The effect of plant proteins and vegetable oils on the sterol metabolism of Atlantic salmon *(Salmo salar)*

Master in Nutrition of Aquatic Organisms in Aquaculture

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Acknowledgments

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The whole food chain project

This master thesis is part of a 3-year project funded by the Norwegian research council (199626). The project objective is to investigate the effect of replacing high portions both fish meal and fish oil with plant protein and vegetable oils on the "whole food chain" of Atlantic salmon farming; from fish feed to fish health and finally health effects on consumer eating salmon fed novel diets (see figure below for a schematic overview over the project). The experimental feeds will be evaluated for their safety and the health of the fish and the health effect of eating salmon fed novel feeds will be studied, focusing on obesity and cardiovascular diseases. By investigating effects of the plant-based feed on the Atlantic salmon and the consumers, the aim is to increase the knowledge about the effect in the whole food chainto consumer health.

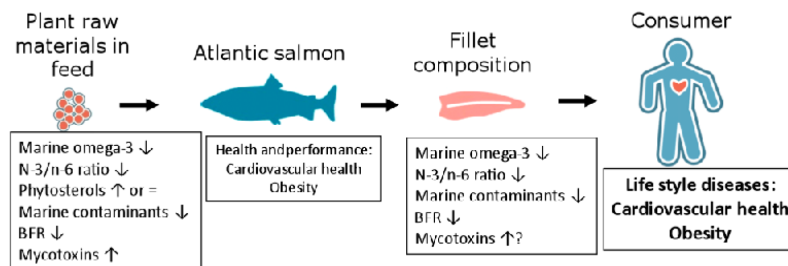


FIGURE 1: OUTLINE OF THE WHOLE FOOD CHAIN PROJECT, BFR: BROMINATED FLAME RETARDANTS.

Abstract

To ensure responsible use of the valuable marine ingredients a major effort is being invested in understanding effects of replacing fish meal and fish oil partially or completely with plant proteins and vegetable oils in aquaculture diets. Decreased dietary n-3/n-6 ratio and cholesterol levels and the introduction of vegetable oil derived phytosterols may affect Atlantic salmon health as well as nutritional product quality.

Atlantic salmon plasma cholesterol is naturally very high being more than twice the upper range for healthy humans (11 mM in salmon vs 5 mM for healthy humans). Increased plasma and LDL cholesterol is a known risk factor for the development of cardiovascular disease in humans and phytosterols are known to lower plasma cholesterol. In Atlantic salmon, however, the role of dietary phytosterols as cholesterol lowering agents is still unexplored.

The aim of the study was to investigate if the cholesterol metabolism and plasma cholesterol levels were altered in seawater phase Atlantic salmon when fed diets with either fish oil (FO) or vegetable oil (VO) based feeds for 6 months. The fish were fed diets with a high and constant inclusion of plant proteins, and either fish oil (FO) or 80 % of the FO replaced by olive oil (OO), rapeseed oil (RO) or soybean oil (SO). These oils were selected for their different levels of phytosterols and n-3/n-6 ratios to make it possible to determine whether it was the sterol composition of the feeds or the fatty acid composition being the main factor affecting fish cholesterol metabolism.

Neither plasma nor lipoprotein cholesterol differed at any sampling point between Atlantic salmon fed the different experimental diets, indicating that cholesterol levels is metabolically regulated also in Atlantic salmon. Phytosterols tended to accumulate in liver, especially in the fish fed RO, which was the diet with the highest content of phytosterols. An increased mRNA expression of genes encoding for proteins involved in cholesterol synthesis (ACAT2, DHCR7 and SREBP2) was observed in VO fed fish. Higher triacylglycerol levels in the liver, as well as a slightly elevated VLDL cholesterol and protein was seen in the RO fed fish compared to the other dietary groups. The changes observed in the RO fed salmon are thought to be caused by lower absorption of dietary cholesterol in this dietary group, caused by an influence of phytosterols on the intestinal sterol-absorber NPC1L1.The results from this study indicate that as long as Atlantic salmon is fed vegetable ingredient based diets low in cholesterol, cholesterol synthesis is up regulated, and dietary phytosterols does not affect the plasma nor lipoprotein cholesterol levels further. However, a high phytosterol-low cholesterol diet, as provided by the RO feed, does have a significant effect on the lipid and sterol metabolism on these fish, possibly due to a cholesterol deficit.

Contents

List of figures 3

List of tables 4

Abbreviations 5

1. Introduction 6

    1.1 General introduction 6

    1.2 Theoretical background 7

    1.3 Effects of phytosterols on humans 19

    1.4 Effects of plant derived feed on sterol metabolism in fish 22

    1.5 Aim of the study 24

    1.6 Method 24

2. Materials and Methods 25

    2.1 Experimental design 25

    2.2 Sampling 28

    2.3 Dietary proximate, fatty acid and lipidclass composition 29

    2.4 Analysis of sterol content 30

    2.5 Separation of lipoproteins 31

    2.6 Plasma and lipoprotein composition 31

    2.7 Real-time Quantitative PCR (qPCR) 31

    2.8 Statistical analysis and calculations 35

    2.9 Methodological considerations 35

3. Results 37

    3.1 Dietary sterols 37

    3.2 Growth & feed intake 38

    3.3 Cholesterol in liver 39

    3.4 Phytosterols in liver 41

    3.5 Liver triacylglycerol (TAG) and hepatosomatic index (HSI) 44

    3.5 Sterols in faeces and apparent digestibility 44

    3.6 Plasma and lipoprotein cholesterol, TAG, ALAT and ASAT 46

    3.7 Transcription of genes related to the cholesterol metabolism 48

4. Discussion 52

    4.1 Effect of vegetable oil and plant protein on growth and feed intake of Atlantic salmon 52

    4.2 Absorption of sterols 53

4.3 Effect of vegetable oils and plant protein on plasma cholesterol and plasma TAG . 55
4.4 Effect of vegetable oils and plant proteins on the sterol metabolism 56
4.5 Accumulation of sterols in liver of Atlantic salmon 58
4.6 Efflux of sterols in Atlantic salmon 60
4.7 Conclusions 62
5. Future perspectives 64
6. References 65
Appendix 76

List of figures

	Page
Figure 1: outline of the whole chain project	-
Figure 1.1: structure of α-cholesten3β-ol	7
Figure 1.2: schematic overview of absorption of sterols	12
Figure 1.3: schematic overview of cholesterol synthesis	13
Figure 1.4: chemical structure of sterols	15
Figure 1.5: schematic overview of the production of bile acid	16
Figure 1.6: lipoprotein structure	16
Figure 1.7: chemical composition of lipoproteins	17
Figure 1.8: schematic overview of cholesterol transport in circulation	18
Figure 2.1: basic composition of experimental diets	25
Figure 3.1: mean sterol content in experimental diets	37
Figure 3.2: bodyweight vs time	38
Figure 3.3: sterol ingested per gram weight gain	39
Figure 3.4: cholesterol contents in liver at 11 and 28 weeks	40
Figure 3.5: cholesterol contents in liver vs time	41
Figure 3.6: phytosterol in feed vs cholesterol in liver	41
Figure 3.7: phytosterol contents in liver at 11 and 28 weeks	42
Figure 3.8: phytosterol contents in liver vs time	42
Figure 3.9: phytosterol in feed vs phytosterol in liver	43
Figure 3.10: relative sterol levels in liver through time (campesterol, β-sitosterol, brassicasterol, sitostanol)	43
Figure 3.11: TAG contents in liver at 28 weeks	44
Figure 3.12: ADC values for cholesterol	45
Figure 3.13: relative amounts in faeces of sterols and values for phytosterol contents in feed (total phytosterol, campesterol, β-sitosterol)	45
Figure 3.14: VLDL cholesterol and protein in plasma, 28 weeks	47
Figure 3.15: normalized expression of genes in liver, 11 weeks	48
Figure 3.16: normalized expression of genes in liver, 28 weeks	49
Figure 4.1: hypothesized effect of high phytosterol-low cholesterol diet on sterol and lipid metabolism	60
Figure 4.2: hypothesized mechanisms of cholesterol regulation by LXR	61
Figure A.1:plot used for data diagnostics, variance of residuals	76
Figure A.2:plot used for data diagnostics, normalized residuals	77

List of tables

	Page
Table 1.1: fatty acid composition of selected vegetable oils	10
Table 1.2: sterol composition of selected vegetable oils	10
Table 1.3: fatty acid composition of fish oils	11
Table 1.4: lipoprotein main mechanisms	17
Table 2.1: formulation and proximate composition of experimental feeds	26
Table 2.2: fatty acid and sterol content in experimental diets	27
Table 2.3: information about primers for RT Q-PCR	32
Table 3.1: relative composition of phytosterols in experimental diets and feed intake	37
Table 3.2: cholesterol contents in plasma and lipoprotein fractions, 11 weeks	46
Table 3.3: cholesterol contents in plasma and lipoprotein fractions, 28 weeks	46
Table 3.4: TAG contents in plasma and lipoprotein fractions, 28 weeks	47
Table 3.5: relative expression of genes in liver tissue, 11 and 28 weeks	50
Table 3.5: relative expression of genes in intestine, 11 and 28 weeks	51

Abbreviations

ABCG5/8: ATP-binding cassette sub-family G member 5/8
a-ACAT2: Acetyl-CoA acetylTransferase 2
c-ACAT2 Acyl-CoA: cholesterol acyltransferase 2
ALA Alfa-linolenic acid
CVD: Cardiovascular disease
CYP7A1: Cholesterol 7-alpha-monooxygenase
DHA: Docosahexaenoic acid
DHCR7: 7-dehydrocholesterol reductase
EPA: Eicosapentaenoic acid
FA: Fatty acid
FCR Feed conversion ratio
FO: Fish Oil
FM Fish meal
HDL: High Density lipoprotein
HDL-C HDL-cholesterol
HMGR: HMG-CoA reductase
IDL: Intermediate density lipoprotein
LA Linoleic acid
LDL: Low density lipoprotein
LDL-C LDL-cholesterol
LXR: Liver X receptor
MVK: Mevalonate kinase
MUFA Monounsaturated fatty acids
NLP: Non-lipoprotein fraction
NPC1L1: Niemann-Pick C1-like 1
OO: Olive Oil
PP Plant protein
PUFA: polyunsaturated fatty acid
qPCR Real-time quantitative PCR
RO: Rapeseed Oil
RT-PCR Reverse transcription PCR
SFA Saturated fatty acids
SO: Soybean Oil
SGR Specific growth rate
SREBP2: Sterol-responsive Element-binding Protein 2
TAG Triacylglycerol
VLDL: Very Low Density Lipoprotein
VO: Vegetable oil

1. Introduction

1.1 General introduction

Replacement of marine raw material in aquaculture feed with plant ingredients

The aquaculture industry in the world is today the fastest growing food sector, growing by an average of 6.6% each year (1970-2008). Worldwide, fish from aquaculture represents 50% of the human fish consumption, and it has been viewed as an alternative to take the pressure of the decreasing fish stocks in our oceans. Already in 2006 the export value of farmed fish in Norway overgrew the value of wild catch fish, and the national production has grown by 10% each year (FAO, 2011). But the pressure on the wild caught fish is not off; about 87% of all the fish oil (FO) produced globally is used by the aquaculture industry as a source of lipids, with the farming of salmonid species accounting for the consumption of as much as 66.4% of the total amount of FO used in aquaculture in 2003 (Tacon et al., 2006). Most of the stocks of the top ten fished species are already fully exploited or overexploited. Because of a growing concern for the marine resources and organic contaminants in the raw material as well as varying availability of marine prime material, a big effort is being put into replacing FO and fish meal in the fish feed partially or completely with alternative material (FAO, 2010; Miller et al., 2008). With an increasing production and competitive prices of vegetable oils (VO), these are viewed as a good as substitutes for FO. Some of the uncertainties concerning this change in the fish's diet have been whether or not the fillet of the farmed fish eating more plant derived food will keep its valued health benefits and if the general health and wellbeing of the fish will change in any way (Turchini et al., 2009).

When replacing the marine raw materials in aquaculture feeds with plant raw materials there are several dietary factors that are changed. Some of the most obvious ones are the fatty acid and indispensable amino acid composition, as well as the possible presence of environmental contaminants new to the aquaculture industry and plant metabolites as phytosterols. Other components that may be introduced into the aquaculture feed with the plant material are antinutritional factors and complex carbohydrates (Francis et al., 2001; Vielma et al., 2003). It has been shown that 90% of the fish meal can be replaced by plant protein mixture with balanced amino acid composition without the fish showing any signs of decrease in growth rate (Espe et al., 2006). Also, replacing the fish oil with a mixture of vegetable oils, but keeping the protein source of marine origin has shown good overall performance (Leaver et al., 2008; Miller et al., 2008; Nanton et al., 2007; Torstensen et al., 2005). In contrast, when both protein and oil fraction was replaced by plant ingredients, lowered growth and increased

overall adiposity was reported (Torstensen et al., 2008; 2011). Most studies done on the effect of different fatty acids and sterols on the organism is performed with mice or other test animals to obtain results related to human health and nutrition. However, an increasing amount of studies within the same field are also performed on fish, both of which are more thoroughly covered in section 1.3 and 1.4 of the introduction. The research done on minor constituents of plant based diets are few and the attention to this field has been enquired (Turchini et al., 2009). It has been shown by microarray studies that VO diets influence the cholesterol metabolism in Atlantic salmon (Leaver et al., 2008; Taggart et al., 2008), although there is a need to better understand the mechanisms of this effect the VO diet exerts on the metabolism. The effect of plant sterols, called phytosterols, on the cholesterol metabolism in Atlantic salmon is the main focus in this master thesis. The effect of changes in fatty acid composition on cholesterol metabolism has also been investigated.

1.2 Theoretical background

Characteristics of sterols

-General information on sterols and their chemical structure

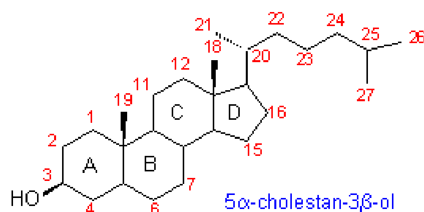


FIGURE 1.1: 5A-CHOLESTEN-3B-OL BASIC STRUCTURE OF WHICH OTHER STEROLS ARE DEFINED
(SOURCE: LIPIDLIBRARY.AOCS.ORG)

The sterols are mainly divided into two groups, the animal sterols, zoosterols, and the plant equivalents, which are called phytosterols. Cholesterol is the main zoosterol and is vital for the human body, being part of cell membranes, and acting as substrate for the production of bile acid and steroid hormones in animals (Schneider, 2008). The biological function of phytosterols is similar to that of cholesterol, as they help stabilising the phospholipid bilayers of the plant cell membranes, and have also been proven the ability to lower levels of harmful cholesterol in human plasma (more details in section 1.3). The structure of sterols is composed of a tetracyclic cyclopentana-phenanthrene structure with *trans* ring junctions, and a side chain at C-17. The basic sterol structure, as seen in figure 1.1, is defined as 5α-cholesten-3β-ol and is the structure of which other sterols are defined. The chemical structures of phytosterols are quite similar to cholesterol, except that the phytosterols always contain some kind of substitution on the C-24 position on the side chain (see figure 1.1 and 1.3).

-Dietary sources of sterols

Sterols are lipid soluble and are therefore found in the fatty portions of food products. Some

of the most common sources of cholesterol in the western diet are cheese, egg yolks, beef, pork, poultry, and shrimp (U.S. Department of Agriculture, 2010). Phytosterols are found in seeds, legumes and unrefined vegetable oils, and are common in cereals (Weihrauch and Gardner, 1978). Phytosterols are only synthesized in plants, so the levels in animals come solely from food via intestinal absorption. The most common phytosterol sources in the western diet are corn, bean and plant oils, and intake can vary from 250 mg per day, as in the USA (Connor, 1968), to about the double for a vegetarian (Cerqueira et al., 1979). Bivalves are also rich in sterols, which reflects their varied phytoplankton food sources (Copeman and Parrish, 2004).

In spite of ingestion of relatively large quantities of foods with phytosterols, the amount of phytosterols absorbed into the human body is low; only about 5% of the phytosterols ingested are absorbed, compared to a 40% for cholesterol (Salen et al., 1989). The difference in absorption between cholesterol and phytosterols is thought to be due to the crucial step in the absorption of sterols, the acyltransferase dependent esterification, is slower for phytosterols than for cholesterol. It has been shown that the acylcoenzyme of cholesterol, A:cholesterol acyltransferase (c-ACAT), is at least 60 times greater than that of β-sitosterol (Ling and Jones, 2005; Miettinen et al., 1990).

-Lipid sources relevant for the aquaculture industry

For a lipid to replace FO in aquaculture feed, it should optimally resemble the composition of FO, which is an oil rich in the highly unsaturated n-3 fatty acids (n-3 HUFA), but with the main portion of the energy suministred by saturated fatty acids and monounsaturated fatty acids (MUFA) (Henderson and Sargent, 1985). The amount of saturated FAs to be used in the feed has to be viewed in context with the temperature of the water for the fish to be fed with the diet, as saturated FAs have a lower digestibility than unsaturated FAs in fish and then especially in cold-water fish (Francis et al., 2007; Menoyo et al., 2003; Ng et al., 2004; Torstensen et al., 2000). Oils have been used singly or as blends in earlier experiments to investigate their suitedness as a replacement for FO (Leaver et al., 2008; Nanton et al., 2007; Torstensen et al., 2008) and the effects of replacing FO with different VOs is reviewed in section 1.4 of this paper. When selecting oils for a fish diet the need for the essential fatty acids, linoleic acid (18:2 n-6, LA) and α-linolenic acid (18:3 n-3, ALA), has to be considered, as these cannot be synthesized by vertebrates (Cunnane, 2003; Turchini et al., 2009). ALA can be converted into n-3 HUFA by specific desaturates and elongases which have shown to increase their activity in Atlantic salmon fed diets where FO has been replaced by VO (Stubhaug et al., 2005; Tocher et al., 2003; 2001). Some of the oils that could be used in the aquaculture industry are described below, as are their FA and sterol compositions.

Rapeseed oil was earlier known for high levels of erucic acid (22:1 n-9) and undesirable levels of glucosinolates. The modern rapeseed (also called canola) has been selected to not have these "defects" and now it is the third largest source of oil for human consumption after soybean and palm oil. A typical rapeseed oil contains less total saturated fatty acid (SFA) than any other commodity oil (about 7% of total fatty acid content, vs. 15% in olive and soybean oil), and is a natural source of plant sterols. In addition, rapeseed oil has been observed to have a high acceptance in Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) compared to other VOs (Geurden et al., 2005 ; 2007)

Olive oil is an important part of the Mediterranean diet and has long been accredited to have positive effects on cardiovascular health in humans. It is characterized by its high levels of oleic acid, 18:1 n-9 (almost 80% of total FA), a monounsaturated fatty acid (MUFA). The virgin oil also contains tocopheroles (vitamin E) and an appreciable amount of squalene (an acyclic C_{30} hydrocarbon, biological precursor of sterols), although many of these health promoting substances get denatured or removed during distillation.

Soybean oil is the second most produced oil after palm oil. Its high levels of unsaturated fatty acids makes it very unstable and prone to oxidation, so it is therefore usual to half the amount of LA by hydrogenation to give it a longer shelf life. The dietary shortcoming with this oil is its lower than recommended n-3/n-6 ratio.

Linseed oil is very rich in PUFA, especially the n-3 PUFAs, and is one of the few VOs that provide ALA in substantial amounts (~50% of total FA). It is, however, not well accepted by Atlantic salmon and rainbow trout compared to other VOs (Geurden et al. 2005; 2007).

Palm oil has high contents of the saturated FA palmitic acid (16:0) and the monounsaturated FA oleic acid. It is naturally rich in carotenes and tocotrienols, but these are often removed during the refinement process. This oil has physical properties valued by many industries, as it is semi-solid at room temperature and can thus give texture to food and cosmetics (Dubois et al., 2007).

Echium oil stands out for its high levels in stearidonic acid (18:4 n-3), which is one step higher than ALA in the elongation and desaturation pathway towards the biosynthesis of n-3 HUFA. Echium oil has been seen to increase the amount of n-3 HUFA in fish tissues when used as lipid source in aquaculture feed for Atlantic cod, *Gadus morhua* (Bell et al., 2006).

Sunflower seed oil contains linoleic and oleic acids as the major FAs, which often make up over 90 % of the total FAs in this oil. High-oleic varieties do also exist for this oil, where up to 90% of the oil is made up of oleic acid.

All vegetable oils are different from marine oils in the sense that they do not have carbon-chains longer than 18-C and have no more than 3 double bonds (table 1.1). The other main difference between plant oils and fish oil is the absence of cholesterol and presence of plant sterols, called phytosterols, which are not synthesized in animals. The rapeseed oil is characterized as a high-phytosterol oil while the olive and soybean oil are viewed as low/intermediate-phytosterol oils (table 1.2).

All information seen here about vegetable oils, when not otherwise cited, from lipidlibrary.org

TABLE 1.1: OVERVIEW OVER TYPICAL FATTY ACIDS COMPOSITIONS OF SELECTED VEGETABLE OILS (GIVEN AS W/W%)

Fatty acid	*Olive oil*	*Rapeseed oil*	*Soybean oil*	*Linseed*	*Palm oil*	*Echium oil*	*Sunflower oil*
Total Saturated	15.3	8.0	15.7	10.0	50.4	11.1	12.8
18:1 n-9	72.5	60.1	23.9	18.4	39.1	15.4	22.1
20:1 n-9	0.3	1.4	0.1	-	0.1	0.8	0.2
22:1 n-9	-	0.4	-	-	-	0.3	0.1
Total MUFA	73.8	76.8	24.2	18.5	39.4	17.2	22.4
18:2 n-6	9.4	21.5	52.1	16.8	10.2	18.8	65.6
18:3 n-3	0.6	9.9	7.8	55.0	0.3	28.4	0.5
Total PUFA	10.0	31.5	59.8	71.8	10.5	71.7	66.0
Total n-6	9.4	21.6	52.1	16.8	10.2	29.8	65.6
Total n-3	0.6	9.9	7.8	55.0	0.3	41.2	0.5
Ratio n-3/n-6	0.06	0.46	0.15	3.27	0.03	1.4	0.007

Source: vegetable oils: (Dubois et al., 2007) ; echium oil: (Surette et al., 2004)

TABLE 1.2: TYPICAL STEROL COMPOSITION OF SELECTED VEGETABLE OILS (MG KG^{-1})

	Olive oil	*Rapeseed oil*	*Soybean oil*	*Linseed oil*	*Palm oil*
Campesterol	28	1530	720	1218	358
Stigmasterol	14	-	720	378	204
β-sitosterol	1310	3549	1908	1932	1894
Δ5-avenasterol	29	122	108	546	51
Δ7-stigmastenol	58	306	108	84	25
Δ7-avenastenol	-	-	36	-	-
Brassicasterol	-	612	-	-	-
Total:	1439	6119	3600	4158	2532

Source: (Harwood et al., 1994)

Fish oil is produced by using the whole fish as a raw material and extracting the fat. It is the edible oil with the highest level of n-3 fatty acids and provides the health promoting n-3 highly unsaturated fatty acids (HUFAs) such as Eicosapentaenoic acid (EPA, 20:5 n-3) and Docosahexaenoic acid (DHA, 22:6 n-3). The FA composition of fish oil varies depending on the fish species used, the season and where it is caught. In table 1.3 FA compositions of oils from various fish species is listed; if possible, the values are taken from the period when the fish is at its fattest period during the year. There is a great variation through the year and between the species in the oil they render, a fact to have in mind when considering different fish oil sources. Fish oils do not contain phytosterols other than in trace amounts, and differ in cholesterol contents which are typically 7100 mg kg^{-1} for sardine oil and 7660 mg kg^{-1} for herring oil (U.S. Department of Agriculture, 2010).

TABLE 1.3: OVERVIEW OVER TYPICAL FATTY ACID COMPOSITIONS OF FISH OIL FROM ANCHOVY AND SARDINE, AND SALMON DIETS BASED ON 100% FISH OIL FROM CAPELIN AND HERRING (DATA GIVEN AS W/W%)

Fatty acid	*Capelin*	*Sardine*	*Herring*	*Anchovy*
Total Saturated	20.8	33.6	25.1	46.6
18:1 n-9	10.9	4.3	21.2	9.3
20:1 n-9	12.2	1.4	4.6	1.9
22:1 n-9	1.9	0.9	0.1	0.6
22:1 n-11	14.5	NA	9.7	NA
Total MUFA	51.0	18.2	48.6	18.2
EPA	9.3	10.3	5.5	11.9
DHA	7.0	18.1	5.4	12.2
18:2 n-6	2.1	1.5	5.9	1.1
18:3 n-3	0.6	-	0.7	-
Total n-6	2.6	4.6	6.5	1.59
Total n-3	22.5	33.0	13.1	25.9
Ratio n-3/n-6	8.6	7.2	2.0	16.3
Total PUFA	25.7	48.2	26.3	35.2

Source: capelin: Torstensen et al. 2000; sardine and anchovy: Zlatanos and Laskaridis, 2007; herring: Hardy et al. 1987

Sterol absorption

The cholesterol absorption in humans takes place in the jejunum of the small intestine, where both dietary and biliary cholesterol is available for absorption. The dietary cholesterol is taken up into the enterocytes by Niemann-Pick C1-like receptors (NPC1L), and its absorbance is dependent on an esterification by Acyl-CoA: cholesterol acyltransferase (c-ACAT) and the subsequent incorporation into chylomicrons by microsomal triglyceride transfer protein (MTP). Cholesterol that is not esterified will not be taken up by the chylomicrons and will be transported back into the intestinal lumen by ATP-Binding Cassette sub-family G (ABCG) transporters, figure 1.4 (Huff et al., 2006; Voelker, 2008). The movement of cholesterol from the intestine into the circulation is therefore directly dependent on the efficacy of c-ACAT. The sterol-absorber, NPC1L1, has a sterol sensing domain, which several other sterol regulating proteins also have (Altmann et al., 2004).

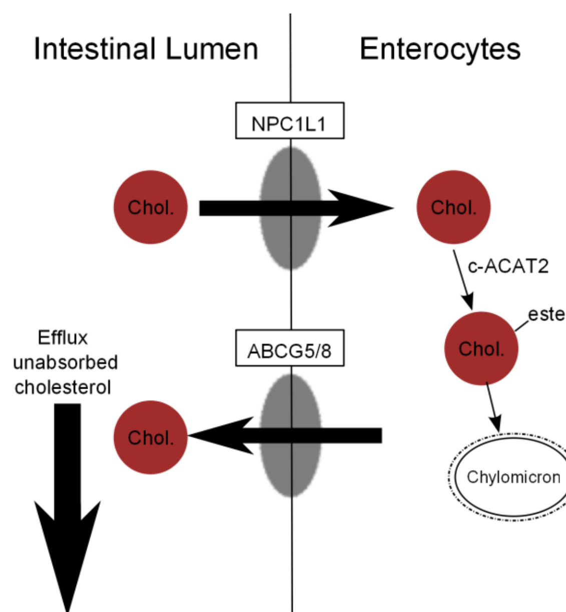


FIGURE 1.2: SCHEMATICAL VIEW OF SOME OF THE COMPONENTS INVOLVED IN THE INTESTINAL ABSORPTION OF STEROLS. SOURCE: AGELLON 2008, VOELKER 2008

Biosynthesis of sterols

Mammalian whole-body cholesterol homeostasis is a highly regulated balance of de novo synthesis, dietary cholesterol absorption, and biliary clearance and excretion (Altmann et al., 2004; Liscum, 2008). Cholesterol is synthesized in basically every tissue from Acetyl-CoA and its levels are controlled by feedback inhibition of cholesterol synthesis and a feed-forward regulation of cholesterol metabolism and catabolism (figure 1.2).

Cholesterol is, as mentioned, synthesized in the tissues from acetyl-CoA which is first interconverted to acetoacetyl-CoA by Acetyl-Coenzyme A Acetyltransferase (a-ACAT) and then condensed by 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase (HMGS) to form HMG-CoA. HMG-CoA reductase (HMGR) catalyzes the reduction of HMG-CoA to mevalonate and is a rate-determining enzyme in the cholesterol biosynthesis, regulated by the supply of cholesterol. A series of reactions, initiated by mevalonate kinase (MVK), transform mevalonate to isopentenyl pyrophosphate and then further to farnesyl-PP by the enzyme

farnesyl-PP synthase. The conversion of farnesyl-PP to squalene is initiated by squalene synthase, which is another one of the links in the cholesterol biosynthesis controlled by the cholesterol levels in the cell. Cyclisation of squalene to form lanosterol, the first sterol in this metabolic pathway, is done by squalene epoxidase and oxidosqualene:lanosterol cyclase, the latter one being a ligand activator for liver X receptor (LXR – mentioned about this under section *sterol metabolism*). Through a series of oxidations, reductions, and demethylations, lanosterol is finally converted to cholesterol by 7-dehydrocholesterol reductase (DHCR7).

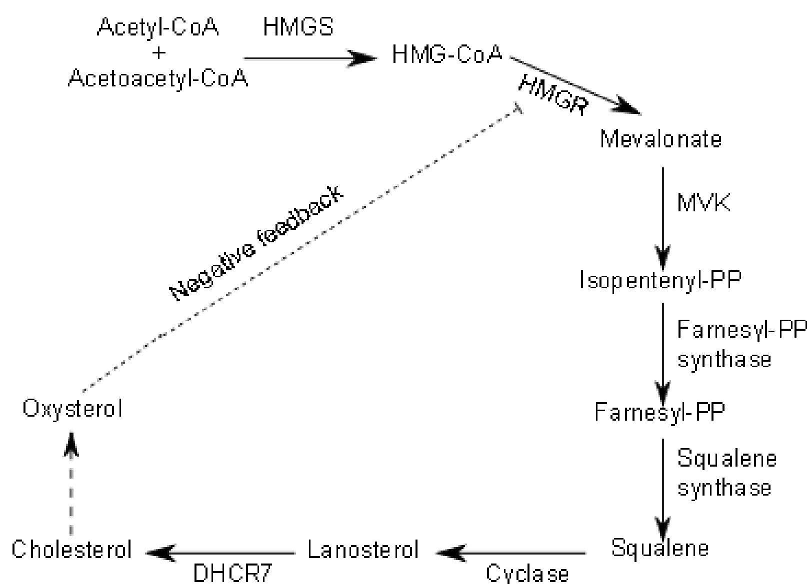


FIGURE 1.3: THE SYNTHESIS OF CHOLESTEROL FROM ACETYL COA AND ITS NEGATIVE FEEDBACK LOOP, WHERE OXYSTEROLS FUNCTION AS A SIGNAL TO THE CELL OF CHOLESTEROL EXCESS. (LISCUM, 2008)

The biosynthetic route of phytosterols is similar to that of cholesterol in many ways as it is the mevalonate pathway in the cytosol that is responsible for the production of sterols also in plants. The difference between animal and plant biosynthesis of sterols lies in the step of cyclisation, which proceeds via lanosterol in animals and fungi and via cycloartenol in photosynthesizing organisms. This is, interestingly, despite the presence of lanosterol in higher plants (Kolesnikova et al., 2006). By a complex series of reactions, including opening of the cycloartenol cyclopropane ring, double bond formation and isomerisation, demethylation of C-rings, and methylation of the side chain a huge variety of plant sterols are produced, see figure 1.3 (Benveniste, 2004). Of the more than 200 reported phytosterols in plants the most common is β-sitosterol (90%), although the sterol composition usually differs from tissue to tissue (Schmid and Ohlrogge, 2008).

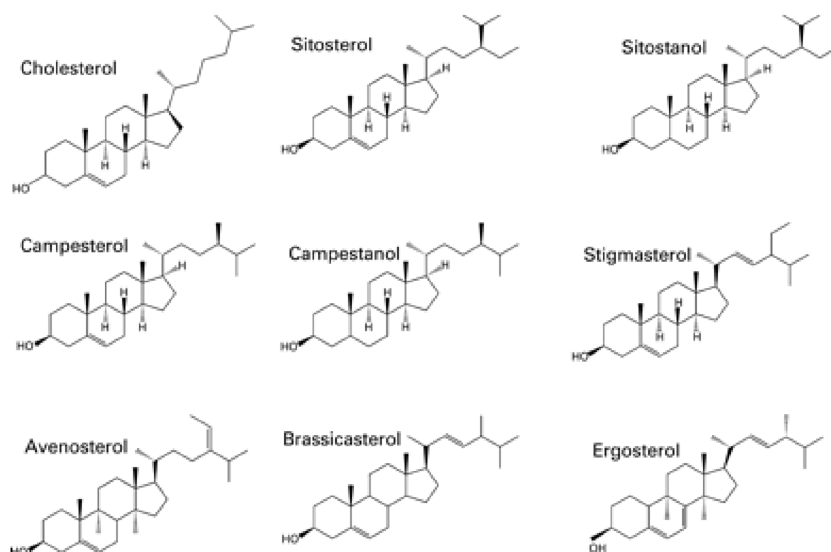


FIGURE 1.4: CHEMICAL STRUCTURES OF CHOLESTEROL AND SOME OF THE MOST COMMON PHYTOSTEROLS. SOURCE: (Kidambi and Patel, 2008)

Sterol metabolism

Excess cholesterol in the tissues is transformed by oxidation into oxysterols, which are suppressors of cholesterol synthesis and also signals of cholesterol excess in a cell (Olkkonen, 2004). These cholesterol metabolites have the ability to diffuse through cell membranes, which removes the need for receptors for the oxysterols to regulate the cholesterol metabolism through negative feedback (Liscum, 2008). The oxysterols activate transcription of many genes important in the metabolism of cholesterol metabolism by binding to the Liver X-receptors (LXR), which belong to the nuclear receptor superfamily of ligand-activated transcription factors (Janowski et al., 1996; 1999). Activation of LXRs increase the expression of CYP7A1 and SREBP1c, which are key genes in the cholesterol and lipid metabolism and control the cholesterol catabolism and the *de novo* synthesis of fatty acids, respectively (Lehmann et al., 1997; Peet et al., 1998; Repa et al., 2002). Two isoforms of LXR are known in humans, LXR-α and LXR-β, where LXR-β is found in metabolically active tissues, as liver and small intestine, whilst LXR-α is found more ubiquitiously (Fan et al., 2008). Only one isoform of LXR is known for Atlantic salmon (Cruz-Garcia et al., 2009), LXRs are nuclear receptors viewed as the main controllers of the sterol metabolism, controlling practically all the aspects of the sterol cycle in the organism by acting on genes involved in the cholesterol efflux as ABCG5/8, CYP7A1, and NPC1L1 and with this protecting the cell from cholesterol overload (Zhao and Dahlman-Wright, 2010).

Sterol regulatory element binding protein (SREBP) is a nuclear receptor and the main regulator of cholesterol synthesis and uptake (Espenshade and Hughes, 2007; Goldstein et al., 2006). There are three known isoforms of SREBP in humans; SREBP-1a, SREBP-1c and

SREBP-2. SREBP2 is the isoform that most actively regulates genes involved in cholesterol homeostasis, like LDLR and sterol biosynthetic enzymes (Horton et al., 1998 ; 2002). Until recently, only one isoform of SREBP, similar to SREBP2 was known in Atlantic salmon. However, a recent publication described two isoforms, similar to SREBP1 and SREBP2 (Minghetti et al., 2011). When the cholesterol levels in a cell are high, SREBP is bound by SREBP-cleavage activating protein (Scap) and attached to the endoplasmic reticulum by the insulin-induced gene, Insig. When cholesterol levels are low, SREBP is split from Insig and released from the ER (Gong et al., 2006). The SREBP/Scap complex is transported to the Golgi complex by COPII-budded vesicles on the ER, made soluble by a cleavage by a protease and then it enters the cell nucleus (Hertzel et al., 2008; Sakai et al., 1998). In the cell nucleus it upregulates the expression of genes involved in the synthesis of cholesterol and fatty acids (Horton et al., 2002). HMGR is one of the genes which is strongly regulated by SREBP2 in mammals, thus making sure that the supply of mevalonate for the production of cholesterol is sufficient, see figure 1.2 (Horton et al., 1998). In Atlantic salmon it is suggested that other mechanisms than transcriptional control may be important for HMGR regulation (Minghetti et al., 2011). In addition to be controlled by SREBP2, HMGR activity is also regulated by products of the sterol and isoprenoid pathways which accelerate its degradation (Ravid et al., 2000; Roitelman and Simoni, 1992). In contrast to in humans, it does not seem as HMGR is as regulated by these pathways in Atlantic salmon (Leaver et al., 2008).

Sterol excretion

The excretion of sterols is regulated by the expression of LXR, which affects the expression of CYP7A1 and ABCG5/8, both involved in the excretion of sterols (Agellon, 2008; Huff et al., 2006). LXR is, as mentioned, activated by oxysterols and LXR agonists are used to prevent accumulation of cholesterol and development of atherosclerosis in humans (Joseph et al., 2002). The body can via the gallbladder get rid of excess cholesterol in the form of bile acids. Two main pathways are known for the production of gall salt from cholesterol; the classical and the alternative (figure 1.5). The classical one functions only in the liver and starts with an α-hydroxylation on C-7 of the cholesterol steroid nucleus, catalyzed by cholesterol 7α-hydroxylase, also called cytochrome P450 7A1 (CYP7A1), which is the limiting step for this pathway. The classical pathway is considered as the main mechanism for production of bile acids by the liver and bile acid output by the liver is correlated with the activity of an increased expression of CYP7A1 (Agellon, 2008). The alternative pathway, where oxysterols are transformed into bile acids in other tissues than liver, is similar to the one of the classical pathway, although two types of enzymes are needed for the complete transformation, CYP7B1 and CYP39A1 (LI-Hakwins et al., 2000).

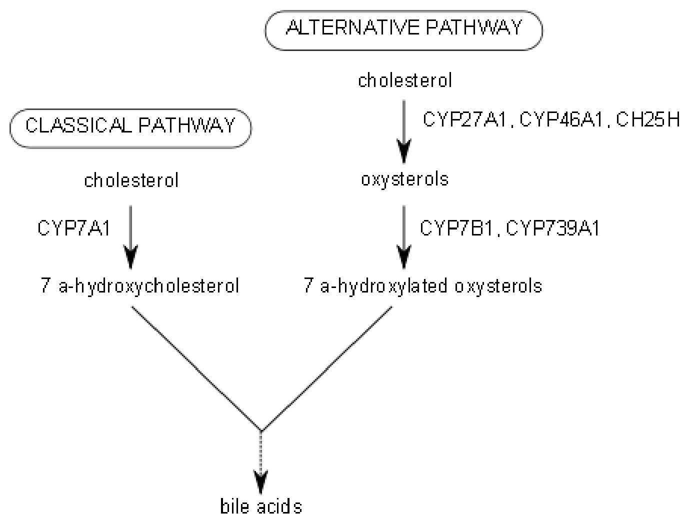


FIGURE 1.5: THE TWO MAIN PATHWAYS FOR THE PRODUCTION OF BILE ACIDS FROM CHOLESTEROL: THE CLASSICAL AND THE ALTERNATIVE. SOURCE: (Agellon, 2008)

Endogenous transportation of sterols

When the triacylglycerides (TAGs), fatty acids (FAs) and other lipidsoluble components are absorbed in the intestine or synthesized in the body, they are, because of their hydrophobic nature, transported in lipoproteins. Lipoproteins are biochemical assemblies that contain TAG and cholesterol esters in their centre covered by a phospholipid layer with free cholesterol embedded in it (figure 1.6). The lipoproteins are covered by apolipoproteins which contribute to the assembly of the lipoprotein, they also provide structural integrity to the same, serve as co-activators of enzymes, and act as receptor ligands for cellular uptake (Jonas and Phillips, 2008).

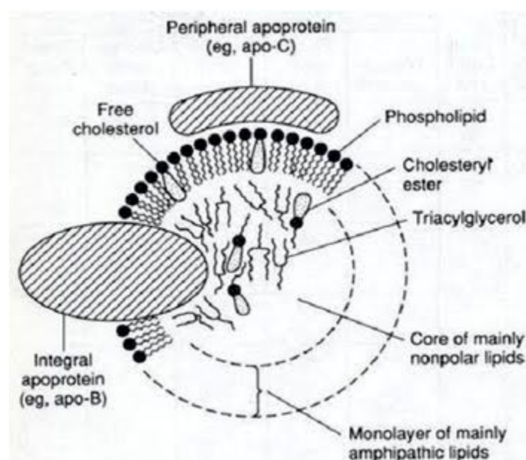


FIGURE 1.6: LIPOPROTEIN STRUCTURE, SOURCE: TOOSOGIE-LIPID-DIAGNOSTICS.BLOGSPOT.COM

There are several classes of lipoproteins, which are all associated with different types of apolipoproteins and categorized according to their density (table 1.4): chylomicrons, high-density lipoproteins (HDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL) and very low-density lipoproteins (VLDL) (figure 1.7). In the intestine the apoB48 helps packaging ingested TAG and cholesterol into the chylomicron, the same apolipoprotein also provides structural support. The apoE in the chylomicron is the ligand for the hepatic

uptake of this structure. The apoCII and CIII, also on the chylomicron, are thought to have an effect on the regulation of triglyceride metabolism.

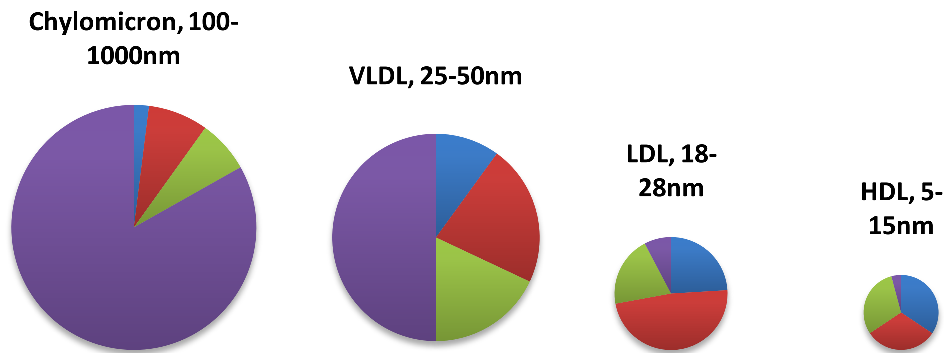


FIGURE 1.7: CHEMICAL COMPOSITION AND SIZE OF LIPOPROTEINS. PURPLE: TRIGLYCERIDES; BLUE: PROTEIN; RED: CHOLESTEROL; GREEN: PHOSPHOLIPIDS. SOURCE: (GARRETT AND GRISHAM, 1999)

TABLE 1.4: THE LIPOPROTEINS, THEIR APOLIPOPROTEINS AND MAIN MECHANISMS OF LIPID DELIVERY. TG: TRIACYLGLYCEROL, CE: CHOLESTEROL ESTER. SOURCE: (GARRETT AND GRISHAM, 1999)

Lipoproteins	Major core lipids	Apolipoproteins	Mechanisms of lipid delivery
Chylomicron	Dietary TG	A1, A2, A4, B48	Hydrolisis by lipoprotein lipase
Chylomicron remnant	Dietary CE	B48, E	Receptor mediated endocytosis by liver
VLDL	Endogenous TG	B100, C, E	Hydrolisis by lipoprotein lipase
IDL	Endogenous CE	B100, E	Receptor mediated endocytosis by liver and conversion to LDL
LDL	Endogenous CE	B100	Receptor mediated endocytosis by liver and other tissues
HDL	Endogenous CE	A1, A2	Transfer of cholesterol esters to liver

After some TAG is released to peripheral tissue through lipolysis, the chylomicron turns into what we call a chylomicron remnant. The chylomicron remnant is taken up by the liver by LDL-R or LDL receptor-related protein (Schneider, 2008). VLDL is synthesized in the liver and contains apoB100, which gives structural support and serves as a ligand for cellular re-uptake, apo E, CII and CIII. VLDL is released into circulation, containing endogenously produced TAG and cholesteryl esters. Lipolysis in the circulation of VLDL results in LDL and IDL, LDL has apoB100 as its only apolipoprotein. The LDL particles are cleared from plasma by LDL-receptors (LDL-R), which are present in all tissues. The densest of the lipoproteins, HDL, is the lipoprotein mainly involved in reverse cholesterol transport, e.g. transport of cholesterol from the periphery to the liver, and contains apoAI as its main apolipoprotein. ApoAI is excreted mainly from the liver in a lipid-poor form and accumulates

cholesterol and phospholipids to form HDL. HDL also serves as a vessel for the exchangeable apolipoproteins (AII, CII, CIII and E) (Fielding and Fielding, 2008). In figure 1.8 the flow of sterols by lipoproteins is shown schematically.

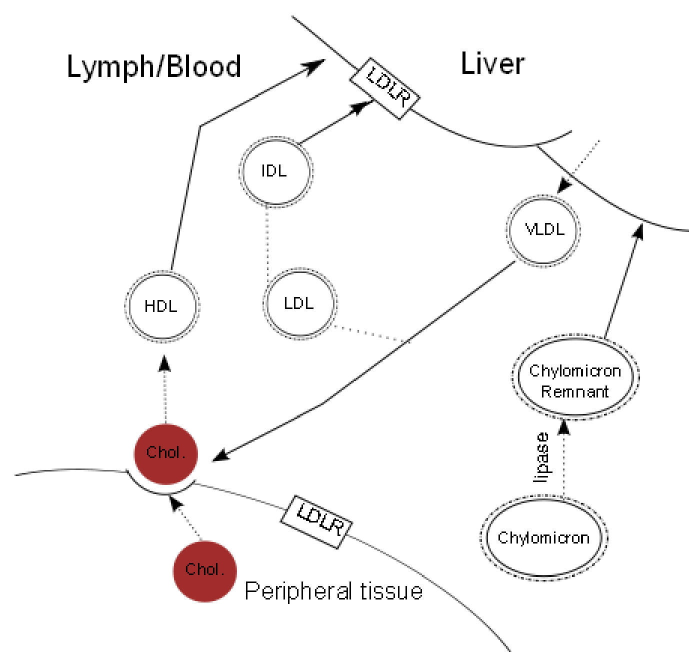


FIGURE 1.8: SCHEMATIC OVERVIEW OF TRANSPORTATION OF CHOLESTEROL IN THE CIRCULATION. THE CHYLOMICRON CONTAINS EXOGENOUS CHOLESTEROL TAKEN UP IN THE INTESTINE. THROUGH LIPASE ACTIVITY IT BECOMES SLIGHTLY DENSER AND IS CLASSIFIED AS A CHYLOMICRON REMNANT, WHICH IS TAKEN UP IN THE LIVER . FORWARD CHOLESTEROL TRANSPORT IS PERFORMED BY VLDL, PRODUCED IN THE LIVER, WHICH DELIVERS CHOLESTEROL TO THE PERIPHERAL TISSUES . THROUGH LIPOLYSIS IN THE CIRUCLATION, VLDL CAN DEVELOP INTO HEAVIER COMPONENTS, CALLED LDL AND IDL, WHICH ARE TAKEN UP IN THE LIVER BY LDL-R . REVERSE CHOLESTEROL TRANSPORT IS EXECUTED BY HDL, WHICH TRANSPORTS CHOLESTEROL FROM THE PERIPHERAL TISSUES TO THE LIVER. FIGURE BASED ON INFORMATION FROM (Fielding and Fielding, 2008).

Lipoproteins and cardiovascular diseases

In today's medicine the ratio of HDL-cholesterol/LDL-cholesterol is given special importance, as LDL-cholesterol is observed to be a factor connected to increasing risks of developing CVDs (Willett and Sacks, 1991) and higher levels of HDL-C is commonly associated with a good cardiovascular health. The main theory is that LDL-C contributes towards atherogenesis by accumulation of LDL-C on the arterial wall, whilst HDL-C has the opposite effect and brings cholesterol from the arterial walls to the liver and the steroid organs (Castelli et al., 1992; Gotto, 2001). The HDL-C also has positive effects on other aspects of the cardiovascular health as it helps inhibit oxidation, inflammation, coagulation and platelet aggregation and it activates the endothelium (Mineo et al., 2006; Navab et al., 2005). The American Heart Association states that a level of total cholesterol level of 5mmol L^{-1} or a LDL-C under 3 mmol L^{-1} is viewed as an optimal level, protecting you from atherosclerosis. Fish, however, has a much higher normal level of total cholesterol, often higher than 10 mmol L^{-1} (Sandnes et al., 1988), with the main proportion of cholesterol as HDL cholesterol (Farrell and Munt, 1983).

Dietary factors have been shown to influence the plasma and lipoprotein cholesterol levels in humans. Some of the factors shown to increase the LDL-C levels are saturated fatty acids (palmitic acid being the SFA increasing LDL-C the most), cholesterol, and excess caloric intake leading to obesity (Grundy and Denke, 1990). Phytosterols have shown to be LDL-C lowering (see section 1.3).

In spite of the common use of HDL-C levels to assess risk of developing CVDs, there are still doubts as to how the CVD-preventing mechanisms of HDL work. The relationship between HDL-C and CVDs is strong but not absolute, as many other factors, genetic and environmental, decide whether or not someone will suffer from CVDs (Chirovsky et al., 2009). The effect of the diet on the CV health is also not clear, and the interindividual variability in effect of dietary treatments has been reported by many researchers to be large (Ordovas, 2006). Atlantic salmon have high levels of plasma cholesterol compared to humans (Jukema et al., 1995; Sandnes et al., 1988) and they also have an important predisposition of suffering from CVDs when they approach their mature age (Seierstad et al., 2008). However, what levels of LDL- and HDL-cholesterol that may provoke an increased risk of CVDs in Atlantic salmon, and if such an association exists for this species, has not yet been determined.

1.3 Effects of phytosterols on humans

Effects of phytosterols on plasma cholesterol

As the studies on the effects of phytosterols on fish are scarce we must rely on some of the results from studies meant to investigate the effect of phytosterols on humans, which are much more abundant. In mammals, the phytosterols are not absorbed as efficiently in the intestine as cholesterol and are also excreted more rapidly from the liver via bile. This leads to mammals usually having low levels of phytosterols in their tissues (Ling and Jones, 2005). Many studies have now shown that dietary phytosterols reduce total plasma cholesterol and LDL cholesterol levels in animals, an attribute it was given already in the early 1950´s (Pollak, 1953). Both phytosterols and their saturated form, phytostanols, have been proven to reduce the uptake of cholesterol and bile acids from the intestine (Berger et al., 2004; Grundy and Mok, 1977; Lichtenstein, 2002; Moreau et al., 2002; Normén et al., 2000).

The absorbance and the plasma-cholesterol lowering effect of phytosterols is affected by the food in which they are incorporated; the efficacy of bread and cereals as plant sterol carriers is three times lower than the efficacy of low fat milk (Noakes et al., 2005). The time of day the phytosterols are eaten and into how many meals it is divides also changes how well they

lower the LDL cholesterol (Abumweis et al., 2006; Doornbos et al., 2006; Hyun et al., 2005; Matvienko et al., 2002; Pineda et al., 2005; Plat et al., 2000). The result for studies looking at the effect of the presence of unsaturated fatty acids are contradictory, as Brown et al. (2010) stated that saturated fatty acids in combination with phytosterols lower solubility of cholesterol in micelles and thus decreasing cholesterol uptake further. Micallef and Garg (2008), however, observed a lowering of plasma TAG, total cholesterol and HDL-cholesterol when patients were given n-3 long chained PUFAS and phytosterols, but not when they were given phytosterols together with saturated fatty acids. It thus seems as if a lower amount of cholesterol in the micelles, not necessarily needs to be connected with lower levels of cholesterol in blood.

Some questions have arisen regarding whether or not the phytosterols present naturally in a diet, e.g. not added from other sources as concentrated or isolated phytosterols, have the same cholesterol-lowering effect as phytosterol supplements do. The dosage of phytosterols at which LDL cholesterol is lowered significantly depends on the type of phytosterols utilized; varying from 1.5g per day, if provided as sitostanol, up to 20 g per day, if provided as β-sitosterol (Heinemann et al., 1988). Lin et al. (2010) gave one group of people a naturally high-phytosterol diet (449 mg phytosterols / 2000 kcal) and another one a low phytosterol diet (126 mg phytosterols / 2000 kcal). An increase in faecal excretion of cholesterol by up to 79% was observed in the phytosterol abundant diets, but there were no visible effects on plasma LDL-cholesterol.

Another effect of ingesting elevated levels of phytosterols, apart from lowering plasma and LDL cholesterol, is an increase in the ratio of liver phytosterol : cholesterol and a decrease in liver-cell membrane fluidity. This was shown by Leikin and Brenner (1989) in rats given high amounts of phytosterols (3% β-sitosterol + 2% campesterol). The effects of phytosterols on lipid metabolism were investigated by Laraki et al. (1993) by feeding rats diets with 12 or 24 mg cholesterol a day and 0 - 96 mg phytosterols. The rats showed lowered liver fatty acid and cholesterol when the ratio of phytosterols : cholesterol was higher than 1 and in the case of cholesterol excess. Phytosterols are also reported to have several additional therapeutic effects on animals; they are anticarcinogenic (Awad et al., 2003; Rao and Janezic, 1992; Rao et al., 1997; von Holtz et al., 1998), are anti-inflammatory (Bouic, 2001) and have anti-oxidative effects (Van Rensburg et al., 2000).

Mechanisms of phytosterols' cholesterol-lowering effect

The mechanisms with which phytosterols reduce plasma cholesterol are not entirely understood, but the parts of the puzzle are starting to fall into place with research discovering different ways phytosterols can affect the sterol balance in the body. Most of the theories until now propose that phytosterols, in some way, inhibit or diminish the uptake of cholesterol through the intestine. Phytosterols replace cholesterol from micelles (Brown et al., 2010; Matsuoka et al., 2010), and with this inhibits the uptake of dietary cholesterol. The ratio of phytosterols : cholesterol does, however, need to be over 1 for the micellar replacement of cholesterol with phytosterol to occur (Brown et al., 2010). Another theory, that has not been properly tested, is that the cholesterol in the intestine is made non-absorbable by the presence of phytosterols and phytostanols (Moreau et al., 2002). ABCG5 and ABCG8 transport cholesterol out of the enterocytes and into the intestinal lumen, thus preventing its absorption (figure 1.4), and they have been hypothesized to be connected to phytosterols' effect on the cholesterol absorption. Increased expression of ABCG5/8 has been seen when phytosterol is given to mice, but has been shown by using $ABCG5^{-/-}$ mice not to be connected with the decreased cholesterol absorption associated with ingestion of phytosterols (Plat and Mensink, 2002; Plösch et al., 2006).

The transporter involved in sterol absorption, NPC1L1 (Altmann et al., 2004), suspected to be regulated by SREBPs (Alrefai et al., 2007), has been shown to be affected by several dietary factors, like PUFAs (Alvaro et al., 2010), DHA (Mathur et al., 2007) and sterols (Jesch et al., 2009). Jesch et al. (2009) showed that cholesterol and β-sitosterol have an ability to reduce both transcription and protein levels of NPC1L1 in a human small intestine epithelial cell line, thus reducing the uptake of sterols into the enterocytes by this transporter.

LXR has also been shown to be affected by sterols, as several phytosterols upregulates the expression of both LXR-α and –β (Kaneko et al., 2003; Plat et al., 2005). But since the ABCG5/8 has been shown to not be the target of phytosterols in the intestine, and the ABCG5/8 are necessary for an LXR induced reduction of cholesterol (Yu et al., 2003), it appears that the LXR pathway is not crucial for the plasma cholesterol-lowering effect of phytosterols.

1.4 Effects of plant derived feed on sterol metabolism in fish

A number of experiments have been performed where the effect of vegetable oils in the diet to fish is investigated, and they generally show that VO can replace FO without compromising growth and feed utilization (Leaver et al., 2008; Miller et al., 2008; Nanton et al., 2007; Torstensen et al., 2005; reviewed by Turchini et al., 2009). Some studies have also replaced the fish meal in the feed with plant protein (PP), showing a better feed utilization, but lower growth because of a decreased feed intake in Atlantic salmon fed a diet with plant protein balanced with crystalline amino acid, compared to the ones fed marine protein (Espe et al., 2006). When replacing both the fish meal and fish oil with plant ingredients, lower feed intake, specific growth rate and lower final weight has been observed when high amounts of both dietary factors are replaced (80% of the fish meal and 70% of the fish oil replaced with plant proteins and vegetable oil, Torstensen et al., 2008).

When changing the marine lipids in the feed with oils from other sources, the FA composition in the dietary lipid is reflected in the FA composition in the tissues of the fish (Jordal et al., 2007; Nanton et al., 2007; Tocher et al., 2001; Torstensen et al., 2000; 2005), generally giving lower contents of highly unsaturated n-3 fatty acids (n-3 HUFA) in VO fed fish than in FO fed fish. Changes in lipid metabolism have been shown on several occasions to be caused by VO diets, often resulting in higher liver TAG when replacing FO with a VO blend (Jordal et al., 2007) or linseed or soybean oil (Leaver et al., 2008).

The increased liver TAG in VO fed fish could be connected to the low levels of HUFA in the VO feeds, since EPA and DHA enriched diets have been observed to decrease hepatic TAG synthesis (Kjær et al., 2008). The lower levels of HUFA in the VO diets is probably also the reason why increased activity of the desaturation and elongation of 18:3 n-3 to HUFAs has been observed many times in Atlantic salmon fed VO feed (Bell et al., 2001; Leaver et al., 2008; Stubhaug et al., 2005; Tocher et al., 2001;2003). An accumulation of phytosterols in salmon muscle and liver is also associated with rapeseed oil based diets, as shown by Miller et al. (2008). Furthermore, the absorption of phytosterols in Atlantic salmon was reported to be poor compared to cholesterol, and some phytosterols were more efficiently absorbed and accumulated than others (Miller et al. 2008).

Some effects on plasma and lipoprotein levels in salmon fed VO diets have been seen, but are somewhat contradictory. Jordal et al. (2007) found a significant decrease in plasma lipids and plasma LDL levels in VO fed fish compared to FO fed fish, whilst others found an increase in plasma lipid and VLDL (Torstensen et al., 2011) or did not find any significant effect of diet on plasma and lipoprotein composition (Torstensen et al., 2000). The study by Torstensen et

al. in 2011 did, however, use a feed with high inclusions of both plant protein and VO, which could have affected the fish more than by just replacing the lipid of their diet. In the Jordal et al. (2007) study the fish were given the VO diets from start feeding, something that could also enhance the effect of the diet change.

VO diets for Atlantic salmon are lower in cholesterol than a traditional fish oil and fish meal based diet, as a regular fish meal/fish oil diet contains about 3 - 4 g cholesterol kg^{-1} feed and a 70% VO 30% FO diet contain less than 1 g cholesterol kg^{-1} feed (Torstensen et al., in prep.). It seems, however, that the Atlantic salmon compensate for low cholesterol levels by upregulating their synthesis of cholesterol, as observed by Leaver et al. (2008) in a microarray study, where VO fed fish had an upregulation of genes involved in cholesterol synthesis in the liver than FO fed fish. They also reported an increase in expression of genes involved in HUFA synthesis in the VO fed fish. VO diets have also shown to lower the expression of LXR in Atlantic salmon, which may lead to a higher catabolism and efflux of cholesterol in the fish (Cruz-Garcia et al., 2009).

Gilman et al. (2003) is one of very few studies specifically focusing on the effect of phytosterol on fish. They tested the effect of the main phytosterol effluent from pulp mills, β-sitosterol, on male brook trout (*Salvelinus fontinalis*) and goldfish (*Carassius auratus*), and found that by giving the fish this sterol in higher concentrations through the water during 20 days, the plasma cholesterol, LDL cholesterol, and plasma triglycerides decreased (Gilman et al., 2003). Some negative effects on fish health of high levels of phytosterols in the water have also been observed, as the phytosterol β-sitosterol functions as a hormone mimic, and thus affected the endocrine and reproductive functions in goldfish and rainbow trout (Maclatchy and Van der Kraak, 1995; Tremblay and Van Der Kraak, 1998).

1.5 Aim of the study

The increasing need for a change from marine towards plant raw materials in aquaculture feed means that a carnivore species will eat vegetable oils and plant proteins to which it is not accustomed to. Vegetable ingredients based diets contain low levels of cholesterol, high levels of phytosterols and an altered fatty acid composition. The first aim of this master thesis was thus to elucidate the effect on sterol metabolism of Atlantic salmon when replacing FO with OO, RO or SO on Atlantic salmon sterol composition, when FM was maintained low.

Low levels of dietary cholesterol and high levels of dietary phytosterols have a plasma cholesterol lowering effect in humans. The second aim of this master thesis was to decide if this is the case also in Atlantic salmon and thus giving a possible change in the cardiovascular health of the fish.

1.6 Method

The sterol metabolism is a complex mechanism and to understand it and the changes it may suffer because of a diet change, one has to take into consideration the absorption of sterol, their flux in the organism and their subsequent efflux. By choosing an array of methods, these three aspects of the sterol metabolism was monitored through the feeding trial. To measure the uptake of the sterols, a digestibility sampling was done where sterol content in faeces was measured, as was the gene expression of receptors for uptake of sterols in the enterocytes. When it comes to the metabolism and storage of sterols in the organism this was investigated by determining the sterol composition of liver, the main organ for maintaining sterol balance in many animals (Dietschy et al., 1993), and the expression of genes involved in the sterol metabolism. Some of the selected genes were found to be changed in their expression in another experiment where fish oil was replaced by vegetable oil (Leaver et al., 2008). The content of cholesterol and other variables in plasma and lipoprotein fractions was also determined, as this is an important factor concerning cardiovascular health and sterol status. As to the efflux of sterols, this was observed through the expression of genes coding for receptors that pump sterols out through the intestine and rate determining enzymes in the bile salt synthesis. Sterol composition was, in every case where it needed to be defined, determined by identification of the specific sterols by a gas-chromatographic method.

2. Materials and Methods

2.1 Experimental design

The effect on Atlantic salmon, *Salmo salar*, of replacing both protein and lipid in the feed with vegetable ingredients was investigated during 6 months on adult fish, 14 months old, to slaughter size. The dietary trial started the 15th of April 2010 at Lerang Research Station, Skretting ARC, Stavanger, Norway, with a final sampling date the 15th of October 2010. In April 2010, 600 fish with a mean weight of 815 ± 28 g (mean ± SD) were distributed equally in 12 tanks (50 fish per tank). The tanks had a diameter of 3m, a volume of 7000L and a flow between 85 - 92 L min^{-1}. The mean water temperature was 9.9 ± 0.6 °C (mean ± SD).

Atlantic salmon was fed in triplicate (n=3) with fish oil (FO) based or vegetable oil (VO) based feeds, all of them with a high plant protein content (figure 2.1 for basic diet designs). 70 % of the fishmeal was replaced by plant protein sources in combination with either 100% fish oil or one of three oil mixes replacing 80% of the fish oil (FO) (table 2.1). The olive oil (OO) and soybean oil (SO) feeds contained a mixture of linseed oil and palm oil to produce a balanced dietary saturated-, monounsaturated- and polyunsaturated fatty acid level (SFA, MUFA and PUFA, respectively). The feed with rapeseed oil (RO) contained no other vegetable oil than RO. Plant protein sources were blended sensibly and necessary crystalline amino acids added to meet known amino acid requirements (National Research Council (U.S.), 1993). Due to a higher feed intake than predicted, a second batch of feed had to be made. All feed was made from the same formulation, but with a slightly different nutrient, fatty acid and phytosterol composition due to raw material batches being used. In table 2.2 the fatty acid composition and sterol content in the two feed batches is shown. The experimental feed containing fish oil had a high n-3 / n-6 ratio and cholesterol level, but low levels of phytosterols compared to the VOs. The vegetable oil diets were formulated to

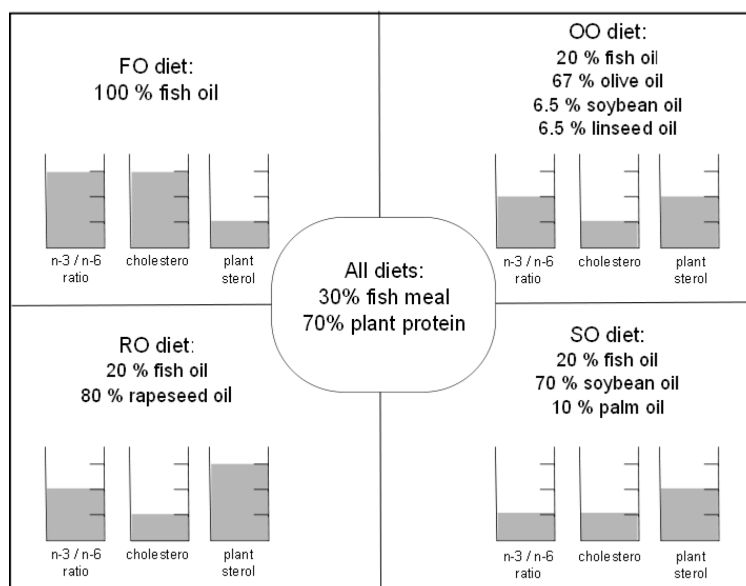


FIGURE 2.1: BASIC COMPOSITION AND OILS USED IN THE EXPERIMENTAL DIETS. MEASURING CUPS INDICATE IF SPECIFIC DIETS CONTAIN LOW, INTERMEDIATE OR HIGH LEVELS OF EACH CHOSEN PARAMETER.

contain similarly low cholesterol levels (table 2.2.). RO was chosen for its high phytosterol levels, OO and SO in the feeds gave intermediate levels of phytosterols. Further, the vegetable oils were chosen to obtain intermediate (OO and RO) and low n-3 / n-6 ratio (SO) (see figure 2.1 for basic design of the diets). Palm oil was added to the SO feed, to increase the amount of saturated fats in the feed. Also, linseed oil and SO was added to the OO diet, to give a more similar fatty acid composition as in the RO diet.

Because of a higher feed intake than predicted, a second feed batch had to be made during the feeding trial. Fish were fed batch 1 diets from the 15th of April till the 1st of September, and then batch 2 was introduced from 1st of September to the 15th of October.

TABLE 2.1: FORMULATION AND PROXIMATE COMPOSITION OF EXPERIMENTAL FEEDS

	FO		*OO*		*RO*		*SO*	
Ingredients ($g\ kg^{-1}$)								
Fish meal[1]	180		180		180		180	
Wheat gluten[2]	162.3		162.3		162.3		162.3	
Hi-pro soya[3]	97.3		97.3		97.3		97.3	
Soya concentrate[4]	150		150		150		150	
Wheat [5]	70		70		70		70	
Fish oil[6]	309.9		62		62		62	
Rapeseed oil [7]	-		-		248		-	
Olive oil [8]	-		208		-		-	
Soybean oil [9]	-		20		-		217.9	
Palm oil [10]	-		-		-		30	
Linseed oil[11]	-		20		-		-	
Premixes[12]	31.8		31.8		31.8		31.8	
Total weight, g	1001.3		1001.3		1001.3		1001.3	
Yttrium oxide premix[13]	1.0		1.0		1.0		1.0	
	Batch 1	**Batch 2**	**Batch 1**	**Batch 2**	**Batch 1**	**Batch 2**	**Batch 1**	**Batch 2**
Composition, $g\ 100\ g^{-1}$								
Fat	35.6	34.1	34.6	33.6	34.2	34.5	35.0	33.2
Protein	40.6	41.1	40.5	40.8	41.3	41.3	39.8	40.6
Moisture	6.6	6.8	6.1	6.9	6.3	6.4	6.3	6.6
Ash	5.2	5.3	5.1	5.2	5.2	5.2	5.2	5.2

1 South American fish meal, Skretting, Stavanger, Norway
2 Cargill Nordic, Charlottenlund, Denmark
3 Felleskjøpet, Stavanger, Norway
4 Imcopa, Araucaria, Brazil
5 Skretting, Stavanger, Norway
6 Northern hemisphere fish oil, Skretting, Stavanger, Noway
7 Skretting, Stavanger, Norway
8 D. Danielsen AS, Stavanger, Norway
9 Denofa AS, Fredrikstad, Norway
10 Fritex 24, Aarhus Karlshamns, Karlshamn, Sweden
11 Elbe Fetthandel GmbH, Geesthacht, Germany
12 Include vitamins and minerals; Trouw Nutrition, Boxmeer, the Netherlands, proprietary composition Skretting ARC
13 Contains 10% Yt_2O_3, Treibacher Auermet, Althofen, Austria

TABLE 2.2: FATTY ACID COMPOSITION AND STEROL CONTENT IN EXPERIMENTAL DIETS

	FO		*OO*		*RO*		*SO*	
Fatty acids (area %)	**Batch 1**	**Batch 2**	**Batch 1**	**Batch 2**	**Batch 1**	**Batch 2**	**Batch 1**	**Batch 2**
14:0	7.4	6.8	1.4	1.5	1.8	2.0	1.5	1.7
16:0	18.4	15.9	12.3	12.4	7.9	7.8	15.1	14.8
18:0	3.7	2.9	3.4	3.3	2.6	2.5	3.7	3.8
Total saturated FA	31.8	26.8	19.0	18.0	14.4	13.2	22.3	21.2
16:1n-7	6.9	5.9	1.7	1.9	1.8	1.8	1.3	1.5
18:1n-7	2.2	2.0	1.8	2.2	2.9	2.7	1.7	1.6
18:1n-9	9.7	11.9	50.2	50.3	44.2	42.7	22.0	21.5
20:1n-9	3.8	5.6	1.0	1.4	1.8	2.4	0.9	1.4
22:1n-9	0.4	0.6	0.1	0.1	0.2	0.4	0.0	0.1
22:1n-11	6.2	10.2	1.1	2.0	1.4	2.8	1.1	2.2
Total monoene FA	30.3	38.5	56.1	58.4	52.4	53.5	26.9	28.6
18:2n-6	2.6	2.7	13.7	12.1	17.3	15.8	40.1	37.5
20:3n-6	0.0	0.9	0.0	1.0	0.0	1.1	0.0	1.3
20:4n-6	1.1	0.5	0.2	0.1	0.2	0.2	0.2	0.1
18:3n-3	1.1	1.4	4.6	4.1	8.3	7.8	4.9	4.4
18:4n-3	2.6	2.6	0.5	0.5	0.6	0.7	0.5	0.6
20:4n-3	0.8	0.7	0.2	0.2	0.2	0.2	0.2	0.2
20:5n-3	11.0	8.1	2.2	1.9	2.7	2.5	2.1	2.0
22:5n-3	2.0	1.1	0.4	0.3	0.5	0.3	0.4	0.3
22:6n-3	8.9	10.0	1.9	2.3	2.3	3.0	1.7	2.4
Sum n-3	29.1	24.9	10.5	9.5	15.2	14.8	10.2	10.1
Sum n-6	4.4	4.7	13.9	13.2	17.5	17.2	40.3	39.0
Sum EPA & DHA	20.0	18.1	4.1	4.2	4.9	5.4	3.8	4.5
Sum PUFA	34.3	30.1	24.6	22.8	32.9	32.2	50.6	49.3
n-3/n-6	6.7	5.3	0.8	0.7	0.9	0.9	0.3	0.3
Sterols (mg kg^{-1} feed)								
Cholesterol	2110.7	2576.2	759.7	909.9	939.7	1114.5	773.1	967.6
Brassicasterol	10.8	57.2	6.0	3.5	195.3	170.1	3.5	0.2
Campesterol	76.3	60.9	170.1	113.1	881.4	583.0	231.3	174.3
Campestanol	19.7	17.5	21.5	21.1	21.4	75.2	25.8	24.6
Stigmasterol	10.2	9.2	38.2	30.5	13.5	17.4	136.8	106.6
β-Sitosterol	208.6	143.3	733.5	564.4	1218.4	821.5	574.1	439.6
Sitostanol	32.4	27.5	123.9	171.8	128.2	183.8	74.9	46.9
Stigmasta-5,24-dienol	0.0	0.0	6.1	10.3	13.4	6.1	9.7	5.1
Stigmast-7-enol	10.6	2.6	175.7	129.5	47.5	6.2	76.0	64.4
Delta-7-avenasterol	3.5	0	4.7	0	6.7	9	15.4	12.6
Total phytosterol	372.1	318.2	1279.7	1044.2	2525.8	1872.3	1147.4	874.3

2.2 Sampling

All the experimental feeds were sampled and stored at -20 °C till the moment of analysis. There were three samplings of the experimental fish: one before the fish were fed the experimental feed, 09th of April 2010, one approximately eleven weeks later, 23 - 24th of June 2010, and one at the end of the feeding trial, 19 - 21st of October 2010 . In addition to this a digestibility sampling was performed at about T = 22 weeks. The final meal prior to the samplings was 24 hours at the initial and second sampling and 48 hours prior to the final sampling. This change in starvation time was due to observed feed in the digestion tract of the fish in the second sampling. The salmon were anaesthetized with MS222 at a concentration of 7 g L^{-1}, killed by a blow to the head and individually weighed and measured.

Initial Sampling:

The first sampling was done before the feeding experiment started. Three random samples were taken from the whole experimental fish population. Liver samples for analysis of phytosterols were collected from five fish (n = 3), pooled and homogenized and stored on dry ice. For the plasma samples, blood from five fish per tank was collected and centrifuged, plasma was pooled to obtain one sample per tank, and then stored on ice/refrigerator until separated into lipoprotein fractions (n = 3).

Intermediate and final Sampling:

The intermediate and final samplings were similar except from the starvation time (see above, under Sampling).

For the RNA samples, three fish from each tank were sampled and ca 1 g of liver per fish was individually put in eppendorf tubes and immediately frozen on liquid nitrogen. The same procedure was followed for the midintestine samples for RNA analysis. For the plasma samples, blood from five fish per tank was collected and centrifuged together, further treatment of plasma samples as in initial sampling. Liver samples for analysis of phytosterols were collected from five fish per tank, in the same manner as in initial sampling.

Digestibility sampling:

The 8th of September 2010, pooled samples of faeces from three fish per tank were taken by opening the intestine of the fish and taking faeces out of the hind gut (n = 3). The fish were fed 12 hours before the sampling, resulting in digested feed in the hind gut.

The samples from all the samplings were stored at -80 °C until further analysis.

2.3 Dietary proximate, fatty acid and lipidclass composition

Diets were analyzed for the content of dry matter, crude protein, total lipid and fatty acids, phytosterols, starch, gross energy, ash and yttrium oxide. Dry matter in the diets was measured gravimetrically after freeze-drying of homogenized samples for 48 h. Ash was determined gravimetrically after flame combustion at 550 °C until constant weight. Total nitrogen was determined on homogenized, freeze-dried samples using a nitrogen determinator (LECO, FP-428 system 601-700-500; Perkin Elmer Coop., CT, USA; crude protein: N × 6.25). Energy content was measured by bomb calorimetry (Parr 1271, Parr, Moline, IL, USA). Yttrium was analyzed by dissolution of ashed samples with hydrochloric acid and nitric acid by heating, and then dissolved in 5% nitric acid. Yttrium was then detected with an ICP-AEF, Optima 3000 V (Perkin Elmer, USA). Total lipid of the diets was measured gravimetrically after ethyl acetate extraction and after acid hydrolysis of the diet samples.

For analysis of fatty acids, lipids from the samples were extracted by adding chloroform/methanol (2:1, v/v). After extraction of lipids, the samples were filtered, an aliquot was removed for determining lipid class composition as described below, and then the remaining samples were saponified and methylated using 12% BF3 in methanol. Fatty acid composition of total lipids was analysed using methods described by (Lie and Lambertsen, 1991) where the methyl esters were separated using a Trace gas chromatograph 2000 (Fison, Elmer, USA) ('cold on column' injection; 60 °C for 1 min 25 °C min^{-1}, 160 °C for 28 min 25 °C min^{-1}, 190 °C for 17 min 25 °C min^{-1}, 220 °C for 10 min), equipped with a 50-m CP-sil 88 (Chromopack) fused silica capillary column (id : 0.32 mm). The fatty acids were identified by retention time using standard mixtures of methylesters (Nu-Chek, Elyian, USA), and the fatty acid composition (area %) were determined. All samples were integrated using the Totalchrom software (ver. 6.2, Perkin Elmer) connected to the Gas Liquid Chromatography (GLC). Amount of fatty acid per gram feed was calculated using 19:0 methyl as internal standard.

The quantification of lipid class composition in diets and liver was determined by high-performance thin-layer chromatography (HPTLC) as described by Bell et al. (1993).Ten microgram total lipid was applied to a 10 cm · 20 cm HPTLC plate that had been prerun in hexane : diethyl ether (1 : 1 v/v) and activated at 110 °C for 30 min. The plates were developed at 5.5 cm in methyl acetate, isopropanol, chloroform, methanol and 0.25% (w/v) aqueous KCl (25 : 25 : 25 : 10 : 9, by volume) to separate phospholipid classes with neutral lipids running at the solvent front (Vitello and Zanetta, 1978). After drying, the plates were developed fully in hexane, diethyl ether and acetic acid (80 : 20 : 2, v/v/v) to separate neutral

lipids and cholesterol. Lipid classes were visualized by charring at 160 °C for 15 min after spraying with 3% copper acetate (w/v) in 8% (v/v) phosphoric acid identified by comparison with commercially available standards. Lipid classes were quantified by scanning densiometry using a CAMAG TLC Scanner 3 and calculated using an integrator (WinCATS-Planar Chromatography, Version 1.2.0). Further, quantitative determination (mg lipid class g^{-1} tissue) of lipid classes was performed by establishing standard equations for each lipid class within a linear area, in addition to including a standard mix of all the lipid classes at each HPTLC plate for corrections of between plate variations.

2.4 Analysis of sterol content

Analysis of phytosterol and cholesterol contents in liver, faeces and feed was performed with gas chromatography as described by Laakso (2005). 500 µl of internal standard, 5-β-cholestan-3-α-ol dissolved in isopropanol to 0.6 mg ml^{-1}, was measured and dried on a heatblock (max. 80 ˚C) with nitrogen flow. The sample was added, exactly about 200 mg of sample if liver; 400 mg if feed; 50 mg if faeces or 20-30 mg if control material, in this case phytosterol enriched margarine of the brand vita pro-aktiv (Mills DA). All the samples except faeces were analyzed without prior freeze-drying.

To saponify the samples 2.5 mL of 2 M KOH solution was added and the tube was put in an ultrasonic bath for an hour at 65-75 ˚C. When chilled to room temperature, 2 mL of water and 3 mL of hexan was added and centrifuged one minute at 3000 rpm. The hexan phase was collected and the extraction process repeated with 3mL hexan. The extract was dried on heatblock (max. 70 °C) with nitrogen flow and then derivatized to trimetyl silyl ether of sterols by adding 100 µl pyridine and 200 µl silylation reagent (Bis[trimethylsilyl]-trifluoracetamid with 1% trimethylchlorosilane) and warmed on a heatblock at 70 °C for 15 min. The samples were diluted 20x in hexan before analysis on GC.

The following instrumentation was used: Thermoquest trace GC 2000 with an autosampler AS2000 (Thermo Finnigan Quest), on-column injector, flame ionization detector and the column Equity® with dimensions 530 m*0.25 mm i.d. (Supelco, Bellefonte, USA). This is not the classic phytosterol-column (which has an internal diameter of 0.32 mm), but it has in the NIFES laboratory shown to give better separation of the phytosterols although it is more sensitive to overloading than the classical one. Helium was used as a carrier gas at 0.9 ml min^{-1}, hydrogen and air were used as detector gases at 35 and 350 ml min^{-1}, respectively. Temperature started at 100 °C, increased by 50 °C min^{-1} to 300 °C and kept for 12 minutes. The peaks were identified with the software Chromeleon® version 6.8.

2.5 Separation of lipoproteins

Separation of lipoproteins was performed by centrifugal flotation as described by Jordal et al. (2007). Potassium Bromide was added to obtain the density intervals of the different lipoproteins. The instrument used was a Beckman Optima XL-100K set to 197600 · g_{av} and 4 °C in all steps of the separation process. The following centrifuge times and densities were used for the separation: VLDL 1.015 g mL^{-1} 20 hours; LDL 1.085 g mL^{-1} 20 hours; HDL 1.21g mL^{-1} 44 hours. The remaining fraction was called non-lipoprotein fraction (NLP). All the fractions were weighed and immediately frozen at -80 °C.

2.6 Plasma and lipoprotein composition

Plasma and lipoprotein were analyzed using a clinical bioanalyzer (Maxmat PL analyzer, Montpellier, France) according to standardized procedures, reagents and controls to find contents of Alaninaminotransferase (ALAT), Aspartataminotransferase (ASAT), cholesterol, glucose, protein and triglycerides. All the reagents, controls and the calibrator solution were from MaxMat S.A. (Montpellier, France). The used reagents were ALAT/GPT 500, ASAT/GOT 1500, Cholesterol 1600, Glucose PAP 800, Total protein 600 and Triglycerides 2000. The control solutions were Maxtrol P (abnormal multiparametric control) and Matrol N (normal multiparametric control). The calibrator was the multiparametric calibrator MaxCal.

2.7 Real-time Quantitative PCR (qPCR)

-Development of qPCR assay

Primers were designed for the genes ACAT2, CYP7A1, ABCG5, and NPC1L1, the LXR primer was found in the PhD thesis of Lourdes Cruz-Garcia (2010), whereas the other primers were previously presented by Leaver et al. (2008) and Torstensen et al. (2011).

The mRNA sequences for ABCG5, ACAT2, and CYP7A1 from zebra fish, *Danio rerio* were found on the NCBI database, the sequences for HMGR and NPC1L1 were found in the human genome. Using a BLAST tool searching in the nucleotide selection, mRNA sequences from *Salmo salar* were found for ACAT2 and CYP7A1. For the rest of the genes, sequences from rainbow trout, *Oncorhynchus mykiss,* were used (table 2.3 for accession numbers).

Using the online primer designing program primer3 (Rozen and Skaletsky, 2000), primers were designed from the reading frame sequence when available, found in the NCBI database. The three best constructs recommended by primer3 were selected and ordered from

TABLE 2.3: GENES, ACCESSION NUMBERS, AND VARIOUS INFORMATION ABOUT THE PRIMERS USED FOR QPCR

Gene name	*Accession N°*	*Amplicon size, BP*	*Anneal. Temp., °C*	*Forward Sequence*	*Reverse Sequence*	*Tissue*	*Mean efficiency of qPCR*
ACAT-2	NM001139949.1	207	60	CAGATGGTGTGTGGATCTGG	GCAGTGGAAAGCATCAGTCA	Liver	1.63
Apo A-1	CB506105	73	60	CCATCAGCCAGGCCATAAA	TGAGTGAGAAGGGAGGGAGAGA	Liver	1.79
Apo B-100	gi:854619	121	60	TTGCAGAGACCTTTAAGTTCATTCA	TGTGCAGTGGTTGCCTTGAC	Liver	2.76
Apo C-2	DN047858	145	60	GGAACCAGTCGCAGATGTTGA	TGAGGACATTCGTGGCCTTC	Liver	1.75
CYP7A1	BT059202.1	178	60	GAAGACCTTGCAGGGTGAAG	GCCGAAGAGTGTCAGGTAGC	Liver	2.18
DHCR7	TC99602	230	60	CTTCTGGAATGAGGCATGGT	-ACAGGTCCTTCTGGTGGTTG-	Liver	1.82
LDLR	AJ003118	78	60	GCATGAACTTTGACAATCCAGTGTAC	TGGAGGAGTGCCTGCTGATAT	Liver	1.84
MVK	EZ861975.1	243	60	GCCTGCAACCGTAGGTATGT	AAGAGTGCCGACAGCTTCAT	Liver	1.74
SREBP2	DY733476	225	60	GACAGGCACAACACAAGGTG	CAGCAGGGGTAAGGGTAGGT	Liver	2.13
HMGR	DW561983	224	60	CCTTCAGCCATGAACTGGAT	TCCTGTCCACAGGCAATGTA	Liver	2.06
LXR	NM001159338	171	60	TGCAGCAGCCGTATGTGGA	GCGGCGGGAGCTTCTTGTC	Liver	2.13
ABCG5	EZ885222.1	165	60	CATGGTCAACACAGGAGTGG	AGGCCATGGAACTCTGTGAC	Intestine	2.04
NPC1L1	EZ788198.1	178	60	GATGGAATAGAGCCGACAGC	GGGTATGAATGCAACCTGCT	Intestine	2.31
β-actin (Reference gene)	BG933897	91	60	CCAAAGCCAACAGGGAGAAG	AGGGACAACACTGCCTGGAT	Ref. gene	Liver: 2.18 Intestine: 2.00
E1ab (Reference gene)	BG933853	59	60	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACTG	Ref. gene	Liver: 2.12 Intestine: 2.14

Invitrogen. All the ordered primers had a melting point of approximately 60.0 ˚C and a product size range between 150 and 250 basepairs. The efficiencies of the primerpairs were between 1.63 and 2.76. The melting point, product size and efficiency of each primer are shown in table 2.3. The two reference genes, β-actin and Elongation factor 1ab (E1ab), were selected based on previous verification by Olsvik et al. (2005).

-One-Step PCR

To verify the efficacy of the primers developed, a one-step PCR was performed. In this reaction, all the reagents needed for a RT-reaction and a PCR were added to have both these reactions in only one step. A master mix was made using the QiaGen Onestep RT-PCR kit and RNase inhibitor was added. The temperature cycler used was a GeneAmp® PCR System 9700 (Applied Biosciences). The PCR was done by starting at 50 °C for 30 minutes for the reverse transcriptase, then 15 min at 95°C for PCR activation, 33 cycles followed with 45 s denaturation at 94 °C, 45 s annealing at 55 °C, 1 min elongation at 72 °C and a 10 min final extension at 72 °C.

The PCR product was run on a 1% agarose gel, using GelRed™ Nucleic Acid Stain (Biotium) as a dye and a 50 bp DNA ladder from Biolabs. The current was set to 80 V and the running time about an hour. The following primers were tested: ABCG5, ACAT2, CYP7A1, MVK and NPC1L1.

-RNA isolation

Liver and midintestine samples from individual fish (n = 9 per dietary treatment) was homogenized in Trizol by using zirconium beads (4 mm) in a Retsch MM 310 homogenizer (Retsch GmbH, Haan, Germany). Subsequent addition of chloroform separates RNA from proteins and DNA. RNA was then precipitated from the water phase by adding isopropanol. Further on, the RNA pellet was cleansed two times in ethanol which then was dissolved in RNase free water. A DNAse treatment with DNA-free™ was performed on the RNA extract to remove the DNA that might be present. The final concentration of RNA was measured with the Nanodrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). To check the quality of the RNA 12 random samples were selected and tested with RNA6000 Nano LabChip kit, run on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The quality was satisfactory, with an RNA Integrity Number (RIN) between 9.4 - 10 for the liver samples and 7.6 - 9.7 for intestine. The RNA extracts were frozen at -80 ˚C and stored until RT-qPCR and qPCR was performed.

-*Reverse transcription-PCR and Real-time Quantitative PCR*

Reverse transcription-PCR (RT-PCR)

The standard curve was made from twofold serial dilutions of a “RNA-pool”, a mix of an equal amount all the samples on the plate. Six serial dilutions (1000-31 ng) were made in triplicates in separate wells on the 96 well plate. The 36 samples from each tissue in duplicates were analyzed on the same plate and the amount of RNA in each well was 500 ng. Negative controls, made from the “RNA-pool”, were non-amplification control without enzymes (nac) and non-template control with RNase free water instead of RNA (ntc). The RT reaction mix was made with the kit TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). The final concentrations in the RT mix were: Taqman RT buffer (1X), magnesium chloride (5.5 mM), 1deoxyNTPs Mixture (500 µM per dNTP), oligo $d(T)_{16}$ (2.5 µM), RNase inhibitior (0.4 U μL^{-1}), Multiscribe reverse transcriptase (1.67 U μL^{-1}). 50 µL reverse transcription reactions were performed during 60 min at 48 °C with the PCR system GeneAmp PCR 9700 (Applied Biosystems, Foster City, CA, USA). The finished RT plates were stored at -80 °C until real-time quantitative PCR was to be run.

Real- time quantitative PCR (qPCR)

The RT plates were defrosted on ice and added RNase free water (1:1 v/v), spun down for 1 minute at 700 rpm and vortexed for 5 minutes at 1500 rpm. A SYBR® Green master mix (Roche Applied Sciences, Basel, Switzerland), which contains gene-specific primers was prepared on a RNA/DNA free room. 8 uL of the master mix was added to each well on a 384 well plate together with 2 uL RNA by a pipetting robot (Biomek® 3000, BeckmanCoulter). The plate was spun down, 1500 rpm during two minutes, and run in the Light Cycler 480 Real-Time PCR System (Roche Applied Sciences, Basel, Switzerland). The PCR was accomplished with a denaturation and enzyme activation at 95 °C for 5 min, followed by 40 cycles of the amplification process which consisted of 10 s denaturation at 95 °C, 20 s annealing at 60 °C and 30 s elongation at 72 °C. A melting curve analysis was performed to make sure only one gene product was produced in the PCR reaction. This was done by maintaining 95 °C for 5 s, then 1 min at 65 °C and finally increasing the temperature to 97 °C. The total time of the real time Q-PCR, included final cooling, was 51 min 15 s. The software Lightcycler® 480 software, version 1.5.0.39, was used for calculating standard curves and efficiency of the reaction.

2.8 Statistical analysis and calculations

For the statistical work with the results, the free software environment R was used (R Development Core Team, 2010). One-way ANOVA was performed on the data on liver and plasma sterols, followed by multiple comparisons using Tukey–Kramer HSD (Hothorn et al., 2008). The data were analyzed for homogeneity in variance using a Levene's test and for normality using a Shapiro Wilk's test. Linear mixed effects models (Pinheiro et al., 2010) were used for the genetic expression and growth results, to be able to take into account the cluster effect each tank has on the individual results. For these results the normality of the residuals and their constant variance from the model was confirmed by graphical analysis. Examples of plots used for this analysis are given in the appendix (figure A.1 and A.2). Data are given as mean ± SD, and a significance level of 5% was used ($p < 0.05$) unless otherwise stated.

The stability of the expression of each reference gene was calculated from the C_t values by using the program geNorm (Vandesompele et al., 2002) according to the geNorm manual. GeNorm determines the stability of each gene within a pool of genes, given as the M value. A normalization factor is made from the geometrical mean by a pairwise comparison of the expression profile of the genes. The M values of both the reference genes were satisfactory, 0.40 - 0.75, when all 36 samples from each tissue were evaluated together. Both of the reference genes were thus included in the normalization factor. The normalized expression of the genes was calculated by dividing each C_t value by its respective normalization value.

The apparent digestibility coefficients (ADC) were calculated as: $ADC (\%) = 100 - [100((Y_{diet}/Y_{faeces}) * ((ST_{faeces}/ST_{diet}))]$, where Y is the percentage of yttrium oxide and ST the percentage of particular sterols. Yttrium oxide has been shown to be valid for this type of digestibility experiment by Carter et al. (2003). The calculation does not take into consideration the possible dealkylation of phytosterols to cholesterol (Miller et al., 2008).

2.9 Methodological considerations

Validity of PCR data

In modern qPCR there are still some dubieties as to which methods are the most correct, especially regarding methods of quantification and normalization of output from the reaction (Adams, 2006). Guidelines have, however, been established for how to perform an optimal qPCR in order to have more comparable results throughout the scientific community and

which information that should be reported when publishing (Bustin et al., 2009; 2010). Considerations to be made when performing PCR are discussed below.

Quantification

The method used for quantification of this qPCR reaction is based on fluorescence of SYBR Green®, which increases its fluorescence when bound to double-stranded DNA. There is no need for a probe in this method, which reduces costs, but the disadvantage is that SYBR Green® is unspecific in its ds-DNA binding and may give false positive signals. Another thing to have in mind when using DNA dyes as this is that they may bind several times to every DNA chain depending on their length, but having approximately the same length of all the amplified sequences reduces this error (Applied Biosystems, 2011). If the RNA Integrity Number (RIN) is insufficient the samples could also come out as a negative (Sproul, 2006).

The efficiency of the qPCR is ideally 2.0, as this is the number of molecules synthesized from every template in the exponential phase of the reaction. The SYBR Green assay is known for showing some inhibition, hence possibilities of giving efficiencies over 2.0. An efficiency of at least 80% (e.g. 1.6) is advised by Wang and Seed (2006).

Normalization

For optimal results from qPCR, an appropriate normalization should be done to control for experimental error (Pfaffl, 2006; Vandesompele et al., 2002). To get reliable results when normalizing data from PCR, a similar sample size should be used for all samples and one should ensure that the amount of RNA in each well is the same. There is also a need for verifying the quality of the RNA as degraded RNA may affect results severely (Bustin and Nolan, 2004).

In this experiment the amplicons had approximately the same length, there was the same amount of RNA in each well and the quality of the RNA was verified and acceptable. This is in accordance with the current guidelines (Bustin et al., 2009; 2010), and should have reduced the possible errors in the qPCR performed.

3. Results

3.1 Dietary sterols

The feeds were analyzed for sterol contents, and the mean sterol levels of the two feed batches made is presented in figure 3.1 (exact values for each feed batch are given in table 2.2). The rapeseed oil (RO) feed is the formulation with the highest content of phytosterols, the fish oil (FO) based feed the one with the highest cholesterol content. The mean ratio phytosterol:cholesterol in the two feed batches were 0.15, 1.42, 2.18 and 1.19 for the FO, OO, RO and SO feed, respectively.

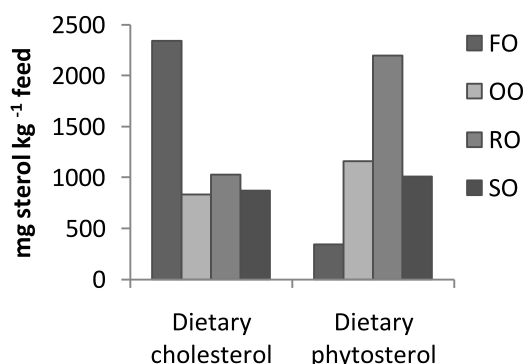


FIGURE 3.1: MEAN PHYTOSTEROL AND CHOLESTEROL, MG KG^{-1}, OF THE TWO FEED BATCHES. FO: FISH OIL. OO: OLIVE OIL, RO: RAPESEED OIL, SO: SOYBEAN OIL.

TABLE 3.1: RELATIVE COMPOSITION OF PHYTOSTEROLS IN THE EXPERIMENTAL DIETS (WT%) AND TOTAL FEED INTAKE IN GRAMS PER FISH DURING EXPERIMENTAL TRIAL (MEAN±SD). SIGNIFICANT DIFFERENCES BETWEEN DIEATRY GROUPS IN FEED INTAKE DENOTED BY DIFFERENT LETTERS (T-TEST, P<0.05).

	Fish oil	*Olive oil*	*Rapeseed oil*	*Soybean oil*
Brassicasterol	10.4	0.3	8.4	0.2
Campesterol	19.8	15.5	33.0	16.6
Campestanol	5.4	2.1	2.4	2.2
Stigmasterol	2.8	7.4	0.7	7.6
β-Sitosterol	50.5	52.0	46.1	53.8
Sitostanol	8.7	11.5	7.4	7.5
Other phyto.	2.3	11.1	1.9	12.0
Total phyto.	100.0	100.0	100.0	100.0
Feed intake, g	2982 ± 79^{a}	2748 ± 169^{ab}	2759 ± 91^{ab}	2573 ± 115^{b}

The three vegetable oil (VO) based feeds had a similar content of cholesterol per kg, but the FO feed had between 2.3 and 2.8 times more cholesterol per kg than the VO feeds (figure 3.1 and table 2.2). The main phytosterols present in the FO feed were β-sitosterol and campesterol at 50.5% and 19.8% of total phytosterols, respectively (table 3.1). In the olive oil (OO) feed phytosterols made out 58.1% of total sterols, β-sitosterol and campesterol being the main ones at 52.0% and 15.5% of total phytosterols, respectively. High contents of β-sitosterol and campesterol also characterized the RO and soybean (SO) based feeds, although the RO feed contained 7.7% more campesterol and 16.4% less β-sitosterol than the SO feed (table 3.1). The RO feed contained the highest ratio of phytosterol/total sterol at 73% and

63% of total dietary sterols in batch 1 and 2, respectively. Dietary fatty acid compositions were close to identical in the two feed batches for all the experimental feeds (table 2.2). Dietary cholesterol contents were higher for all the feeds in batch 2 than in batch 1, being on average 21% higher in feed batch 2 than in feed batch 1 , whereas the phytosterol levels were lower in batch 2 than in batch 1 (15% lower in the FO, 18% lower in OO, 26% lower in RO and 24% lower in the SO diet, table 2.2).

3.2 Growth & feed intake

The fish started out with an average weight of 815 ± 28 g, and after 28 weeks of feeding the weight was 3395 ± 534 g (figure 3.2). The mean length and condition factor at the final sampling, T = 28 weeks, were 59 ± 3 cm and 1.60 ± 0.15, respectively. There were no significant differences in weight, length or condition factor between the dietary groups in the final sampling, although the soybean oil (SO) fed fish were slightly shorter than the other fish and nearly significantly shorter than the FO fed fish ($p = 0.066$) with a mean length of 60.2 cm in the FO group and 58.9 in the SO group.

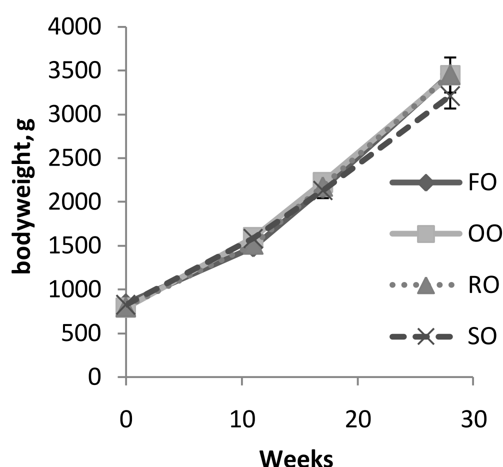


FIGURE 3.2: BODYWEIGHT DURING THE 28 WEEKS OF THE DIETARY TRIAL, MEAN VALUES FOR EACH DIETARY GROUP ± SD

The mean feed intake for all the dietary groups throughout the dietary trial was 2765 ± 182 g per fish (table 3.1), being significantly larger in the FO group than the SO group. The mean weight gain per fish was 2558 ± 79, 2655 ± 164, 2683 ± 90, 2442 ± 99 in the FO, OO, RO and SO group, respectively. The SO group had a slightly smaller weight gain than the other groups, being significantly smaller then the RO group ($p = 0.035$, t-test). The data for sterol amounts in feed were used together with the growth data for calculating the amount of sterols each fish consumed per gram of weight gain through the feeding trial (figure 3.3). The sterol ingested per gram of weight gain reflects the cholesterol and phytosterol composition in the feed (figure 3.1).

The feed conversion ratio (FCR) and specific growth rate (SGR) was calculated for all the dietary groups. The FO group had the highest FCR of all the dietary groups, being significantly higher than the VO groups ($p < 0.001$). The FCR value for FO was 1.17 ± 0.03 and 1.04 ± 0.01 in the VO groups. SGR values for FO, OO, RO and SO were 0.75 ± 0.02,

0.78 ± 0.01, 0.79 ± 0.03 and 0.74 ± 0.01, respectively, and was significantly lower in SO compared to both OO and RO (p = 0.02 and p = 0.05, respectively, t-test).

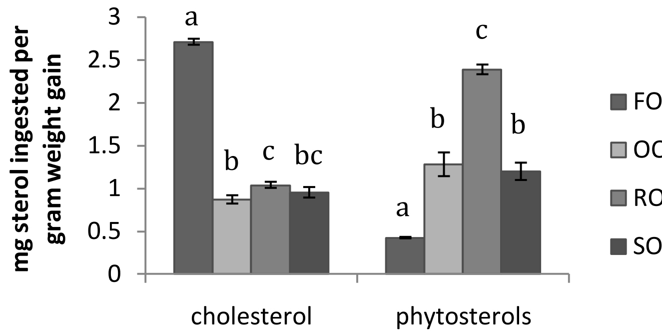


FIGURE 3.3: MG CHOLESTEROL AND PHYTOSTEROLS INGESTED PER GRAM WEIGHT GAIN IN ATLANTIC SALMON FED DIFFERENT EXPERIMENTAL FEEDS DURING 28 WEEK. DATA ARE PRESENTED AS MEAN ±SD (N=3). SIGNIFICANT DIFFERENCES ARE DENOTED BY DIFFERENT LETTERS.

3.3 Cholesterol in liver

There was a tendency of RO fed Atlantic salmon having lower liver cholesterol after 11 weeks of feeding than all the other dietary groups (figure 3.4a), but there was only a significant difference between the RO and the SO group (p = 0.04). At the final sampling, e.g. after 28 weeks, the liver cholesterol levels were significantly higher in the OO and SO group compared to the FO group, with OO containing 3073 ± 35 and SO 3052 ± 66 mg cholesterol per kg liver tissue and the FO fed fish 2844 ± 56 mg cholesterol per kg liver tissue. Due to non-homogeneity in the variance of the data a t-test was performed instead of an ANOVA in this case, which told us that the OO and SO groups were significantly different from the FO group (p = 0.007 and p = 0.015, respectively, figure 3.4b). The content of cholesterol in the RO group at the final sampling was varying, ranging from 2742 and 3028 mg kg^{-1} (figure 3.4b). A significant increase in liver cholesterol levels was seen from the initial sampling to the final sampling for all the groups ($p < 0.008$, t-test) except for the RO group where the variance was very big and thus the changes not significant (figure 3.5). The RO group shows a different behaviour in its liver cholesterol levels than the other groups, as it has a clear decrease from the initial to the second sampling ($p < 0.001$, t-test) which is not present for the other groups (figure 3.5).

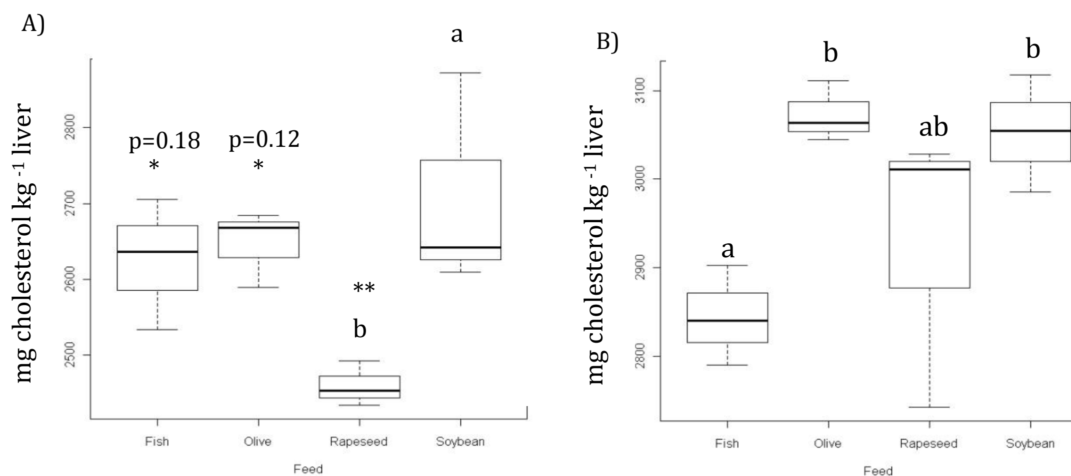


FIGURE 3.4: CHOLESTEROL CONTENTS IN LIVER SAMPLED FROM ATLANTIC SALMON FED DIFFERENT OILS IN DIET, SAMPLED AFTER A: 11 WEEKS AND B: 28 WEEKS. VALUES PORTRAYED ARE THE STANDARD BOX-AND-WHISKER PLOT IN R, I.E. MEDIAN, AND FIRST AND THIRD QUARTILE (Q1 AND Q3). WHISKERS ARE MIN. AND MAX. VALUES. STATISTICAL DIFFERENCES BETWEEN DIETARY GROUPS ARE DENOTED BY DIFFERENT LETTERS. BORDDERLINE SIGNIFICANCE IS DENOTED BY DIFFERENT NUMBER OF STARS AND P-VALUE IS SHOWN.

From the second to the final sampling all the dietary groups had a significant increase in liver cholesterol ($p < 0.001$, t-test; figure 3.5). Even though FO was the group with the highest levels of cholesterol in the feed, it was the group that had the smallest increase in liver cholesterol from the initial to the final sampling. The biggest increase in liver cholesterol from the intermediate to the final sampling was seen in the RO group (+467 mg kg^{-1} tissue), closely followed by OO (+426 mg kg^{-1}) and SO (+344 mg kg^{-1}). The FO group had the smallest change in liver cholesterol from the intermediate to the final sampling (+219 mg kg^{-1}) and also had the lowest cholesterol levels in the liver at the final sampling at 2844 ± 56 mg kg^{-1}. The OO and SO fed groups had the highest levels of liver cholesterol at the final sampling, with 3073 ± 35 and 3052 ± 66 mg kg^{-1}, respectively.

To look for effects of phytosterols in feed on cholesterol levels in liver, only the VO groups were used for the calculations as they have approximately the same cholesterol levels. By excluding the FO group from these calculations, one makes sure that it is only the effect of phytosterols in feed one investigates, and not the possible effect the cholesterol in feed might have on the cholesterol levels in the liver. A correlation of -0.72 between the phytosterol in feed and the cholesterol amounts in liver indicates that an increasing phytosterol amount in the feed is connected to decreasing amounts of cholesterol in liver when considering both samplings (figure 3.6).

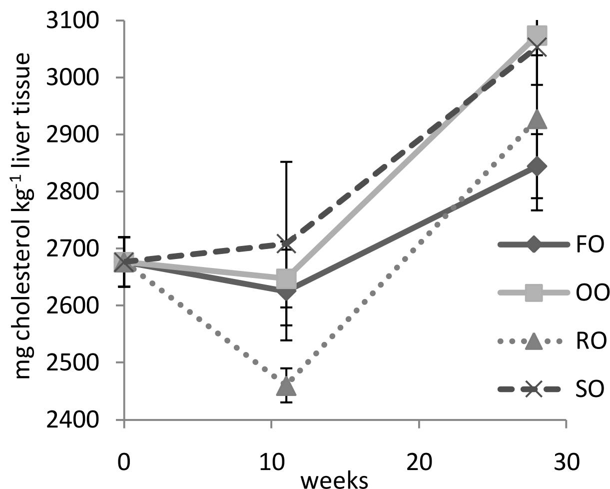


FIGURE 3.5: CHOLESTEROL CONTENTS IN THE LIVER OF ATLANTIC SALMON FED DIFFERENT OILS IN DIET. SEEN IN A TIME PERSPECTIVE, T=0 VALUE IS THE SAME FOR EVERY GROUP. FO: FISH OIL. OO: OLIVE OIL, RO: RAPESEED OIL, SO: SOYBEAN OIL. DATA IS PRESENTED AS MEAN ± SD.

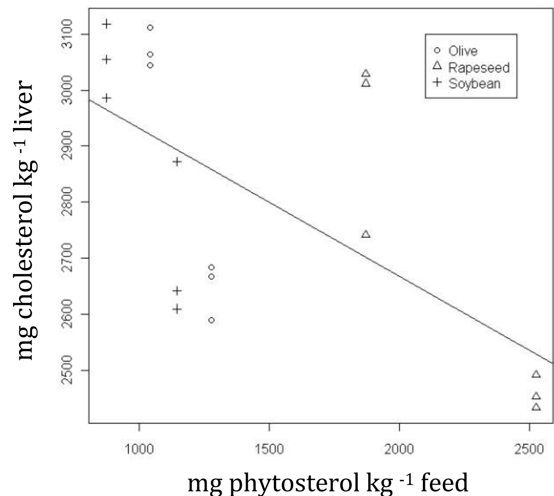


FIGURE 3.6: RELATIONSHIP BETWEEN THE PHYTOSTEROL AMOUNT IN FEED AND CHOLESTEROL IN LIVER, DATA FROM FISH SAMPLED AT INTERMEDIATE AND FINAL SAMPLING (T=11 AND 28 WEEKS, RESPECTIVELY). THE LINEAR MODEL PLOTTED EXPLAINS 40% OF THE VARIABILITY IN THE DATA (MULTIPLE R-SQUARED = 0.40). THE FISH OIL GROUP IS LEFT OUT BECAUSE OF THE HIGH CHOLESTEROL CONTENTS IN THE FO FEED COMPARED TO THE VO FEEDS, THIS MAKES IT POSSIBLE TO SEE THE EFFECT OF ONLY PHYTOSTEROLS ON THE CHOLESTEROL LEVELS IN THE LIVER.

3.4 Phytosterols in liver

The liver phytosterol content in the RO fed fish was significantly higher than in the fish fed the other feeds at both the intermediate and final sampling ($p < 0.003$, figure 3.7a and 3.7b). There was an observed change in time where the RO fed fish had an increase in liver phytosterols from the initial to the final sampling, whilst the rest of the dietary groups had a decrease in the phytosterol levels during the same period (see figure 3.8). There was a general trend that when the amount of phytosterol in the feed increased, the amount of phytosterols in the liver also increased (correlation = 0.77, figure 3.9). Although, this could merely be due to the strong influence the RO fed group's data exerts on the rest, the trend of increasing phytosterol in liver due to increased phytosterol in the feed might just be the case only for concentrations as high as in the RO feed. All the experimental feeds had different phytosterol/cholesterol ratios, being: FO: 0.15; OO: 1.51; RO: 2.18; and SO: 1.25. The groups fed FO, OO and SO feed had approximately the same ratio of phytosterols : cholesterol in the liver at 0.02, the RO fed fish had a ratio at 0.06.

The composition of phytosterols in the liver did also change during the feeding trial. The main phytosterols in the liver before the experiment started were campesterol, at 78 wt% of

the phytosterols. The percentage of campesterol of total phytosterols did decrease with time (figure 3.10a), but it remained the main liver phytosterol in all the dietary groups throughout the whole experiment. The percentage of β-sitosterol of total phytosterols increased at each sampling (figure 3.10b), whilst both brassicasterol and sitostanol increased at the intermediate sampling and decreased to approximately original levels at the final sampling (figure 3.10c and 3.10d). The amount of a sterol in the liver did not always reflect the amount of that particular sterol in the feed they were given. The sterols that had the most dramatic difference between ratios of '*particular phytosterol:total phytosterol'* were campesterol, which occurred in ratios up to 5 times higher in the liver than in the feed, and β-sitosterol which was found in ratios up to 3 times lower in the liver than in the feed.

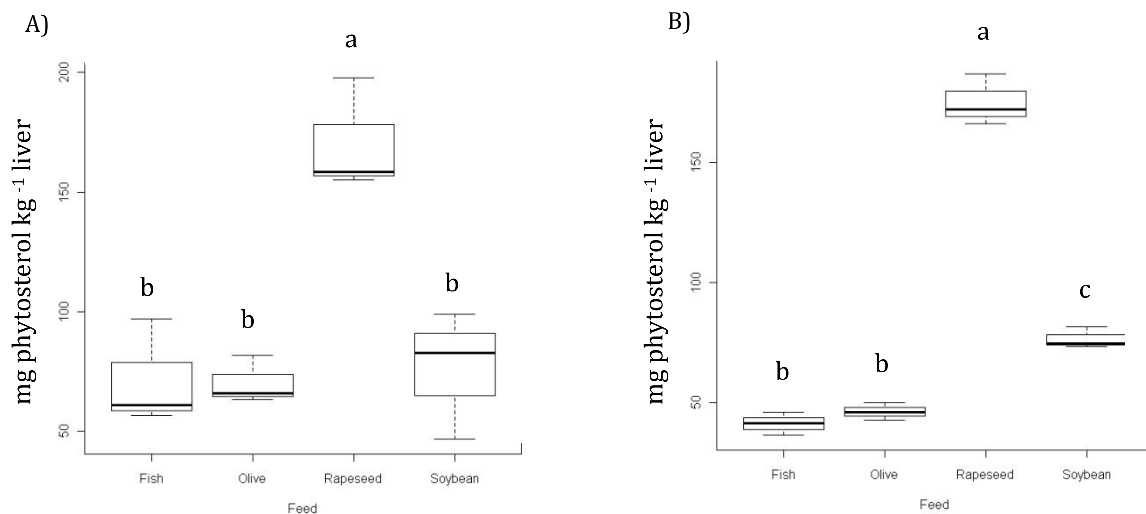


FIGURE 3.7: PHYTOSTEROL CONTENTS IN LIVER SAMPLED FROM ATLANTIC SALMON FED DIFFERENT OILS IN DIET, SAMPLED AFTER A: 11 WEEKS AND B: 28 WEEKS. VALUES PORTRAYED ARE THE STANDARD BOX-AND-WHISKER PLOT IN R, I.E. MEDIAN, AND FIRST AND THIRD QUARTILE (Q1 AND Q3). WHISKERS ARE MIN. AND MAX. VALUES. STATISTICAL DIFFERENCES BETWEEN DIETARY GROUPS ARE DENOTED BY DIFFERENT LETTERS.

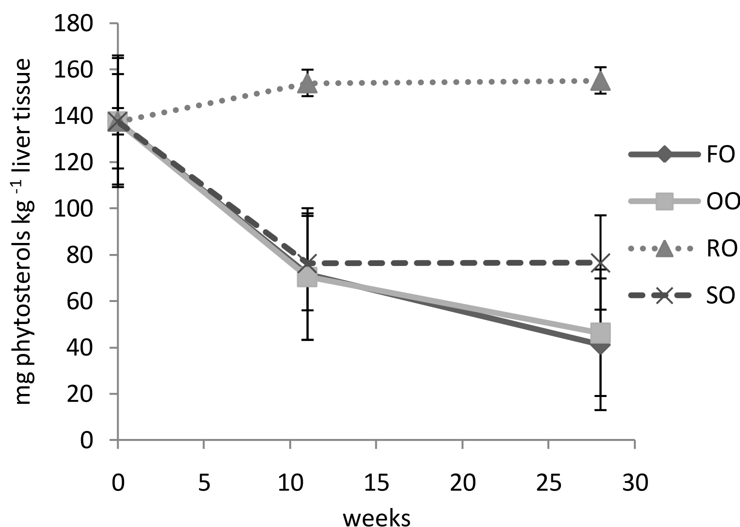


FIGURE 3.8: PHYTOSTEROL CONTENTS IN THE LIVER OF ATLANTIC SALMON FED DIFFERENT OILS IN THEIR FEEDS SEEN IN A TIME PERSPECTIVE, T=0 VALUE IS THE SAME FOR EVERY GROUP. FO: FISH OIL. OO: OLIVE OIL, RO: RAPESEED OIL, SO: SOYBEAN OIL.

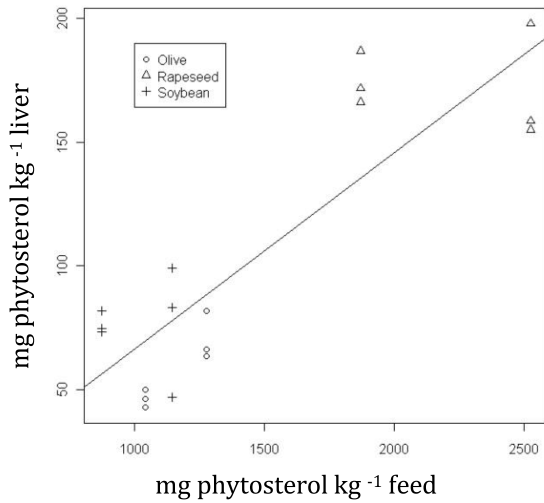
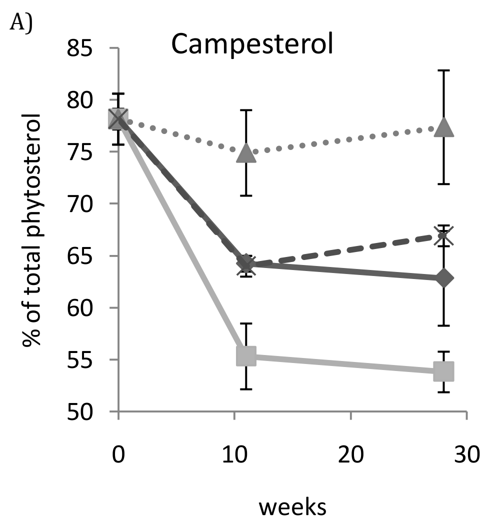
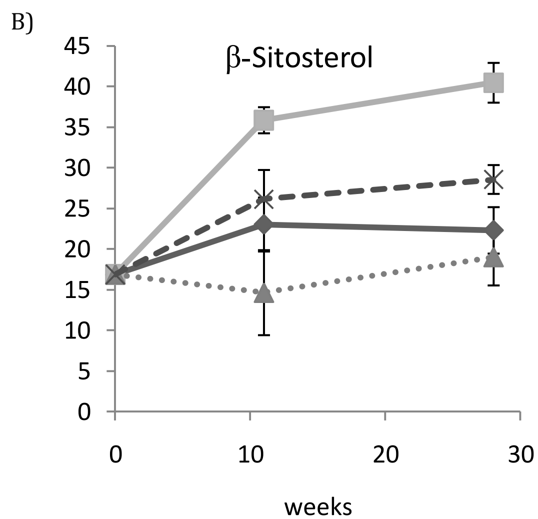
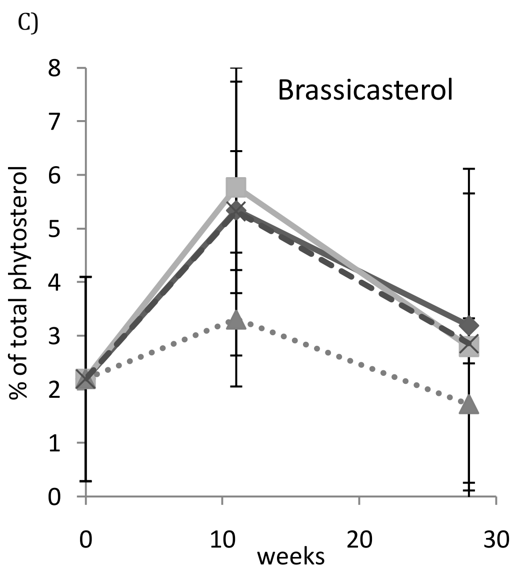


FIGURE 3.9: RELATIONSHIP BETWEEN PHYTOSTEROL AMOUNT IN FEED AND PHYTOSTEROL IN LIVER OF ATLANTIC SALMON FED FEEDS WITH DIFFERENT LIPID SOURCES. DATA FROM FISH SAMPLED AT INTERMEDIATE AND FINAL SAMPLING (T=11 AND 28 WEEKS RESPECTIVELY). THE LINEAR MODEL PLOTTED EXPLAINS 75% OF THE VARIABILITY IN THE DATA (MULTIPLE R-SQUARED = 0.75). THE FISH OIL GROUP IS LEFT OUT BECAUSE OF THE HIGH CHOLESTEROL CONTENTS IN THE FO FEED COMPARED TO THE VO FEEDS, THIS MAKES IT POSSIBLE TO SEE THE EFFECT OF ONLY PHYTOSTEROLS ON THE PHYTOSTEROL LEVELS IN THE LIVER.







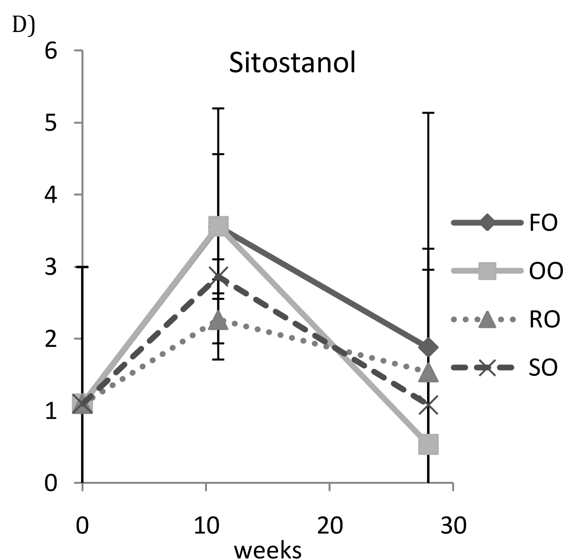


FIGURE 3.10: RELATIVE LIVER STEROL LEVELS DURING THE 28 WEEKS OF FEEDING ATLANTIC SALMON THE EXPERIMENTAL DIETS. A: CAMPESTEROL. B: B-SITOSTEROL, C: SITOSTANOL, D: BRASSICASTEROL. RESULTS GIVEN AS WT% OF TOTAL PHYTOSTEROLS ± SD (N=3).

3.5 *Liver triacylglycerol (TAG) and hepatosomatic index (HSI)*

Liver TAG at the final sampling was significantly higher in the RO fed fish compared to the FO group (p = 0.04), and borderline significantly different from the SO group (figure 3.11).

Hepatosomatic index was calculated as: (liver weight * 100) / body weight, and did not differ significantly between the dietary groups with a mean value at 1.08 ± 0.14.

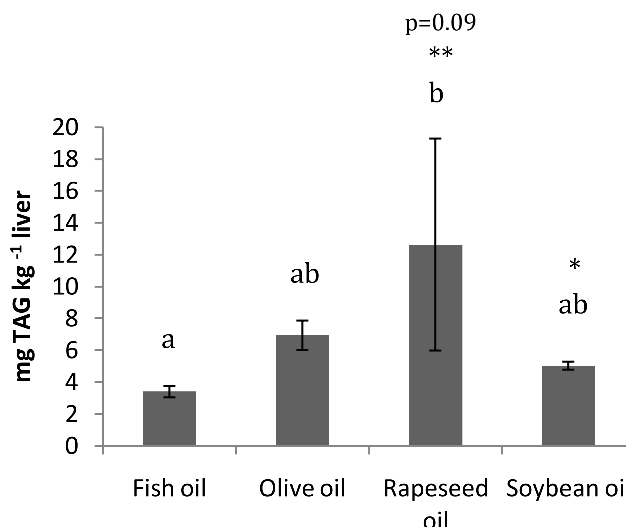


FIGURE 3.11: CONTENTS OF TRIACYLGLYCEROLS (TAG), MG TAG PER KG LIVER, IN ATLANTIC SALMON FED DIFFERENT EXPERIMENTAL DIETS DURING 28 WEEKS. SHOWN AS MEAN ± SD. STATISTICAL DIFFERENCES BETWEEN DIETARY GROUPS ARE DENOTED BY DIFFERENT LETTERS, BORDERLINE SIGNIFICANCE DENOTED BY DIFFERENT NUMBER OF STARS AND P-VALUE IS SHOWN (N=3).

3.5 *Sterols in faeces and apparent digestibility*

The faeces samples were analyzed for phytosterol contents and the apparent digestibility coefficient (ADC) was calculated for each sterol. The ADC values for the specific phytosterols and total phytosterols were very varying and often undetectable, only the ADC values for cholesterol were detectable. Because of non-homogeneous variation in the ADC cholesterol values, non-parametric tests were performed for these data. The FO fed fish had a significantly higher digestibility of cholesterol than the RO fed fish (p = 0.025, t-test- figure 3.12).

To look at the relative amounts of phytosterols excreted and compare this between the groups, the following calculation was done: amount of specific sterol in faeces/ yttrium in faeces for that sample. Comparing the phytosterol content in each diet with the relative amount of phytosterols in faeces, it was apparent that the levels of phytosterols in faeces generally varied with the levels in the feed given to them (figure 3.13).

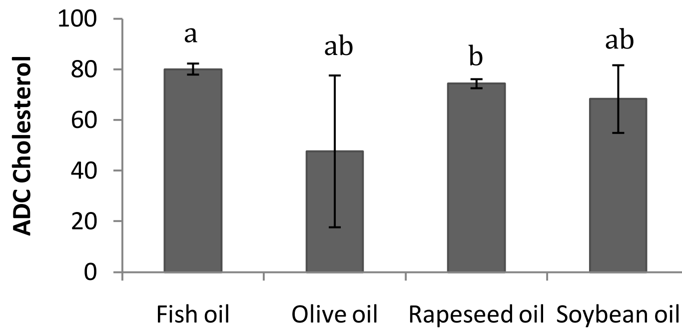
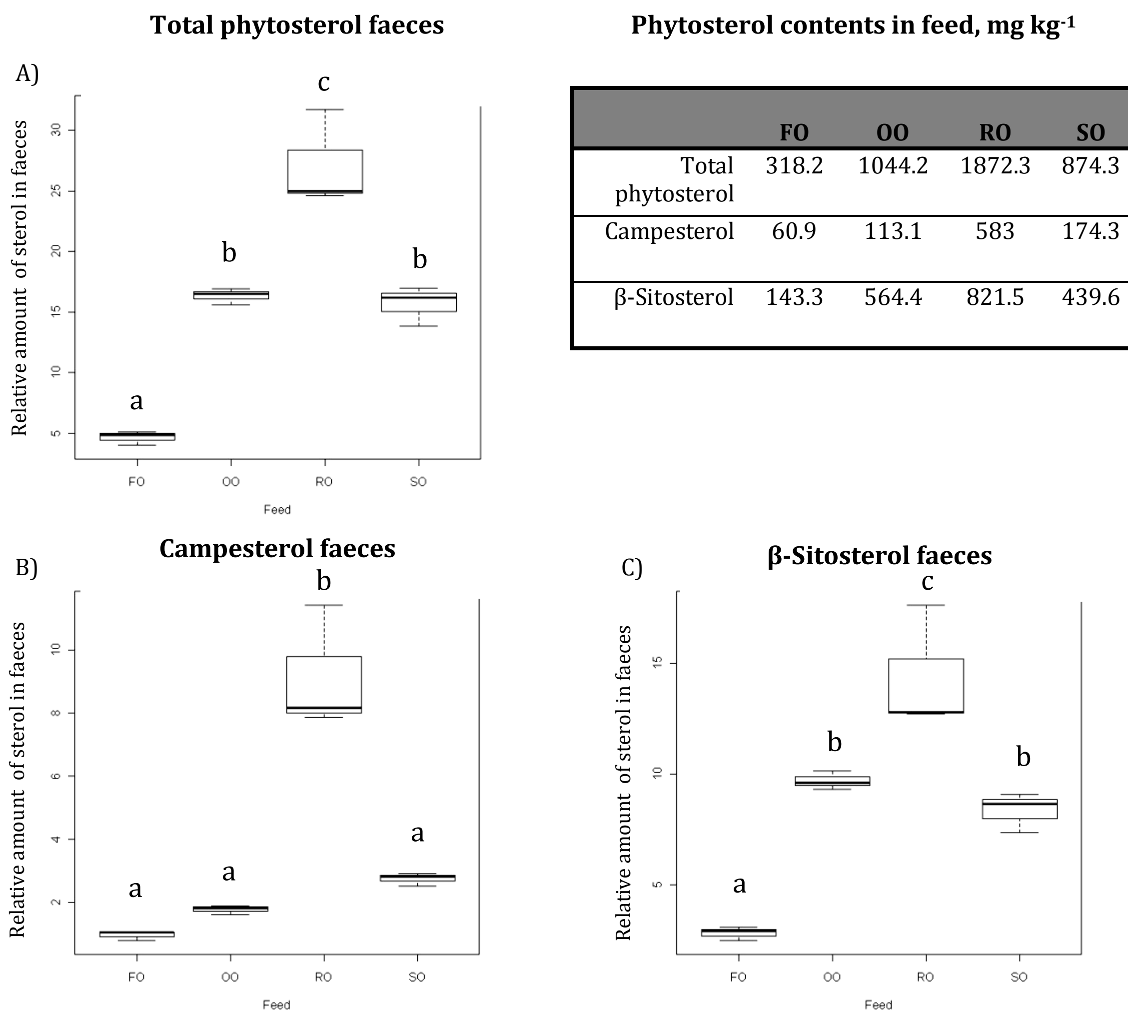


FIGURE 3.12: APPARENT DIGESTIBILITY COEFFICIENT (ADC) VALUES OF CHOLESTEROL IN ATLANTIC SALMON GIVEN DIFFERENT LIPID SOURCES IN THEIR DIET. SHOWN AS MEAN VALUES FOR EACH DIETARY GROUP± SD (N=3). STATISTICAL DIFFERENCES BETWEEN DIETARY GROUPS ARE DENOTED BY DIFFERENT LETTERS.



Phytosterol contents in feed, mg kg^{-1}

	FO	OO	RO	SO
Total phytosterol	318.2	1044.2	1872.3	874.3
Campesterol	60.9	113.1	583	174.3
β-Sitosterol	143.3	564.4	821.5	439.6

FIGURE 3.13: RELATIVE AMOUNTS IN FAECES OF A: TOTAL PHYTOSTEROLS; B: CAMPESTEROL; C: SITOSTEROL (MG STEROL KG FAECES^{-1} / MG YTTRIUM KG FAECES^{-1}) IN ATLANTIC SALMON FED DIFFERENT DIETS. TABLE IS SHOWING CONTENTS IN FEED OF PHYTOSTEROLS (MG KG^{-1}). FO: FISH OIL. OO: OLIVE OIL, RO: RAPESEED OIL, SO: SOYBEAN OIL. STATISTICAL DIFFERENCES BETWEEN DIETARY GROUPS ARE DENOTED BY DIFFERENT LETTERS. VALUES PORTRAYED ARE THE STANDARD BOX-AND-WHISKER PLOT IN R, I.E. MEDIAN, AND FIRST AND THIRD QUARTILE (Q1 AND Q3). WHISKERS ARE MIN. AND MAX. VALUES. NO SIGNIFICANT DIFFERENCES BETWEEN DIETARY GROUPS.

3.6 Plasma and lipoprotein cholesterol, TAG, ALAT and ASAT

The cholesterol content in plasma and the different lipoprotein fractions did not differ significantly between the dietary groups in any of the samplings (table 3.2 and 3.3), although when plotting the data there was a trend of higher VLDL cholesterol levels in the OO and RO group than in the two other groups at the intermediate sampling (figure 3.14a). The VLDL protein was also slightly higher in the RO group (see figure 3.14b), which indicates that this dietary group had a higher number of VLDL particles in plasma than the others, whilst the OO group merely had an increase of the cholesterol amount in each VLDL particle. No significant differences were seen in the HDL protein levels. A change with time was seen in the lipoprotein levels of HDL and NLP cholesterol in all the dietary groups. The mean level of HDL cholesterol decreased with 53% from the intermediate sampling to the final sampling, and a 1.98 times increase was seen in the NLP cholesterol level during the same period (table 3.2 and 3.3). This change is, however, most likely caused by differences in sampling time after last meal at the two samplings (see section 4.3).

TABLE 3.2: CHOLESTEROL CONTENTS IN PLASMA AND LIPOPROTEIN FRACTIONS IN ATLANTIC SALMON FED DIETS WITH DIFFERENT OILS, SAMPLED AT T=11 WEEKS. RESULTS GIVEN AS MG CHOLESTEROL PER 100G PLASMA. DATA GIVEN AS MEAN ± SD (N=3). FISH WERE FED 24 HOURS PRIOR TO SAMPLING.

	Fish oil			*Olive oil*			*Rapeseed oil*			*Soybean oil*		
Plasma	282.8	±	20.6	304.1	±	12.4	272.9	±	19.0	312.6	±	33.0
HDL	124.0	±	11.3	143.8	±	9.9	126.2	±	8.1	134.4	±	13.6
LDL	27.5	±	2.5	35.9	±	8.0	29.1	±	3.9	34.0	±	8.0
VLDL	11.6	±	1.1	23.2	±	9.6	15.4	±	3.1	15.1	±	4.5
NLP	53.3	±	1.2	45.6	±	4.7	44.6	±	9.0	57.6	±	9.3

TABLE 3.3: CHOLESTEROL CONTENTS IN PLASMA AND LIPOPROTEIN FRACTIONS IN ATLANTIC SALMON FED DIETS WITH DIFFERENT OILS, SAMPLED AT T=28 WEEKS. RESULTS GIVEN AS MG CHOLESTEROL PER 100G PLASMA. DATA GIVEN AS MEAN ± SD (N=3). FISH WERE FED 48 HOURS PRIOR TO SAMPLING.

	Fish oil			*Olive oil*			*Rapeseed oil*			*Soybean oil*		
Plasma	290.0	±	22.8	331.0	±	16.5	305.1	±	33.2	324.1	±	25.1
HDL	61.9	±	16	53.7	±	2.4	64.7	±	20.9	66.6	±	14
LDL	26.0	±	7.5	34.9	±	9.8	24.7	±	3.4	29.8	±	2.6
VLDL	8.7	±	2.7	17.1	±	4.7	19.3	±	6.9	11.2	±	3.0
NLP	89.0	±	12.2	126.9	±	20.7	87.1	±	12.9	96.0	±	15.1

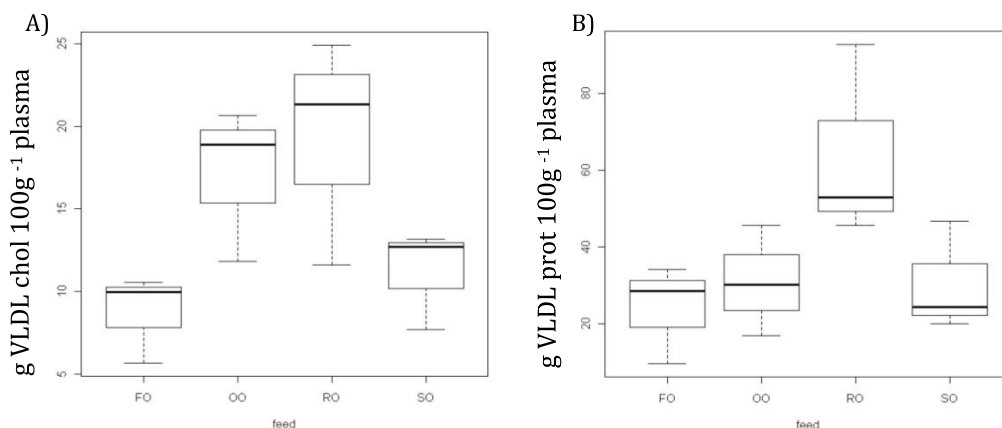


FIGURE 3.14: A: VLDL CHOLESTEROL AND B: VLDL PROTEIN IN PLASMA FROM ATLANTIC SALMON FED DIETS WITH DIFFERENT OILS, SAMPLED 28 WEEKS INTO THE FEEDING TRIAL. VALUES PORTRAYED ARE THE STANDARD BOX-AND-WHISKER PLOT IN R, I.E. MEDIAN, AND FIRST AND THIRD QUARTILE (Q1 AND Q3). WHISKERS ARE MIN. AND MAX. VALUES. NO SIGNIFICANT DIFFERENCES BETWEEN DIETARY GROUPS.

No significant differences were found between the dietary groups in plasma TAG levels at the intermediate sampling, e.g. after 11 weeks of feeding, but after 28 weeks of feeding there were some significant differences as shown in table 3.4. The RO fed fish had a higher content of TAG in plasma than the other groups at 2.5 ± 0.3 µmol TAG g^{-1} plasma, the FO group had the lowest mean value of plasma TAG at 1.6 ± 0.3 µmol TAG g^{-1} plasma (table 3.4). Significant differences were also found between the RO and FO groups in the HDL and VLDL fractions and between the SO and RO groups in the VLDL fraction of plasma sampled 28 weeks into the feeding trial. No significant differences between the dietary groups were found in the plasma levels of alaninaminotransferase (ALAT) or aspartataminotransferase (ASAT), which had means of 0.1 ± 0.1 and 46.2 ± 3.3 UI per 100 g plasma at the initial sampling; 1.1 ± 2.4 and 16.3 ± 11.4 UI per 100 g plasma at the intermediate sampling; and 19.0 ± 17.3 and 68.9 ± 67.1 UI per 100 g plasma at the final sampling, respectively.

TABLE 3.4: TRIACYLGLYCEROL (TAG) CONTENTS IN PLASMA AND LIPOPROTEIN FRACTIONS IN ATLANTIC SALMON FED DIETS WITH DIFFERENT OILS, SAMPLED AT T=28 WEEKS. RESULTS GIVEN AS µMOL TAG PER GRAM PLASMA. DATA GIVEN AS MEAN ± SD (N=3). STATISTICAL DIFFERENCES BETWEEN DIETARY GROUPS ARE DENOTED BY DIFFERENT LETTERS.

	Fish oil			Olive oil			Rapeseed oil			Soybean oil		
Plasma	1.58[b]	±	0.27	2.10[ab]	±	0.14	2.51[a]	±	0.34	1.74[b]	±	0.19
HDL	0.32[b]	±	0.07	0.40[ab]	±	0.06	0.52[a]	±	0.04	0.39[ab]	±	0.06
LDL	0.22	±	0.01	0.29	±	0.08	0.36	±	0.18	0.24	±	0.04
VLDL	0.44[b]	±	0.15	0.68[ab]	±	0.13	0.91[a]	±	0.14	0.47[ab]	±	0.17
NLP	0.43	±	0.11	0.62	±	0.16	0.52	±	0.20	0.44	±	0.05

3.7 Transcription of genes related to the cholesterol metabolism

A series of genes involved in the biosynthesis, metabolism, uptake and excretion of cholesterol and other sterols were selected for performing qPCR on RNA extracted from liver and intestine of Atlantic salmon fed different experimental feeds. Some of the genes showing changes in expression were the genes involved in the biosynthesis of cholesterol from acetyl-CoA (a-ACAT2, DHCR7 and MVK), which had a general upregulation of their expression in the VO fed fish compared to the FO fed fish (figure 3.15 and 3.16). The upregulation of these genes was more pronounced in the intermediate sampling than at the final sampling. At the intermediate sampling (figure 3.15), the normalized expression of these three genes was between 1.8-3.3 times higher in the VO groups than in the FO group and they were significantly upregulated at five occasions (see table 3.5 for relative expressions and p-values), as opposed to at the final sampling (figure 3.16) when they were between 0.8 - 7.1 times higher and upregulated only three times. The gene that maintained its high expression is a-ACAT2, which had a 7.2 times higher expression in the OO fed fish than in the FO fed fish at the final sampling.

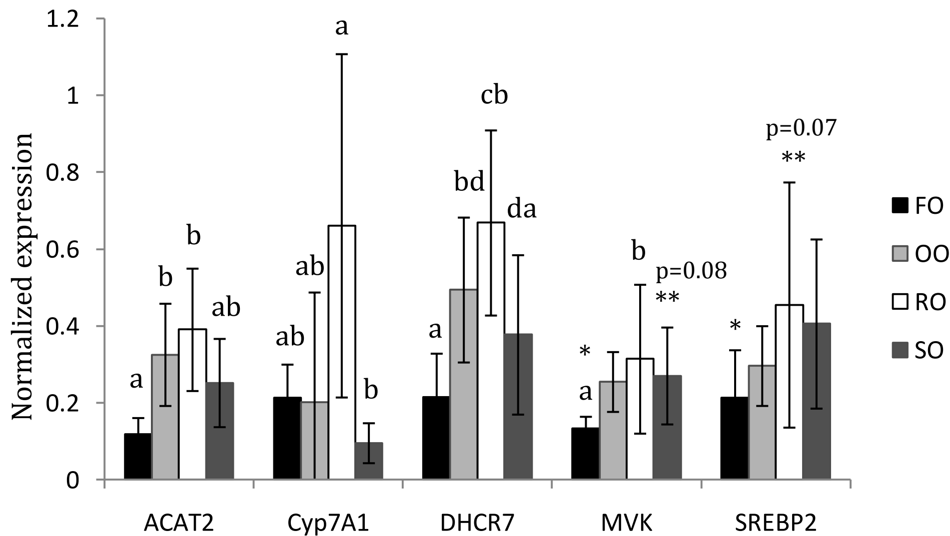


FIGURE 3.15: NORMALIZED EXPRESSION OF GENES IN LIVER FROM ATLANTIC SALMON FED DIFFERENT EXPERIMENTAL DIETS FOR <u>11 WEEKS</u>. FO: FISH OIL. OO: OLIVE OIL, RO: RAPESEED OIL, SO: SOYBEAN OIL. STATISTICAL DIFFERENCES BETWEEN DIETARY TREATMENTS ARE DENOTED BY DIFFERENT LETTERS, BORDERLINE SIGNIFICANCE IS DENOTED BY DIFFERENT NUMBERS OF STARS AND P-VALUE IS SHOWN.

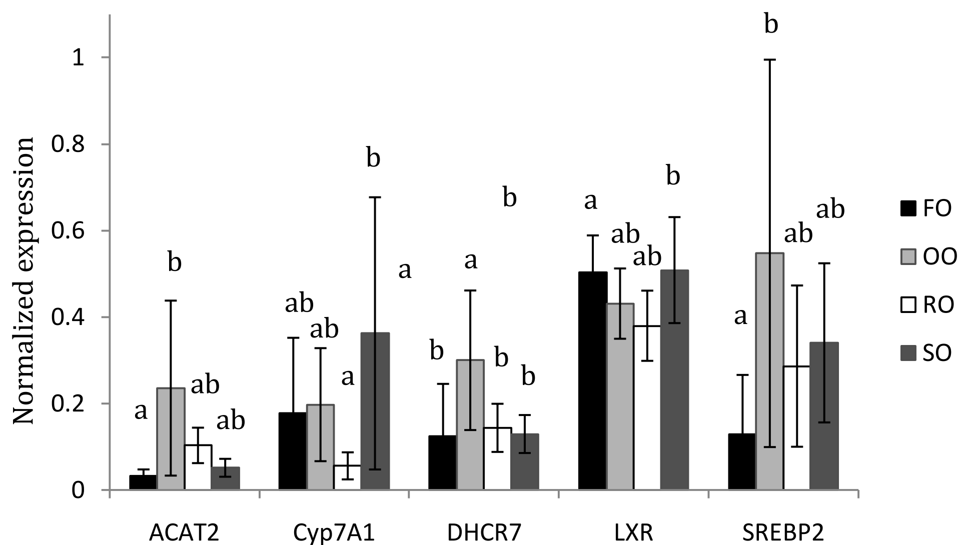


FIGURE 3.16: NORMALIZED EXPRESSION OF GENES IN LIVER FROM ATLANTIC SALMON FED DIFFERENT EXPERIMENTAL DIETS FOR 28 WEEKS. FO: FISH OIL. OO: OLIVE OIL, RO: RAPESEED OIL, SO: SOYBEAN OIL. STATISTICAL DIFFERENCES BETWEEN DIETARY TREATMENTS ARE DENOTED BY DIFFERENT LETTERS.

Genes coding for nuclear receptors involved in the regulation of the cholesterol metabolism (LXR and SREBP2) did show some changes in expression during the dietary trial. At the intermediate sampling the expression of LXR remained very stable in all the dietary groups, whilst the SREBP2 expression was found to be 2.1 and 1.9 times higher and borderline significantly different in the RO and SO groups compared to the FO group, respectively (table 3.5 and figure 3.15). At the final sampling the differences between the VO and FO group in the expression of these two genes had increased. The LXR was now significantly downregulated in the RO fed fish compared to the FO group (p=0.028) and SREBP2 was highly upregulated in the VO groups (from 2.2 – 4.3 times higher in VO than FO, figure 3.16).

Expression of genes coding for proteins transporting sterols between the intestinal lumen and the enterocytes, ABCG5 and NPC1L1, had not been affected by the diets after 11 weeks of being fed the experimental diet. At the final sampling, T=28 weeks, there had been an upregulation in the SO group of the gene coding for the protein NPC1L1 (p=0.02, table 3.6), which is a protein that transports sterols from the lumen to the enterocytes. There was also a tendency of the OO group having this gene higher expressed, and for RO having it lower expressed at T=28 weeks.

The expression of genes coding for the different apolipoproteins were expressed quite stable through the whole feeding trial. The only exception from this was ApoA1, the apolipoprotein commonly found in chylomicrons and HDL particles, which was expressed 1.66 times higher in the OO fed fish compared to the FO fed fish in liver from the final sampling (p=0.084).

The LDL-receptor, involved in the lipoprotein metabolism, was not fluctuating in its expression (table 3.5).

Significant differences were seen in the expression of CYP7A1, rate-determining enzyme in the conversion of cholesterol to bile salts. CYP7A1 was expressed 3.1 times higher in the RO group than in the FO group at the intermediate, but strongly downregulated in the same dietary group at the final sampling. The SO group had a development in expression of CYP7A1 contrary of RO, being low at the intermediate sampling and high at the final sampling (table 3.5 and figure 3.15 and 3.16).

TABLE 3.5: RELATIVE EXPRESSION OF GENES INVOLVED WITH CHOLESTEROL SYNTHESIS, LIPOPROTEINS AND CHOLESTEROL EFFLUX IN LIVER TISSUE FROM ATLANTIC SALMON FED DIFFERENT OILS IN THEIR FEED. TISSUE FROM THE INTERMEDIATE AND FINAL SAMPLING (T=11 AND 28 WEEKS, RESPECTIVELY). THE NUMBERS INDICATE FOLD CHANGE RELATIVE TO THE FISH OIL GROUP. THE GENES WHICH HAD A SIGNIFICANTLY DIFFERENT EXPRESSION IN VO FED FISH COMPARED TO FO FED FISH ARE INDICATED IN BOLD (P-VALUES <0.05, NESTED ANOVA AND TUKEY HSD).

		Gene	*Olive oil*		*Rapeseed oil*		*Soybean oil*	
			expression	*p-value*	*expression*	*p-value*	*expression*	*p-value*
T= 11 weeks	Chol. synth.	ACAT2	**2.739**	**0.014**	**3.286**	**<0.001**	2.122	0.210
		DHCR7	**2.298**	**0.012**	**3.108**	**<0.001**	1.755	0.282
		HMGR	0.866	0.973	1.379	0.617	1.144	0.967
		MVK	1.915	0.152	**2.360**	**0.010**	2.032	0.083
		SREBP2	1.392	0.143	2.137	0.059	1.905	0.052
	Lipo. prot.	ApoA1	0.912	0.986	1.479	0.252	1.152	0.936
		ApoB100	0.922	0.987	1.331	0.498	0.893	0.969
		Apo C2	0.884	0.982	1.381	0.601	0.922	0.994
		LDLR	0.864	0.881	0.972	0.999	0.987	1.000
	Chol. efflux	CYP7A1	0.943	0.906	**3.098**	**0.025**	**0.449**	**0.006**
		LXR	0.873	0.968	1.205	0.883	1.072	0.994
T= 28 weeks	Chol. synth.	ACAT2	**7.139**	**0.017**	**3.125**	**0.001**	1.557	0.067
		DHCR7	**2.404**	**0.009**	1.152	0.986	1.037	0.999
		HMGR	1.268	0.713	1.091	0.984	1.140	0.945
		MVK	1.389	0.308	0.846	0.915	0.977	0.999
		SREBP2	**4.241**	**0.034**	2.220	0.060	**2.637**	**0.020**
	Lipo. prot.	ApoA1	1.663	0.084	1.357	0.580	1.309	0.687
		ApoB100	1.989	0.305	0.800	0.990	1.574	0.813
		Apo C2	1.284	0.445	1.038	0.997	1.250	0.559
		LDLR	1.386	0.787	0.957	0.999	1.468	0.671
	Chol. efflux	CYP7A1	1.106	0.806	0.313	0.087	2.029	0.176
		LXR	0.856	0.363	**0.755**	**0.028**	1.010	0.999

TABLE 3.6: EXPRESSION OF GENES EXPRESSING INTESTINAL STEROL TRANSPORTERS IN INTESTINE OF ATLANTIC SALMON FED DIFFERENT OILS IN THEIR FEED. TISSUE FROM THE INTERMEDIATE AND FINAL SAMPLING (T=11 AND 28 WEEKS, RESPECTIVELY). THE NUMBERS INDICATE FOLD CHANGE RELATIVE TO THE FISH OIL GROUP. THE GENES WHICH HAD A SIGNIFICANTLY DIFFERENT EXPRESSION IN VO FED FISH COMPARED TO FO FED FISH ARE INDICATED IN BOLD (P-VALUES <0.05, NESTED ANOVA AND TUKEY HSD).

	Gene	*Olive oil*		*Rapeseed oil*		*Soybean oil*	
		expression	*p-value*	*expression*	*p-value*	*expression*	*p-value*
T=11 weeks	ABCG5	1.068	*0.824*	0.754	*0.426*	1.058	*0.849*
	NPC1L1	1.178	*0.664*	1.423	*0.314*	1.171	*0.675*
T=28 weeks	ABCG5	0.812	*0.294*	0.704	*0.115*	0.976	*0.887*
	NPC1L1	1.480	*0.131*	0.770	*0.443*	**1.804**	***0.022***

4. Discussion

4.1 Effect of vegetable oil and plant protein on growth and feed intake of Atlantic salmon

The fish fed soybean oil (SO) showed a tendency of lower final weight and were borderline significantly shorter ($p = 0.066$) compared to the fish oil (FO) fed group. In addition, the SO fed fish had a lower specific growth rate (SGR) than the fish fed olive oil (OO) and rapeseed oil (RO). Feeding trials where plant oils have replaced marine oils in the feed, but where the protein portion was fishmeal, have mostly shown no significant differences in final weight or growth between the FO and vegetable oil (VO) fed fish (Leaver et al., 2008; Miller et al., 2008; Torstensen et al., 2005), although Torstensen et al. (2005) observed reduced growth in the FO fish during the winter season. This reduced growth was thought to be due to decreased digestibility of saturated fatty acids (SFA), abundant in FO, at low temperatures.

A lower final weight has been reported in fish given high replacement of both protein and lipids (80% PP and 70% VO) (Torstensen et al., 2008). This lower weight was explained by significantly decreased feed intake during the first period, which is similar to what we observed in our results where the SO fish had significantly lower feed intake compared to FO. The higher feed conversion ratio (FCR) in the VO fed groups than in the FO fed group in our dietary trial is also in accordance to what was reported by Torstensen et al. (2008).

In feed preference studies where voluntary feed intake has been investigated, VOs have been reported as less accepted by the Atlantic salmon resulting in lower feed intake and subsequently lower growth rate (Geurden et al., 2005; 2007). SGR was affected by the oil source and it appears that, in the case of high inclusion of plant protein in the diet of Atlantic salmon, the type of lipid utilized does indeed influence the growth of the fish. The FO and RO diets gave high final weights, but FO fed fish had a less efficient feed utilization (higher FCR), hence the FO fed fish had to eat more to obtain the same final weight. The OO fed fish had intermediate growth and feed intake compared to the other dietary groups. SO seems to be the oil less suited for replacing FO in feed for Atlantic salmon, in the sense that it gave slightly lower final weight, probably caused by the low feed intake by this dietary group. The higher digestibility of the VO diets compared to the FO diet (unpublished data), probably balance this lowered feed intake, making it the reason that the decrease in growth is not as dramatical as the lowered feed intake would suggest. Based on current and previous studies, it seems that replacement of FO with VO in diet does not severely affect the growth and feed intake of Atlantic salmon.

4.2 Absorption of sterols

Feeding Atlantic salmon with OO and SO diets during 28 weeks led to an upregulation of the expression of NPC1L1 compared to the FO fish ($p = 0.022$ and $p = 0.131$, respectively). The RO fed fish had a slightly lowered expression of NPC1L1 in the intestine compared to the FO group. Clinical trials with mildly hypercholesterolemic and sitosterolemic humans have given indications of NPC1L1 being important for the absorption of both cholesterol and phytosterols (Garcia-Calvo et al., 2005; Salen et al., 2004; Sudhop et al., 2002). The expression of NPC1L1 mRNA has been shown to correlate with the amount of NPC1L1 protein in the intestine of rat, mouse and human, and to be strongly correlated with the absorption of sterols as mice deficient in NPC1L1 reduce their cholesterol absorption by ~70% (Altmann et al., 2004). Assuming that Atlantic salmon NPC1L1 has a translation and subsequent function as the mammalian NPC1L1, the sterol absorbing ability was higher in the OO and SO fed fish than in the FO and RO fed fish. It has, however, been indicated that NPC1L1 resides intracellularly and moves to the cell membrane in case of cellular cholesterol depletion (Yu et al., 2006); if this is the case also in Atlantic salmon is not known. However, if the latter is true in Atlantic salmon, the expression of NPC1L1 in the intestine is not necessarily reflecting the ability to absorb sterols at a given moment, but may rather be expressed to build and intracellular NPC1L1 pool.

The NPC1L1 protein has a sterol sensing domain (Altmann et al., 2004), and its activity can thus be affected by sterols. Accordingly, Jesch et al. (2009) showed that the presence of β-sitosterol and cholesterol, but not stigmasterol, lowered the expression of NPC1L1 in a human enterocytic cell-line. Our results are in line with this, as the SO and OO feeds were the ones with the lowest dietary levels of β-sitosterol and cholesterol and the highest expression of NPC1L1. It therefore seems that the β-sitosterol and cholesterol contents in the SO and OO diets were below the levels needed to lower the expression of the NPC1L1 transporter, and consequently giving higher levels of NPC1L1 in these groups due to no suppression of expression. The level of β-sitosterol in the RO diet appears to have been high enough to suppress the expression of NPC1L1 in this dietary group, giving this group a lower expression of NPC1L1 than the other dietary groups. The FO fed fish also had a lower expression of NPC1L1 than the OO and SO fed fish, which could be due to the high cholesterol levels in the FO feed. The fact that the RO fed fish had an even lower expression of NPC1L1 than the fish fed FO indicate that phytosterols may have a stronger inhibiting effect on this receptor than cholesterol in Atlantic salmon. It appears that the FO diet contained sufficient cholesterol and the RO diet sufficient β-sitosterol to lower the expression

of NPC1L1 in Atlantic salmon, but that the OO and SO diet had too low levels of both these sterols to have an effect on NPC1L1 expression.

Fatty acids are also reported to affect the expression of NPC1L1, with DHA lowering the expression of NPC1L1 in hamster proximal intestine compared to oleic acid (Mathur et al., 2007). In accordance, EPA and DHA have shown to decrease the expression of NPC1L1 in enterocytic membranes *in vitro* (Alvaro et al., 2010). In contrast, varying levels of saturated or monounsaturated fatty acids did not alter the expression of NPC1L1 (Alvaro et al., 2010). Considering dietary fatty acid composition (table 2.2), this could explain the expression pattern of NPC1L1 in the FO, OO and SO fed fish after 28 weeks, but the low expression of NPC1L1 in the RO fed fish after 28 weeks of feeding cannot be explained directly by the amounts of EPA and DHA in the feed. It could be that HUFA lowers the expression of NPC1L1 in the FO fed fish, however phytosterols seem more important for the control of the NPC1L1 expression in the VO fed fish.

Some indications of how well the sterols are absorbed compared to each other, can be found by comparing the sterol ratios in the diet to the ones it the liver. The ratio of phytosterol : cholesterol was higher in the feed than in the liver for all the dietary groups, indicating a less efficient absorption of phytosterols than of cholesterol. The ratio of campesterol : total sterols was higher in the liver of all feed groups than in the feed, whilst β-sitosterol was found in lower proportions in the liver than in the feed, meaning that campesterol was accumulated in the liver more than β-sitosterol. These results are in line with earlier research on salmon parr, which showed that C_{29} phytosterols, as β-sitosterol, have reduced digestibility compared to C_{28} phytosterols, as campesterol, and were consequently less accumulated in liver and white muscle (Miller et al., 2008). This difference in absorption and hepatic retention could be connected to the suspected existence of differences in efficiency of the various sterol ACATs (Hamada et al., 2007; Sabeva et al., 2009). The absorption of particular sterols in Atlantic salmon thus seems to be selective, absorbing campesterol more efficiently than β-sitosterol.

The apparent digestibility coefficient (ADC) was undetectable for nearly all the sterols, except for the cholesterol ADC values which were significantly lower in the RO fed fish than in the FO fed fish. These results are in accordance to what has been observed for humans, as a naturally high-phytosterol diet give an increase in the faecal excretion of cholesterol (Lin et al., 2010). Summarizing the results on sterol absorption, RO fed fish had a lower expression of the sterol absorbing NPC1L1 and an increased intestinal efflux of sterols. It therefore seems that the effect of a high-phytosterol diet on Atlantic salmon is similar to the effect observed in humans, consisting in an increased efflux of cholesterol caused by a decreased intestinal absorption of the same sterol.

4.3 Effect of vegetable oils and plant protein on plasma cholesterol and plasma TAG

There were no significant changes between the dietary groups in the cholesterol of plasma and lipoprotein fractions, revealing that a plant based feed, formulated as in this experiment, did not severely affect the plasma cholesterol. In humans, phytosterol and phytostanols given in dosages between 0.8 - 4.0 g daily give reductions of LDL-cholesterol between 10 - 15% (Katan et al., 2003). The intake of sterols per VO fish through the whole period ranged from 2.6 g (SO) to 6.1 g (RO). This translates into approximately 13 mg (SO) to 31 mg (RO) of phytosterols per day, which, when taking into account the normal weight of a human (~70 kg), compared to the weight of the salmon (~4 kg at slaughter), is a level that could be comparable to what would lower the LDL-cholesterol in a human.

The plasma cholesterol levels in our experiment started at 6.15 mmol L^{-1} (initial sampling, April) and had increased to 8.16 mmol L^{-1} at the final sampling in October. This can be compared with the normal plasma cholesterol ranges of Atlantic salmon (1.4 - 4.2 kg) fed traditional feed with fish oil and fish meal, found to vary between 9.3 mmol L^{-1} (May) and 12.8 mmol L^{-1} (December) (Sandnes et al., 1988), however current plasma cholesterol was consistently lower compared to this. The feed given to the fish investigated by Sandnes et al. (1988) was not analyzed for cholesterol, but the traditional aquaculture diets usually have a cholesterol contents at about 3000-4000 mg kg $feed^{-1}$ (Torstensen et al., in prep.). This is somewhat higher than in our experimental diets, where the FO feed contained 2111 and 2576 mg of cholesterol per kg feed in batch 1 and batch 2, respectively. In dietary trials where both traditional feed with fish oil and fish meal as well as diets with 80% PP and 70% VO (80PP70VO) replacement are given to Atlantic salmon, lower plasma cholesterol has been observed in the fish given the high replacement feed (Torstsensen et al., in prep.). Based on the fact that all the dietary groups from this feeding trial had lower plasma cholesterol than traditionally fed Atlantic salmon and that no difference were seen between the dietary groups, the earlier observed effects of plant ingredients in feed are probably due to the high and equal plant protein inclusion in all experimental diets.

The RO fed fish had a significantly higher plasma TAG level than the SO and FO fed fish, which is similar to the results seen by Torstensen et al. (2011) where a 80PP70VO-diet resulted in increased plasma TAG in adult Atlantic salmon. It has been shown that both EPA and DHA lower plasma TAG (Frøyland et al., 1996), which explains the low levels of plasma TAG in the FO fed fish but not in the SO and OO fed fish. A trend of higher VLDL protein and VLDL cholesterol in the RO group, supports the theory proposed by Torstensen et al. (2011), where they suggest that the increased plasma TAG levels is a results of increased

secretion of TAG-rich VLDL from the liver. An increased expression of ApoB100 has also been seen as a result of the 80PP70VO diet (Torstensen et al., 2011); this was however not supported by the current data. The high TAG levels in the liver of the RO fed fish, agrees with the increased levels of VLDL and transport of TAG to the tissues. At the same time as forward sterol and lipid transport by VLDL is increased it could be that also the reverse cholesterol transport is affected by plant derived feed, as suggested by Cruz-Garcia et al. (2010). This hypothesis was made due to the changes seen in the expression of LXR, which regulates ABCA1; the latter one having an essential role in the formation of HDL (Fielding and Fielding, 2008).

A pronounced change in the ratio between NLP and HDL cholesterol was observed from the intermediate sampling to the final sampling. These changes are, however, most likely caused by the different feeding statuses in the two samplings, the fish from the final sampling having had 48h since their last meal, to ensure that the fish were in a post-absorptive phase when sampled, and the ones from the intermediate sampling only 24h. This could lead to the fish sampled at the final sampling having more heavy HDL particles, e.g. containing less fat and more protein, which means that they could have been centrifuged out in the NLP phase and with this causing the increased size of the NLP fraction. The plasma cholesterol levels from the two samplings can thus not be directly compared.

Plasma cholesterol was not affected by dietary phytosterols or EPA and DHA, although a tendency of higher VLDL cholesterol and protein was seen in the RO group. In humans, sitostanol lowers the circulating cholesterol if both cholesterol and phytosterol is above a threshold level (Jones et al., 1997). How Atlantic salmon would react to high levels of both phytosterols and cholesterol is not known and was not included in our experimental design. However, it seems that when the cholesterol level in their diet is between 759 - 2576 mg per kg feed, Atlantic salmon have sufficient mechanisms for regulating their plasma cholesterol.

4.4 Effect of vegetable oils and plant proteins on the sterol metabolism

Cholesterol is produced endogenously in the tissues or taken up through the enterocytes, whilst phytosterols cannot be synthesized in animals and can only be accumulated by ingesting plant material. When sterols are taken up from the intestine, they are transported by chylomicrons to the blood where they are transported further to the tissues for either assimilation into cell membranes or brought to the liver for transformation into bile acids (Altmann et al., 2004; Liscum, 2008). In our results there was a clear trend of increasing expression of genes involved in the biosynthesis of cholesterol in liver tissue (SREBP2, MVK, DHCR7, and a-ACAT2) of the Atlantic salmon. This is in accordance with Leaver et

al. (2008), who showed that VO fed Atlantic salmon upregulated the expression of SREBP2, MVK and other genes involved in cholesterol biosynthesis compared to FO fed fish. The fact that the expression of HMGR was not affected in the VO fed fish, also supports the findings by Leaver et al. (2008), verifying that cholesterol is not a key regulator of salmon-HMGR as it is of mammalian-HMGR (Horton et al., 1998; Ravid et al., 2000; Roitelman and Simoni, 1992). An increase of genes in the cholesterol biosynthetic pathway suggests that there is a higher biosynthesis of cholesterol in the VO fed fish than in the FO fed fish. This is in line with mammalian findings, which show that a decreased cholesterol absorption caused by phytosterol lead to increased hepatic cholesterol synthesis (Moghadasian et al., 1999; Shefer et al., 1973). This could indicate an increased need for endogenous production of cholesterol in the VO fed fish possibly caused by cholesterol depletion. Phytosterols can exert an effect on the cholesterol metabolism through the activity of acetyl-CoA carboxylase and malic enzyme if the ratio of phytosterol : cholesterol is larger than 1 (Laraki et al., 1993). The ratio of phytosterol : cholesterol in our experimental VO diets ranged from 1.19 in the SO diet to 2.18 in the RO diet, indicating that the changes we see in the expression of genes involved in cholesterol biosynthesis could, in fact, be due to dietary phytosterols.

Special importance is given to the observed upregulation of SREBP2, nuclear receptor activated by low levels of cholesterol in the cytosolic compartment of cells (Gong et al., 2006), which activates genes involved in cholesterol biosynthesis and uptake of sterols (Espenshade and Hughes, 2007; Goldstein et al., 2006; Horton et al., 2002). The stable upregulation of SREBP2 in all the VO groups compared to FO, could imply that the liver cells of Atlantic salmon fed VO diets are in a state of low or deficient cholesterol. Another possibility is that the phytosterols, directly or indirectly, manipulate the expression of SREBP2 in some way. In mice it has been shown that an increased expression of SREBPs achieved by genetic techniques, is joined by an increase in hepatic synthesis of cholesterol and fatty acids (Horton et al., 1998; Zheng et al., 2005) and can give an enlarged or fatty liver (Shimano et al., 1996). In spite of high transcription of SREBP2 in several of the dietary groups compared to FO, the hepatosomatic index (HSI) did not differ between them. There were, however, higher liver TAG levels in the RO fed group than in the other dietary groups. It thus appears that one of the results of feeding Atlantic salmon with high phytosterol/low cholesterol feed is an increase in the endogenous synthesis of cholesterol and perhaps also fatty acids, triggered by SREBP2 and possibly caused by cholesterol depletion in the hepatocytes.

The synthesis of cholesterol is an energy demanding process, so the observed increase in expression of genes in the cholesterol biosynthetic pathway in the VO fed groups, could

imply an increased energy-demand of the cells of the VO fish. Leaver et al. (2008) reported an upregulation of several genes involved in NADPH producing systems and HUFA biosynthesis in Atlantic salmon smolt fed VO diets. Based on our data and the observed changes by Leaver et al. (2008), this could mean that an increased amount of energy is required for the cholesterol and HUFA synthesis and that this energy is spent on the expense of other energy-demanding processes. If the energy is administered in a similar way in Atlantic salmon as in humans, these energy-demanding processes would, in the case of energy depletion in the cell, be inhibited by a sensor of energy status cellular called AMP-Activated Protein Kinase (AMPK). The activity of AMPK makes sure that catabolic processes generating ATP are prioritized over anabolic processes that demand energy and that are not essential for the short-term survival of a cell (Hardie et al., 2003). Which processes that are prioritized over others in Atlantic salmon is not known. Since cholesterol synthesis is observed to be higher in the VO fed salmon, it seems as if the fish are able to handle the increased energy-demand these diets impose without compromising growth, possibly due to the high dietary energy content.

4.5 Accumulation of sterols in liver of Atlantic salmon

Liver cholesterol content in increased in all the dietary groups from the initial to the final sampling. Interestingly, in spite of the FO group being the dietary group with the highest levels of dietary cholesterol, the FO fed fish was the dietary group with the lowest liver cholesterol levels after 28 weeks of feeding. The same tendencies, where the FO group increased the least in liver cholesterol and the VO groups more, is also reported for Atlantic salmon smolt (Miller et al., 2008). Based on this comparison of results, it looks as if the same mechanisms for accumulating cholesterol in the liver are valid for adult Atlantic salmon as for juvenile fish of the same species.

The interesting development in the liver cholesterol of the RO fed fish, which decreased from the initial to the intermediate sampling and increased to the levels of the other groups at the final sampling, is presumably connected with the phytosterol levels. This presumption is based on the negative correlation between the phytosterol contents in feed and the cholesterol in the liver, implying that the more phytosterol a fish ingests, the less cholesterol it will accumulate in its liver. This is, however, in contrast to what one would expect based on the discussion in the former passage. When looking closer at the data, it is quite clear that this correlation can be showing skewed results. The RO fed fish is the only group with very high phytosterol levels, and it therefore becomes the sole dietary group giving the slope of the line representing the linear relationship between the phytosterol in feed and cholesterol in liver

(figure 3.6). Because of this, no conclusions will be based on the correlation between feed and liver phytosterol.

Decreased levels of liver phytosterols from the initial to the final sampling were observed in all the groups except for in the RO fed fish, which had an increase in liver phytosterols during the same period. A commercial feed containing rapeseed oil was used prior to the dietary trial, and was most likely the reason why all the fish had high levels of liver phytosterol at the start of the dietary trial and a subsequent decrease in phytosterol levels in the same organ. The RO feed was the one with the highest content of phytosterols, and since this feed was the only one giving accumulation of phytosterols in the liver, all though OO and SO diets also contained phytosterols, it could be that the dietary phytosterol has to reach a threshold before it is absorbed and accumulated in Atlantic salmon liver. Such a threshold could be connected with the phytosterol : cholesterol ratio in the feed or with the phytosterol concentrations in the intestine at the moment of absorption. The higher phytosterol : cholesterol ratio in the liver of the RO fed fish compared to in the other dietary groups indicate that this threshold limit in the phytosterol : cholesterol ratio would lie between 1.42 (ratio in the OO feed) and 2.18 (ratio in the RO feed). Since the RO feed was also the one with the highest concentrations of phytosterols, a threshold in the amount of total or specific phytosterols present in the intestinal lumen after a meal may be determining phytosterol uptake.

The sterol levels in liver was positively correlated to the TAG levels in this organ, which maybe be due to sterols being lipid soluble and a tissue with a higher fat content thus being able to store more phytosterols (Miller et al., 2008). In our experiment, the RO fed fish had a significantly higher liver TAG level than the fish fed FO. A connection between a VO diet and increased liver lipid in Atlantic salmon was established in Atlantic salmon fed a VO blend of rapeseed oil, palm oil and linseed oil during 22 months (Jordal et al., 2007), and confirmed by Torstensen et al. (2011) when both dietary FO and fishmeal were replaced by plant raw material. Miller et al. (2008) reported that liver lipid levels were significantly higher in salmon smolt fed canola oil in their diet compared to salmon smolt fed diets with fish oil during 9 weeks, a change that was accompanied by an increase of liver phytosterols in this dietary group.

TAG synthesis in liver is shown to be upregulated by oleic acid and suppressed by EPA (Berge et al., 1999; Ranheim et al., 1994) and increased expression of SREBP1-a and -c are associated with FA synthesis (Horton et al., 2002) and increased liver lipids (Shimomura et al., 1998). This shows that EPA possibly lowers the expression of SREBP1, leading to lower FA synthesis. The two isomers of SREBP known in Atlantic salmon respond differently to the exposure to EPA and DHA; decreasing the expression of SREBP1 and increasing the

expression of SREBP2 in macrophage cell lines (Minghetti et al., 2011). Since both RO and SO diets are low in HUFA, we could explain parts of the slightly elevated TAG levels in these two groups by the FA composition in feed. The high liver TAG found in the RO group, could be also be partially explained by the lack of EPA and DHA in the RO diet, but could, in addition, be caused by a cholesterol deficit that activates both SREBPs and thus also FA synthesis (Horton et al., 2002). It thus seems that a high phytosterol-low cholesterol diet like the RO feed reduces cholesterol uptake, which leads to an upregulation of SREBPs triggered by a cholesterol deficit. This upregulation of SREBPs is thought to result in an increased synthesis of cholesterol and fatty acids in liver and, consequently, an accumulation of phytosterols in this organ (figure 4.1).

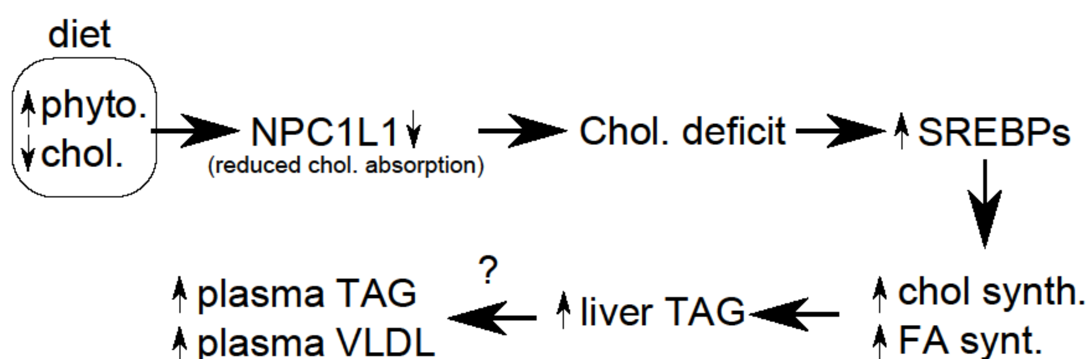


FIGURE 4.1: HYPOTHESIZED EFFECT OF A HIGH PHYTOSTEROL – LOW CHOLESTEROL DIET, LIKE THE RO DIET IN THIS EXPERIMENT, ON THE STEROL AND LIPID METABOLISM

4.6 Efflux of sterols in Atlantic salmon

LXR, a nuclear receptor important for controlling the homeostasis of cholesterol, was expressed in a similar manner in the liver of all the dietary groups during the feeding trial, but with a significantly lowered expression in the RO fed fish compared to the FO fed fish at the final sampling. LXR controls many genes related to the efflux of cholesterol, amongst them CYP7A1, and has been shown to be upregulated by β-sitosterol and campesterol in intestinal caco 2 cells (Plat et al., 2005) and by cholesterol in Atlantic salmon HSK-1 cell lines (Minghetti et al., 2011). Lowered levels of LXR have been reported in Atlantic salmon fed diets with a VO blend (Cruz-Garcia, 2010). The downregulation of LXR in the liver of RO fed fish after 28 weeks of feeding, is the contrary of what we would expect based on the mentioned results in human cell lines (Plat et al., 2005), since the RO feed had high levels of both β-sitosterol and campesterol. Current results are, however, in line with the results in Atlantic salmon (Minghetti et al., 2011; Cruz-Garcia, 2010). The effect of phytosterols on LXR expression thus seems to be quite different in Atlantic salmon liver than in human

enterocytes, although it could be that the modulating effect of phytosterols on the efflux of sterols only functions in the case of a high cholesterol diet.

The lowered expression of both LXR and CYP7A1 in the RO fed fish at the final sampling, indicates a low sterol efflux in this dietary group compared to the others. The situation was different at the intermediate sampling, where LXR expression in the RO fed fish was slightly upregulated compared to FO fed fish and CYP7A1 was highly upregulated, both indicating a high sterol efflux through bile acid synthesis. Increased bile production (De Castro-Orós et al., 2011; Moghadasian et al., 1999) and increased LXR expression (Plat et al., 2005) are known reactions to high levels of phytosterols in humans, supporting current findings in Atlantic salmon. Concordantly, a dramatical increase in LXR expression was reported in Atlantic salmon macrophage cell lines when exposed to surplus of cholesterol (Minghetti et al., 2011). At 11 weeks, the same time as the expression of LXR and CYP7A1 in the RO fed group was high, the cholesterol levels in the liver of this dietary group was the lowest, possibly due to a high efflux of cholesterol as bile acids, mediated by LXR. At the final sampling the liver cholesterol of the RO fed fish had increased up to the level of the other groups and LXR and CYP7A1 levels had decreased their expression. This indicates a lowered bile acid synthesis during this period between the samplings, accompanied by accumulation of cholesterol in liver tissue. An inverse relation between the hepatic cholesterol levels and the expression of LXR and CYP7A1 thus seems to exist. Based on this, it appears that the cholesterol levels in the liver are more affected by cholesterol synthesis and excretion than by dietary cholesterol. And as LXR is shown to increase its expression in Atlantic salmon as a response to cholesterol (Minghetti et al., 2011), it could be that LXR functions as a cholesterol sensor, activating cholesterol efflux when cholesterol levels in the tissues exceed a certain level (figure 4.2).

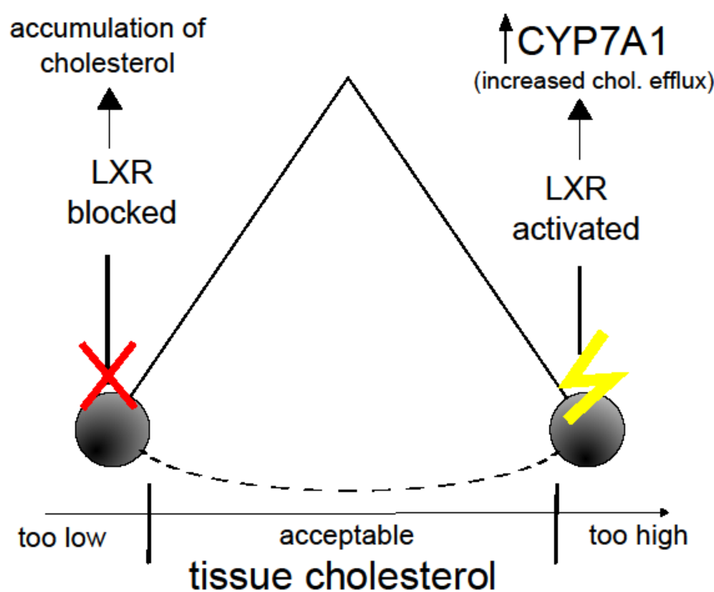


FIGURE 4.2: HYPOTHESIZED MECHANISMS OF CHOLESTEROL REGULATION BY LXR. ILLUSTRATED AS A PENDULUM SWINGING BETWEEN CHOLESTEROL SYNTHESIS AND EXCRETION ACCORDING TO TISSUE LEVELS OF CHOLESTEROL.

While the liver cholesterol levels varied in the RO fed fish, the amount of phytosterols in the liver of the same dietary group was quite stable throughout the dietary trial, indicating that the absorption of phytosterols was unchanged. The decrease in expression of LXR from the intermediate to the final sampling in the RO group could have been triggered by the low levels of liver cholesterol seen in the fish from this group at the intermediate sampling. It therefore appears that a cholesterol deficit will override the upregulating effect phytosterols might have on LXR. Alternatively, the regulation of LXR by phytosterols is different in Atlantic salmon compared to in mice and human or only functions in the case of excess dietary cholesterol.

The ABCG5/8 and ABCA1 are involved in hepatobiliary transport of cholesterol in humans, and ABCG5/8 is shown to be crucial for the removal of cholesterol from the body through the intestine (Sabeva et al., 2009). No significant change in expression of ABCG5 was seen in the intestine of the Atlantic salmon from this feeding trial. In wildtype mice, however, exposure to high concentrations of cholesterol and plant stanols increased the expression of ABCA1 in liver and intestine (Plat and Mensink, 2002). It has been shown that the ABCG5/8 and ABCA1 proteins are under control of the LXR pathway, which means that they are activated by high tissue cholesterol concentrations (Plat and Mensink, 2005; Yu et al., 2003). The unfluctuating expression of ABCG5 in the intestinal samples from this feeding experiment, indicate that the sterol concentrations in the experimental feeds have been too low for an upregulation of ABCG5 to occur.

4.7 Conclusions

A replacement of marine raw materials in Atlantic salmon diet with 70% plant protein and 80% vegetable oils, significantly affected the cholesterol and lipid metabolism.

The sterol balance was specially affected in the RO fed fish, as they showed tendencies of higher VLDL protein and cholesterol, as well as significantly higher plasma TAG. In addition, the liver of the RO fed fish had high TAG levels and an accumulation of phytosterols, something that was not seen in the other VO or FO fed fish. Based on the expression of the transporter protein NPC1L1, the uptake of cholesterol in intestine could be lower in the FO and RO fed groups compared to the OO and SO fed groups, possibly because of a depression of the receptor's expression by cholesterol and β-sitosterol in the FO and RO fed fish, respectively. This decreased absorption through NPC1L1 combined with low dietary cholesterol in the RO fed fish is suspected to cause a cholesterol deficit. The increased TAG content in the liver of the RO fed fish is suggested to be caused by increased expression

SREBPs, which are activated by low levels of cholesterol in tissue and trigger the endogenous synthesis of cholesterol and fatty acids.

The plasma cholesterol and lipoprotein cholesterol levels were stable in all the dietary groups throughout the dietary trial, demonstrating that this parameter is closely controlled by the sterol metabolism of Atlantic salmon. Based on the lack of differences in the current plasma cholesterol and differences between dietary groups in other feeding trials (Torstensen et al., in prep.), it is probable that it is the plant protein and not the vegetable oil that causes changes in plasma and lipoprotein cholesterol.

Since no effects of the VO diets was seen on plasma and lipoprotein cholesterol, used as biomarkers for CVDs in humans, no change in cardiovascular health is expected in VO and PP fed fish.

Different phytosterols were absorbed differently, campesterol being absorbed and stored in liver to a higher extent than β-sitosterol.

The increased expression of SREBP2 in liver of all the VO fed groups could be an indication of low cholesterol in the hepatocytes, and is hypothesized to be the trigger of the increased expression of genes involved in cholesterol synthesis.

Liver cholesterol levels varied, and there was a relationship between increased expression of genes involved in bile acid synthesis and decreased levels of cholesterol in this organ, and *vice versa*. Liver cholesterol levels increased more with time in the VO fed fish than in the FO fed fish, in spite of the FO diets containing more cholesterol.

Based on the fluctuating expression of genes involved in the production of bile acid, the varying liver cholesterol and the stable levels of plasma cholesterol, Atlantic salmon seems to manage its corporal cholesterol levels actively through synthesis and excretion of bile acids from the liver. Maintaining a stable plasma cholesterol appears to be prioritized over stabilising the sterol contents in the liver.

5. Future perspectives

In this experiment we have seen the effect on the cholesterol metabolism of changing the fatty acid and sterol composition by varying the dietary lipid sources. To obtain a full overview over the sterol metabolism in the salmon, it would be of interest to have a closer look at where cholesterol and phytosterols are stored in adult salmon if in excess and which tissues are prioritized for receiving cholesterol when feed cholesterol is deficient. To establish some guidelines as to how much cholesterol should be present in the feed could also be necessary, since plant lipids already are used in a major extent in the aquaculture industry.

The cholesterol in all the feeds used in this feeding trial were equal to or lower than what a fish would get in a marine diet, and the lack of effects on the cholesterol levels in the plasma could have been a result of that. In the future, replacing marine lipids with lipids from terrestrial animals can become a possibility for the fish feed industry (Bureau and Meeker, 2011). With prices of fish oil and protein rising, it seems tempting for the aquaculture industry to take use of the relatively affordable raw material from terrestrial animal by-products. These lipid sources might challenge the nutritional status of the fish as they have a low content of unsaturated fatty acids as well as a high cholesterol content. In this case, an increase in plasma cholesterol and thus a possible increase in risk for aquaculture fish developing CVDs, might be a consequence of the diet change. In a society where the food industry produces increasing amounts of animal fats and the supply of marine oils is unstable and getting increasingly expensive (Turchini et al., 2009), this might just be a real situation in the not-too-distant future.

6. References

Abumweis, S.S., Vanstone, C.A., Ebine, N., Kassis, A., Ausman, L.M., Jones, P.J.H., Lichtenstein, A.H., 2006. Intake of a single morning dose of standard and novel plant sterol preparations for 4 weeks does not dramatically affect plasma lipid concentrations in humans Journal of Nutrition 136, 1012-1016.

Adams, P.S., 2006. Data analysis and reporting, in: M.T. Dorak (Ed.), Real-time PCR. Taylor & Francis, Newcastle-upon-Tyne, 39-62.

Agellon, L.B., 2008. Metabolism and function of bile acids, in: D.E. Vance, J.E. Vance (Eds.), Biochemistry of lipids, lipoproteins and membranes, 5th ed. Elsevier, Amsterdam, 423-438.

Alrefai, W.A., Annaba, F., Sarwar, Z., Dwivedi, A., Saksena, S., Singla, A., Dudeja, P.K., Gill, R.K., 2007. Modulation of human Niemann-Pick C1-like 1 gene expression by sterol: role of sterol regulatory element binding protein 2. American Journal of Physiology - Gastrointestinal and Liver Physiology 292, 369-376.

Altmann, S.W., Davis, H.R., Zhu, L.-j., Yao, X., Hoos, L.M., Tetzloff, G., Iyer, S.P.N., Maguire, M., Golovko, A., Zeng, M., Wang, L., Murgolo, N., Graziano, M.P., 2004. Niemann-Pick C1 Like 1 Protein Is Critical for Intestinal Cholesterol Absorption. Science 303, 1201-1204.

Alvaro, A., Rosales, R., Masana, L., Vallvé, J.-C., 2010. Polyunsaturated fatty acids down-regulate in vitro expression of the key intestinal cholesterol absorption protein NPC1L1: no effect of monounsaturated nor saturated fatty acids. The Journal of Nutritional Biochemistry 21, 518-525.

Applied Biosystems, 2011. TaqMan® and SYBR® Green Chemistries. (Webpage)

Awad, A.B., Williams, H., Fink, C.S., 2003. Effect of phytosterols on cholesterol metabolism and MAP kinase in MDA-MB-231 human breast cancer cells. The Journal of Nutritional Biochemistry 14, 111-119.

Bell, J.G., Dick, J.R., Mc Vicar, A.H., Sargent, J.R., Thompson, K.D., 1993. Dietary sunflower, linseed and fish oils affect phospholipid fatty acid composition, development of cardiac lesions, phospholipase activity and eicosanoid production in Atlantic salmon. Prostaglandins, leukotrienes, and essential fatty acids 49, 665-673.

Bell, J.G., McEvoy, J., Tocher, D.R., McGhee, F., Campbell, P.J., Sargent, J.R., 2001. Replacement of Fish Oil with Rapeseed Oil in Diets of Atlantic Salmon (Salmo salar) Affects Tissue Lipid Compositions and Hepatocyte Fatty Acid Metabolism. The Journal of Nutrition 131, 1535-1543.

Bell, J.G., Strachan, F., Good, J.E., Tocher, D.R., 2006. Effect of dietary echium oil on growth, fatty acid composition and metabolism, gill prostaglandin production and macrophage activity in Atlantic cod (Gadus morhua L.). Aquaculture Research 37, 606-617.

Benveniste, P., 2004. Biosynthesis and accumulation of sterols. Annual Review of Plant Biology 55, 429-457.

Berge, R.K., Madsen, L., Vaagenes, H., Tronstad, K.J., Göttlicher, M., Rustan, A.C., 1999. In contrast with docosahexaenoic acid, eicosapentaenoic acid and hypolipidaemic derivatives decrease hepatic synthesis and secretion of triacylglycerol by decreased diacylglycerol acyltransferase activity and stimulation of fatty acid oxidation. Biochemical Journal 343, 191-197.

Berger, A., Jones, P.J., Abumweis, S.S., 2004. Plant sterols: factors affecting their efficacy and safety as functional food ingredients. Lipids in Health and Disease 3, 5.

Bouic, P.J.D., 2001. The role of phytosterols and phytosterolins in immune modulation: a review of the past 10 years. Current Opinion in Clinical Nutrition & Metabolic Care 4, 471-475.

Brown, A.W., Hang, J., Dussault, P.H., Carr, T.P., 2010. Phytosterol Ester Constituents Affect Micellar Cholesterol Solubility in Model Bile. Lipids 45, 855–862.

Bureau, D.P., Meeker, D.L., 2011. Terrestrial animal fats, in: G.M. Turchini, W.-K. Ng, D.R. Tocher (Eds.), Fish oil replacement and alternative lipid sources in aquaculture feeds. CRC Press, Boca Raton, 245-267.

Bustin, S.A., Beaulieu, J.-F., Huggett, J., Jaggi, R., Kibenge, F.S.B., Olsvik, P.A., Penning, L.C., Toegel, S., 2010. MIQE précis: Practical implementation of minimum standard guidelines for fluorescencebased quantitative real-time PCR experiments. BMC Molecular Biology 11.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE Guidelines:Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry 55, 611-622.

Bustin, S.A., Nolan, T., 2004. Pitfalls of Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction. Journal of biomolecular techniques 15, 155-166.

Carter, C.G., Lewis, T.E., Nichols, P.D., 2003. Comparison of cholestane and yttrium oxide as digestibility markers for lipid components in Atlantic salmon (Salmo salar L.) diets. Aquaculture 225, 341-351.

Castelli, W.P., Anderson, K., Wilson, P.W.F., Levy, D., 1992. Lipids and risk of coronary heart disease The Framingham Study. Annals of Epidemiology 2, 23-28.

Cerqueira, M.T., Fry, M.M., Connor, W.E., 1979. The food and nutrient intakes of the Tarahumara Indians of Mexico. American journal of clinical nutrition 32, 905-915.

Chirovsky, D.R., Fedirko, V., Cui, Y., Sazonov, V., Barter, P., 2009. Prospective studies on the relationship between high-density lipoprotein cholesterol and cardiovascular risk: a systematic review. Journal of Cardiovascular Risk 16, 404-423.

Connor, W.E., 1968. Dietary sterols: their relationship to atherosclerosis. Journal of the American Dietetic Association 52, 202–208.

Copeman, L.A., Parrish, C.C., 2004. Lipids Classes, Fatty Acids, and Sterols in Seafood from Gilbert Bay, Southern Labrador. Journal of agricultural and food chemistry 52, 4872-4881.

Cruz-Garcia, L., 2010. Regulation of lipid metabolism in fish: characterization and physiological role of LXR, PhD thesis, Department of physiology. University of Barcelona, Barcelona, 288p.

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

Cunnane, S.C., 2003. Problems with essential fatty acids: time for a new paradigm? Progress in Lipid Research 42, 544-568.

De Castro-Orós, I., Pampín, S., Cofán, M., Mozas, P., Pintó, X., Salas-Salvadó, J., Rodríguez-Rey, J.C., Ros, E., Civeira, F., Pocoví, M., 2011. Promoter variant −204A > C of the cholesterol 7α-hydroxylase gene: Association with response to plant sterols in humans and increased transcriptional activity in transfected HepG2 cells. Clinical nutrition 30, 239-246.

Dietschy, J.M., Turley, S.D., Spady, D.K., 1993. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. Journal of Lipid Research 34, 1637-1659.

Doornbos, A.M.E., Meynen, E.M., Duchateau, G., Van der Knaap, H.C.M., Trautwein, E.A., 2006. Intake occasion affects the serum cholesterol lowering of a plant sterol-enriched single-dose yoghurt drink in mildly hypercholesterolaemic subjects. European Journal of Clinical Nutrition 60, 325-333.

Dubois, V., Breton, S., Linder, M., Fanni, J., Parmentier, M., 2007. Fatty acid profiles of 80 vegetable oils with regard to their nutritional potential. European journal of lipid science and technology 109 710–732.

Espe, M., Lemme, A., Petri, A., El-Mowafi, A., 2006. Can Atlantic salmon (Salmo salar) grow on diets devoid of fish meal? Aquaculture 255, 255-262.

Espenshade, P.J., Hughes, A.L., 2007. Regulation of sterol synthesis in eukaryotes. Annu. Rev. Genet. 41, 401-427.

Fan, X., Kim, H.-J., Bouton, D., Warner, M., Gustafsson, J.-Å., 2008. Expression of liver X receptor β is essential for formation of superficial cortical layers and migration of later-born neurons. Proceedings of the National Academy of Sciences 105, 13445-13450.

FAO, 2010. Technical Guidelines for Responsible Fisheries on the Ecosystem approach to aquaculture (EAA), in: Doris Soto (Ed.), Fao technical guidelines for responsible fisheries. Food and agriculture organization of the united nations (FAO), Rome, 68p.

FAO, 2011. State of World Aquaculture 2010, in: FAO (Food and Agriculture Organization of the United Nations) (Ed.), Fisheries Technical Paper No. 500. . FAO Fisheries Department, Rome, 218p.

Farrell, A.P., Munt, B., 1983. Cholesterol levels in the blood of Atlantic salmonids. Comparative Biochemistry and Physiology Part A: Physiology 75, 239-242.

Fielding, C.J., Fielding, P.E., 2008. Dynamics of lipoprotein transport in the circulatory system, in: D.E. Vance, J.E. Vance (Eds.), Biochemistry of lipids, lipoproteins and membranes, 5th ed. Elsevier, Amsterdam, 533-554.

Francis, D.S., Turchini, G.M., Jones, P.L., De Silva, S.S., 2007. Effects of fish oil substitution with a mix blend vegetable oil on nutrient digestibility in Murray cod, Maccullochella peelii peelii. Aquaculture 269, 447-455.

Francis, G., Makkar, H.P.S., Becker, K., 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture 199, 197-227.

Frøyland, L., Vaagenes, H., Asiedu, D., Garras, A., Lie, Ø., Berge, R., 1996. Chronic administration of eicosapentaenoic acid and docosahexaenoic acid as ethyl esters reduced plasma cholesterol and changed the fatty acid composition in rat blood and organs. Lipids 31, 169-178.

Garcia-Calvo, M., Lisnock, J., Bull, H.G., Hawes, B.E., Burnett, D.A., Braun, M.P., Crona, J.H., Davis, H.R., Dean, D.C., Detmers, P.A., Graziano, M.P., Hughes, M., MacIntyre, D.E., Ogawa, A., O'Neill, K.A., Iyer, S.P.N., Shevell, D.E., Smith, M.M., Tang, Y.S., Makarewicz, A.M., Ujjainwalla, F., Altmann, S.W., Chapman, K.T., Thornberry, N.A., 2005. The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). Proc. Natl. Acad. Sci. U. S. A. 102, 8132-8137.

Garrett, R.H., Grisham, C.M., 1999. Biochemistry, 2nd ed. Harcourt Brace, Boston, 851 p.

Geurden, I., Corraze, G., Boujard, T., 2007. Self-feeding behaviour of rainbow trout, Oncorhynchus mykiss, offered diets with distinct feed oils. Applied Animal Behaviour Science 108, 313-326.

Geurden, I., Cuvier, A., Gondouin, E., Olsen, R.E., Ruohonen, K., Kaushik, S., Boujard, T., 2005. Rainbow trout can discriminate between feeds with different oil sources. Physiology & Behavior 85, 107-114.

Gilman, C.I., Leusch, F.D.L., Carl Breckenridge, W., MacLatchy, D.L., 2003. Effects of a phytosterol mixture on male fish plasma lipoprotein fractions and testis P450scc activity. General and Comparative Endocrinology 130, 172-184.

Goldstein, J.L., DeBose-Boyd, R.A., Brown, M.S., 2006. Protein Sensors for Membrane Sterols. Cell 124, 35-46.

Gong, Y., Lee, J.N., Lee, P.C.W., Goldstein, J.L., Brown, M.S., Ye, J., 2006. Sterol-regulated ubiquitination and degradation of Insig-1 creates a convergent mechanism for feedback control of cholesterol synthesis and uptake. Cell Metab. 3, 15-24.

Gotto, A.M., 2001. High-density lipoprotein cholesterol: an updated view. Current Opinion in Pharmacology 1, 109-112.

Grundy, S.M., Denke, M.A., 1990. Dietary influences on serum lipids and lipoproteins. Journal of Lipid Research 31, 1149-1172.

Grundy, S.M., Mok, H.Y.I., 1977. Determination of cholesterol absorption in man by intestinal perfusion. Journal of Lipid Research Volume 18, 263-271.

Hamada, T., Egashira, N., Nishizono, S., Tomoyori, H., Nakagiri, H., Imaizumi, K., Ikeda, I., 2007. Lymphatic Absorption and Deposition of Various Plant Sterols in Stroke-Prone Spontaneously Hypertensive Rats, a Strain Having a Mutation in ATP binding cassette transporter G5. Lipids 42, 241-248.

Hardie, D.G., Scott, J.W., Pan, D.A., Hudson, E.R., 2003. Management of cellular energy by the AMP-activated protein kinase system. FEBS Letters 546, 113-120.

Hardy, R.W., Scott, T.M., Harrell, L.W., 1987. Replacement of herring oil with menhaden oil, soybean oil, or tallow in the diets of Atlantic salmon raised in marine net-pens. Aquaculture 65, 267-277.

Harwood, J.L., Padley, F.B., Gunstone, F.D., 1994. Ocurrence and characteristics of oils and fats, in: J.L. Harwood, F.B. Padley, F.D. Gunstone (Eds.), The Lipid handbook, 2nd ed. Chapman & Hall, London, 722p.

Heinemann, T., Pietruck, B., Kullak-Ublick, G., von Bergmann, K., 1988. Comparison of sitosterol and sitostanol on inhibition of intestinal cholesterol absorption. Agents and Actions Supplements 26, 117-122.

Henderson, R.J., Sargent, J.R., 1985. Chain-length specificities of mitochondrial and peroxisomal b-oxidation of fatty acids in livers of rainbow trout (Salmo gairdneri). Comparative Biochemistry and Physiology 82, 79-85.

Hertzel, A.V., Thompson, B.R., Wiczer, B.M., Bernlohr, D.A., 2008. Lipid Metabolism in adipose tissue, in: D.E. Vance, J.E. Vance (Eds.), Biochemistry of lipids, lipoproteins and membranes, 5th ed. Elsevier, Amsterdam, 277-303.

Horton, J.D., Goldstein, J.L., Brown, M.S., 2002. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J. Clin. Invest. 109, 1125-1131.

Horton, J.D., Shimomura, I., Brown, M.S., Hammer, R.E., Goldstein, J.L., Shimano, H., 1998. Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. J. Clin. Invest. 101, 2331-2339.

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous Inference in General Parametric Models. Biometrical Journal 50, 346-363.

Huff, M.W., Pollex, R.L., Hegele, R.A., 2006. NPC1L1: Evolution From Pharmacological Target to Physiological Sterol Transporter. Arteriosclerosis, thrombosis, and vascular biology 26, 2433-2438.

Hyun, Y.J., Kim, O.Y., Kang, J.B., Lee, J.H., Jang, Y., Liponkoski, L., Salo, P., 2005. Plant stanol esters in low-fat yogurt reduces total and low-density lipoprotein cholesterol and low-density lipoprotein oxidation in normocholesterolemic and mildly hypercholesterolemic subjects. Nutrition Research 25, 743-753.

Janowski, B.A., Grogan, M.J., Jones, S.A., Wisely, G.B., Kliewer, S.A., Corey, E.J., Mangelsdorf, D.J., 1999. Structural requirements of ligands for the oxysterol liver X receptors LXRα and LXRβ. Proceedings of the National Academy of Sciences 96, 266-271.

Janowski, B.A., Willy, P.J., Devi, T.R., Falck, J.R., Mangelsdorf, D.J., 1996. An oxysterol signalling pathway mediated by the nuclear receptor LXR[alpha]. Nature 383, 728-731.

Jesch, E.D., Seo, J.M., Carr, T.P., Lee, J.-Y., 2009. Sitosterol reduces messenger RNA and protein expression levels of Niemann-Pick C1-like 1 in FHs 74 Int cells. Nutrition Research 29, 859-866.

Jonas, A., Phillips, M.C., 2008. Lipoprotein structure, in: D.E. Vance, J.E. Vance (Eds.), Biochemistry of lipids, lipoproteins and membranes, 5th ed. Elsevier, Amsterdam, 485-505.

Jones, P.J.H., MacDougall, D.E., Ntanios, F., Vanstone, C.A., 1997. Dietary phytosterols as cholesterol-lowering agents in humans. Canadian Journal of Physiology and Pharmacology 75, 217-227.

Jordal, A.E.O., Lie, Ø., Torstensen, B.E., 2007. Complete replacement of dietary fish oil with a vegetable oil blend affect liver lipid and plasma lipoprotein levels in Atlantic salmon (Salmo salar L.). Aquaculture Nutrition 13, 114-130.

Joseph, S.B., McKilligin, E., Pei, L., Watson, M.A., Collins, A.R., Laffitte, B.A., Chen, M., Noh, G., Goodman, J., Hagger, G.N., Tran, J., Tippin, T.K., Wang, X., Lusis, A.J., Hsueh, W.A., Law, R.E., Collins, J.L., Willson, T.M., Tontonoz, P., 2002. Synthetic LXR ligand inhibits the development of atherosclerosis in mice. Proceedings of the National Academy of Sciences 99, 7604-7609.

Jukema, J.W., Bruschke, A.V.G., van Boven, A.J., Reiber, J.H.C., Bal, E.T., Zwinderman, A.H., Jansen, H., Boerma, G.J.M., van Rappard, F.M., Lie, K.I., 1995. Effects of Lipid Lowering by Pravastatin on Progression and Regression of Coronary Artery Disease in Symptomatic Men With Normal to Moderately Elevated Serum Cholesterol Levels : The Regression Growth Evaluation Statin Study (REGRESS). Circulation 91, 2528-2540.

Kaneko, E., Matsuda, M., Yamada, Y., Tachibana, Y., Shimomura, I., Makishima, M., 2003. Induction of Intestinal ATP-binding Cassette Transporters by a Phytosterol-derived Liver X Receptor Agonist. Journal of Biological Chemistry 278, 36091-36098.

Katan, M.B., Grundy, S.M., Jones, P., Law, M., Miettinen, T., Paoletti, R., Stresa Workshop, P., 2003. Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. Mayo Clin. Proc. 78, 965-978.

Kidambi, S., Patel, S.B., 2008. Sitosterolaemia: pathophysiology, clinical presentation and laboratory diagnosis. Journal of Clinical Pathology 61, 588-594.

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

Kolesnikova, M.D., Xiong, Q., Lodeiro, S., Hua, L., Matsuda, S.P.T., 2006. Lanosterol biosynthesis in plants. Archives of Biochemistry and Biophysics 447, 87-95.

Laakso, P., 2005. Analysis of sterols from various food matrices. European Journal Of Lipid Science And Technology 107 402-410.

Laraki, L., Pelletier, X., Mourot, J., Debry, G., 1993. Effects of Dietary Phytosterols on Liver Lipids and Lipid Metabolism Enzymes. Annals of Nutrition & Metabolism 37, 129-133.

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

Lehmann, J.M., Kliewer, S.A., Moore, L.B., Smith-Oliver, T.A., Oliver, B.B., Su, J.-L., Sundseth, S.S., Winegar, D.A., Blanchard, D.E., Spencer, T.A., Willson, T.M., 1997. Activation of the Nuclear Receptor LXR by Oxysterols Defines a New Hormone Response Pathway. Journal of Biological Chemistry 272, 3137-3140.

Leikin, A.I., Brenner, R.R., 1989. Fatty-acid desaturase activities are modulated by phytosterol incorporation in microsomes. Biochimica et Biophysica Acta (BBA) 1005, 187-191.

LI-Hakwins, J., Lund, E.G., Turley, S.D., Russell, D.W., 2000. Distruption of the oxysterol 7α-hydroxylase gene in mice. Journal of Biological Chemistry 275, 16536-16542.

Lichtenstein, A.H., 2002. Plant sterols and blood lipid levels. Current Opinion in Clinical Nutrition and Metabolic Care 5, 147-152.

Lie, Ø., Lambertsen, G., 1991. Fatty acid composition of glycerophospholipids in seven tissues of cod (Gadus morhua), determined by combined high-performance liquid chromatography and gas chromatography. Journal of Chromatography: Biomedical Applications 565, 119-129.

Lin, X., Racette, S.B., Lefevre, M., Spearie, C.A., Most, M., Ma, L., Ostlund, R.E., 2010. The effects of phytosterols present in natural food matrices on cholesterol metabolism and LDL-cholesterol: a controlled feeding trial. European Journal of Clinical Nutrition 64, 1481-1487.

Ling, W.H., Jones, P.J.H., 2005. Dietary phytosterols: A review of metabolism, benefits and side effects. Life Sciences 57, 195-206.

Liscum, L., 2008. Cholesterol Biosynthesis, in: D.E. Vance, J.E. Vance (Eds.), Biochemistry of lipids, lipoproteins and membranes, 5th ed. Elsevier, Amsterdam, 399-420.

Maclatchy, D.L., Van der Kraak, G.J., 1995. The Phytoestrogen [beta]-Sitosterol Alters the Reproductive Endocrine Status of Goldfish. Toxicology and Applied Pharmacology 134, 305-312.

Mathur, S.N., Watt, K.R., Field, F.J., 2007. Regulation of intestinal NPC1L1 expression by dietary fish oil and docosahexaenoic acid. Journal of Lipid Research 48, 395-404.

Matsuoka, K., Kajimoto, E., Horiuchi, M., Honda, C., Endo, K., 2010. Competitive solubilization of cholesterol and six species of sterol/stanol in bile salt micelles. Chemistry and Physics of Lipids 163, 397-402.

Matvienko, O.A., D.S., L., Swanson, M., Arndt, B., Rainwater, D.L., Stewart, J., Alekel, D.L., 2002. A single daily dose of soybean phytosterols in ground beef decreases serum total cholesterol and LDL cholesterol in young, mildly hypercholesterolemic men. The American journal of clinical nutrition 76, 57-64.

Menoyo, D., Lopez-Bote, C.J., Bautista, J.M., Obach, A., 2003. Growth, digestibility and fatty acid utilization in large Atlantic salmon (Salmo salar) fed varying levels of n-3 and saturated fatty acids. Aquaculture 225, 295-307.

Micallef, M.A., Garg, M.L., 2008. The lipid-lowering effects of phytosterols and (n-3) polyunsaturated fatty acids are synergistic and complementary in hyperlipidemic men and women. Journal of Nutrition 138, 1086-1090.

Miettinen, T.A., Tilvis, R.S., Kesaniemi, Y.A., 1990. Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. American Journal of Epidemiology 131, 20-31.

Miller, M.R., Nichols, P.D., Carter, C.G., 2008. The Digestibility and Accumulation of Dietary Phytosterols in Atlantic Salmon (Salmo salar L.) Smolt Fed Diets with Replacement Plant Oils Lipids 43, 549-557.

Mineo, C., Deguchi, H., Griffin, J.H., Shaul, P.W., 2006. Endothelial and Antithrombotic Actions of HDL. Circulation research 98, 1352-1364.

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

Moghadasian, M.H., Mcmanus, B.M., Godin, D.V., Rodrigues, B., Frohlich, J.J., 1999. Effects of dietary phytosterols on cholesterol metabolism and atherosclerosis: clinical and experimental evidence Circulation 109, 588-594.

Moreau, R.A., Whitaker, B.D., Hicks, K.B., 2002. Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. Progress in Lipid Research 41 457–500.

Nanton, D.A., Vegusdal, A., Rørå, A.M.B., Ruyter, B., Baeverfjord, G., Torstensen, B.E., 2007. Muscle lipid storage pattern, composition, and adipocyte distribution in different parts of Atlantic salmon (Salmo salar) fed fish oil and vegetable oil. Aquaculture 265, 230-243.

National Research Council (U.S.), 1993. Nutrient Requirements of Fish. National Academy Press, Washington D.C. 116p.

Navab, M., Anantharamaiah, G.M., Fogelman, A.M., 2005. The Role of High-Density Lipoprotein in Inflammation. Trends in Cardiovascular Medicine 15, 158-161.

Ng, W.-K., Sigholt, T., Gordon Bell, J., 2004. The influence of environmental temperature on the apparent nutrient and fatty acid digestibility in Atlantic salmon (Salmo salar L.) fed finishing diets containing different blends of fish oil, rapeseed oil and palm oil. Aquaculture Research 35, 1228-1237.

Noakes, M., Clifton, P.M., Doornbos, A.M.E., Trautwein, E.A., 2005. Plant sterol ester-enriched milk and yoghurt effectively reduce serum cholesterol in modestly hypercholesterolemic subjects. European Journal of Nutrition 44, 214-222.

Normén, L., Dutta, P., Lia, Å., Andersson, H., 2000. Soy sterol esters and ß-sitostanol ester as inhibitors of cholesterol absorption in human small bowel. The American journal of clinical nutrition 71, 908-913.

Olkkonen, V.M., 2004. Oxysterol binding protein and its homologues: new regulatory factors involved in lipid metabolism. Current Opinion in Lipidology 15, 321-327.

Ordovas, J.M., 2006. Nutrigenetics, Plasma Lipids, and Cardiovascular Risk. Journal of the American Dietetic Association 106, 1074-1081.

Peet, D.J., Turley, S.D., Ma, W., Janowski, B.A., Lobaccaro, J.-M.A., Hammer, R.E., Mangelsdorf, D.J., 1998. Cholesterol and Bile Acid Metabolism Are Impaired in Mice Lacking the Nuclear Oxysterol Receptor LXR[alpha]. Cell 93, 693-704.

Pfaffl, M.W., 2006. Relative quantification, in: M.T. Dorak (Ed.), Real-time PCR. Taylor & Francis Abingdon, 65-66.

Pineda, J.A., Ranedo, M.J.C., Anda, J.A., Terreros, S.F., 2005. Hypocholesteremic effectiveness of a yogurt containing plant stanol esters. Revista de la Sociedad Española de Química Clínica 205, 63-66.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., R Development Core Team, 2010. nlme: Linear and Nonlinear Mixed Effects Models, R package, 3.1-97 ed.

Plat, J., Mensink, R.P., 2002. Increased intestinal ABCA1 expression contributes to the decrease in cholesterol absorption after plant stanol consumption. FASEB 16, 1248-1253.

Plat, J., Nichols, J.A., Mensink, R.P., 2005. Plant sterols and stanols: effects on mixed micellar composition and LXR (target gene) activation. Journal of lipid research 46, 2468-2476.

Plat, J., Van Onselen, E.N.M., Van Heugten, M.M.A., Mensink, R.P., 2000. Effects on serum lipids, lipoproteins and fat soluble antioxidant concentrations of consumption frequency of margarines and shortenings enriched with plant stanol esters. European Journal of Clinical Nutrition 54, 671-677.

Plösch, T., Kruit, J.K., Bloks, V.W., Huijkman, N.C.A., Havinga, R., Duchateau, G.S.M.J.E., Lin, Y., Kuipers, F., 2006. Reduction of Cholesterol Absorption by Dietary Plant Sterols and Stanols in Mice Is Independent of the Abcg5/8 Transporter. The Journal of Nutrition 136, 2135-2140.

Pollak, O.J., 1953. Reduction of Blood Cholesterol in Man. Circulation 7, 702-706.

R Development Core Team, 2010. R: A language and environment for statistical computing., R Foundation for Statistical Computing,Vienna, Austria, http://www.R-project.org/.

Ranheim, T., Gedde-Dahl, A., Rustan, A.C., Drevon, C.A., 1994. Effect of chronic incubation of CaCo-2 cells with eicosapentaenoic acid (20:5, n-3) and oleic acid (18:1, n-9) on triacylglycerol production. Biochem J. 303, 155-161.

Rao, A.V., Janezic, S.A., 1992. The role of dietary phytosterols in colon carcinogenesis. Nutrition and Cancer 18, 43-52.

Rao, A.V., Janezic, S.A., Koratkar, S.A., 1997. Anticarcinogenic Effects of Saponins and Phytosterols, in: F. Shahidi (Ed.), Antinutrients and Phytochemicals in Food. American Chemical Society, Washington D.C., 313-324.

Ravid, T., Doolman, R., Avner, R., Harats, D., Roitelman, J., 2000. The Ubiquitin-Proteasome Pathway Mediates the Regulated Degradation of Mammalian 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase. Journal of Biological Chemistry 275, 35840-35847.

Repa, J.J., Berge, K.E., Pomajzl, C., Richardson, J.A., Hobbs, H., Mangelsdorf, D.J., 2002. Regulation of ATP-binding Cassette Sterol Transporters ABCG5 and ABCG8 by the Liver X Receptors α and β. The Journal of Biological Chemistry 277, 18793-18800.

Roitelman, J., Simoni, R.D., 1992. Distinct sterol and nonsterol signals for the regulated degradation of 3-hydroxy-3-methylglutaryl-CoA reductase. Journal of Biological Chemistry 267, 25264-25273.

Rozen, S., Skaletsky, H.J., 2000. Primer3 on the WWW for general users and for biologist programmers (source code available at: http://fokker.wi.mit.edu/primer3/.), Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology., 365-386.

Sabeva, N.S., Liu, J., Graf, G.A., 2009. The ABCG5 ABCG8 sterol transporter and phytosterols: implications for cardiometabolic disease. Current Opinion in Endocrinology, Diabetes and Obesity 16, 172-177.

Sakai, J., Rawson, R.B., Espenshade, P.J., Cheng, D., Seegmiller, A.C., Goldstein, J.L., Brown, M.S., 1998. Molecular Identification of the Sterol-Regulated Luminal Protease that Cleaves SREBPs and Controls Lipid Composition of Animal Cells. Molecular Cell 2, 505-514.

Salen, G., Shore, V., Tint, G.S., Forte, T., Shefer, S., Horak, I., Dayal, B., Nguyen, L., Batta, A.K., Lindregn, T., Kwiterovich, P.O., 1989. Increased sitosterol absorption, decreased removal, and expanded body pools compensate for reduced cholesterol synthesis in sitosterolemia with xanthomatosis. Journal of lipid research 30, 1319-1330.

Salen, G., von Bergmann, K., Lütjohann, D., Kwiterovich, P., Kane, J., Patel, S.B., Musliner, T., Stein, P., Musser, B., 2004. Ezetimibe Effectively Reduces Plasma Plant Sterols in Patients With Sitosterolemia. Circulation 109, 966-971.

Sandnes, K., Lie, O., Waagbo, R., 1988. Normal ranges of some blood-chemistry parameters in adult farmed atlantic salmon, salmo-salar. Journal of Fish Biology 32, 129-136.

Schmid, K.M., Ohlrogge, J.B., 2008. Lipid metabolism in plants, in: D.E.Vance, J.E. Vance (Eds.), Biochemistry of lipids, lipoproteins and membranes, 5th ed. Elsevier, Amsterdam, 98-129.

Schneider, W.J., 2008. Lipoprotein receptors, in: D.E. Vance, J.E. Vance (Eds.), Biochemistry of lipids, lipoproteins and membranes, 5th ed. Elsevier, Amsterdam, 556-577.

Seierstad, S.L., Svindland, A., Larsen, S., Rosenlund, G., Torstensen, B.E., Evensen, Ø., 2008. Development of intimal thickening of coronary arteries over the lifetime of Atlantic salmon, Salmo salar L., fed different lipid sources. Journal of Fish Diseases 31, 401-413.

Shefer, S., Hauser, S., Lapar, V., Mosbach, E.H., 1973. Regulatory effects of sterols and bile acids on hepatic 3-hydroxy-3-methylglutaryl CoA reductase and cholesterol 7α-hydroxylase in the rat. Journal of Lipid Research 14, 573-580.

Shimano, H., Horton, J.D., Hammer, R.E., Shimomura, L., Brown, M.S., Goldstein, J.S., 1996. Overproduction of Cholesterol and Fatty Acids Causes Massive Liver Enlargement in Transgenic Mice Expressing Truncated SREBP-1a. J. Clin. Invest. 98, 1575-1584.

Shimomura, I., Shimano, H., Korn, B.S., Bashmakov, Y., Horton, J.D., 1998. Nuclear Sterol Regulatory Element-binding Proteins Activate Genes Responsible for the Entire Program of Unsaturated Fatty Acid Biosynthesis in Transgenic Mouse Liver. Journal of Biological Chemistry 273, 35299-35306.

Sproul, A.M., 2006. Real-time PCR applications in hematology, in: M.T. Dorak (Ed.), Real-time PCR. Taylor & Francis, Newcastle-upon-Tyne, 284-285.

Stubhaug, I., Tocher, D.R., Bell, J.G., Dick, J.R., Torstensen, B.E., 2005. Fatty acid metabolism in Atlantic salmon (Salmo salar L.) hepatocytes and influence of dietary vegetable oil. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1734, 277-288.

Sudhop, T., Lütjohann, D., Kodal, A., Igel, M., 2002. Inhibition of Intestinal Cholesterol Absorption by Ezetimibe in Humans. Circulation 106, 1943-1948.

Surette, M.E., Edens, M., Chilton, F.H., Tramposch, K.M., 2004. Dietary Echium Oil Increases Plasma and Neutrophil Long-Chain (n-3) Fatty Acids and Lowers Serum Triacylglycerols in Hypertriglyceridemic Humans. The Journal of Nutrition 134, 1406-1411.

Tacon, A.G.J., Hasan, M.R., Subasinghe, R.P., 2006. Use of fishery resources as feed inputs to aquaculture development: trends and policy implications. FAO, Rome. 114p.

Taggart, J.B., Bron, J.E., Martin, S.A.M., Seear, P.J., Høyheim, B., Talbot, R., Carmichael, S.N., Villeneuve, L.A.N., Sweeney, G.E., Houlihan, D.F., Secombes, C.J., Tocher, D.R., Teale, A.J., 2008. A description of the origins, design and performance of the TRAITS–SGP Atlantic salmon Salmo salar L. cDNA microarray. Journal of Fish Biology 72, 2071-2094.

Tocher, D.R., Bell, J.G., Dick, J.R., Crampton, V.O., 2003. Effects of dietary vegetable oil on Atlantic salmon hepatocyte fatty acid desaturation and liver fatty acid compositions. Lipids 38, 723-732.

Tocher, D.R., Bell, J.G., MacGlaughlin, P., McGhee, F., Dick, J.R., 2001. Hepatocyte fatty acid desaturation and polyunsaturated fatty acid composition of liver in salmonids: effects of dietary vegetable oil. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 130, 257-270.

Torstensen, B.E., Espe, M., Sanden, M., Stubhaug, I., Waagbo, R., Hemre, G.I., Fontanillas, R., Nordgarden, U., Hevroy, E.M., Olsvik, P., Berntssen, M.H.G., 2008. Novel production of Atlantic salmon (Salmo salar) protein based on combined replacement of fish meal and fish oil with plant meal and vegetable oil blends. Aquaculture 285, 193-200.

Torstensen, B.E., Espe, M., Stubhaug, I., Lie, Ø., 2011. Dietary plant proteins and vegetable oil blends increase adiposity and plasma lipids in Atlantic salmon (Salmo salar L.), British Journal of Nutrition.

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

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

Tremblay, L., Van Der Kraak, G., 1998. Use of a series of homologous in vitro and in vivo assays to evaluate the endocrine modulating actions of [beta]-sitosterol in rainbow trout. Aquatic Toxicology 43, 149-162.

Turchini, G.M., Torstensen, B.E., Ng, W.-K., 2009. Fish oil replacement in finfish nutrition. Reviews in Aquaculture 1, 10-57.

U.S. Department of Agriculture, 2010. Cholesterol (mg) content of selected foods USDA National Nutrient Database for Standard Reference, Release 23. United States Department of Agriculture.
Van Rensburg, S.J., Daniels, W.M., Van Zyl, J.M., Taljaard, J.J., 2000. A comparative study of the effects of cholesterol, beta-sitosterol, beta-sitosterol glucoside, dehydroepiandrosterone sulphate and melatonin on in vitro lipid peroxidation. Metabolic brain disease 15, 257-265.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3.

Vielma, J., Koskela, J., Ruohonen, K., Jokinen, I., Kettunen, J., 2003. Optimal diet composition for European whitefish (Coregonus lavaretus): carbohydrate stress and immune parameter responses. Aquaculture 225, 3-16.

Vitello, F., Zanetta, J.P., 1978. Thin-layer chromatography of phospholipids. Journal of chromatography 166, 637-640.

Voelker, D.R., 2008. Lipid assembly into membranes, in: D.E.Vance, J.E. Vance (Eds.), Biochemistry of lipids, lipoproteins and membranes, 5th ed. Elsevier, Amsterdam, 441-484.

von Holtz, R.L., Fink, C.S., Awad, A.B., 1998. β-sitosterol activates the sphingomyelin cycle and induces apoptosis in LNCaP human prostate cancer cells. Nutrition and Cancer 32, 8 -12.

Wang, X., Seed, B., 2006. High-throughput primer and probe design, in: M.T. Dorak (Ed.), Real-time PCR. Taylor & Francis, Newcastle-upon-Tyne, 103-104.

Weihrauch, J.L., Gardner, J.M., 1978. Sterol content of foods of plant origin. Journal of the American Dietetic Association 73, 39-47.

Willett, W., Sacks, F.M., 1991. Chewing the Fat How Much and What Kind. New England Journal of Medicine 324, 121-123.

Yu, L., Bharadwaj, S., Brown, J.M., Ma, Y., Du, W., Davis, M.A., Michaely, P., Liu, P., Willingham, M.C., Rudel, L.L., 2006. Cholesterol-regulated Translocation of NPC1L1 to the Cell Surface Facilitates Free Cholesterol Uptake. Journal of Biological Chemistry 281, 6616-6624.

Yu, L., York, J., von Bergmann, K., Lutjohann, D., Cohen, J.C., Hobbs, H.H., 2003. Stimulation of Cholesterol Excretion by the Liver X Receptor Agonist Requires ATP-binding Cassette Transporters G5 and G8. Journal of Biological Chemistry 278, 15565-15570.

Zhao, C., Dahlman-Wright, K., 2010. Liver X receptor in cholesterol metabolism. Journal of Endocrinology 204, 233-240.

Zheng, X., Torstensen, B.E., Tocher, D.R., Dick, J.R., Henderson, J., Bell, J.G., 2005. Environmental and dietary influences on highly unsaturated fatty acid biosynthesis and expression of fatty acyl desaturase and elongase genes in liver of Atlantic salmon (Salmo salar). Biochimica and biophysica acta: Molecular and cell biology of lipids 1734, 13-24.

Zlatanos, S., Laskaridis, K., 2007. Seasonal variation in the fatty acid composition of three Mediterranean fish - sardine (Sardina pilchardus), anchovy (Engraulis encrasicholus) and picarel (Spicara smaris). Food Chemistry 103, 725-728.

Appendix

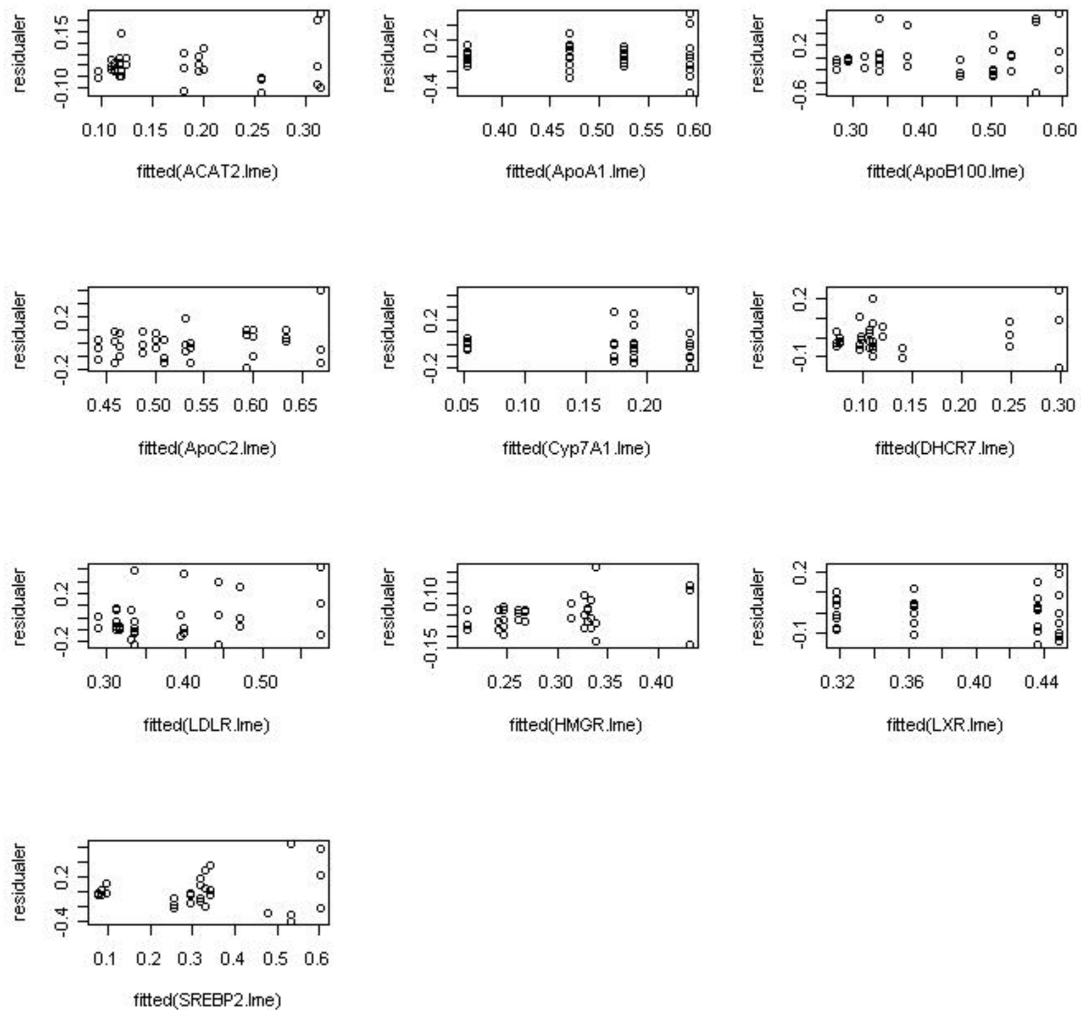


Figure A.1: Diagnostic plots testing the assumption of homoscedasticity, i.e. constant variance along predicted values of the chosen model. Each plot is showing residuals of the model chosen versus model predictions. A plot should not show a strong pattern. For instance, a fan shaped pattern would mean that the variance increases or decreases along increased model predictions. Data used for the example is qPCR results from final sampling.

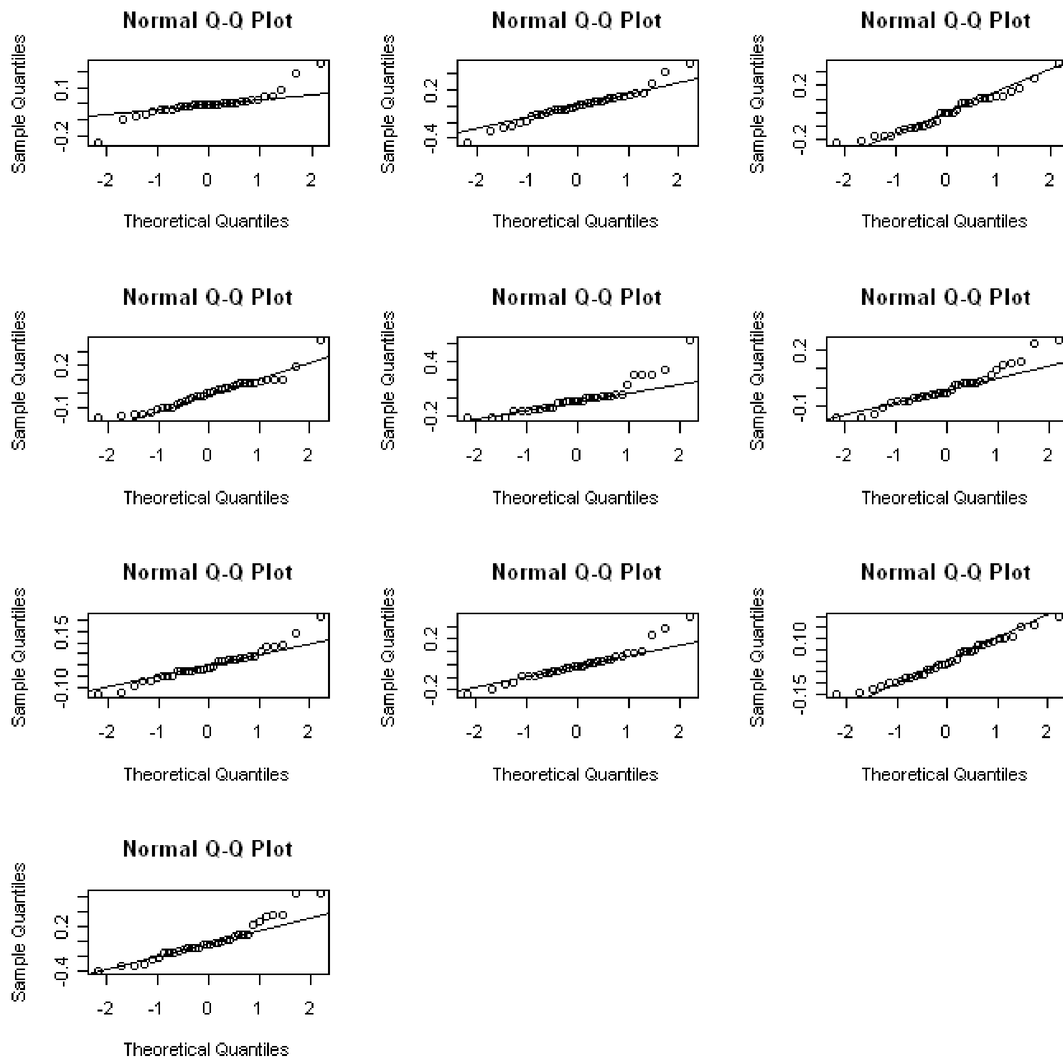


Figure A.2: Normal Q-Q plots testing the assumption of normality, i.e. normal distribution of residuals of the chosen model. The plots are comparing quantiles from the model data on the vertical axis against theoretical quantiles from a standard normal population on the horizontal axis. If data points show strong deviations from the line, the chosen model is assumed to violate the assumption of normally distributed residuals. The data used here is the same and mentioned in the same order as in A.1.