increasing body weight and adiposity rapidly in periods of high food-availability, and might thus not have the same negative health effects of visceral lipid storage as mammals. In contrast to what appeared to be the case for liver lipid metabolism, the largest effects on inflammation were seen as changes in gene expression and plasma concentrations of PGE_2; not at tissue level (CLS in visceral adipose tissue, Paper IV). The optimal endpoint to be measured to find the early effects of diets is thus dependant on the metabolic systems being studied.

Dietary lipids affect the immunity of fish (section 3.3.3), but the mechanisms responsible for these effects are not completely understood (Trichet, 2010, Waagbø, 2006). Head kidney cells were isolated (Paper IV) to investigate whether the dietary lipids would affect the response to addition of bacterial lipopolysaccharide (LPS, a component of the bacterial cell wall), as has been shown to due earlier (Holen *et al.*, 2011). This is an *in vitro* method for assessing the response to a bacterial infection, and can give information on how the diet affects immune response. The gene expressions of several inflammatory markers (*cox2*, *il8, tnfα* and *il1β*) were measured, but there were no clear trends indicating that the disease resistance was compromised by reducing the dietary concentrations of EPA + DHA from 1.8 % of diet to 0.5 % of the diet.

5.8 Lipids and lipid-soluble contaminants affect cell membranes

FAs, sterols and lipid soluble contaminants accumulate in the cell membranes and alter their physical properties in humans (Cornell and Separovic, 1983, Endo *et al.*, 2011, Murphy, 1990) as well as in Atlantic salmon (Bell *et al.*, 1996, Ruyter *et al.*, 2006, Waagbø *et al.*, 1993). Typical VO FAs are shorter than the marine VLC n-3 PUFAs and thus make thinner membranes, whilst cholesterol thickens the membrane (Mouritsen and Zuckermann, 2004). It is thus possible for the dietary lipids to change the thickness of the cell membranes. This can have a severe effect on cell viability, since many membrane-embedded proteins have optimal function at a defined membrane thickness (Mouritsen and Zuckermann, 2004). Additionally, some contaminants, such as benzo[a]pyrene and phenanthrene, might increase the fluidity of the membranes (Paper V). This was seen in work performed on model membranes, and measured as changes in enthalpy and the temperature at which the lipids in the membrane go from a solid to a liquid-crystalline state (Figure 7). This fluidizing effect was, however, only seen for the two largest molecules investigated in the cited work, and not for the smallest of the compounds, naphthalene. The reported morphological changes in fish intestine after feeding diets with PAHs (Bravo *et al.*, 2011), may be related to this fluidizing effect of PAHs (Paper V). The smaller PAHs, like naphthalene, have earlier shown higher toxicity in fish when delivered via water, than the larger PAHs (Schirmer *et al.*, 1998). This could simply be due to a more efficient delivery of the smaller PAHs owing to their lower hydrophobicity compared to the larger PAHs. When these compounds are delivered via the dietary lipids, instead of via the water, it is possible that the delivery efficiency of the larger and extremely hydrophobic PAHs could increase (Castelli *et al.*, 2002, 2001), resulting in a stronger toxic effect.

Phytosterols can also be embedded in the membranes, and increased phytosterols in cell membranes can have negative effects on membrane properties, such as more rigid erythrocytes resulting in reduced life-span in stroke-prone rats (Ratnayake *et al.*, 2000). An increased membrane phytosterol concentration is also suspected to interfere with the proper functioning of membrane-embedded proteins (Clayton Md *et al.*, 1998). The

PAHs have a similar structure as cholesterol, consisting of several ring structures (Figure 1 in Paper V), and were therefore hypothesised to have cholesterol-like structuring effects on membranes. In the model system used (Paper V), there were, however, no signs of the PAHs having any cholesterol-like effect on the membranes. This confirms earlier studies, where even very small changes in the molecular structure of cholesterol remove its unique membrane-ordering properties (Mouritsen and Zuckermann, 2004). The only effect seen of PAHs on the membranes was the earlier mentioned fluidizing effect of phenanthrene and benzo(a)pyrene (Paper V), a commonly reported effect of small lipid-soluble compounds capable of embedding in membranes (Duelund *et al.*, 2012). When the membrane-perturbing effects of phytosterols is combined with the fluidizing effect of the PAHs (Paper V), the result could be cells more prone to rupture due to changes in external factors such as temperature or chemical exposure. Additional alterations induced by changes in membrane FA composition, could further strengthen these effects. Both individual and interaction effects of such typical VO components, like FAs, phytosterols and contaminants, on membrane properties are therefore interesting topics for further investigation.

6. Conclusions

- Diets low in dietary EPA and DHA can lead to increased lipid depositions in liver, but mainly in interaction with other dietary lipids. Low saturated fat (<5 % of diet) and high phytosterol concentrations (>1100 mg kg^{-1}) in combination with dietary EPA + DHA under ~2 % of diet increases the risk of depositing more lipids in the liver. It is, however, not clear at which concentrations the liver lipids negatively affect the health of the fish.

-The sterol and lipid metabolism, mediated through transcription factors SREBP and LXR, are affected by accumulations of phytosterols in the liver. The increased liver lipid concentrations in fish fed diets high in phytosterols might thus be partly due to changes in the activity of these transcription factors and their target genes.

- Inflammation was increased in Atlantic salmon fed diets low in dietary EPA + DHA (0.5 % of diet), leading to increased plasma PGE_2 concentrations in fish reared at 12 °C and increased expression of inflammatory markers in the visceral fat in fish reared at 6 °C. There were no signs of the low EPA + DHA diets (0.5 – 1.8 % of diet) changing the Atlantic salmon resistance to bacterial infections *in vitro*. The dietary concentrations of n-6 FA did not affect inflammation, and neither low dietary EPA + DHA nor varying n-3/n-6 ratios affected adiposity or the density of inflammatory cells in visceral fat.

- The highly lipophilic contaminants benzo[a]pyrene and phenanthrene affected the fluidity of model membranes. This could be related to size or lipophilic properties, as the smaller naphthalene did not show the same effects. The use of solid-supported lipid bilayers as models for biological membranes show a potential for further use within aquaculture lipid research.

7. Future Perspectives

Defining the nutrient requirements for Atlantic salmon is important for the growing salmonid aquaculture. With changing water temperatures and constant disease and parasite pressure, it is crucial for the fish to be robust and able to handle stressors without reducing its performance of welfare. The feed is a major factor to improve fish robustness to handle these challenges. The optimal dietary levels of cholesterol, saturated fats and EPA and DHA in various life stages need to be quantified.

The concentrations of liver lipids considered harmful for the fish health needs to be decided. This would make it easier to compare results between dietary trials and enabling the use of this parameter as a health estimate. There is also a need for more knowledge on the mechanisms regulating the activity of SREBP and LXR in Atlantic salmon.

The inflammatory system is a complex mechanism, involving a variety of eicosanoids from both n-3 and n-6 VLC PUFAs. It is important to get a better picture of which eicosanoids and cytokines are involved in which inflammatory processes in Atlantic salmon, and of how dietary factors can affect inflammation and immunity. The normal range of plasma eicosanoids in Atlantic salmon should also be determined, to enable the use of this parameter as a measure of inflammatory status.

Environmental contaminants are suspected to have a negative impact on human metabolic health (Thayer *et al.*, 2012). Considering the current knowledge on the effects of high-plant diets on Atlantic salmon metabolic health, this is a potentially interesting connection to investigate. Additionally, the interaction effects between nutrients and contaminants on Atlantic salmon metabolic health should be a studied topic in the years to come.

With the experiences made in the area of physical chemistry, a clear potential has been seen in the use of model membranes to assess effect of lipid soluble dietary components on fish health. This is a fast and precise method, able to give valuable information about

the physico-chemical effects of changes in the diets. Reduced saturated fat could have consequences for membrane fluidity, especially together with PAHs or other fat-soluble contaminants. Also, membrane thickness as a result of typical VO FAs, contaminants and other lipid soluble compounds should be addressed, as this can have effects on the function of membrane-embedded proteins and thus affect processes involving cell signalling, osmoregulation and immunity.

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