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INVESTIGATING ALTERNATIVE RAW MATERIALS AND DIET FORMULATIONS ON GROWTH PERFORMANCE, LIPID METABOLISM AND GENE EXPRESSION IN ATLANTIC SALMON (*Salmo salar* L.)

This thesis submitted for the degree of Doctor of Philosophy
University of Stirling

By

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To my parents

Declaration

I hereby declare that this thesis has been composed entirely by myself and has not been submitted for any other degree. Except where specifically acknowledged, the works described in this thesis is the result of my own investigations.

Jarunan Pratoomyot

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Abstract

Fish meal (FM) and fish oil (FO) have traditionally been central in aquaculture feed formulation but the finite global supply situation limiting future use along with issues of contaminant levels in these feed ingredients have become critical issues. The objectives of the present study were to investigate alternative feed ingredients as substitutes for both FM and FO in feeds for Atlantic salmon (*Salmo salar*) to ensure optimal growth, feed efficiency and health of the fish as well as maintaining the nutritional quality of the fish product to the human consumer, especially the levels of n-3 highly unsaturated fatty acid (HUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in the flesh. The results of the present study revealed that there were no negative effects on growth performance, feed utilisation and apparent digestibility in Atlantic salmon when FO was substituted with vegetable oil (VO) but these parameters were affected when FM was replaced with alternative protein sources from plants and animals at high levels, despite dietary supplementation with crystalline amino acids and lecithin. Reduction in feed intake was a factor affecting growth retardation when FM inclusion decreased. However, replacing FM with alternative plant and animal proteins along with partial replacement of FO had no major effect on nutritional quality, particularly n-3 HUFA content of salmon tissues. Replacing Northern FO with decontaminated FO or blends of southern hemisphere FO and VOs strategies to reduce POP contaminants and retain high nutritional values in flesh were very successful. Dietary treatments and genetic origin of fish both had effects on tissue compositions and gene expression. All fish groups (strain/family), consist of CAL, LEAN and FAT strains, fed a diet containing VO showed significant differential expression of lipid metabolism-related genes compared to fish fed a FO diet with LEAN strain appearing to adapt to VO inclusion better than FAT strain. This thesis has demonstrated dual replacement of FM and FO with alternative raw materials in salmon feeds without a major negative impact on nutritional quality.

Abbreviations and Acronyms

ADC	Apparent Digestibility Coefficient
ALA	Alpha-linolenic acid
ANF	Antinutritional factor(s)
ANOVA	Analysis of Variance
ARA	Arachidonic acid (20:4n-6)
BSAL	Bile salt - activated lipase
CO	Camelina oil
DHA	Docosahexaenoic acid (22:6n-3)
DL-PCB	Dioxin-like polychlorinated biphenyls
EAA	Essential amino acid
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid (20:5n-3)
FA	Fatty acid(s)
FABP	Fatty acid binding protein
FAME	Fatty acid methyl esters
FAS	Fatty acid synthase
FCR	Feed Conversion Ratio
FFA	Free fatty acid
FM	Fish meal
FO	Fish oil
HDL	High-density lipoprotein
HUFA	Highly unsaturated fatty acid(s)
LA	Linoleic acid
LO	Linseed oil
LDL	Low - Density Lipoprotein
LPC	Lyso-phosphatidylcholine
LPL	Lipoprotein lipase
NFO	Northern fish oil
NQC	Norwegian Quality Cut
OFN	Oxygen-free Nitrogen
OO	Olive oil
PC	Phosphatidylcholine
PCB	Polychlorinated biphenyls
PBDE	Polybrominated diphenyl ethers
PCDD	Polychlorinated dibenzo- p- dioxins

PCDF	Polychlorinated dibenzofurans
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PO	Palm oil
POP	Persistent organic pollutant (s)
PUFA	Poly unsaturated fatty acid(s)
PS	Phosphatidylserine
RO	Rapeseed oil
SFA	Saturated fatty acid
SGR	Specific Growth Rate
SM	Sphingomyelin
SO	Soybean oil
SFO	Southern fish oil
TEF	Toxicity equivalent factor
TAG	Triacylglyceride
TEQ	Toxicity equivalent
TGC	Thermal Growth Coefficient
VLDL	Very low - density lipoprotein
VO	Vegetable oil(s)

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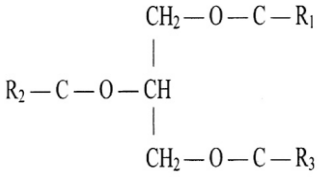
Chapter 1 - General Introduction

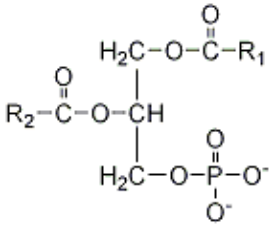
1.1 Fatty acid structure and nomenclature

Lipids are a large group of chemically heterogeneous compounds, having in common the property of insolubility in water, but a ready solubility in organic solvents such as chloroform, hydrocarbons or alcohols (Gurr & Harwood 1991). The basic units or building blocks for many lipids are fatty acids (FA). Lipids that contain FA are thus termed complex lipids, whereas lipids that do not contain FA, and so cannot be saponified, are termed simple lipids (Gurr & James 1975; Kjorsvik *et al.* 2004).

In vertebrate species, FA contain a long hydrocarbon chain ranging from 14 to 24 carbon atoms with a carboxylic acid group at one end and a methyl group at the opposite end (Gurr & Harwood 1991). They most commonly contain an even number of carbons in a straight chain, but can be odd numbered and branched. FAs are classified as saturated and unsaturated depending on the whether the carbons are fully combined with hydrogen and so a saturated FA (SFA) contains the maximum number of hydrogen atoms and no carbon double bonds, whereas unsaturated FA have C-C double bonds. The unsaturated FA can be further divided into monounsaturated FA (MUFA) with one double bond with Z (cis) configuration, and polyunsaturated FA (PUFA) with two or more double bonds. Highly unsaturated FA (HUFA) is a subgroup of PUFA distinguished by having chain lengths of $\geq$ C20 and with $\geq$ 3 double bonds up to 6 double bonds (Gunstone 1991; Jobling 1993). The structure of some lipids and FA are shown in Figure 1.1.



Triacylglycerol (TAG)



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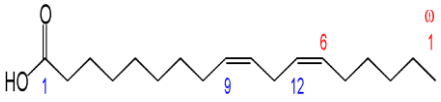
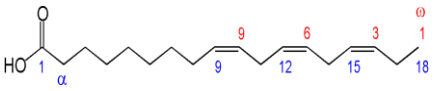
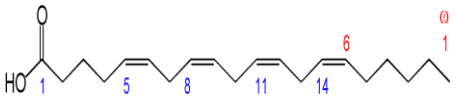


Linoleic acid (18:2n-6)

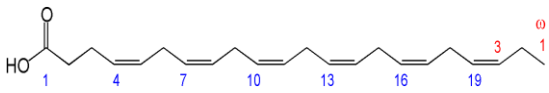


Linolenic acid (18:3n-3)



Arachidonic acid (ARA,20:4n-6)

Eicosapentaenoic acid (EPA, 20:5n-3)



Docosahexaenoic acid (DHA, 22:6n-3)

Figure 1.1. Structures of triacylgylcerol, phosphatidic acid, the basic phospholipid, and some fatty acids. Numbering of carbon atoms from the methyl terminus, is indicated as numbers above the structures. Source: Gurr & Harwood 1991.

FA nomenclatures are designed to denote the chain length, the degree of unsaturation and the position of the double bonds, according to the International Union of Pure and Applied Chemistry (IUPAC). Shorthand systems (the omega/n-designation system) and the delta (Δ) configuration are two accepted nomenclature systems used in lipid chemistry and aquaculture. Both systems are similar in that they consist of two numbers separated by a colon. The number before the colon gives the number of carbons in the chain and the figure after the colon denotes the number of double bonds. However, the nomenclatures differ in the way they describe the double bond position. The delta configuration uses numerical values to indicate the position of each double bond in relation to the carboxyl terminus of the chain. For example, α-linolenic acid, 18:3Δ 9,12,15 contains a carbon chain length of 18 and 3 double bonds, which are situated at carbon 9,12 and 15 from the carboxylic end of the chain. The shorthand systems designate the location of the first double bond from the methyl end of FA. For example, 16:0 represents a FA with 16 carbons and no double bonds, and α-linolenic acid is 18:3n-3 representing an 18 carbon chain with 3 double bonds, and n-3 defines the position of the first double bond with respect to the methyl end of the chain (Gurr *et al.* 2002). Examples of FA denoted by trivial name and their relative nomenclatures are illustrated in Table 1.1.

Table 1.1. The systematic, trivial names and designations of fatty acids.

Systematic name	Trivial name	Δ-designation	n-designation
Saturated			
Tetradecanoic	Myristic	14	14
Hexadecanoic	Palmitic	16	16
Octadecanoic	Stearic	18	18
Eicosanoic	Arachidic	20	20
Docosanoic	Behenic	22	22
Monounsaturated			
cis-9-octadecenoic	Oleic acid	18:1Δ9	18:1n-9
cis-9-eicosenoic	Gadoleic	20:1Δ9	20:1n-11
cis-11-eicosenoic	Godonic	20:1Δ11	20:1n-9
cis-11-docosenoic	Cetoleic	22:1Δ11	22:1n11
cis-13-docosenoic	Erucic	22:1Δ13	22:1n-9
n-6 family			
9,12-octadecanoic	Linoleic	18:2Δ9,12	18:2n-6
8,11,14-eicosatrienoic	Dihomo-γ- linolenic	20:3Δ8,11,14	20:3n-6
5,8,11,14-eicosatetraenoic	Arachidonic	20:4Δ5,8,11,14	20:4n-6
n-3 family			
9,12,15-octadecatrienoic	α-linolenic	18:3Δ9,12,15	18:3n-3
5,8,11,14,17-eicosapentaenoic	Timnodonic	20:5Δ5,8,11,14,17	20:5n-3
7,10,13,16,19-docosapentaenoic	Clupanodonic	22:5Δ7,10,13,16,19	22:5n-3
4,7,10,13,16,19-docosahexaenoic	Cervonic	22:6Δ 4,7,10,13,16,19	22:6n-3

Source: Gunstone & Norris (1983)

1.2 Lipid classes in fish

As mentioned above, lipids are a chemically heterogeneous group of compounds, having a range of different functions and roles in cells (Gurr and James, 1975). Lipids serve as storage, transport of energy, structural components of biological membranes and precursors of vitamins and hormones (Zubay 1993; Turner *et al* 2006; Nelson & Cox 2008). Dietary lipids provide energy and essential FA (EFA), and they also assist the absorption of fat-soluble vitamins. Generally, lipids can be divided into two broad classes depending on their polarity, specifically neutral lipids, that are completely soluble in non-polar solvents, and polar lipids that are generally more amphipathic (Gurr & Harwood 1991).

The main neutral lipids in fish are triacylglycerols (TAG), wax and steryl esters and sterols. TAG and wax esters are important primarily as energy stores, while sterols have a structural role in cell membranes and also function as a precursor of steroid hormones with steryl esters generally being a store of sterol rather than FA. TAG consists of three molecules of FA esterified to the three alcohol groups of glycerol. In land animal TAG, the FA is saturated with have no double bonds, so the chains are linear due to the molecules can pack tightly and the resulting fats are solid. Plant and fish oils contain unsaturated FAs with one or more double bonds, and so the chains are not straight. The angled structures of these chains prevent close packing so they tend have low melting point and to be liquids at room temperature (Turner *et al* 2006). The stereochemistry of TAGs can be described by the"stereospecific numbering"(sn) system (IUPAC-IUB system), with the carbon atoms of the glycerol moiety numbered 1-3. In a natural L-glycerol derivative, the secondary hydroxyl group is shown to the left of C-2. The carbon above this then becomes C-1, that below becomes C-3 and the prefix sn is placed before the stem name of the compound (Gurr *et al.* 2002). Generally, SFA and MUFA are preferentially located at the sn1 and sn3 positions of glycerol while PUFA are preferentially located at the sn2 position of glycerol (Gurr & James 1975) (Figure 1.1). Wax esters can be abundant in some aquatic animals, marine zooplankton, plant and microorganisms, but not in land animals (Hanahan 1960). Wax esters consist of a single molecule of FA esterified to a single molecule of a long-chain fatty alcohol (Christie 2003). Sterols are tetracyclic hydrocarbon compounds that are integral to membranes of animal tissues. The major sterol in animal, including fish, tissues is cholesterol. It can exist non-esterified as free cholesterol as in cell membranes where it has a role in maintaining membrane fluidity, or it can be esterified to FA as steryl (cholesterol) esters, that can be found in blood plasma and steroidogenic tissues such as

the adrenal cortex (Sargent *et al.* 2002). Free fatty acid (FFA) are also classed as neutral lipids but may be liberated from any of the complex (FA containing) lipids by post-mortem enzymatic hydrolysis (Gurr & Harwood 1991).

The polar lipids of fish are principally composed of a group of compounds generally, and sometimes incorrectly, termed phospholipids. Phospholipids are amphipathic molecules that have important functions of bilayer cell membrane structure and fluidity, and they may act as precursors for the synthesis of a series of biologically active compounds (Jobling 1993; Sargent 2001). The glycerophospholipids or phosphoglycerides are the major group of phospholipids that have a common backbone of phosphatidic acid (Figure 1.1), which consists of a glycerol molecule esterified to two FAs in sn1 and sn2 and a phosphate group esterified at sn3. The phosphate group at the sn-3 is then esterified to the "bases" choline, ethanolamine, serine and inositol to form the major phosphoglycerides of animals, including fish, namely phosphatidylcholine (PC) or lecithin, phosphatidylethanolamine (PE) or cephalin, phosphatidylserine (PS) and phosphatidylinositol (PI). PC and PE are generally the most abundant phospholipids in fish tissues (Hanahan 1960; Jobling 2004) reflecting their important function in membrane structure (Spector 1999). In fish, the highest levels of DHA (22:6 n-3) are usually contained in PE (Sargent *et al.* 2002). PC commonly has the lowest levels of DHA in fish tissues, being rich instead in 16:0 and 18:1n-9, and appears to be more easily influenced by dietary FA than the other phosphoglycerides. The amino phosphoglyceride, PS, and PI are present in smaller quantities in cells, with PS rich in DHA and PI having the highest level of ARA (20:4n-6) of all the fish phosphoglycerides (Sargent *et al.* 1989) and is also rich in 18:0 (Spector 1999). Indeed the sn*2* position of fish PI has a distinct preference for C20 PUFA. Thus, 20:5n-3 and 20:3n-6 can also be found in PI (Sargent *et al.* 2002).

Lysophosphatidylcholine (LPC), a lipolysis product of PC with one FA removed by hydrolysis, can be found in small amounts in fish tissues (Gunstone 1991). Another class of polar lipids is the sphingolipids (Christie 2003). The most abundant member of sphingolipids is sphingomyelin (SM) that contains phosphocholine esterified to the alcohol group of sphingosine and is thus also a phospholipid (Sargent *et al.* 2002).

1.3 Fatty acid metabolism

1.3.1 Fatty acid digestion, absorption, transport and esterification

The digestion process in fish is thought to be essentially the same as occurs in mammals. Digestion, absorption and transport of lipids occurs in three different sites, i.e. intestinal lumen, enterocytes (intestinal epithelial cell), and lymph or blood. The apparent digestibility of dietary lipids depends on their degree of unsaturation and temperature, generally increasing as water temperature rises (Leger 1985). The greatest lipolytic activity occurs in intestine, both proximal intestine and pyloric caecum (Tocher 2003). Pyloric caeca aids digestion in fish by prolonging exposure time to digestive enzymes and providing greater surface area for absorption (Buddington & Diamond 1987).

Lipid digestion is accomplished in the small intestine by the action of hydrolytic enzymes, including pancreatic lipase, bile salt-activated lipase (BSAL) and phospholipases, which act on dietary TAG and phospholipids, respectively (Kjorsvik *et al.* 2004). The products of pancreatic lipase hydrolysis of dietary TAG are FA and diacylglycerols and, especially, monoacylglycerols (Lie & Lambertsen 1991). Pancreatic lipase exhibits low hydrolytic activity towards TAG with HUFA at sn-1 and sn-3 (Olsen & Ringo 1997). Alternatively, BSAL is capable of hydrolysing wax esters and steryl esters as well as TAG (Leger 1985). The intestinal phospholipases hydrolyse

the ester bonds between FA and the glycerol backbone. Thus, phospholipase A_2 hydrolyses the fatty acyl ester bonds at the sn*2* position while phospholipase A_1 hydrolyses the fatty acyl ester bonds specifically at the sn1 position with the products of phospholipase action are lyso-PL and FA (Henderson *et al.* 1995).

The products of digestion, including FA, glycerol, monoacylglycerol and lyso-phospholipids penetrate the brush-border of enterocytes in a monomolecular form. FA absorption rate increases with decreasing melting point, and so MUFA and PUFA are better absorbed than SFA (Leger 1985). As in mammals, the absorption of lipids in fish takes place mainly in the proximal intestine or in pyloric caeca. However, with high dietary lipid loads, absorption may extend to the distal intestine. In the mucosal epithelial cells of the mid gut, FFAs are re-esterified with monoacylglycerol and glycerol to reform TAG in the smooth endoplasmic recticulum and become temporarily deposited as large lipid droplets in the cells (Kjorsvik *et al.* 2004).

After passage across the intestinal cell wall, dietary lipids are mainly recovered in the lymph, but also in the blood, in the form of specific lipoproteins, namely chylomicrons and very low-density lipoprotein (VLDL)-like particles (Hames & Hooper 2000). Most lipids are present in plasma in the form of the combination of lipid and protein in variable amounts, collectively termed lipoproteins (Nelson & Cox 2008). The lipoproteins provide an efficient system for transport of lipids into the vascular system from the site of absorption (enterocytes) and/or biosynthesis (mainly hepatocytes and enterocytes) to the sites of conversion, storage or energy utilisation. The three main classes of plasma lipoproteins, VLDL, low-density lipoprotein (LDL), and high-density lipoprotein (HDL), are all present in fish (Leger 1985). In humans, VLDL contains mainly 50% TAG, LDL contains mainly 37% cholesterol ester, HDL contains mainly 55% protein (Nelson & Cox 2008). VLDL carries cholesterol,

cholesterol ester and TAG from liver to other tissues (Zubay 1993). LDL carries cholesterol to peripheral tissue (Stryer 1988). HDL removes cholesterol from the blood carrying it to the liver (Nelson & Cox 2008). HDL is generally the most predominant class of lipoproteins present in the plasma of fish suggesting an important role for HDL in the transport of lipids in most fish (Leger 1985).

FFA are normally found in only small amounts in fish tissues as FA exist mainly in their esterified form as components of the complex, saponifiable lipids (Henderson & Tocher 1987). Therefore, after FA are transported into the cytosol, via FA binding proteins (FABP), they are esterified in structural lipid of membranes (phospholipids), or assimilated in TAG. In adipose tissue, when energy supply exceeds expenditure, excess FA is deposited as TAG and stored in lipid droplets. In other tissues, usually associated with steroid hormone production, a small quantity of FA can be esterified to cholesterol to form cholesterol ester, which is the storage form of cholesterol in cells (Buzzi 1996). Esterification, however, is a selective process in fish, based mainly on degree of unsaturation, such that HUFA are preferentially incorporated into PL compared to TAG. For example, SFA and MUFA, as well as 18:3n-3 and 18:2n-6, are generally more esterified into TAG, whilst ARA, EPA and DHA are found in higher concentrations in PL (Henderson & Tocher 1987).

1.3.2 Fatty acid biosynthesis

The basic pathways of FA synthesis from non-lipid precursors are believed to be similar in fish to those in animals (Henderson & Sargent 1985a; Henderson 1996). FA in fish can ultimately be derived from two sources: synthesis *de novo* from non-lipid carbon sources within the fish, or obtained directly from dietary lipid (Henderson 1996). Glucose from carbohydrate is generally used as the main carbon source for FA synthesis

in mammals (Stryer 1988). However, carbohydrate is very low in the natural diet of many species, such as marine and carnivorous fish, that have diets rich in protein, resulting in amino acids (AA) generally being the main carbon source for endogenous FA synthesis in fish (Henderson & Sargent 1981). Both liver and adipose tissue are capable of FA and TAG synthesis to different extents that vary with species (Henderson 1996). In salmonids, on a whole body basis, cytoplasm in liver is the principal site of FA synthesis *de novo* whilst adipose tissue is adapted for the uptake and storage of FA originating from the diet or hepatic synthesis (Henderson 1996).

The pathway of FA biosynthesis is initiated when the two-carbon acetyl-CoA unit is carboxylated by acetyl-CoA carboxylase (ACC) to form a three-carbon intermediate, malonyl-CoA. Saturated FA are now synthesised by successive additions of 2-carbon subunits, supplied by malonyl-CoA, to an initial acetyl group attached to acyl carrier protein in the FA synthase (FAS) multienzyme complex (Baeverfjord & Krogdahl 1996). The reaction proceeds via a series of condensation, reduction and dehydration steps involving the utilisation of NADPH (Henderson & Sargent 1985a; Gurr & Harwood 1991). Therefore, with each cycle two saturated carbon atoms from malonyl-CoA are added to the original acetyl-CoA moiety, ultimately forming 16:0, palmitic acid, which in turn can be further elongated to 18:0, stearic acid (Stryer 1988; Nelson & Cox 2008). The SFA products of FAS are then able to undergo separate elongation or desaturation reactions in order to yield longer-chain SFA and MUFA, respectively (Henderson 1996).

1.3.3 Fatty acid desaturation and elongation

As described above, SFA produced *de novo* and dietary FA can be modified by desaturation and/or elongation (Gurr & James 1975). Fatty acyl desaturation enzymes

include Δ9, Δ6 and Δ5 desaturases, which introduce cis double bonds at the 9th, 6th and 5th carbon atom counting from the carboxylic end, respectively (Gurr & Harwood 1991). All animals, including fish, contain a Δ9 desaturase, sometimes termed stearoyl-CoA desaturase, that acts on SFA 16:0 and 18:0 (Tocher 2003). Therefore, fish can produce 16:1$^{\Delta 9}$ = 16:1n-7 (palmitoleic acid) and 18:1$^{\Delta 9}$ = 18:1n-9 (oleic acid) from the endogenously synthesised 16:0 and 18:0 FA, respectively (Sargent *et al.* 2002). However, vertebrates, including fish, lack the Δ12 and Δ15 desaturases which are generally found only in plants and some invertebrates and other lower animals (Tocher *et al.* 1998). The Δ12 desaturase is responsible for converting 18:1n-9 into 18:2n-6 (linoleic acid, LA) and the Δ15 desaturase converts the 18:2n-6 to 18:3n-3 (α-linolenic acid, ALA), and thus the LA and ALA are often termed as EFA since most animals are unable to manufacture these *de novo*, and they must be obtained through the diet (Sargent *et al.* 2002). In vertebrates, including fish, the three families of unsaturated FA, n-9, n-6 and n-3, can be acted upon by a common enzyme system of alternating desaturases and elongases to give a series of FA of increasing length and unsaturation (Henderson & Sargent 1985a). Animals have the ability to convert ALA to EPA and ultimately to DHA, and LA to ARA (Nakamura & Nara 2004). Thus, once obtained from the diet, LA and ALA acids can be further desaturated, by Δ6 and Δ5 desaturases, and elongated to produce the LC-PUFA, ARA, and EPA and DHA, respectively (Tocher 2003).

The HUFA biosynthetic pathway in fish appears to be similar to that in mammals (Buzzi 1996; Tocher 2003). HUFA biosynthesis, as shown in Figure 1.2, can be summarised as the following; a) the elongations and desaturations only occur within the same family of FA and cannot interconvert between families. Thus, n-6 FA cannot give rise to n-3 FA and *vice versa*, b) as there is competition for the same enzymes,

whether elongases or desaturases, between the n-3 and n-6 series, a dietary excess of, say, LA will depress the further desaturation and elongation of ALA and vice versa, c) synthesis of DHA does not occur through direct Δ4 desaturation of 22:5n-3, which must be further elongated to 24:5n-3, followed by desaturation to 24:6n-3 by Δ6 desaturase. The 24:6n-3 is then chain shortened to DHA by limited peroxisomal β-oxidation (Voss *et al.* 1991; Buzzi 1996). Similarly, the same pathway is able to produce 22:5n-6 via elongation of ARA to 24:4n-6, followed by Δ6 desaturation to 24:5n-6 and chain shortening (Tocher 2003), d) Δ5 fatty acyl desaturase acts at only one step in the pathway, involving 20:3n-6 or 20:4n-3, whereas Δ6 FA desaturase occurs at two steps, firstly involving the C18 PUFA, LA or ALA, and secondly involving the C24 HUFA, 24:4n-6 or 24 :5n-3 (Sargent *et al.* 2002). Direct synthesis of DHA from 22:5n-3, as has been described in the marine microheterotrope, *Thraustochytrium* sp. requires desaturation of PUFA at the Δ4 position (Qui *et al.* 2001), but this activity has not been demonstrated in any vertebrate (Tocher *et al.* 1998; Tocher 2003). It has been reported that Zebrafish (*Danio rerio*) has no Δ4 desaturase activity but was able to desaturate 24:5n-3 to 24:6n-3 suggesting that a single Δ6 desaturase may be responsible for the desaturation of both C18 and C24 substrates (Tocher *et al.* 2003a). The synthesis of DHA proceeded via C24 PUFA intermediates through a pathway requiring two sequential elongations of 20:5n-3 to 24:5n-3, which is then desaturated by a Δ6 desaturase to 24:6n-3, and that this intermediate is chain shortened to 22:6n-3 in rat liver (Sprecher 2000) and in fish (Tocher *et al.* 2003a)

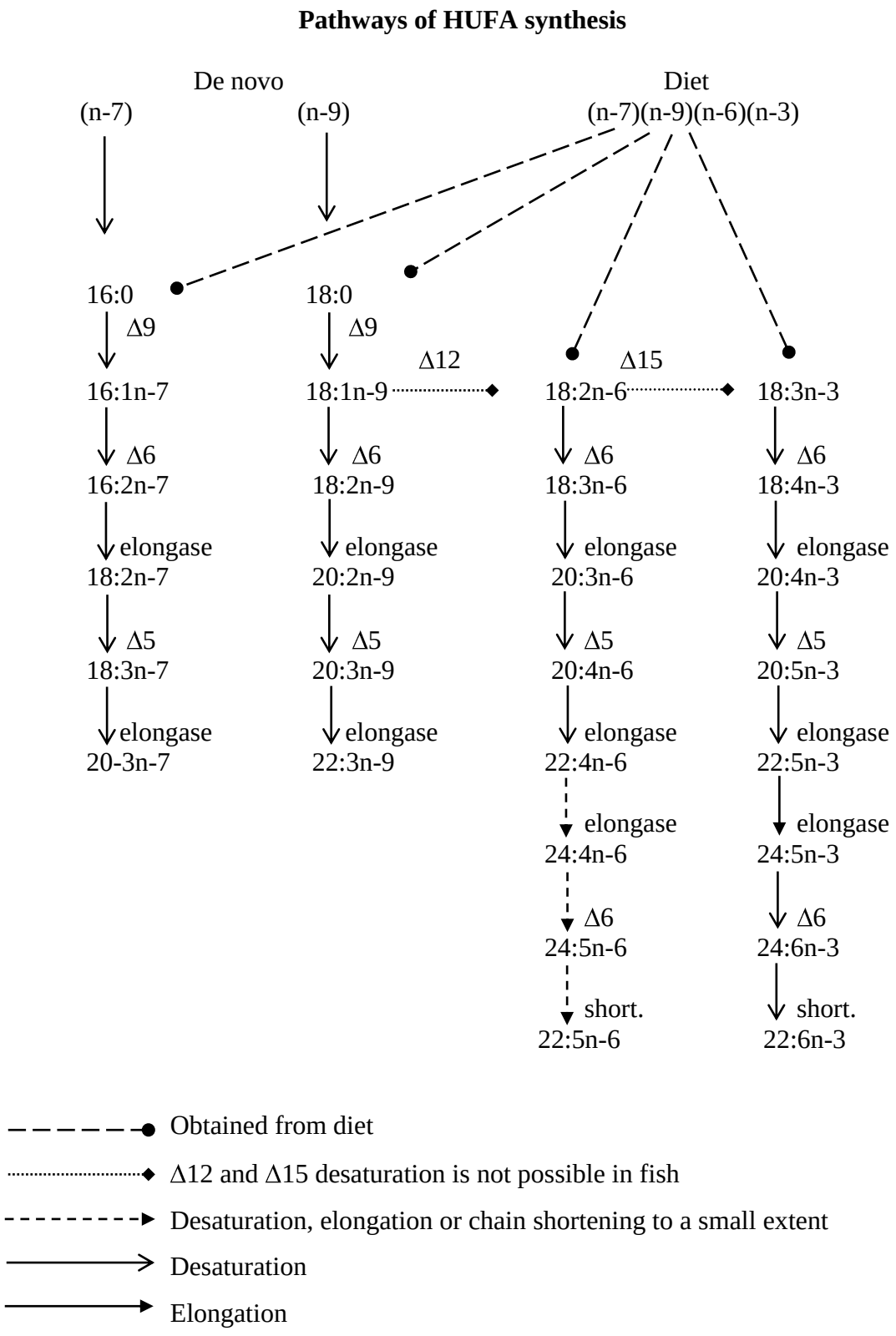


Figure 1.2. Pathways of chain elongation and desaturation of fatty acid families. Sources: Jobling (1993); Sargent *et al* (2002).

1.3.4 Fatty acid beta-oxidation

A major role of lipids is as an energy store to fuel future energy requirements. Excess dietary lipid is generally deposited as TAG in adipose tissue and other sites depending upon species and then provides a source of FA for catabolism (Nelson and Cox 2008). Metabolic energy is recovered from lipid by the catabolism of the FA via beta-oxidation, a process that involves the sequential removal of two-carbon units by oxidation at the beta-carbon position of the fatty acyl-CoA molecule, occurring in both mitochondria and peroxisomes, although mitochondrial β-oxidation is quantitatively more important and can utilise a wide range of FA substrates whereas peroxisomal oxidation is restricted to one round reducing longer-chain FA substrates (Henderson 1996).

Mitochondrial β-oxidation of FA requires four reactions; oxidation, hydration, second oxidation, and thiolysis to yield acetyl-CoA (Gurr and James 1975). The acetyl-CoA enters the citric acid cycle yielding $FADH_2$ and NADH, which are transferred to the electron transport chain to provide the energy for oxidative phosphorylation and ATP production. The fatty acyl-CoAs >12C require an acyl-carnitine to penetrate mitochondrial membranes which is mediated by carnitine palmitoyltransferases (CPT-I and CPT-II) and translocase. In peroxisomal β-oxidation, it produces the same two carbon acyl-CoA as mitochondrial β-oxidation, however, it produces hydrogen peroxide in place of $FADH_2$. The peroxisomal β-oxidation system is incapable of oxidising long-chain fatty acyl-CoAs completely (Gurr *et al.* 2002), and may therefore act as a chain shortening system with the medium chain FA being transferred via acyl-carnitines to the mitochondria for further oxidation (Gurr and Harwood 1991; Henderson 1996).

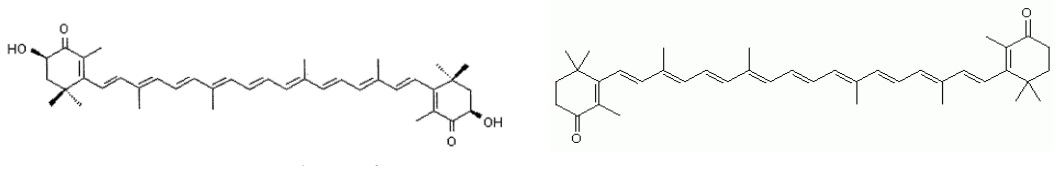
Mitochondrial β-oxidation dominated in all tissues of adult Atlantic salmon (*Salmo salar* L.) while peroxisomal β-oxidation prevailed in liver (Henderson 1996).

The rate of oxidation decreased with increasing FA chain length (Henderson & Sargent 1985a). In fish, mitochondrial β-oxidation preferred SFA and MUFA such as 16:0 18:1n-9, 20:1n-9 and 22:1n-11, over PUFA with EPA usually oxidised to a lesser extent and DHA mainly selectively retained in tissues rather than catabolised (Sargent *et al.* 1989; Tocher 2003)

1.4 Lipid-soluble substances

1.4.1 Carotenoids - structure and function

Carotenoids are classified as non-polar lipids (Hanahan 1960). Most carotenoids are polyunsaturated hydrocarbons, containing 40 carbon atoms, and comprising two terminal ring systems, resulting in as symmetrical molecular structure, which has in the region of 7-15 conjugated double bonds as shown in Figure 1.3. Carotenoids that are composed entirely of carbon and hydrogen are thus hydrocarbons and known as carotenes, such as alpha-carotene, beta-carotene, while those that contain oxygen are termed xanthophylls such as astaxanthin, canthaxanthin, lutein and zeaxanthin (Armstrong & Hearst 1996).



Astaxanthin Canthaxanthin

Figure 1.3. The structure of astaxanthin and canthaxanthin that have been used as pigment sources in salmon feed. Source: Metzler (1977).

Apart from serving to pigment the flesh, carotenoids have been reported to have possible metabolic functions in fish e.g. antioxidant protection against free radical damage (Edge *et al.* 1997) possibly encompassing UV protection in fish eggs, pro-vitamin A activity (Craik 1985), associated with the egg yolk protein lipovitellin (Ando & Hatano 1986), respiration function: the oxygen supply of pelagic eggs is rich and the presence of a large amount of pigments is not required (Pavlov *et al.* 2004). It has been suggested that carotenoids, including β-carotene, astaxanthin, and canthaxanthin, are potent antioxidants and that they operate synergistically with vitamin E (Nishigaki *et al.* 1994; Bell *et al.* 2000). Astaxanthin has been shown to be one hundred times more effective than vitamin E as an antioxidant (Miki 1991). Carotenoids have been shown to be important in normal embryonic development and could affect hatching rates and larval survival (Torrissen 1984; Craik 1985; George *et al.* 2001). It has been demonstrated that carotenoid supplementation of broodstock diets resulted in increased carotenoid in eggs (Torrissen 1989), improving egg quality and larval production in red sea bream (*Pagrus major*) (Watanabe & Miki 1993), yellowtail (*Seriola quinqueradiata*) (Verakunpiriya *et al.* 1997), and Atlantic cod (*Gadus morhua*) (Sawanboonchun *et al.* 2008). Dietary carotenoids also showed a positive relationship between egg pigmentation and fertilisation and survival in rainbow trout eggs (Harris 1984). Cold-water fishes, like salmon, have a high level of PUFA in their membranes, and protection of lipid tissue from peroxidation is likely to be a major metabolic role that dietary carotenoids such as astaxanthin could play (Bell *et al.* 2000).

1.4.2 Carotenoids - absorption and deposition

The duodenal intestine of fish contains a large number of pyloric caecum and is the main site of lipid absorption in salmonids (Ostos Garrido *et al.* 1993). The

carotenoids, however, are lipid-soluble and thus the ingested carotenoids are mainly absorbed in the midgut (from pylorus to hindgut) and hindgut (the posterior, folded part of the intestine) section (Torrissen *et al.* 1990), and transported in the blood in lipoproteins and ultimately deposited in the tissues (Torrissen 1986). Carotenoids cannot typically be synthesised by fish, including salmonids; therefore, salmon must obtain carotenoids from dietary sources (NRC 1993). In wild salmon, the carotenoids are present as a mixture of astaxanthin and its esters in a number of prey organisms consumed by the fish including zooplankton and crustaceans, and this naturally pigments the flesh (Storebakken & No 1992). The absorption of astaxanthin and canthaxanthin in salmonids is 10 to 20 times more efficient than lutein and zeaxanthin, and free astaxanthin is absorbed more efficiently than the astaxanthin ester (Schiedt *et al.* 1986). In farmed salmon, flesh colour is a very important commercially as consumers prefer pigmented products similar to wild salmon (Nickell 1997), therefore, dietary formulations must be supplemented with carotenoids by adding pigment-containing natural products or chemically-synthesised pigments (Jobling 2004). Pigments supplied in the diet are relatively poorly retained in salmonid muscles (Jobling 1993). In order to meet the consumer preference for red colouration, salmonid flesh should contain at least 3-4 mg of astaxanthin / kg (Torrissen *et al.* 1989), and astaxanthin and other alternative forms of carotenoids such as canthaxanthin, have been added to the formulated feed as a pigment source for farmed salmonids at the levels of 40-100 mg/kg dry diet (Jobling 1993). A combination of astaxanthin and canthaxanthin in the diet gave a higher total carotenoid deposition in the flesh than either astaxanthin or canthaxanthin alone (Torrissen *et al.* 1989), and this is deposited as the free form in flesh (Storebakken *et al.* 1987) and in esterified form, mono- or di- ester astaxanthin, in skin (Schiedt *et al.* 1986). The deposition of astaxanthin in the fish fillet occurs at

different rates depending on dietary sources (Choubert *et al.* 1995), dietary pigment concentration (Torrissen *et al.* 1995), size of fish (Torrissen 1985), stage of maturation (Bjerkeng *et al.* 1992), feeding period (Torrissen *et al.* 1995), genetic factors (Iwamoto *et al.* 1990; Storebakken & No 1992), digestibility of carotenoids (Choubert *et al.* 1995), absorption from intestine (Torrissen *et al.* 1990) and transport in blood by lipoprotein and metabolism (Nickell 1997).

The quantity and distribution of lipid around the fillet and its effects on pigmentation are also important to flesh quality tissues in salmonids. Astaxanthin binds non-specifically to hydrophobic binding sites in the white muscle of salmonids (Hemni *et al.* 1990). The number and size of white muscle fibre varies in different parts of the muscle of fish which could lead to variability in the number of astaxanthin binding sites around the fillets, and could explain the differences in astaxanthin concentrations between fillet areas (Kiessling *et al.* 1991).

Salmonid muscle shows a preferential accumulation of astaxanthin. In rainbow trout, it was reported that astaxanthin was the preferred carotenoid absorbed followed by canthaxanthin, while zeaxanthin, lutein and β-carotene were poorly absorbed (Guillou *et al.* 1992). Although dietary dose can affect flesh carotenoid concentration to some extent, increased dietary dose of carotenoids has been shown to have no significant effect on the pigment variation in Atlantic salmon, while an increase in the duration of feeding dietary astaxanthin has been reported to reduce variation of flesh pigment concentration in Atlantic salmon (Torrissen *et al.* 1995) and in chinook salmon (*Oncorhynchus tshawytscha*) (March & MacMillan 1996) by producing a more homogenous distribution of pigment around the fillet (Torrissen *et al.* 1995).

1.5 Organic contaminants

Persistent Organic Pollutants (POPs), chemical substances persisting in the environment, bioaccumulate through the food web and causing harmful effects to human health and the environment (Walker 2001). The POPs include dioxins, including polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans (PCDD/Fs), polychlorinated biphenyls (PCB), dioxin-like PCBs (DL-PCBs), polybrominated diphenyl ethers (PBDEs), dichlorodiphenyltrichloroethane (DDT), dieldrin among others (Lundebye *et al.* 2004). Generally, these compounds are only slightly soluble in water and are more stable in the presence of sunlight, moisture, air, and heat, which make them persistent in the environment (Walker 2001). Aquatic organisms take up these organic compounds directly from water, probably through the gills, or via the food chain (Edward 1970).

The persistent nature of POPs results in the processes of bioaccumulation, with concentration increasing in an organism throughout its lifespan and bioaccumulation, and the concentrations increasing higher up the food chain (Hendricks 2002). The contamination of aquaculture products with POPs due to human activity has become an issue of public concern particularly after the recent publication of information referring to the contaminant levels of farmed salmon. The source of the contaminants was related to the traditional practice of feeding marine FM and FO that can be contaminated with dioxins, PCBs and other chemicals including pesticides (Jacobs *et al.* 2002a, 2002b; Hites *et al.* 2004a). The contamination of FM and FO with POPs therefore has potential implications in fish nutrition, quality of farmed fish as final products and consequently to public health.

The POPs are not distributed equally in all tissues of the fish, but are concentrated in the fatty tissues. In the case of an "oily" fish like salmon that stores much of its fat in the flesh, it means that the contaminants are passed on to the human consumers. This is the paradox with salmon, as an oily fish they are an excellent source of the beneficial n-3 HUFA but, as with all oily carnivorous fish, the oil-rich flesh can accumulate lipophilic organic pollutants, including dioxins/furans, PCBs and PBDEs derived largely from their feed (Bell *et al.* 2005a). As POPs are only slightly soluble in water, when there is exposure the blood, it is rapidly saturated and, as a consequence of this saturation, the POP compounds partition into lipid, in which they are much more soluble, and are deposited in the fatty tissues where they accumulate as they do not then return to the aqueous phase (Walker 2001). Several studies have reported the levels of contaminants and their accepted limits, as regulated by the European Union (EU) and the World Health Organisation (WHO), in FM and FO (Jacobs *et al.* 2002a), in farmed and wild fish (Hites *et al.* 2004a, 2004b), especially oily fish like Atlantic salmon, as well as their potential effects on human health (Jacobs *et al.* 2002b; Mozaffarian & Rimm 2006). Jacobs *et al* (2002a) investigated the PCBs, organochlorine pesticides, and PBDEs in farmed and wild Atlantic salmon, aquaculture feeds, and FOs used to supplement the feeds. The results showed that the concentrations of POPs varied among the samples, and showed an increasing in concentration in the order salmon flesh > feed > FO. The DL-PCBs appear to be more efficiently transferred than dioxins from the feed to the flesh of Atlantic salmon (Lundebye *et al.* 2004). However, reducing the level of dietary FO in fish feed by replacement of FO with VOs decreased the organic contaminant levels in farmed salmon while still providing a good dietary source of marine n-3 HUFA (Bell *et al.* 2005a; Bethune *et al.* 2006).

1.6 Essential fatty acids in nutrition

1.6.1 Essential fatty acids in human nutrition

In recent years the possible beneficial roles of marine lipids, particularly n-3 HUFAs, in human development and health have been extensively considered (Jobling 2004). Deficiency of dietary n-3 HUFA, particularly DHA, associated with an over high dietary intake of 18:2n-6 causes the occurrence of pathologies and health disorders in developed societies especially those involving inflammatory responses e.g. inflammatory bowel diseases (Calder 2008), breast cancer (Bougnoux *et al.* 1994), cardiovascular disease (CVD), (Von 2004; Holub & Holub 2005), atherosclerosis, (Whyte *et al.* 2007), diabetes (Das 2001), Alzheimer's disease (Das 2008), cognitive impairement (Fontani *et al.* 2005), mental health (Mamalakis *et al.* 2002; Ross *et al.* 2007) and performance (learning and mood) (Richard 2007). There are now accumulating evidences in humans that cardiac pathologies are associated with an over-high dietary ratio of n-6/n-3 PUFA, and that the developing embryo is vulnerable to an over-high maternal ratio of dietary n-6/n-3 PUFA during pregnancy (Sargent 2001).

SFA and n-6 PUFA are the major FA constituents of fat consumed in developed countries. It has been advised that 5:1 is a desirable ratio of n-6 PUFA : n-3 PUFA for the UK national diet (Anon 1992), compared to the current estimates of dietary n-6/n-3 ratio in western diets of 10:1 to 25:1, averaging around 15:1 (Simopoulos 1999). A major beneficial effect of n-3 HUFA is that they provide a better metabolic counterbalance to this dietary imbalance than short-chain n-3 PUFA as well as having specific roles such as DHA being essential for normal neurological and visual development in humans and vertebrates in general (Uauy *et al.* 1999; Lauritzen *et al.* 2001; Richard 2007).

There is considerable variation in recommended intakes for PUFA in adults. Recommended intakes for LA ranges from 4.4-20g/day: ALA ranges from 1.35-2.2 g/day; and LC-PUFA range from 0.16-1.6 g/day (Meyer *et al.* 2003). The daily intakes of EPA/DHA in most European countries are in region of 0.1 to 0.5 g/day, and the current intake in the UK is reported to be at the lower end of this range, at 0.18 g/day. It was noted that an intake of 0.85 g/day of EPA/DHA reduced mortality from subsequent myocardial infarction and recent recommendations have advised daily intakes of 0.45 g EPA/DHA per day for healthy adults (Williams 2007).

Ensuring adequate dietary intake of HUFA from fish or other aquatic animal products is therefore important to optimal nutritional health in humans. In addition to the essential role of DHA in neural development and function, dietary supplementation with FO provides EPA, which can be beneficial by damping down possible excess eicosanoid production from ARA when diets are otherwise rich in n-6 PUFA. Increasing the amount of oily fish in the human diet will help re-balance the n-6: n-3 PUFA ratio and reduce diseases associated with dietary n-3 PUFA deficiencies, which can be most effectively reversed by providing the biologically active n-3 HUFA, EPA and DHA. In addition, reducing the intake of SFAs, unsaturated FAs and cholesterol in the diet and partly substituting them with MUFA and PUFA reduced both serum cholesterol and the risk of developing coronary heart disease (CHD) (Nordoy 1999).

As the appropriately balanced intake of n-3 and n-6 PUFA and HUFA partly determines the relative production of eicosanoids, the amounts and balance of these FA in the human diet will affect the body's eicosanoid-controlled functions. The eicosanoids are oxygenated derivatives of a group of C20 HUFA produced by the action of cyclooxygenase and lipoxygenase enzymes. Almost every tissue in the body produces eicosanoids and they have a wide range of physiological actions, e.g. in blood

clotting, the immune response, the inflammatory response (predominantly those of the joints, skin and eyes), cardiovascular tone, and renal, neural and reproductive functions (Sargent *et al.* 2002; Tocher 2003). Eicosanoid production is generally associated with stressful situations, and the excessive eicosanoid production often occurring in pathological conditions. In mammals, ARA is the main substrate for synthesis of eicosanoids whilst the EPA and to a lesser extent dihomo-γ-linolenic acid (20:3n-6) competitively depress the production of eicosanoids from ARA (Funk 2001). Thus, eicosanoid actions are determined by the ratio of ARA to EPA in cellular membranes, this in turn being determined by the dietary intake of n-6 and n-3 FA (Sargent *et al.* 1989, 2002).

1.6.2 Essential fatty acids in fish nutrition

Lipids and their constituent FA are very important as a source of metabolic energy in fish, particularly carnivorous fish which do not utilise carbohydrate as efficiently as herbivorous fish (Tocher 2003). TAG is the dominant energy and carbon storage product in higher animals, including fish. Apart from being an energy source, FA are functionally essential for normal growth, development and reproduction in all vertebrates. Thus, dietary lipids are also a source of FA required for the synthesis of new cellular lipid for growth and reproduction and for turnover of existing lipid (Sargent *et al.* 1989, 2002). Phospholipids together with cholesterol and sphingolipids are the structurally and functionally important lipids as ubiquitous constituents of cell membranes (Kjorsvik *et al.* 2004). Aquatic cold-blooded animals are characterised by having phospholipids containing a high proportion of PUFA. Like all other vertebrates, fish can synthesise SFA and MUFA *de novo*, but they cannot synthesise FA with two or more double bonds (PUFA). These FA must be obtained from the diet and are therefore

known as EFAs (Kjorsvik *et al.* 2004). However, vertebrates, including fish, have varying abilities to convert dietary C18 PUFA, LA and ALA, by desaturation and elongation, to the main biologically active HUFA, ARA, EPA, and DHA (Henderson 1996; Sargent *et al.* 2002). Therefore, in some species of fish there is some synthesis of EPA from ALA, but the conversion of ALA and/or EPA to DHA appears somewhat limited in most fish species (Tocher *et al*. 1992).

As alluded to above, the specific EFA requirement of fish differs between species that can be classified as essentially freshwater or marine, and this difference appears to be associated with the FA compositions of the natural diets of the fish species and also, to some extent, whether the species is herbivorous, omnivorous, or carnivorous (Sargent *et al.* 1989). Although the lipid and FA compositions in freshwater environments are perhaps less well defined than in the marine environment, it is clear that C18 PUFA are at least as well represented as C20 PUFA at the base of the freshwater food webs and that both LA and ALA are present in relatively high concentrations (Sargent 2001). This correlates with the broad ability of freshwater fish in converting C18 PUFA to the biologically active C20 and C22 HUFA and with freshwater fish having substantial dietary requirements for both n-6 and n-3 PUFA (Sargent *et al.* 2002).

In contrast to freshwater and/or herbivorous fish that have the ability to convert C18 PUFA to C20 and C22 homologues, the ability of marine and/or carnivorous/piscivorous fish to perform these conversions is very limited probably related to the fact that their natural diets invariably contain DHA (Sargent 2001). Certainly, the PUFA in the marine food-web and the carnivorous lifestyle of the main marine species are dominated by n-3 HUFA. The predominantly carnivorous marine species consume smaller fish that are rich in EPA and DHA derived ultimately from the

primary producers, the phytoplankton, and passed up through the food chain to zooplankton, and thus they have no need to convert ALA to EPA and DHA (Tocher 2003). The biochemical mechanism of this inability of marine fish to convert C18 PUFA to HUFA is based on enzyme deficiencies within the desaturation/elongation pathway (Olsen *et al.* 2004). Due to the abundance of EPA and DHA in the marine environment, there has been no evolutionary pressure for marine fish to retain the capacity to elongate and desaturate C18 PUFA and so the activities are inferior to freshwater fish where LA and ALA are abundant in natural diets (Sargent *et al.* 2002).

1.6.3 Quantitative essential fatty acid requirements

EFA supplied in the food are crucial for a) growth or *de novo* formation of tissues and differentiation, b) membrane activity and metabolism: membrane transport, respiration and enzyme activity c) regulation of metabolism: prostaglandins and their hormonal function (Olsen *et al.* 2004). The principal signs of EFA deficiency are known that it interferes with growth, reproduction and causes increased mortality (Castell *et al.* 1972). The signs of EFA deficiency can be prevented by adding n-3 FA in the diet, a level of 5% or more of marine FO in the diet usually provides sufficient dietary input of n-3 FA for salmonids (Lovell 2002). Most fish species, including salmonids, have a dietary requirement for n-3 FA, but can grow well on diets low in n-6 (Jobling 1993). In general, freshwater fish require either dietary LA or ALA, or both, whereas stenohaline marine fish require dietary EPA and/or DHA (NRC 1993).

The EFA requirements of salmonids are 1% LA (Castell *et al.* 1972; Takeuchi & Watanabe 1982), 0.8-1% ALA (Watanabe *et al.* 1974; Takeuchi & Watanabe 1982) or 20% of lipid as ALA or 19% of lipid as EPA and DHA (Takeuchi & Watanabe 1977). The EFA requirement of adult Atlantic salmon is about 1.0 % of ALA or perhaps

0.5 – 1.0 % of n-3 HUFA, although it has not been precisely defined (Sargent *et al.* 2002). The requirements of salmon parr may be different, because at the parr stage Atlantic salmon naturally consumes a diet consisting largely of freshwater invertebrates rich in C18 PUFA. Atlantic salmon parr may perform better on diets containing both ALA and LA than on diets with high levels of n-3 HUFA (Bell *et al.* 1997). In juvenile salmon fed semipurified diets with graded amounts of methyl esters of either 18:2 n-6, 18:3n-3 or a 1:1 mixture of 20:5n-3 and 22:6 n-3, increasing levels up to 1% of dietary dry matter improved growth and survival, and no requirement has been established for n-6 FA (Storebakken 2002).

1.7 Current Issues in Aquaculture

1.7.1 Atlantic salmon aquaculture

Aquaculture has showed potential of increasing production in satisfying the demand for fish and fishery products for human consumption. Global aquaculture (fish, crustaceans and molluscs) has contributed to this increase and reached 140 million tonnes in 2007. Aquaculture production excluding China has continued to increase at an average annual growth rate of 6.5% from 36.8 million tonnes in 2002 to 50.3 million tonnes in 2007 (FAO 2009a). Moreover, the growth rate of aquaculture production including China is increasing at 8.5% per year (FAO 2009b). In 2007, the share of aquaculture production was estimated at 44% of total fish food supply (FAO 2009a). Traditional wild capture fisheries for Atlantic salmon have not increased in recent years and so the demand for fish production has been met by a great expansion of salmon aquaculture as shown in Figure 1.4 (FAO 2009b). Atlantic salmon and rainbow trout production account for more than 80 % of total European aquaculture production with Norway being the major producer of salmon. Salmon aquaculture became commercial

in the 1970s (Jobling 1993). Salmon aquaculture production grew more than ten-fold during the 25 years from 1982 to 2007. Total salmon production in 2007 was almost 3.2 million tonnes of which farmed salmon contributed 69 % and wild salmon 31 %. Geographically, salmon production was dominated by Norway with 33 %, Chile with 31 %, and other European producers including Scotland with 19 % (FAO 2009b). Among all fish, Atlantic salmon production has trend to increases for years, and in Europe the production of Atlantic salmon has grown to over 1.6 million tonnes in 2006 (FAO 2007). In Scotland, Shetland has been the largest regional producer of Atlantic salmon since 1996, and it currently accounts for 34 % of Scottish output, and the remainder of Scottish production is divided between the following regions: NW Highlands (28%), Western Isles (15%), SW (Argyll and the Inner Hebrides not including Skye) (18%), and Orkney (5%). (Henserson & MacBean 2004).

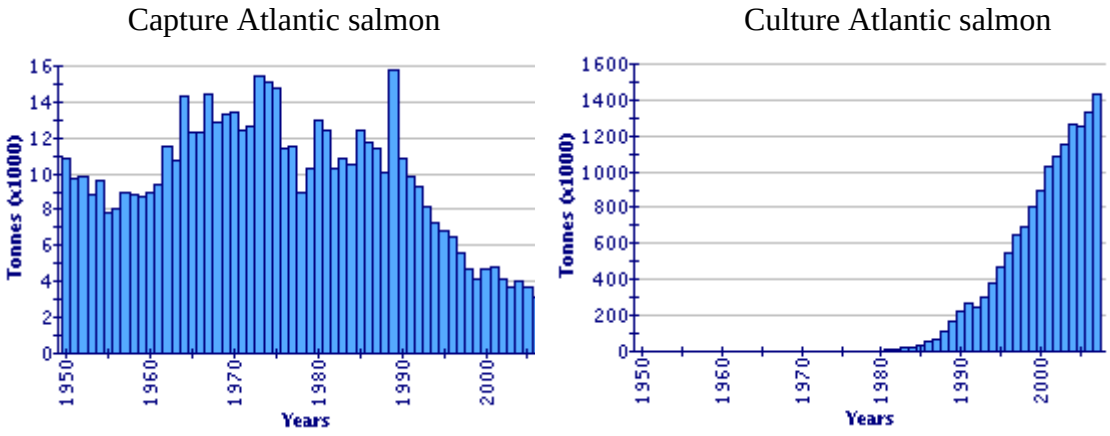


Figure 1.4. Global capture and global aquaculture of Atlantic salmon.
Source:FAO (http://www.fao.org/fishery/species).

Atlantic salmon, brown trout (*Salmo trutta*), chinook salmon, chum salmon (*Oncorhynchus keta*), coho salmon (*Oncorhynchus kisutch*), pink salmon (*Oncorhynchus gorbuscha*), rainbow trout, and sockeye salmon (*Oncorhunchus nerka*) belong to the family of Salmonidae (Jobling 1993). In the wild, Atlantic salmon are

found on both the European and North American sides of the North Atlantic (FAO 2004a). It is an anadromous species that lives in the ocean after spending 1-3 years in freshwater, and then returns as an adult to breed in freshwater. Thus, spawning and smoltification take place in freshwater prior to the main growing period, which takes place in the sea. During the adult period, which can take up to 4 years, wild Atlantic salmon feed on pelagic species such as herring, sprat and squid (FAO 2004a). Once large enough, Atlantic salmon are ready to return to the precise freshwater in which they hatched. If this is after one sea winter the fish are termed grilse but it is normally after two or more sea winters when they are called salmon (Laid & Needham 1991).

In aquaculture, Atlantic salmon are usually farmed in two stages. The salmon are hatched from eggs and raised in freshwater tanks to the smolt stage. When they are 6 to 18 months old, the smolts are transferred to seawater where fish are ongrown in floating sea cages or net pens (Purser & Forteath 2003). They are fed formulated, pelleted feed for another 12 to 24 months until they attain marketable size, which may vary from 2-6 kg, and they are harvested (FAO 2004a). The pellet diets of Atlantic salmon are traditionally based on FM and FO, used as the main sources of protein and lipid. Atlantic salmon feeds contain high amounts of protein, lipid and energy (Helland *et al.* 1991; Lovell 2002). As global FM and FO production is limited, these marine products are being increasingly substituted with vegetable protein and oil sources in salmon feeds at the same time as the trend to increase dietary lipid level continues with the aim to enhance protein sparing (Helland *et al.* 1991; Hillestad & Johnsen 1994; Einen & Roem 1997; Lovell 2002; Solberg 2004).

1.7.2 Dietary protein/energy

Fish require dietary energy for maintenance of bodily functions, activity and reproduction. Once these energy requirements are mets, any addition dietary input can be utilised for growth (Jobling 1993). All carbohydrates, proteins and lipids can be used as energy sources by fish, but these organic compounds are not equally well suited in providing energy for promotion of tissue synthesis and growth (Jobling 2004). Lipid rather than protein or carbohydrate is the favoured source of metabolic energy in most species of fish and can have a major protein-sparing effect in many fish species (Sargent 2001). The application of high-energy lipid in fish feeds is mainly designed to increase the growth performance of the fish for a given amount of feed intake by maximising the utilisation of lipids for energy with a consequence of protein-sparing effect allowing as much of the dietary protein as possible to be converted into flesh protein instead of energy production (Sargent *et al.* 2002). Some protein is essentially utilised for energy through direct oxidation of AAs via the tricarboxylic cycle, or after conversion of AAs to glucose via gluconeogenesis, but these processes can be diminished by including a large quantity of energy-providing nutrients in the diet. Salmonids can utilise lipids efficiently, an increased fat content in diet allows protein sparing and has an effect on growth and feed utilisation in rainbow trout and salmonids, and also contributes to an improvement in utilisation of protein (Hillestad *et al.* 1998; Bendiksen *et al.* 2003). Thus, high dietary fat levels are commonly used in salmonid diets as an important source of energy to promote protein sparing and to decrease nitrogenous losses. However, the high dietary lipid level can also lead to increased lipid deposition in fish fillet and a marked reduction in the proportion of body moisture rather than protein (Schreckenbach *et al.* 2001).

The culture of marine and anadromous carnivorous fish species, such as salmon, mainly has depended on the utilisation of FM and FO as the protein, energy and protein sparing sources in diet (FAO 2002). However, as global supplies of FM and FO are becoming more and more limited, it is therefore crucial for the aquafeed industry to optimise the use of feed protein and to improve protein utilisation in salmon diets. One problem in considering the effects of high-energy diets in aquaculture is that what precisely constitutes a high-energy or high-fat content in diet is rather undefined (Sargent *et al.* 2002). It was known that lipid in the range of 10-20 % of the dry weight of the diet is sufficient to allow protein to be effectively utilised for fish growth without depositing excessive lipid in the fish tissues (Watanabe, 1982 reviewed by Sargent *et al* 2002). Rainbow trout fed dietary lipid at 21 % increased growth over than that of fish consuming diets with 8 and 11 % dietary lipid (Luzzana *et al.* 1994). Atlantic salmon fed diets containing 38 and 47% lipid showed great growth in comparison to fish fed a diet containing 31 % lipid (Hemre & Sandnes 1999). However, limits to the effectiveness of feeding high-energy diets and growth-promotion were also found, and vary with the stage of development of fish. In sea bass larvae (*Dicentrachus labrax*), no effect on growth was observed when fish were fed diets containing lipid varying from 12 to 20 % (Salhi *et al.* 1994). However, the growth rate of sea bass juveniles was higher in fish fed a diet containing 24 % lipid than that of fish fed diets containing 12, 18 %, and 30 % lipid (Pereira & Oliva-Teles 2002). However, the 'optimum' protein/energy ratios are not rigid values. The actual value can vary according to fish species, fish stage, water temperature, and feed utilisation etc. For instance, the African catfish, *Clarius gariepinus*, cultured in high water temperature requires a higher protein/energy ratio, and larger fish showed a higher rate of energy expenditure per unit gain than small fish (Ng et al, 2003). Moreover, diets containing lower protein at a

constant dietary energy level inclusion resulted in an increase in energy expenditure (Steffens 1989).

1.7.3 Fish oil and fish oil replacement

FOs are virtually unique sources of the n-3 HUFA, predominantly EPA and DHA, which comprise 10% to 30% of total FA in most FOs. Moreover, FOs are excellent sources of energy owing to their high content of 16:0 and 18:0 as the predominant SFA and substantial amounts of the MUFA 18:1n-9, 20:1n-9 and 22:1n-11 (Sargent *et al.* 2002). The abundance of 20:1n-9 and 22:1n-11 FAs is a characteristic of all commercial FOs produced from northern Atlantic fisheries, which are currently used extensively in aquaculture (Tocher 2003). The precise FA compositions of the FOs are ultimately derived from the diets of the wild fish from which the oils are obtained. The FA profiles of marine phytoplankton and zooplankton tend to be dominated by n-3 PUFA, particularly EPA and DHA, and this pattern is generally retained at higher levels in the food chain (Jobling 1993). Zooplankton – consuming fish in northern latitudes, e.g. herring, menhaden and capelin accumulate body TAGs whose FA compositions very largely reflect the composition of the zooplankton diets including the calanoid copepods who deposit large amount of lipid as wax esters. Therefore, the fish TAGs are rich in 20:1n-9 and 22:1n-11 MUFA that are derived from the corresponding 20:1n-9 and 22:1n-11 fatty alcohols present in the zooplankton wax esters (Sargent *et al.* 1989). In contrast, wax ester-containing zooplankton do not form a major part of the diet in fish from the southern latitudes such as sardine, anchovy and pilchard, and so these species only contain low levels of 20:1 and 22:1 and have higher proportions of n-3 HUFA. FO from livers tend to have lower EPA and DHA and intermediate levels of 20:1 and 22:1 (Tocher 2003).

FO, a world-wide commodity, is presently used mainly in the feeds for farmed fish, and is used in only relatively small quantities in land animal feed (FAO 2002). The main global FO producing countries are Peru, Chile, China, Thailand, USA, Japan, Denmark, Norway, Iceland and South Africa (Fishmeal Information Network (FIN). 2007). In general, FO falls into two broad categories. First, the body oils produced by the processing of mainly pelagic species that deposit oil in the flesh including sardines, anchovy, menhaden, capelin, mackerel and herring. Second, the liver oils produced from mainly demersal species that deposit oil in the liver, predominantly cod and halibut liver oils (Tocher 2003). Irrespective of source, global total FO production has, at best, reached its sustainable limit in line with declining or static FM production worldwide. The limiting global production means current forecasts predict that the supply of traditional marine FO resources for fish feed will be exceeded by the demand of the aquaculture industry by 2010 (FAO 2004b).

Due to the situation of limited supply, high demand continues to make the prices of FO upwards, causing the production of feeds using the marine sources to become critical (FAO 2006). It is estimated that in 2006 the global aquaculture region consumed 835 thousand tonnes of FO (88.5% total global FO in 2006) or equivalent of 16.6 million tonnes of small pelagic forage fish (Tacon & Metian 2008). For the continued future development and expansion of aquaculture activities, alternative oil sources are required to be developed for aquafeeds, and also to contribute to the long-term sustainability of the world aquaculture industry (Hardy 2008). As the feed costs account for over 50% of the fish production cost (Lovell 2002), any dietary changes using alternative plant-derived materials must ensure that the growth of the fish, and the quality of the final product, including its highly beneficial properties for human health, should not be compromised. Thus, the strategy to develop alternative dietary oils using

a variety of oil sources should be focused on obtaining FA compositions in the final fish similar to the FA profile of current marine oil sources. The replacement of FO with VO can thus be rather challenging due to the fact that VOs are devoid of the n-3 HUFA which are abundant in FO including EPA and DHA that are essential for optimal growth and development in salmon (Sargent *et al.* 2002). The n-3 HUFA in FO is generally around 15 – 30 % of total FAs, while VO generally contain no HUFA at all and, in most cases, only moderate levels of the C18 n-3 PUFA, 18:3n-3. Furthermore, FOs also contain low levels of the n-6 HUFA, ARA, and also 18:2n-6, whereas VO contain no ARA and are usually rich sources of the n-6 PUFA, especially 18:2n-6 (Hertrampf & Piedad-Pascual 2000). However, although anything but ideal in terms of EFA, VOs are the most obvious candidates for FO replacement as the global production of the major seed oils in recent years has steadily increased such that the price and availability of VOs have been relatively constant.

The substitution of FO with alternative oils should provide sufficient energy in the form of the preferred FA in order to minimise any reduction in growth rate. SFA and MUFA are preferred over PUFA as substrates for energy production in fish e.g. 16:0, 18:1n-9 (Henderson 1996). Oil and fat alternatives to FO can be rich in energy-providing FA. Thus, fats from land animals, beef and pork lards, mutton and poultry fats, are rich in SFAs and MUFAs, but they generally lack EFAs, especially n-3 HUFA (Jobling 2004). Oilseeds can contain high levels of unsaturated FAs, but no HUFA. Palm oil (PO) is rich in SFA, particularly 16:0 (Bell *et al.* 2002), whereas olive oil (OO) and rapeseed oil (RO) are rich in MUFA, more specifically 18:1n-9 (Torstensen *et al.* 2004b). The very high content of 16:0 in PO with some 18:1n-9, and the high 18:1n-9 and moderate SFA in OO, along with relatively low levels of 18:2n-6, means that PO and OO provide sufficient energy for adequate/good growth (Ng 2002a, 2002b). Other

VOs are rich in PUFA, with sunflower oil, corn oil and soybean oil (SO) all very rich in 18:2n-6 (Bell *et al.* 1993; Rora *et al.* 2005). RO and SO have often been considered as possible alternative oil sources for salmonid production, as they are high volume, relatively low-cost oils and, although SO is particularly rich in n-6 PUFA, especially 18:2n-6, they also contain some 18:3n-3 (Caballero *et al.* 2002). Moreover, RO contains high levels of MUFA, especially 18:1n-9 (Bell *et al.* 2001). High levels of 18:3n-3 are not common in VOs, with LO the most common exception (Bell *et al.* 2003b, 2004). However, Camelina seed is a promising new lipid source of 18:3n-3 for aquafeeds and it also has other good nutritional qualities. Camelina seed contains 42.5 % crude protein containing considerable amounts of essential amino acids (EAA), and also 43.3 % crude oil (Zubr 2003). Camelina oil (CO) is less unsaturated than LO and more unsaturated than SO or canola oil. The balance of SFA and unsaturated fats is similar to that of SO, but it contains significantly higher proportions of 18:3n-3 (Putnam *et al.* 1993). CO is characterised by a high content of LA and ALA, together constituting about 52.6% (Zubr 2003). Moreover, it contains 12 % SFA, 34% MUFA primarily oleic acid (18:1) and eicosenoic acids (20:1), and 54 % PUFA, primarily 18:3n-3 and 18:2n-6 (Putnam *et al.* 1993).

The adequate balance of EFA and FAs for energy in aquafeeds is a key requirement in the replacement of FO with VO in order to sustain high growth and survival, feed conversion efficiency, immune competence, disease resistance, and high-quality of fillet (Peng *et al.* 2008). The use of VOs in the diets of salmonids has been investigated, with regards to the growth and feed utilisation efficiency, and changes in tissue FA composition and FA metabolism, for years (Bell *et al.* 1997; Turchini *et al.* 2003; Torstensen *et al.* 2008). Although it has been reported that it is possible to replace dietary FO with single VO as the added lipid source in aquafeeds without compromising growth in various fish, flesh FA composition and subsequent quality of

the fillet products in all fish, including salmonids, largely modified irrespective of the capacity of the fish to convert 18:3n-3 to EPA and DHA (Greene & Selivonchick 1990). Hence, replacement of FO with blended VOs is possibly more likely to provide a balanced dietary FA composition both for the requirement of the fish and to maintain flesh n-3 HUFA as high as possible.

Replacement of marine FO with VO in aquafeeds should consider not only the supply of lipid at the correct level with a balance of EFA to sustain optimum growth, but also to maintain normal immune function in fish (Montero *et al.* 2003). Substitution of FO with blend of VOs had no affects on fish histology while replacement with a single VO showed some affect on fish health. Thus, 60 % of FO can be replaced by a blend of different VOs without affecting gilthead sea bream (*Sparus aurata*) health, but when 60 % of FO was replaced by a single VO it affected the immunosuppression or stress response. For instance, RO affected head kidney macrophage activity, SO affected serum alternative complement pathway activity, and LO altered the stress response of fish (Montero *et al.* 2003). In European sea bass, partial substitution of single VOs, LO, RO and OO at 60% replacement of FO altered some immune parameters including the number of circulating leucocytes and macrophage respiratory burst activity, and promoted accumulation of large amounts of lipid droplets in hepatocytes, although no signs of cellular necrosis were evident (Mourente *et al.* 2005). Similarly, in rainbow trout, partial substitution of dietary FO with 60% VOs resulted in increasing lipid deposition in hepatocytes (Caballero *et al.* 2002). Other authors have also described histological changes, mainly large vacuoles in liver of Arctic charr (*Salvelinus alpinus*), and effects on the immune/stress response of gilthead sea bream and European sea bass when fish were fed dietary VOs (Olsen *et al.* 1999, 2000; Benedito-Palos *et al.* 2008). However, in other studies no histological signs of liver and

intestine damage were observed when gilthead sea bream were fed blended VOs (RO, SO, PO) replacing 60 – 69 % of dietary FO (Benedito-Palos *et al.* 2008; Fountoulaki *et al.* 2009). Similarly, Atlantic salmon fed dietary VO at up to 100 % replacement of FO showed no reduction in growth performance, feed conversion or development of histology (Bell *et al.* 2001, 2003a).

The nutritional quality of the final flesh product in terms of content of n-3 HUFA, particularly EPA and DHA, supplied to the human consumer must also be taken into account. Hence, the inclusion of VOs in the feeds for farmed fish can lead to alteration of the FA profile and reductions of EPA, DHA and the n-3/n-6 FA ratio (Martinez-Llorens *et al.* 2007), with a direct effect on the fish fillet quality (Regost *et al.* 2004) and sensory characteristics (Guillou *et al.* 1995; Izquierdo *et al.* 2005). Since it is desirable to produce fish fillets with a high content of HUFA, feeding fish previously fed VO with a 100 % FO diet (finishing diet) during the last part of their ongrowing period before harvest in order to allow recovery of the n-3 HUFA levels in their fillets is well documented (Bell *et al.* 2003b, 2004; Benedito-Palos *et al.* 2009).

1.7.4 Fish meal and fish meal replacement

FM, a light brown flour or meal, is an excellent and rich source of high quality protein containing the ten EAAs that animals, including fish, cannot synthesise. It is also a rich source of energy, EFA, vitamins and minerals, and also enhances the palatability of diets and feed efficiency in fish (NRC. 1993). FM is produced from fresh raw fish and fish trimmings by cooking followed by pressing, separation, drying and milling (Jobling 1993). The chemical composition of different FM may vary significantly depending on the source of fish used for its production (Hertrampf & Piedad-Pascual 2000). The quality of FM is influenced by fish species, seasonal

variations in compositions of the fish and the freshness of raw materials used (Jobling 1993, 2004). High quality FM show protein contents of 70-72 % in herring, capelin and sand eel meals, 65% in anchovy and horse mackerel, 60-62 % in menhaden, and 55-60 % in fish processing waste (Hardy 1996).

The top main FM-producing countries are Peru, Chile, China, Thailand, USA, Japan, Denmark, Norway, Iceland and South Africa (Fishmeal information network, FIN, 2007). The source of the FM thus varies geographically and includes a variety of fish species caught for the sole purpose of FM production, by-catches from another fisheries, or fish trimmings/offals from the food fish processing industry (Jobling 1993). Most FM is produced from small pelagic fish species e.g. anchovies, blue-whiting, herrings, mackerels, capelin, pilchard, menhaden, sardines, sand eel, Norway pout, sprat, and other (Jobling 2004). There are four different grades/qualities of FM products available: High quality - usually for small-scale aquaculture units (trout farms) or marine species; LT (low temperature) meal - is highly digestible and used in salmon and piglet production; prime and FAQ (fair average quality) - lower protein content feed ingredient for pigs and poultry (Fishmeal Information Network (FIN). 2007). Similar to FO production, FM manufacture has been critical for aquafeeds production (FAO 2006). The aquaculture sector consumed 3724 thousand tonnes of FM in 2006 (68.2% total global FM production) (Tacon & Metian 2008). Hence, aquaculture feed manufacturers are searching for suitable sources of plant materials rather than marine materials in their fish feed formulas, as the plant materials have a more stable supply and often much cheaper price compared to the FM. Animal by-products can also possibly be used in aquafeeds as substitutes for FM. Meals produced from a range of animals by-products e.g. meat and bone meal, blood meal, feather meal, poultry by-product meals are available and have been included in formulated feed for fish,

although the use of many of these is not currently permitted in the EU (Jobling 2004). However, their nutritional quality is variable depending upon the specific products. Meals derived from various animal by-products have a relatively high ash content and often low digestibility. Animal by-product meals can be rich sources of the EAAs lysine and histidine, but they may be deficient in the sulphur-AAs (cysteine, cystine and methionine) (Jobling 1993).

Substantial effort is being applied in investigation the use of plant proteins as substitutes for FM and this presents several challenges. Many plant protein products (meals etc) have the potential to be used as sustainable substitutes for FM because plant ingredients have high global availability at competitive prices compared to FM, and they generally have nutritional properties that can largely satisfy the requirements of the fish (NRC 1993). However, there are several issues with plant proteins that must be considered. First, the AA compositions of plant proteins can differ significantly from FM and feeding such diets may induce EAA deficiencies that would restrict growth and protein utilisation unless supplemented (Espe *et al.* 2006). Furthermore, many if not most plant protein sources contain anti-nutritional factors (ANF) that may affect growth, nutrient utilisation and fish welfare in general (Francis *et al.* 2001). Thus, cereals generally contain high carbohydrate and thus lower protein contents with a poor AA profile, often deficient in lysine, that limits their potential use as protein sources (Jobling 1993). Legumes such as peas, beans, lupins and lentils have been used as ingredients in feed ingredients of domestic animals, and some of these have been tested for suitability in fish feeds (Hertrampf & Piedad-Pascual 2000). However, possibly the most important plant protein sources are the oilseed meals, for example soybean, derived from the dried residues after the oil has been extracted. Oilseed meals may also be deficient in some AAs, particularly methionine and lysine (Jobling 1993).

One potentially major disadvantage of using plant materials in aquafeeds is the presence of ANFs. These substances are naturally occurring toxins or antimetabolites, whose main function in plants is as natural insecticides or, generally, as part of survival mechanisms that prevent grazing and consumption of plants by insects and other herbivores (Hendricks 2002). Common ANFs include trypsin inhibitors, hemagglutinins (Refstie *et al.* 1998), phytic acid, gossypol, phytoestrogens, cyclopropenoic FAs, glucosinolates, erucic acid, alkaloids and thiaminase (NRC 1993). However, some of the ANFs can be destroyed or inactivated by the heating and drying procedures involved in the production of oilseed meal (Jobling 1993). Another disadvantage of utilisation of plant products in aquafeeds is that they may contain up to 70 % of their phosphorus bound in phytic acid (phytate) (Selle *et al.* 2000; Francis *et al.* 2001). The phosphorus in phytate has low bioavailability to non-ruminant animals, including fish (Selle *et al.* 2000). Phytic acid can both reduce the availability of minerals and reduce the protein digestibility of salmonid diets (Jobling 1993). Thus, the presence of such substances in aquafeeds may affect their nutritional value that may result in a variety of physiological effects on fish (Francis *et al.* 2001).

The levels of inclusion of alternative plant protein ingredients in fish diets should be set such that they do not cause harm to fish, and the level of each ANF in the diet should be low. The use of plant protein concentrates such as soybean protein concentrates or canola protein concentrate (60-65 % protein) can overcome many of the problems associated with the presence of ANFs. The plant protein concentrates provide a source of protein with an EAA profile that, with the exception of methionine, would seem to fulfill the requirement for fish growth (Jobling 1993, 2004). Generally, the replacement of up to 20 – 40 % of FM with single plant meals does not normally compromise fish growth (Nengas *et al.* 1996; Refstie *et al.* 2000). However, complete

replacement of FM by plant protein has not been very successful and growth has been compromised (Refstie *et al.* 2000, 2001; De Francesco *et al.* 2004; Sitja-Bobadilla *et al.* 2005; Espe *et al.* 2006).

The palatability and digestibility of the plant protein-based diets both appear to be factors that affect growth performance of fish. In salmonids, using plant protein ingredients in feeds with and without AA supplementation resulted in lower growth performance that was related to reduced feed intake (Gomes *et al.* 1995; De Francesco *et al.* 2004; Espe *et al.* 2006). In Atlantic cod, SGR was reduced and FCR increased when 75 % of dietary FM was replaced with plant protein consisting of wheat gluten, soy protein concentrate and bioprocessed soybean, and reduced appetite of the fish was found when total substitution of FM was applied (Hansen *et al.* 2007a).

Growth and histology are good indicators of the adverse affects of plant-based ingredients on fish health and welfare. Although the full range of potential effects of ANFs has yet to be established, there have been several studies of the effects of dietary ANFs on fish growth and health. In salmonids, ANFs can decrease digestion and reduce utilisation of proteins (Krogdahl *et al.* 1994). In addition, high levels of dietary soybean products damage the distal part of the gut in salmon, where cells show extensive endocytotic activity and high numbers of intracellular vacuoles. Damage is basically characterised by alters in the number of mucus-producing goblet cells, intracellular absorptive vacuoles, amount of connective tissue, cellularity of the laminar propria, and degrees of mucosal folding and infiltration of the epithelium or lamina propria by inflammatory cells (Baeverfjord & Krogdahl 1996). The dietary effect on goblet cells may be caused by phytate or fibre in diets (Olsen *et al.* 2007). In extreme cases, massive necrosis was also found, a condition that referred to as soybean-induced enteritis (Baeverfjord & Krogdahl 1996).

Substitution of up to 75 % of dietary FM with plant protein ingredients appears not to affect health to any major degree in Atlantic cod (Olsen *et al.* 2007). However, total replacement of FM with plant protein meals may induce enteritis-like conditions in the guts of some fish and also activate heat shock protein (HSP) 70 gene mRNA expression in the intestine tissue. HSP70 is known as a biomarker of stress (Iwama *et al.* 1998). Plant proteins do not seem to induce HSP activation in cod liver (Hansen *et al.* 2006), but the HSP70 expression was found up-regulation in hindgut of Atlantic salmon fed diets with full fat soybean meal (Sagstad *et al.* 2007). Nevertheless, using the plant protein products (wheat gluten, soybean protein and bio-processed soybean meal) up to 75 % of diet protein produced only marginal effects on fish growth and fish health (Olsen *et al.* 2007).

1.8 General objectives

Due to the limited and, at best, static availability of the marine raw materials, FO and FM, and the ecological concerns of using non-sustainable marine resources, along with issues of POP contaminant levels in these feed ingredients, substitution of FO and FM must be investigated for development and expansion of aquaculture to continue. The overall aim of the present study was to develop alternative, sustainable diets for farming Atlantic salmon. More specifically, the aim was to investigate alternative feed ingredients as replacements/substitutes for both FO and FM in Atlantic salmon diets, and different strategies for their application and use, to ensure optimal growth, feed efficiency and health of the fish at the same time as maintaining, as much as possible, the nutritional quality of the fish product to the human consumer, especially the levels of n-3 HUFA, EPA and DHA, in the flesh.

To achieve this overall aim, the study involved four dietary trials with specific objectives. In trials 1 & 2, the focus was primarily on FO replacement. In trial 1, the objective was to investigate and compare the effects on n-3 HUFA and POP contaminant levels following substitution of standard northern FO (NFO) with either decontaminated northern FO (DFO) or with VOs blended with southern FO (SFO) as two alternative strategies. In trial 2, the effects of long-term (> 1 yr) replacement of 100 % of dietary NFO with blended VOs followed by a subsequent FO "finishing" diet period was investigated as an alternative strategy. This study was performed in different families (strains) of Atlantic salmon to determine genetic effects on the adaptation to alternative diets. In both trials 1 and 2, FM was also partially replaced as the diets were formulated to be practical and thus reflect current feed formulation practices. In trials 3 & 4, the focus was on substitution of FM. In trial 3, a regression design was employed to investigate the effect of graded substitution of FM with plant products. In trial 4, the effects of alternative protein substitute blends as replacement for FM were investigated. In line with the ethos that the diets investigated should be practical, in trials 3 & 4, the maximum level of FM inclusion used was 25 %, and 60 % of added oil was supplied by VO. Therefore, all trials in this study were examples of dual FM and FO substitution. Overall therefore, the study had a range of specific objectives as described below:

1) To investigate the effects of the total replacement of NFO with decontaminated NFO on growth, nutrients digestibility, tissue proximate and FA composition, tissues contaminant levels and liver gene expression in Atlantic salmon.

2) To evaluate the effects of the total replacement of NFO with SFO/VO blends on growth, nutrients digestibility, tissue proximate and FA composition, tissues contaminant levels and liver gene expression in Atlantic salmon.

3) To elucidate the effects of long term dual replacement of 100 % FO in dual-substituted diets (25% FM, 45 % PPs) in three families/strains of Atlantic salmon on growth, tissue proximate and FA composition, and liver gene expression in Atlantic salmon.

4) To determine the effects of a 100 % FO finishing diet in the three strains of salmon on tissue proximate and FA compositions in Atlantic salmon.

5) To elucidate the effects of graded substitution of FM in dual-substituted diets on growth, feed efficiency, nutrient digestibility and FA composition in Atlantic salmon.

6) To investigate the effects of different blends of replacement proteins in dual-substituted diets with high replacement of FM on growth, feed efficiency and tissue FA composition in Atlantic salmon.

Chapter 2 - Materials and Methods

The materials used and the procedures followed that represent techniques used throughout this study are presented below.

2.1 Materials

All reagents used for chemical, biochemical and molecular analysis in this study were HPLC grade. The reagents and materials used consist of cupric acetate, potassium hydrogen carbonate ($KHCO_3$), sodium hydrogen carbonate ($NaHCO_3$), iodine, neutral and polar lipid standards, butylated hydroxytoluene (BHT), 17:0 free fatty acid standard were obtained from Sigma (Poole, UK.). Chloroform, methanol, toluene, iso-hexane, diethyl ether, ethyl acetate, ethanol, acetonitrile, acetone, propan-2-ol, methyl acetate, potassium chloride, sulphuric acid and glacial acetic acid were obtained from Fisher Scientific UK, (Loughborough, England). Herring sperm DNA, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS) and dimethyl sulfoxide (DMSO), TriReagent, SSC buffer, Poly(A) (10 mg/ml in H_2O) were obtained from Sigma (poole, UK.). Ultra HyB buffer and Amino Allyl Messenger Amp II aRNA Amplification kit were obtained from Ambion (Ambion, Huntingdon, UK.). Cy3 monoreactive dye, Cy5 monoreactive dye, and Illustra AutoSeq G-50 columns were obtained from GE Healthcare (Chalfont, UK). Pre-coated silica gel 60 thin-layer chromatography (TLC) (20 × 20 × 0.25 cm) and high-performance thin-layer chromatography (HPTLC) (10 × 10 × 0.15 cm) plates, without fluorescent indicator were obtained from Merck (Darmstadt, Germany). All compressed gases: oxygen-free nitrogen, hydrogen, oxygen and helium were obtained from the British Oxygen Company (Glasgow, UK).

2.2 Experimental fish and diets

The experimental fish, Atlantic salmon, and diets used in this thesis are described in detail in the relevant chapters. The experimental diets were all formulated to satisfy the nutritional requirements of salmonid fish (National Research Council, 1993). The diets for trials 1,3 and 4 were manufactured at the BioMar TechCentre, Brande, Denmark while the diet for trial 2 was produced at the Skretting Aquaculture Research Centre (ARC) Stavanger, Norway. The specific dietary formulations and experimental conditions are described in the appropriate chapters.

2.3 Experimental sampling and processing

2.3.1 Biometric measurements

For all weighings, fish were anaesthetised with metacaine sulphonate (MS222). (50 mg/L) and, if not required for further sampling, fish were placed in clean aerated water and allowed to recover before being returned to the cage/pen. Weighing under anaesthesia and recovery was supervised by fish farm staff who had obtained the appropriate Home Office training and licenses. In some cases fish were bulk weighed but when fish were measured individually they were weighed to the nearest 0.5 g, and the total fork length was measured to the nearest millimeter. After the fish were killed by a blow to the head, the gut cavity was opened and entire livers and intestines were weighed to the nearest 0.1g.

2.3.2 Calculations of growth performance and carcass composition

Growth performance and carcass composition of fish were evaluated according to the followed calculations (Carter and Hauler 2000; Opstvedt *et al*. 2003; Torstensen *et al*. 2008).

Feed conversion ratio (FCR) = feed intake (g) / wet weight gain (g)

Feed consumption (g/day) = feed intake (g)/ (number of fish × day)

Weight gain = final weight – initial weight

Specific growth rate, SGR %day^{-1} = 100 × (ln final mean weight - ln initial mean weight)/ days

Thermal growth coefficient,TGC = 1000 × ((final weght)$^{1/3}$- (initial weight)$^{1/3}$)/ (days × temp.)

Hepatosomatic Index (HSI,%) = 100 × (weight of liver (g) /weight of fish(g))

Visceromatic Index (VSI,%) = 100 × (weight of viscera (g) /weight of fish(g))

Protein efficiency ratio, PER = (final mean weight (g) – initial mean weight (g))/ protein fed (g)

2.3.3 Sampling fish and tissues for biochemical and molecular analyses

Sampling for all biochemical and molecular analyses was carried out initially at random. At the time of sampling fish from each treatment, the runt fish were first removed and then only those fish that were around the mean weight of the remaining sample of fish were used in the biometrics and subsequent analyses. When required, whole fish for proximate analyses were immediately frozen and stored at -20^{o}C prior to analysis. Tissue samples (e.g. liver and viscera) were dissected and immediately frozen in liquid nitrogen and stored at - 70^{o}C prior to analyses. For flesh samples, Norwegian quality cuts (NQC) were taken, frozen and stored at -20^{o}C before analysis. The flesh samples for contaminant analyses were wrapped in aluminum foil before being frozen at -20^{o}C. The processing of specific samples from each trial is described in detail in the appropriate chapter.

2.4 Chemical analysis

The gross chemical compositions of the experimental diets, fish tissues and faeces samples were determined by proximate analyses based on official methods of the Association of Official Analytical Chemists (AOAC 2000). Moisture content was

determined directly from wet samples whereas protein, lipid, fibre and ash content were determined from dry samples. Samples of the dried experimental diets were finely ground, and the dry tissue samples were blended into homogeneous crumbles prior to proximate composition analysis.

2.4.1 Proximate analysis of diet, tissues and faeces

2.4.1.1 Moisture content (drying oven) and dry matter

Approximately 1.0 g of the wet samples (diets and tissues) were placed into a drying oven at 110^{o}C for 24 h or until constant weight was achieved (AOAC 2000).

2.4.1.2 Moisture content (freeze drying)

The faeces samples were freeze dried before analysis using a CHRIST Alpha 1-4 LSC freeze dryer (Osterode am Hartz, Germany). Fish faeces were stored at -70^{o}C for at least 6 h prior to place into a freeze dryer at -50^{o}C under vacuum for at least 16 h until constant weight.

2.4.1.3 Crude protein content

Crude protein content was determined from the nitrogen content of each sample, which assumes that protein contains 16% nitrogen, using automated Kjeldahl analysis (Tecator Kjeltec TM 2300 analyser, Foss, Warrington, UK.) according to the standard method (AOAC 2000) and manufacture's protocol.

2.4.1.4 Crude lipid content

Crude lipid content was determined according to the Soxhlet method (Tecator Soxtec system 2050 Auto Extraction apparatus, Foss, Warrington, U.K). Dry samples approximately 1.0 - 3.0 g were extracted with petroleum ether (40 – 60^{o}C boiling point).

2.4.1.5 Crude lipid extraction with acid hydrolysis

In some samples a portion of the fat is bound as lipoproteins and liposaccharides, which interferes with efficient solvent extraction. Thus, boiled samples with 3N hydrochloric acid for 1 h (Hotplate 2022, Foss, UK) and then extracted using a Tecator Soxtec Unit according to the manufacture's protocol. The samples were re-weighed and calculated as in the above formula.

2.4.1.6 Ash content

The total ash content was determined by incineration of the samples in a muffle furnace at 600^{o}C for 16 h (AOAC 2000).

2.4.1.7 Crude fibre content (Foss fibercap)

The fibre content was determined by removing all the digestible materials from the sample by sequential boiling with acid and alkali solutions, drying and incinerated in a furnace using foss fibercap according to foss fibercap manufacture's protocol and the standard method (AOAC 2000).

2.4.2 Nitrogen Free extract (NFE)- carbohydrate equivalent

Nitrogen free extract was determined by substraction the crude protein, crude lipid, crude fibre, ash and moisture from 100%.

2.4.3 Energy content of diets

The gross energy contents of the diets were determined by Bomb Calorimetry (Foss, Warrington, UK) where dry samples are completely combusted in an oxygen filled container, and the heat released measured and energy content calculated (Manufacture's manual).

2.4.4 Apparent digestibility using yttrium trioxide (Y_2O_3) as an indicator

Dried diets and faeces samples were analysed for dry matter, protein and lipid according to the standard method (AOAC 2000). FA contents of diets and feaces were analysed and quantified followed the method of Folch *et al.* (1957) and Christie (2003). Quantification of yttrium trioxide was performed at the University of Plymouth. In short, approximately 0.2 - 0.5 g of diet or 0.1 g freeze-dried faeces were digested with concentrated nitric acid overnight under a fome cupboard. A blank was prepared in the same manner. The quantity of yttrium trioxide was analysed by inductively couple plasma-optical emission spectrometry (ICP-OES) using a Liberty 200 instrument (Varian, Surrey, UK.). Values for faeces and diets were calculated using a series of calibration standards prepared from a 10,000 ppm stock solution (BDH, Poole, UK).

$$ADC\% = 100 - 100 \times ((Y\ feed / Y\ faeces) \times (N\ faeces / N\ feed))$$

Where Y feed is yttrium oxide in feed, Y faeces is yttrium oxide in feed, N faeces is yttrium oxide in faeces, N feed is yttrium oxide in feed.

2.4.5 Lipid analyses

2.4.5.1 Total lipid extraction and quantification

Total lipid for biochemical analyses was extracted from diets, tissues and faeces by homogenisation in chloroform/methanol following a standard protocol (Folch *et al.* 1957). Approximately 1 g of sample was homogenised in 30 ml glass tubes with 16 ml ice-cold chloroform/methanol (2:1,v/v) containing 0.01 % (w/v) BHT using a tissue disrupter at 8500-9000 rpm (Ultraturrax ^{TM} system, Fisher Scientific, Loughborough, England). The homogenates were kept on ice for 1 h prior to adding 4 ml of aqueous 0.88 % (w/w) KCl, followed by mixing and being left on ice for a further 5 min. The

tubes were centrifuged at 1500 rpm (400 g_{ave}) for 4 min (Joauan C312, France) and the supernatant non-lipid phase was aspirated and discarded. The lower organic phase was filtered through a pre-washed (chloroform/methanol, 2:1, v/v) 9 cm filter paper (Whatman no.1, Schleicher & Schuell, Maidstone, UK) into a clean pre-weighed 10 ml test tube. Solvents were evaporated under a stream of oxygen-free nitrogen (OFN) on an N-Evap evaporator (Organomation Associates Inc., Berlin, MA, USA). The lipid weight was determined gravimetrically after overnight desiccation in a vacuum. The total lipid was re-dissolved in chloroform/methanol (2:1,v/v) to a concentration of 10 mg/ml and stored under nitrogen at -20 ^{o}C for further analysis.

2.4.5.2 Determination of lipid class composition

Separation of lipid classes was performed by single-dimension double-development high performance thin-layer chromatography (HPTLC). A 10 x 10 cm HPTLC plate, pre-coated with silica gel 60 was pre-washed by fully developing in 10 ml chloroform/methanol (2:1, v/v) and activated at 100^{o}C for 20 min. Approximately 10 μg (1 μl of a 10 mg/ml solution) of total lipid was applied in a 2 mm streak 1 cm above of the bottom edge of the plate using a 10 μl syringe (Hamilton, Fisher, Loughborough, UK.). The plate was developed to 5.5 cm in a freshly prepared solvent mix which consist of methyl acetate/isopropanol/chloroform/methanol/0.25%KCL (25:25:25:10:9, v/v) in an HPTLC tank. The HPTLC plate was desiccated in a vacuum desiccator for 15 min prior to being fully developed to 9.0 cm in a freshly prepared solvent mix consisting of iso-hexane/diethyl ether/ glacial acetic acid (80:20:1, v/v) and desiccated for a further 15 min. The lipid classes were visualised by charring the plates at 160^{o}C for 20 min after spraying with 3 % (w/v) copper acetate in 8 % phosphoric acid reagent. The lipid classes were quantified by densitometry with a tungsten lamp at 370 nm using a Camag 3 TLC Scanner (Camag, Muttenz, Switzerland) with winCATS Planar

Chromatography Manager software, version 1.2.0 (Henderson & Tocher 1992). The identities of individual lipid classes were confirmed by comparison with reference to Rf values of authentic class standards that were run alongside the samples on the HPTLC plates and developed in the above solvent solutions.

2.4.5.3 Determination of fatty acid compositions

Fatty acid methyl esters (FAME) were obtained from total lipid subjected to acid-catalysed transesterification according to a standard method, and quantified by gas chromatography (Christie 2003). Approximately 1 mg of total lipid (100 μl of 10 mg/ml solution) and 0.1 mg of 17:0 free fatty acid internal standard (100 μl of a 1 mg/ml solution in chloroform/methanol, 2:1,v/v) were transferred to a clean test tube and the solvent evaporated to dryness under a stream of OFN. Acid-catalysed transesterification was performed by adding 1 ml of toluene and 2 ml of freshly prepared 1% sulphuric acid in methanol, gassing tubes with OFN, lightly stoppering followed by incubation at 50^{o}C for 16 h. FAME were extracted, after neutralising the acid with 2 ml of 2 % $KHCO_3$, with 5 ml iso-hexane/diethyl ether (1:1, v/v) containing 0.01% (w/v) BHT. The solutions were mixed well and centrifuged at 1500 rpm (400g_{ave}) for 3 min. The upper organic layer was transferred to a new test tube and the lower layer extracted with a further 5 ml iso-hexane/diethyl ether (1:1, v/v) without BHT. After centrifugation, the upper layer was combined with the previous one and the solvent evaporated under a stream of OFN. The FAME extract was re-suspended in 100 μl isohexane and applied to a 20 × 20 cm thin-layer chromatography (TLC) plate in 2.5 cm streaks, 1.5 cm from the bottom of the plate using a glass microsyringe (Hamilton, UK.). The plates were developed to within 2 cm of the top of the plate in iso-hexane/diethyl ether/acetic acid (90:10:1, v/v). After separation, the plate was partially sprayed with 1 % iodine in chloroform (w/v) to visualise the FAME (Tocher & Harvie 1988). The bands of silica

containing FAME were scraped from the TLC plate with a scalpel and transferred to a clean test tube. To elute FAME from the silica gel, 1 ml iso-hexane/diethyl ether (1:1, v/v) containing 0.01% BHT and 9 ml iso-hexane/diethyl ether (1:1, v/v) without BHT were added, and the tubes vortexed and centrifuged at 1500 rpm (400 g_{ave}) for 3 min. The solvent was transferred to a clean 15 ml glass test tube and the solvent evaporated under OFN. The FAME were re-dissolved in an appropriate volume of iso-hexane containing BHT to produce a 1 mg/ml solution of FAME. The FAME were separated and quantified by gas-liquid chromatography using a Fisons GC 8600 gas chromatograph (ThermoFisher Scientific, Hemel Hempstead U.K.) equipped with on-column injection and a wall-coated capillary column (CPWAX52CB column, 30 m × 0.32 mm id, 0.25µm film thickness, Chrompack Ltd., London, U.K.) and a flame ionisation detector (FID) at 250^{o}C. Hydrogen gas was used as carrier at a constant flow rate 2.0 ml/min. The temperature programming was initially at 50^{o}C to 180^{o}C at a rate of 40^{o}C/min and then to 225^{o}C at 2^{o}C/min and then held at 225^{o}C for 5 min. Individual FAME were identified by comparison to known standards and by reference to published data (Ackman 1980). Data were collected and processed using Chromcard for windows, version 1.19 computer package (Thermoquest Italia S.p.A., Italy).

2.4.6 Determination of carotenoid pigments

2.4.6.1 Pigment composition of diets

Diet carotenoids were extracted and analysed according to the method of Barua *et al*. (1993). Approximately 1 g of ground diet was digested with 110 mg of Maxatase enzyme (International Biosynthetics, Rijswijk, Netherlands) in 10 ml distilled water for 30 min at 50^{o}C. Ten ml of ethyl acetate/ethanol (1:1, v/v) were added and the mixtures centrifuged at 1500 rpm (400 g_{ave}) for 4 min. Five ml iso-hexane and 2 ml of ethanol

were then added and the combined solvents mixed and centrifuged as above. The solvent was transferred into a 25 ml glass tube before a final extraction with ethyl acetate/ethanol/ iso-hexane (5:2:10,v/v). The mixture was centrifuged as above, and the upper solvent layer combined with the previous extract. The pooled solvent extracts were evaporated under OFN and desiccated overnight under darkness. The residues were resuspended in 40 µl iso-hexane, and mixed well before transferring into microcentrifuge tubes and being centrifuged at 12,000 x g for 5 min (MH-2/18100, Degaspeed, Sarsted Ltd, Leicester, UK). The clear solvent was pipetted into 2 ml glass vials, flushed under OFN and stored in darkness. The carotenoids were separated by HPLC (Waters, St Quentin, France) using a stainless steel column 150 mm x 4.6 mm i.d., stationary phase 5µ Ascentis ™ silica gel HPLC column (Phenomenex, Macclesfield, UK) with iso-hexane/acetone (86:14, v/v) as a mobile phase at a flow rate 1 ml/min., and detected at 470 nm using a Waters™ 486 multiwavelength UV/vis detector (Millipore, Watford, UK) and quantified by comparison with authentic external standards (Roche, Heanor, UK). Data was processed using Millennium software (Waters, St Quentin, France) and calculation according to the following formula.

Calculation the concentration of standard from the spectrophotometer

$$\text{Standard concentration } (\mu g/ml) = [\text{Abs@470 nm}] \times \frac{10{,}000}{2{,}100}$$

Where10,000 is dilution factor of the standard astaxanthin and 2,100 is the standard absorption of a 1% all -E-extinction solution (w/v) in a 1 cm cuvette at 470 nm in iso-hexane. (Preparation of the standard see Appendix I).

Calculation total and individual pigment concentration from the HPLC

$$\text{Concentration (mg/kg)} = \frac{\text{SC} \times \text{peak area} \times \text{dilution factor}}{\text{std area} \times \text{weight of sample (g)}}$$

2.4.6.2 Pigment composition of flesh

Pigments were extracted from flesh by homogenising approximately 1g of tissue in 10 ml of ice-cold ethyl acetate/ethanol (1:1,v/v) using a tissue disrupter (Ultra-Turrax T25, Fisher, Loughborough, England). The homogenate was centrifuged at 1500 rpm (400 g_{ave}) for 4 min and the upper layer removed to a clean glass tube. The pellet was re-extracted, with 5 ml ethyl acetate, and then 5 ml iso-hexane, and in both cases, centrifuged and combined with the previous extracts. The solution was evaporated to dryness under OFN and desiccated overnight in darkness. The residues were resuspended in 4 ml iso-hexane, transferred into 2 ml plastic microcentrifuge tubes followed by centrifugation at 12,000 x g for 5 min. Carotenoids were separated by HPLC using a stainless steel column 150 mm × 4.6 mm i.d., stationary phase SYNERGI 4µ MAX-RP 80A column (Phenomenex, Macclesfield, UK) with acetonitrile/methanol (95:5, v/v) as a mobile phase at a flow rate of 0.8 ml/min, detected at 470 nm using a Waters^{TM} 490 programmable multiwavelength detector (Millipore UK, Watford) and quantified by comparison with authentic external astaxanthin standards (Roche, Heanor, UK). Data was processed using Millennium software.

2.4.7 Molecular analyses

2.4.7.1 RNA extraction

Approximately 100 mg of frozen liver was weighed and homogenised on ice in 1 ml TRIzol® in a 1.5 ml microcentrifuge tube using a hand-held Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, England). The homogenised sample left at room temperature for 5 min before 200 µl of chloroform (Sigma, Dorset, UK) was added, then shaken vigorously for 15 s and incubated for a further 3 min at room temperature. The sample was centrifuged at 12,000 g for 15 min at 4 ^{o}C (Sigma 4K15,

Osterode am Hartz, Germany), the supernatant phase transferred to a clean 1.5 ml microcentrifuge tube, and 500 µl of isopropanol (Sigma) added and the tube gently shaken and left at room temperature for a further 10 min to precipitate the RNA. The sample was centrifuged at 12,000 g for 10 min at 4 ^{o}C, the supernatant discarded and the RNA pellet washed by addition of 1 ml 75 % ethanol (v/v) in MilliQ-H_2O and vortexing. The tube was centrifuged at 7500 g for 5 min at 4^{o}C, the supernatant discarded, and the pellet allowed to dry at room temperature for 5 min. The pelleted RNA was re-dissolved in an appropriate volume of MilliQ-H_2O and the RNA quantity and purity were determined at absorbance wavelength ratios of 260/280 nm and 260/230 nm by spectrophotometry (NanoDrop, ND 1000, Thermo Scientific, Wilmington, USA). The quality of the RNA was confirmed by agarose gel electrophoresis using 1 % agarose gel in 1 × TAE/TBE buffer containing 10 ng ethidium bromide (see Appendix I) and UV fluorescent detection (INGENIUS, syngene bio imaging, Cambridge, UK). The RNA solution was stored at -70 ^{o}C prior to further analysis.

2.4.7.2 Transcriptomic (microarray) analysis

To obtain sufficient material for array hybridisation and to improve the reliability of array results, RNA amplification was used. The total RNA was reverse transcribed to first strand and second strand complimentary DNA (cDNA), which was subjected to *in vitro* transcription to synthesise anti-sense RNA (aRNA). The aRNA amplification, aRNA purification, aRNA dye coupling reaction, and dye labeled aRNA purification were performed according to the instruction manual for the Amino Allyl message Amp II aRNA amplification kit, RNA amplification for array analysis (Ambion, UK.).

2.4.7.2.1 First strand and second strand cDNA reverse transcription and purification

First strand and second strand cDNA were prepared by reversed transcription following the instruction manual (Ambion, UK.). In short, 500 ng of total RNA was mixed with 1 μl of T7 oligo (dT) primer in a nonstick, sterile, RNase-free, 0.5 ml tube and adjusted with nuclease-free water to a final volume of 12 μl, vortexed for 5 s and then briefly centrifuged to collect the mixture at the bottom of the tube. The mixture was incubated in a thermocycler (PCR machine, Thistle Scientific, Luton, UK.) at 70^{o}C for 10 min to denature the RNA followed by brief centrifugation, and placed on ice. First strand cDNA synthesis was performed by adding 8 μl of reverse transcription master mix (including 2 μl 10 × first strand buffer, 4μl dNTP mix, 1μl RNase inhibitor and 1μl ArrayScript enzyme), mixing well and centrifuging briefly prior to incubation at 42^{o}C for 2 h in an incubator. Proceeding immediately to the second strand cDNA synthesis, 80 μl of second strand master mix (including 10 μl 10 × second strand buffer, 4μl dNTP mix, 2μl DNA polymerase, 1μl RNase H and 63 μl nuclease-free water) was added to the first strand cDNA reaction, centrifuged briefly, and the mixture incubated at 16^{o}C for 2 h in a thermal cycler and then placed on ice. The second strand cDNA was purified by adding 250 μl of cDNA binding buffer, and mixing thoroughly before being transferred into a cDNA filter cartridge, placed in a 1.5 ml collection tube, prior to centrifuging for 1 min at 12,000 × g (260D, Denville Scientific Inc., Metuchen, NJ, USA.). A volume of 500 μl wash buffer was then added to the filter cartridge and centrifuged as above before transferring the filter cartridge into a new 1.5 ml collection tube (Ambion, UK.). The cDNA on the filter was eluted into the collection tube by adding 9 μl of warm (50-55^{o}C) nuclease-free water and centrifuging as above. The elution step was repeated once and the purified cDNA placed on ice prior to aRNA synthesis and amplification.

2.4.7.2.2 aRNA synthesis and amplification

The aRNA was synthesised by *in vitro* transcription (IVT) kit, following the manufacturers instructions (Ambion, UK.). For this, 26 μl of *in vitro* transcription double strand (ds) master mix (including 3μl of aaUTP, 12μl of 25 mM ATP/CTP/GTP mix, 3μl of 50 mM UTP solution, 4μl of T7 10 × reaction buffer and 4μl of T7 enzyme mix) was added to the sample prior to incubation at 37 ^{o}C for 16 h. The reaction was then stopped by adding 60 μl of nuclease-free water to bring the final volume to 100 μl before aRNA purification. For purification, 350 μl of aRNA binding buffer (containing β-mercaptoethanol) was added, immediately followed by 250 μl of 100 % ethanol, with thorough mixing. The sample was transferred to the centre of an aRNA filter cartridge, placed in a 1.5 ml collection tube, centrifuged for 1 min at 12,000 ×g and the flow-through discarded. For washing the filter, 650 μl of wash buffer was added to the aRNA filter cartridge, centrifuged and the flow-through discarded. The purified aRNA was eluted by applying 100μl of warm (50 - 55^{o}C) nuclease-free water into the center of the aRNA filter cartridge, incubated at room temperature for 2 min and then centrifuging at 12,000 × g for 1.5 min. The concentration of purified aRNA was determined spectrophotometrically using the NanoDrop spectrophotometer (Wilmington, DE, USA) and the aRNA then stored at -70 ^{o}C.

2.4.7.3 aRNA labelling

The aRNA was labelled for microarray analysis by the NHS-ester containing dye coupling reaction. Thus, 10 μl of each aRNA biological replicate were combined by transfer into a clean tube to prepare a pooled reference, with the final concentration verified spectrophotometrically (Nanodrop 1000). The aRNA samples were labelled with either Cy3 for treatment samples or Cy5 for the pooled reference (GE healthcare, UK). A volume containing 1.25 μg of each aRNA treatment sample and the pooled

sample was transferred into another microcentrifuge tube prior to the appropriate nuclease-free water was added to bring the volume to 4 μl. One μl of 0.5 M $NaHCO_3$ (2× coupling buffer) was applied into the tube and then vortexing briefly. A 5 μl aliquot of Cy dye dissolved in 100 μl dimethyl sulfoxide (DMSO) was added to the aRNA and gently mixed by pipetting before incubation at room temperature (25^{o}C) for 1.5 h in the dark. To remove the un-incorporated dye, the labeled aRNA (total volume 10 μl) was pipetted directly onto the resin-bed surface of the pre-spin Illustra AutoSeq G-50 dye terminator (GE Healthcare, UK) and centrifuged at 2000 × g for 1 min. The eluted purified labeled aRNA was then quantified spectrophotometrically (Nanodrop 1000) at microarray setting (RNA-40). In addition, the dye-labeling was assessed by electrophoresis of 0.4 μl of sample in a thin-agarose gel (1 % agarose gel in 0.5 × TAE buffer without ethidium bromide) and subsequently visualising the fluorescent products using a Typhoon fluorescent scanner (GE Healthcare, Chalfont, UK.). The purified labeled product was stored at -70^{o}C prior to hybridisation.

2.4.7.3.1 Microarray hybridisation and scanning

For hybridisation, 40 pmol of each Cy3 and Cy5 labelling sample was combined and brought to a final volume of 25 μl with nuclease-free water, before denaturing at 95 ^{o}C for 3 min and cooling to 4 ^{o}C. The mixture was then added to 225 μl freshly prepared and heated (60^{o}C) hybridisation buffer master mix (Ultrahyb, Ambion, UK.) (containing 185 μl of 0.7 × ultra-hybridisation buffer, 20 μl of 10 mg/ml in H_2O poly (A), 10 μl of herring sperm DNA (*ca* 10 mg/ml), and 10 μl of ultra pure BSA). The hybridisation mixtures, treatment (Cy3-labelled) and pooled reference (Cy5-labelled) samples, were incubated at 60^{o}C in a hot block in the dark before being applied to the 17 K salmon cDNA TRAITS/SGP microarray. For hybridisation, 200 μl of the hybridisation mixtures were injected using a microsyringe (Hamilton, UK.) into

hybridisation chambers (Lucidea slidepro, Amersham Biosciences, Chalfont, UK), where the cDNA microarrays were previously inserted. The chambers were heated to 70 ^{o}C for 10 min and cooled to 42^{o}C before hybridisations were performed at 60^{o}C for 17 h. The slides were automatically washed with first wash 1 buffer (1.0 × SSC; 0.1% SDS solution) and second wash 2 buffer (0.3× SSC; 0.2% SDS). The microarray slides were then removed from the Lucidea hybridiser after the chambers were cooled down to 40 ^{o}C, and subsequently washed manually twice in wash 2 buffer at 45^{o}C for 3 min, and three times in wash 3 buffer (0.2 × SSC; 0.2% SDS) for 2 min, in an incubator set at 125 rpm. The slides were finally washed in wash 4 buffer (0.1 × SSC) for 20 s at room temperature and then placed in a dry EasyDip container and centrifuged at 500 × g for 5 min at room temperature (Sigma, 4K15) to remove residual buffer. The hybridised slides were autoscanned at 10 μm resolution with wavelength 635 nm and 532 nm for Cy 3 and Cy 5 respectively, by using Gene Pix autoloader 420042A2 (Axon Instruments, Inc, Union city, CA, USA.) at the Roslin Institute Edinburgh. The hybridisation data were obtained in GenePix Array List (GAL) file.

2.4.7.4 Quantitative PCR (qPCR) analysis

2.4.7.4.1 First strand cDNA synthesis by reverse transcription (RT)

First strand cDNA synthesis of total RNA from the liver was performed using the Verso^{TM} cDNA kit (Thermo scientific, Fisher Scientific, Loughborough, England). Briefly, 1 μg RNA of each sample was brought to a volume of 11 μl with nuclease-free water in a PCR plate, denatured at 70^{o}C for 5 min in a PTC-100 thermocycler PCR machine (MJ Research, MA, USA.) and then cooled on ice for 5 min. To each sample, 9 μl of the reverse transcription mix, containing 4 μl of 5× cDNA synthesis buffer, 2 μl of dNTP mix, 0.75 μl of random hexamers (400 ng/μl) and 0.25 μl of anchored oligo-dT primer (500 ng/μl), 1μl of RT enhancer and 1μl of verso enzyme mix, was added. The

plate was then briefly mixed and centrifuged before being placed in a PCR thermocycler to synthesis cDNA at 42^{o}C for 60 min followed by enzyme inactivation at 95^{o}C for 2 min (manufacturer's protocol). The cDNA was stored at -20^{o}C prior to further analysis.

2.4.7.4.2 qPCR determination of gene expression

Gene expression was determined by quantitative real-time polymerase chain reaction (qPCR) using a Quantica thermocycler (Techne, Cambridge, UK). Quantitative PCR analysis for each gene was performed on 6 biological replicates, 2 technical replicates, in a total volume of 20 µl containing 5 µl of 10 ^{20} dilution of 1st strand synthesis cDNA, 1 µL (10 pmol) of each forward and reverse PCR primer, 10 µl of 2 × Absolute QPCR SYBR Green Mix (ABgene Ltd, Epsom, UK), and 3µL of nuclease-free water. A non-template control (NTC) containing no cDNA was included in each run to control genomic DNA contamination and the formation of primer dimer. The primer sequences of each gene are given in the appropriate chapter. Thermal cycling was initiated with incubation at 95°C for 15 min to activate the Thermo-Start^{TM} DNA polymerase, and forty cycles of PCR were performed. Each PCR cycle consisted of heating at 95°C for 15 s for denaturing, followed by 15 s at 60°C and 30 s at 72°C for annealing and extension. Cycle threshold (CT) corresponded to the number of cycles at which the fluorescence emission monitor in real time exceeded the threshold limit. PCR melting curve analysis was performed to confirm the production of a single product in these reactions. Standard dilution curves were established using five different serial dilutions (in triplicate) of pooled cDNA sample solutions and plotting the CT values against the $\log_{10}$ of the five dilutions of cDNA. Real-time efficiency was thus determined for each gene from the slopes given by Quantsoft software, applying the equation $E = 10^{(-1/slope)}$. The relative expression ratio of each gene was calculated using REST© software (http://www.gene-quantification.info). The relative expression ratio

for a considered gene is based on the PCR efficiency (E) and the CT of the sample compared with a control, and expressed in relation to reference genes. In this work, normalised relative expression was obtained by using the genomic mean expression of the following references genes: β-actin, elongation factor-1α and an unresponsive EST. The unresponsive liver EST is an anonymous cDNA feature selected from a salmon cDNA microarray study and identified as a suitable flatliner reference gene on the basis of constant expression between different dietary treatments and sampling time points (Taggart *et al.* 2008). The relative expression in comparison to the reference genes was determined according to Pfaffl's mathematical model (Pfaffl, 2001):

$$\text{Ratio} = (E_{\text{target}})^{\Delta CT\ \text{target(control-sample)}} / (E_{\text{reference}})^{\Delta CT\text{reference(control-sample)}}$$

2.4.8 Statistical Analysis

2.4.8.1 Biochemical data analysis

All the biological data are presented as means ± SD, and the significance of differences ($P < 0.05$) among the dietary treatments were determined by one-way or two-way analysis of variance (ANOVA) according to the experimental design, followed when appropriate by Turkey's post-hoc test. Data that were identified as non-homogeneous were subjected to transformation using log10, natural log, square root prior to ANOVA (Zar, 1999). All calculations performed by SPSS 14.0 for windows (SPSS Inc., 2005).

2.4.8.2 Microarray data analysis

The BlueFuse software version 3.2 (BlueGenome, Cambridge,U.K.) was used to analyse the TIFF images obtained from scanning the microarrays, and to thus identify features and extract fluorescence intensity data. A manual spot removal procedure was applied to eliminate obvious artefactual spots and the software used proprietary

algorithms to fuse duplicate spots. The BlueFuse software was also used to filter data using its own quality metrics and to perform "block LOWESS" data normalisation (block lowess normalisation does correcting the brightness/lowest intensity of spots in each local square block on the array). The resulting fluorescence intensity and quality data were transferred into GeneSpring GX version 10.0.2 (Agilent Technologies, Cheshire, UK). All control features, including positive and negative spots, were excluded from the analysis. Data transformation, normalisation, and quality control filtering were as follows: (a) all intensity values < 1 were set to 1 (threshold = 1), (b) the block LOWESS normalisation carried out in BlueFuse was verified by intensity with whisker box-plots, which showed that the median intensities and range of all the arrays used in this were similar such that no further normalisation was required, (c) data were quality control filtered using a BlueFuse spot quality of ≥ 0.5 in 75 % of the arrays in 2 out of 4 conditions, (where the 4 conditions comprise: FO-FAT, FO-LEAN, VO-FAT and VO-LEAN), and a BlueFuse confidence of ≥ 0.3 in 75 % of the arrays in 2 out of 4 conditions, to filter out features which were below the threshold. The 14772 genes out of the initial 16725 genes remaining following quality filtering were eligible for statistical analysis. The hybridisation data were analysed by both two-way and one-way ANOVA without multiple test correction, which was not employed in this study as it was found to be too conservative for this type of data, in previous studies. The two- way ANOVA examined the effect of the variables "diet" and "strain" and the interaction between the diet and strain at a p-value cut off $P < 0.05$. The one-way ANOVA was performed without multiple test correction, after the hybridisation data were filtered on the volcano plot at the p-value cut off at $P < 0.05$ and at the fold change cut off at 1.2, respectively. All the Atlantic salmon expression sequence tags (ESTs) were identified by basic local alignment tool, BLASTN and BLASTX homologs searching against

Tentative Consensus (TC) contigs from the Atlantic salmon gene index (ASGI) (http://compbio.dfci.harvard.edu/tgi/tgipage.html). In addition, the sequences of the blasted TCs were used to interrogate the nr NCBI (non-redundant sequences) database. Gene ontology (GO) analysis was used as a visualising tool to assist allocation of biological function.

2.4.8.3 qPCR analysis

The relative expression results obtained on liver RNA by microarray analysis were verified by quantitave qPCR on the same liver RNA. The statistical differences in gene expression between the control and the samples were evaluated in group means by randomisation tests (Pfaffl *et al.* 2002) using REST© software (REST, 2005). Fifty thousand allocations were performed and the significant differences were considered at $P \leq 0.05$.

Chapter 3 - Comparison of effects of vegetable oils blended with southern hemisphere fish oil and decontaminated northern hemisphere fish oil on growth performance, composition and gene expression in Atlantic salmon (*Salmo salar* L.)

3.1 Introduction

FOs are the most important source of lipid providing essential PUFA especially n-3 HUFA, EPA and DHA for both humans and fish (Sargent *et al.* 2002). The n-3 HUFA have been shown to have beneficial effects in a number of imflammatory and pathological conditions, including cardiovascular and neurological diseases in human being. Demand for fish and seafood is increasing such that an increasing proportion of these are derived from aquaculture production (Tidwell & Allan 2001). However, aquaculture production itself has been dependent upon FO for providing n-3 HUFA, as well as other FAs required for energy production, for feeds (Sargent & Tacon 1999). Marine FO production depends on wild fisheries that have, at best, reached their sustainable limit whereas the demand of FO for aquaculture and human consumption continues to increase (FAO 2006). Lipids are a major source of energy and FOs have been the main source of dietary lipid in fish farming. Thus FOs, derived from the industrial fisheries e.g. capelin, herring, sand eel, mackerel, anchovy and sardine among others, have been major components of the feed for intensively farmed fish for many years (Sargent & Tacon 1999). However, as alluded to above, FO supplies are finite and at their sustainable limited (FAO 2006) and so continued aquaculture development requires replacement of FO with alternative nutritionally suitable and sustainable oils. As well as providing essential PUFA, especially the EPA and DHA, to promote normal

growth and development (Sargent *et al.* 2002), FO can also contain undesirable contaminants (e.g. persistent organic pollutants, POPs) that may affect human health, and the composition of FO can vary among sources both in terms of n-3HUFA and POP concentrations (Hites *et al.* 2004b).

Successful replacement of FO requires retention of the health-promoting properties of the end product for the consumer, which means retaining as much as possible the current high levels of EPA and DHA in farmed fish (Sargent & Tacon 1999). Presently, VOs are the only viable, cost competitive alternative lipid source for aquaculture diets, and a number of VOs have been used as partial and complete replacements for FO (Bell *et al.* 2005b). Several research papers reported that the partial replacement of FO by VO was possible without compromising the growth of fish including studies with oils such as sunflower oil (Bell *et al*. 1993), LO (Bell *et al*. 2003b; Menoyo *et al*. 2005), PO (Bell *et al*. 2002), RO (Bell *et al*. 2003a; Jordal *et al*. 2005), and SO (Regost *et al*. 2003). Nevertheless, VO are generally rich in C18 PUFA, such as 18:2n-6 and 18:3n-3 but devoid of n-3 HUFA and thus they have a major impact on the FA composition of the final fish products if added at high inclusion levels. For that reason, some studies have investigated the use of "finishing diets" containing FO to restore levels of n-3HUFA in the flesh (Caballero *et al*. 2004; Torstensen *et al*. 2005; Mourente & Bell 2006). However, the use of FO finishing diets has a conceptual drawback in that, in addition to adding back the valuable and highly desirable n-3HUFA, it could be challenged as a potential mechanism for increasing POPs in a previously low contaminant load product as VO do not normally contain the POPs concentrations found in marine FO (Bell *et al*. 2005b).

Consumer awareness of the potential contaminants in fish products has increased in the last few years. Most POPs are lipophilic and so accumulate in the fatty

tissues particularly in oil rich aquatic animals (Walker 2001). Atlantic salmon is such an oil-rich species and so, as with all other oily fish, can accumulate lipophilic undesirable compounds, including polychlorinated dibenzo-*para*-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) (collectively known as dioxins or PCDD/F), dioxin-like non-*ortho* and mono-*ortho* polychlorinated biphenyls (PCB), known collectively as dioxin-like (DL-PCB) and the polybrominated diphenyl ether (PBDEs) flame retardants. All fish, whether oily or not, accumulate the undesirable compounds from their food e.g. pelagic marine fish in the wild or feeds containing FM and FO in aquaculture production (Jacobs *et al.* 2002a). Although there are around 210 dioxin congeners and 209 PCB congeners (isomers) that have been identified only 17 dioxins and furans and 12 DL-PCBs have known toxicity and have been assigned toxic equivalency factors (TEFs) by the World Health Organisation (WHO), according to their relative toxicity compared to the reference, 2,3,7,8 tetra chlorinated dibenzo dioxin (TCDD). The concentration of the toxic POPs is expressed as toxic equivalents (TEQ), where a TEF is applied to the concentration of the individual congeners and summed to generate the total TEQ in a mixture of the 17 congeners (Van den berg *et al.* 1998). For these reasons there is currently considerable interest to investigate the transfer of POPs from feed to farmed fish as well as assessing the potential for transfer to humans and any consequences for human health.

With the introduction of the revised EU limits for dioxins and DL-PCBs in 2006, some of the FOs previously used in aquafeeds would no longer comply with these new limits and so would have to be removed from the food chain. Although the content of POPs in farmed fish, including salmon, has been shown to be below all national and international limits (FDA, UK, and European FSA, WHO, EU), their presence in farmed fish, especially salmon, has received some negative reporting (Hites *et al.*

2004a). Therefore, it may be desirable to formulate finishing diets with decontaminated FO in order to restore n-3 HUFA levels without "adding back" the undesirable POPs. Although the current availability of decontaminated FO is still limited, market demand for using this cleaned oil in aquaculture may change this situation in the near future to reduce POPs levels in the farmed product, and so it is important to evaluate potential effects of the decontamination process on the nutritional quality of the oil and how it may affect growth performance and product quality.

In recent years a number of studies have been conducted where FO was replaced by terrestrial VO in salmon feeds. The fish produced using diets with a high VO content contained significantly lower levels of POPs compared to fish grown on diets with a high marine FO content, but also had significantly lower n-3 HUFA (Berntssen *et al.* 2005; Bell *et al.* 2005a). At lower inclusion levels, dietary VOs have correspondingly less impact on flesh FA compositions (Robin *et al.* 2003). Therefore, an alternative to high VO replacement followed by FO finishing diets would be the use of lower levels of FO substitution, particularly if the FO and VO blends used are carefully chosen to limit n-3 HUFA reduction (Torstensen *et al.* 2004a). For instance, southern hemisphere FO contain higher levels of n-3HUFA, especially EPA, and so can deliver similar levels of n-3HUFA at lower inclusion levels than the northern hemisphere FOs traditionally used in salmonid diets in Europe (Sargent *et al.* 2002). On the other hand, the southern hemisphere FOs are also lower in C20 and C22 monoenes, traditionally regarded as excellent energy sources for salmonids (Tocher 2003) as well as containing higher levels of SFA (Karalazos 2007). Different VOs also have contrasting nutritional and economic qualities. RO is nutritionally balanced but is relatively expensive, whereas SO is readily available and its price is favorable, although it contains high levels of 18:2n-6, which limits the use of this oil due to greater reduction of the flesh n-3/n-6 ratio when

using this product. In addition, EU legislations limit the commercial use of SO to non-GM products containing a maximum of 1.0 mg kg^{-1} endosulfane (pesticide residue).

In the present study, two hypotheses were tested. Firstly, that the blending of RO and SO with southern hemisphere FO is a strategy that may result in lower impact upon tissue n-3HUFA levels in Atlantic salmon. Secondly, that the decontamination (stripping) of FO will have no major effects on the nutritional quality of the oil as a feed ingredient for Atlantic salmon. Specifically, we investigated the effects of replacement of northern hemisphere FO by decontaminated northern FO or blends of southern hemisphere FO with RO and SO on growth performance and composition of Atlantic salmon. The use of decontaminated FO and VO substitution as two alternative methods for reducing contaminant loads in the flesh of farmed Atlantic salmon was also investigated.

3.2 Materials and Methods

3.2.1 Experimental fish

Atlantic salmon of initial mean weight 0.78 ± 0.01 kg were fed one of the five diets for 10 weeks in triplicate 5 m^3 net pens (n = 3 per treatment), with 120 fish per pen, at the Fjord Research Station, Dønna, Norway. The experiment was performed between July 2006 and October 2006 under natural photoperiod and when the mean seawater temperature was 12.7 ± 1.5^oC, the mean salinity was 31.9 ± 0.8 ppt. and the average water visibility by Secchi disk was 11.5 ± 2.7 m. Feed was supplied manually to apparent satiation twice a day with waste feed collection via an up-lift system. Feed fed, waste feed and the resulting net feed intake and mortality were registered daily.

3.2.2 Experimental diets

The diets were formulated to satisfy the nutritional requirements of salmonid fish (NRC. 1993), and to contain 33 % crude protein and 34 % crude lipid. The five experimental diets (9 mm pellets) had the same basal composition but were coated with five different oil sources that were 100 % northern hemisphere FO (diet NFO) as control, 100 % decontaminated northern hemisphere FO (diet DFO), and the three blends of the VOs with southern hemisphere FO (collectively termed the SVO diets), specifically 40 % southern FO/60 % RO (diet SRO), 40 % southern FO/30 % RO/30 % SO (diet SRO/SO) and 40 % southern FO/60 % SO (diet SSO). All diets were formulated and manufactured at the BioMar TechCentre, Brande, Denmark. The northern FO was sprat (*Sprattus sprattus*) oil obtained from FF Skagen, Skagen, Denmark. Decontamination of the sprat oil to produce decontaminated northern FO was a two-step procedure that included treatment with activated carbon followed by thin film deodorisation to remove POPs. An initial adsorption using activated carbon that was designed to remove ~90% of the PCDD/Fs. The second step was a thin-film deodourisation step that should remove up to 95% of PCBs as well as pesticides and other contaminants, FFA and peroxides. The DFO oil was representative of decontaminated oil currently commercially available in Europe. The southern hemisphere FO was from anchovetta (*Engraulis ringens*), and the RO (low in erucic acid and glycosinolates, the latter responses for the bitter or sharp taste of plants) and SOs were both non-GM, degummed quality. All diets contained yttrium oxide (Y_2O_3) as an indicator to determine the ADC of nutrients. The feed formulation is given in Table 3.1.

Table 3.1. Formulations of experimental diets fed to Atlantic salmon for 10 weeks.

Components	Diets				
	NFO	DFO	SRO	SRO/SO	SSO
Feed ingredients (g/kg)					
LT fish meals [1]	378.0	378.0	378.0	378.0	378.0
Legume/oil seed meals	149.0	149.0	149.0	149.0	149.0
Northern fish oil (sprat oil)	325.0	-	-	-	-
Decontaminated sprat oil	-	325.0	-	-	-
Southern fish oil (anchovetta)	-	-	130.0	130.0	130.0
Rapeseed oil [2]	-	-	195.0	98.0	-
Soybean oil [3]	-	-	-	98.0	195.0
Binders	140.0	140.0	140.0	140.0	140.0
Vitamins and minerals	9.2	9.2	9.2	9.2	9.2
Yttrium oxide	0.1	0.1	0.1	0.1	0.1
Proximate composition (%)					
Protein	32.6	32.2	31.4	32.8	32.6
Lipid	33.6	34.3	34.5	34.2	34.7
Moisture	6.0	5.5	6.3	5.5	5.2
Fibre	2.1	2.2	1.9	2.0	2.0
Ash	7.8	7.7	7.6	7.8	7.8
DE (kJ g-1)	25.3	25.3	25.2	25.2	25.3
Pigment Content (mg/kg)					
Total pigment	24.0	19.2	18.4	22	21.1
Astaxanthin	22.6	18.1	15.2	20.1	18.5
Astacene	1.1	0.7	0.7	0.6	0.6
Lutein	0.3	0.4	2.4	2.4	2.0

Results are means of triplicate analysis. DFO; decontaminated northern fish oil, NFO; northern fish oil, SFO/RO; southern fish oil and rapeseed oil 60%, SFO/RO/SO; southern fish oil, rapeseed oil 30% and soybean oil 30%, SFO/SO; southern fish oil and soybean oil 60%.

[1] Blend of Peruvian super prime and standard fishmeals produced from Anchoveta.

[2] low erucic acid and glycosinolates.

[3] Non-GM, degummed quality.

3.2.3 Experimental sampling protocol

At the start and end of the trial, all the fish in each pen were bulk weighed. At the end of week 10, six fish per pen (18 per dietary treatment) were anaesthetised by metacaine sulphonate (MS222; 50 mg/L). The fish were then killed by a blow to the

head and samples taken for compositional analyses. The gut cavity was opened on three fish per pen and any remaining contents of the intestine removed before each whole fish was cut into pieces and minced thoroughly and samples collected and stored at -20^{o}C prior to proximate analysis.

The remaining three fish per pen were used for biochemical and organic contaminant (POP) analyses. Flesh was sampled by taking the NQC and each NQC steak was cut in half; one side was immediately frozen at -20^{o}C for lipid and FA analyses, and the other side was wrapped in foil and then immediately frozen at -20^{o}C for contaminant analysis. The livers were removed from the same fish and three samples were collected from each. Approximately 0.5 g of liver was placed into a glass vial (8 ml) containing ice-cold chloroform/methanol (2:1, by vol.) and stored at -20^{o}C prior to lipid class analysis. A further piece of < 0.5 g was collected into a microcentrifuge tube (1.5 ml) for gene expression analysis, and the remaining liver for FA analysis was placed in a plastic test tube (10 ml), with both these samples immediately frozen in liquid nitrogen before being stored at -70^{o}C and -20^{o}C, respectively, prior to analyses.

At the end of the trial faeces samples were obtained by stripping according to Austreng method (Austreng 1978). The fish were anaesthetised as above and faeces collected by gently squeezing the hindgut of the fish. Faeces were collected from at least three fish per pen and the faeces from each fish were pooled providing one sample per pen. Ethoxyquin (ETQ; 400 mg/L, 1 ml/60g wet faeces) was added to each faeces sample before being stored at -20 °C until analysis. The faeces samples were freeze dried prior to gross nutrients and FA analysis as described in Chapter 2.

3.2.4 Proximate composition analysis

The moisture, crude protein, and ash contents of the diets and minced whole fish were determined by standard procedures (AOAC 2000). Crude fat of the experimental diets were acid hydrolysed before fat extraction. The gross energy content of the diets was determined by Bomb Calorimetry (Foss, UK.). Yttrium oxide (Y_2O_3) was analysed to determine the nutrient and FA digestibility values. The methods followed for the above extractions and the analyses are described in detail in Chapter 2.

3.2.5 Pigment composition analysis

Pigment content and composition were determined as described in detail in Chapter 2. Diets and flesh samples were homogenised and prepared according to the method of Barua *et al* (1993), with diet samples extracted using the same solvent mixtures as used for tissue samples but following enzymatic digestion with Maxatase enzyme. Measurement of individual carotenoids was carried out by HPLC.

3.2.6 Lipid extraction and analysis

The liver and flesh tissues from all three fish per pen were combined to prepare pooled homogenates for lipid analyses. Thus, lipid analyses were based on n = 3 with each replicate being pooled from 3 fish from one cage. Total lipids of tissue and diet samples were extracted by homogenising in 20 volumes of ice-cold chloroform/methanol (2:1, by vol.) according to Folch *et al.* (1957). Lipid class composition of total lipid was determined by densitometry after separation of lipid classes by HPTLC (Henderson & Tocher 1992). FAME were prepared from total lipid by acid-catalysed transesterification (Christie 1993), extracted and purified by TLC (Tocher & Harvie 1988), separated and quantified by gas-liquid chromatography and

individual FAME identified by comparison to known standards (marinol oil, Sigma) and by reference to published data (Ackman 1980). The methods are all described in detail in Chapter 2.

3.2.7 Dioxin, DL-PCB and PBDE analysis

Samples of the NFO, DFO and SRO/SO diets and salmon flesh samples from fish fed these diets were selected for the analysis of POPs. Determinations of the contents of dioxins, DL-PCBs and PBDEs of flesh tissue and diet samples were carried out according to the method of the United States Environmental Protection Agency (USEPA), methods 1613 and 1668 (USEPA 1994, 1999) and conducted by the Nutrition Analytical Service, Institute of Aquaculture, University of Stirling.

Samples of approximately 10 g diet were weighed and ground, and weighed samples of approximately 50 g of flesh were freeze-dried for at least 12 h before grinding and extraction. For analysis of dioxins and DL-PCBs, the samples had a solution of 2,3,7,8-chloro-substituted $^{13}C12$ – labeled PCDD/Fs internal standard (1 ml of a 2 ng/ml standard; EPA-1613-LCS; Wellington Laboratories, Guelph, Canada) prior to extraction with isohexane at 125^{o}C and 1500 pounds per square inch (psi) in an accelerated solvent extractor (ASE™, Dionex, Camberley, UK). Fat and organic matter were removed from the 100 ml ASE^{TM} extract by sulphuric acid treatment using 50-100 ml of 95-97% sulphuric acid for 72 h. The extract obtained was purified using a Power Prep automated purification System™ (Fluid Management Systems, Waltham, MA, USA) via a series of columns (H_2SO_4 on silica, multi-layered silica, basic alumina and carbon column, in sequence; as per Fluid Management protocols). This procedure separated and purified the dioxin/furan and DL-PCB congener fractions. The different fractions were reduced on a rotary evaporator before a further extraction with

concentrated sulphuric acid in isohexane was used to remove any traces of residual lipid. At this stage 10 µl of recovery standard (EPA-1613-ISS for dioxins & 68A-IS for PCBs; Wellington Laboratories, Guelph, Canada) was added. The dioxin and non-*ortho* PCB fraction was finally resuspended in nonane and evaporated to 10-50 µl subsequent to gas chromatography/ion trap tandem mass spectrometry (GC/MS/MS, Thermo Fisher Scientific Polaris Q, Hemel Hempstead, UK). The mono-*ortho* PCB fraction was treated similarly but only the PCB recovery standard was added and the nonane evaporated to 100 µl prior to analysis. Analysis of the 29 dioxin and DL-PCB congeners with WHO TEFs was conducted using GC/MS/MS on a Trace GC 2000 coupled to a Polaris Q ion trap MS/MS (Thermo Finnegan, Hemel Hempsted, UK). The chromatographic separations were conducted on a Rxi-5ms (5% phenyl/95% dimethyl polysiloxane) fused silica column (Thames Restek Ltd., Saunderton, England) 30 m x 0.25 mm i.d. x 0.25 mm film thickness with helium as carrier gas at 0.8 ml/min. Injector temperature was kept at 250^{o}C and samples and standards were injected in the splitless mode. MS conditions were in positive electron ionisation mode (EI+) using automatic gain control with electron energy of 70eV and emission current of 250 µA. The transfer line and ion source were kept at 305 and 250^{o}C respectively. Xcalibar version 1.3 software was used for data acquisition and processing of results. Non-detected congeners were set at limit of quantification, which was the concentration that produced an instrument response of monitored ions with 3 times a signal to noise ratio of 3:1 (limit of detection, LOD). Quantification of each congener is based on the isotope dilution method of the USEPA methods 1613 and 1668 of the United States Environmental Protection Agency (USEPA 1994, 1999). The range of limits of quantification for whole fish sample were 0.03-0.18 pg g^{-1} wet weight for PCDD/Fs, 0.03-0.06 pg g^{-1} wet weight for non-ortho PCBs and mono-ortho PCBs. The congeners analysed included the 17 PCDD/Fs and 12 dioxin-

like PCBs for which the World Health Organisation has established TEFs from 1997 (Van den Berg *et al.* 2006).

For PBDE analysis, 2.5 g of dried ground flesh or diet was extracted with isohexane using the ASE™ apparatus at 40^{o}C and 1500 psi after addition of 1 ml of 10 ng of internal standard (PBDE 119; Wellington Laboratories, Guelph, Canada). The extract from the ASE™ is reduced to ~5ml by rotary evaporation and then reduced to 1ml under nitrogen. Five ml of isohexane and 2ml of concentrated sulphuric acid were added and vortex mixed and centrifuged (1000 x g, 15 min). The organic phase was transferred to a clean tube and the procedure repeated. The combined organic phases were reduced to 1 ml under nitrogen and 100 µl of concentrated sulphuric acid added, mixed and centrifuged (500 x g, 4 min). The iso-hexane layer was then removed and added to 0.5 ml nonane and the solvent evaporated to the nonane mark. The nonane is transferred to an autosampler vial prior to analysis by GC/MS (Thermo Fisher Scientific Trace DSQ, Hemel Hempstead, UK). Analysis of the seven PBDE congeners (28, 47, 99, 100, 153, 154 & 183) was conducted using a Thermo Finnegan Trace GC Ultra equipped with a ZB5-MS column (30m x 0.25 mm i.d. x 0.25 micron phase; Phenomenex, Macclesfield, England). Helium was used as carrier gas and coupled to a Trace DSQ MS, (Bremen, Germany) in negative chemical ion (CI-) mode and methane as reagent gas at a flow rate of 2.0 ml/min. Xcalibar version 1.4 software was used for data acquisition and processing of results. The range of limits of quantification for whole fish samples were 0.06-0.15 pg g^{-1} wet weight for PBDEs.

3.2.8 Gene expression

Effect of dietary treatments on the expression of selected candidate genes was analysed in six biological replicates per treatment determined by real time qPCR. The

methodology is described in detail in Chapter 2 but, in summary, total RNA was extracted from individual liver samples by homogenising in TriReagent, and first strand cDNA synthesis of total RNA performed followed by quantification of gene expression by qPCR (Quantica thermocycler, Techne). The PCR primer sequences for the three target genes (Δ5 and Δ6 fatty acyl desaturases and fatty acyl Elovl5 elogase) and house keeping genes were used successfully in previously studies, and are given in Table 3.2.

Table 3.2. Primer sequences for target and housekeeping genes used in real-time qPCR.

Gene	Primer	Sequence 5'-3'	Size (bp)
Desaturase 5	F	GTGAATGGGGATCCATAGCA	192
	R	AAACGAACGGACAACCAGA	
Desaturase 6	F	CCCCAGACGTTTGTGTCAG	181
	R	CCTGGATTGTTGCTTTGGAT	
Elongase	F	TGATTTGTGTTCCAAATGGC	219
	R	CTCATGACGGGAACCTCAAT	
Elongation factor-1alpha (Housekeeping)	F	CTGCCCCTCCAGGACGTTTACAA	175
	R	CACCGGGCATAGCCGATTCC	
Beta-actin (Housekeeping)	F	ACATCAAGGAGAAGCTGTC	141
	R	GACAACGGAACCTCTCGTTA	

3.2.9 Calculations and statistical analysis

Feed Conversion Ratio (FCR), Specific Growth Rate (SGR) and Thermal Growth Coefficient (TGC) were calculated according to the formulae defined in Chapter 2. The effects of dietary treatment were determined by one-way ANOVA as described in Chapter 2. Statistical analyses were performed using SPSS 14 (SPSS Inc, 2005), and differences were regarded as significant when $P < 0.05$ (Zar, 1999).

3.3 Results

3.3.1 Lipid class and fatty acid compositions of the oil sources

There were some relatively minor differences in lipid class compositions of the dietary oil sources (Table 3.3). All the oils contained only neutral lipid with TAG as the dominant lipid class, with the VOs containing about 81 % and the FOs containing around 77 % TAG. The VOs had lower levels of FFA compared to the FOs, and within the FOs, FFA was higher in southern hemisphere FO than in northern FO. Within the VOs, sterol and FFA were higher in RO than in SO (Table 3.3).

Table 3.3. Lipid class composition (percentage of total lipid) of dietary oil sources.

Lipid class	NFO	SFO	RO	SO
Total PL	n.d.	n.d.	n.d.	n.d.
Total NL	100	100	100	100
Triacylglycerol	76.8	76.6	81.8	81.4
Sterol	11.1	9.1	10.3	7.7
Free fatty acid	12.1	14.3	7.9	5.9
Unidentified NL	n.d.	n.d.	n.d.	5.0

Values are means of triplicate analysis. n.d., not detected; NL; neutral lipid; PL; polar lipid

The FA compositions of the dietary oil sources are shown in Table 3.4. The FOs showed some differences compared to the VOs. The FOs contained high total saturated and n-3 HUFA, particularly EPA and DHA, while the VOs were rich in n-6 PUFA and lacked the n-3 HUFA. However, the VOs contained 18:3n-3, the precursor of n-3 HUFA. Total monoenes were high in both FO and VO sources, with RO showing the highest level (60 % of total FA). The major FA of SFA, MUFA and n-6 PUFA in all oils were 16:0, 18:1n-9 and 18:2n-6 respectively, while the major n-3 FA were EPA and DHA in FOs and 18:3n-3 in VOs. The rank order of the FA composition in NFO was

total monoenes > total saturated > total n-3 PUFA > total n-6 PUFA, whereas the rank order of FA in SFO was total saturated > total n-3 PUFA > total monoenes > total n-6 PUFA. The rank order of the FA composition in RO was total monoenes > total n-6 PUFA > total n-3 PUFA> total saturated, and the rank order of the FA composition in SO was total n-6 PUFA > total monoenes > total saturated > total n-3 PUFA (Table 3.4).

Table 3.4. Fatty acid compositions (percentage of total fatty acids) of dietary oil sources.

Parameters	NFO	SFO	RO	SO
14:00	5.2	9.5	0.1	0.1
16:00	19.5	22.8	4.6	11.1
18:00	2.2	4.3	1.7	3.8
20:00	0.3	0.5	0.6	0.4
22:00	0.5	0.5	0.6	0.7
Total saturated[1]	28.4	38.3	7.6	16.5
16:1n-7	6.1	8.3	0.2	0.1
18:1n-9	25.4	7.3	60.0	26.4
18:1n-7	2.3	3.1	3.1	1.6
20:1n-9	2.0	1.0	1.2	0.3
22:1n-11	1.9	0.6	0.1	tr.
24:1n-9	1.6	0.4	0.1	n.d.
Total monoenes[2]	40.3	21.3	64.8	28.6
18:2n-6	4.0	1.3	17.4	49.3
18:3n-6	0.1	0.3	0.6	0.2
20:2n-6	0.6	0.2	0.1	n.d.
20:4n-6	0.6	1.3	n.d.	n.d.
22:5n-6	0.3	0.4	n.d.	n.d.
Total n-6 PUFA	5.7	3.9	18.1	49.6
18:3n-3	2.8	0.8	9.5	5.4
18:4n-3	2.3	2.9	n.d.	n.d.
20:4n-3	0.5	0.8	n.d.	n.d.
20:5n-3	7.1	18.3	n.d.	n.d.
22:5n-3	0.7	2.0	n.d.	n.d.
22:6n-3	12.0	11.1	n.d.	n.d.
Total n-3 PUFA[3]	25.6	36.5	9.5	5.4
Total PUFA	31.3	40.4	27.6	55.0

Results are means of triplicate analysis. [1] includes 15:0 up to 0.6%, [2] includes 20:1n-7 up to 0.2%. [3] includes 20:3n-3 up to 0.2%. FO, fish oil; n.d., not detected; PUFA, polyunsaturated fatty acids; tr,< 0.05.

3.3.2 Proximate and pigment composition of the diets

There were some slight differences in proximate compositions and pigment contents of the five experimental diets (Table 3.1). The experimental diets contained protein in the range 32 – 33 % and lipid in the range 34 – 35 %. The NFO control diet, contained the highest total pigment, particularly astaxanthin and astacene, compared to the four other dietary treatments. In contrast, the SVO diets contained higher lutein than the NFO and DFO diets (Table 3.1).

3.3.3 Lipid class and fatty acid compositions of the diets

The lipid class composition of the DFO diet showed few differences compared to the NFO diet. DFO diet contained a slightly higher level of TPL, PC and TAG but lower level of sterol and FFA compared to diet NFO (Table 3.5). The SRO and SRO/SO diets had a slightly lower level of TPL and PC while the SSO showed the opposite trend compared to the NFO diet. However, the SVO diets showed higher levels of TAG and lower levels of sterol and FFA compared to the NFO diet. Within the SVO diets, the SSO diet had a slightly higher level of PC but lower level of TNL and TAG compared to the SRO diet (Table 3.5).

The FA composition of the DFO diet showed some small differences compared to the NFO diet including lower levels of total MUFA, 18:1n-9 and 18:3n-3, but higher levels of 18:0, 20:1, 22:1, 18:2n-6 and EPA (Table 3.6). However, most of these differences were relatively minor. The SVO diets, the three blends of the VOs with southern hemisphere FO, all showed increased 18:0, 18:2n-6 and 18:3n-3, and lower levels of 14:0, 16:0, ARA, EPA and DHA compared to the NFO and DFO diets. Within the SVO diets, 16:0, 18:0 and 18:2n-6 increased with increasing inclusion of SO so that the rank order for their levels was SSO > SRO/SO > SRO, whereas 18:1n-9 and 18:3n-3

increased with increasing inclusion of RO such that the rank order for their levels was SRO > SRO/SO > SSO. Similarly the total SFA, n-6 PUFA and PUFA increased in the SSO diet while the total MUFA and n-3 PUFA increased in the SRO diet (Table 3.6).

Table 3.5. Lipid class composition (percentage of total lipid) of the experimental diets fed to Atlantic salmon for 10 weeks.

Lipid class	NFO	DFO	SRO	SRO/SO	SSO
PC	2.8	3.1	2.1	2.6	3.3
Total PL	2.8	3.1	2.1	2.6	3.3
Total NL	97.2	96.9	97.9	97.4	96.7
TAG	72.3	82.5	80.0	79.9	75.2
Sterol	13.0	10.2	8.9	8.5	9.2
Free fatty acids	11.9	4.2	9.0	9.0	9.5
Unidentified NL	n.d.	n.d.	n.d.	n.d.	2.8

Results are means of triplicate analysis. DFO, decontaminated northern fish oil; n.d., not detected; NFO, northern fish oil; NL, neutral lipid; PC, phosphatidylcholine; PL, polar lipid; SRO, southern fish oil and rapeseed oil (40/60); SRO/SO, southern fish oil, rapeseed oil and soybean oil (40/30/30); SSO, southern fish oil and soybean oil (40/60). TAG, triacylglycerol.

Table 3.6. Fatty acid compositions (percentage of total fatty acid) of the experimental diets fed to Atlantic salmon for 10 weeks.

Parameters	NFO	DFO	SRO	SRO/SO	SSO
14:00	5.2	5.6	2.9	2.9	2.9
16:00	19.2	18.8	10.9	13.5	15.0
18:00	2.2	3.3	3.8	4.4	4.8
20:00	0.3	0.4	0.7	0.6	0.5
22:00	0.3	0.6	1.5	1.4	1.4
Total saturated	27.8	29.3	20.0	23.1	24.9
16:1n-9	0.4	0.3	tr.	n.d.	n.d.
16:1n-7	6.0	5.8	2.7	2.7	2.6
18:1n-9	25.9	21.6	41.2	29.5	21.2
18:1n-7	2.5	2.5	2.9	2.6	2.2
20:1n-9	2.1	3.2	1.2	0.9	0.6
22:1n-11	2.4	4.1	0.3	0.3	0.4
22:1n-9	0.4	0.5	0.2	0.2	0.2
24:1n-9	2.0	1.6	0.3	0.3	0.2
Total monoenes	42.0	40.0	48.9	36.5	27.5
18:2n-6	3.8	4.3	12.6	23.1	32.0
18:3n-6	0.1	0.1	0.1	0.1	0.1
20:2n-6	0.6	0.5	0.1	0.1	0.1
20:3n-6	0.1	0.1	0.1	0.1	0.1
20:4n-6	0.6	0.6	0.4	0.4	0.4
22:5n-6	0.3	0.3	0.1	0.1	0.1
Total n-6 PUFA	5.6	5.9	13.4	23.9	32.7
18:3n-3	2.7	2.3	6.3	5.1	3.7
18:4n-3	2.2	2.3	0.9	0.9	0.9
20:3n-3	0.2	0.2	n.d.	n.d.	n.d.
20:4n-3	0.5	0.5	0.2	0.2	0.2
20:5n-3	6.7	7.2	5.4	5.2	5.1
22:5n-3	0.7	0.7	0.4	0.6	0.6
22:6n-3	11.6	11.4	4.3	4.3	4.2
Total n-3 PUFA	24.7	24.7	17.7	16.5	14.9
Total PUFA	30.3	30.6	31.1	40.4	47.7

Values are means of triplicate analysis. Values within a row with different superscript letters are significantly different ($P < 0.05$) as determined by ANOVA. DFO, decontaminated northern fish oil; n.d., not detected; NFO, northern fish oil; PUFA, polyunsaturated fatty acids; SFO/RO, southern fish oil and rapeseed oil (40/60); SFO/RO/SO, southern fish oil, rapeseed oil and soybean oil (40/30/30); SFO/SO, southern fish oil and soybean oil (40/60); tr, < 0.05.

3.3.4 Contaminant (dioxins, DL-PCBs and PBDEs) content of diets

The NFO diet contained 17.4 ng toxicity equivalents (TEQ)/kg of the 29 dioxin and DL-PCB congeners, and these values were decreased to 0.45 and 0.53 ng TEQ/kg in the DFO and SRO/SO diets, respectively. (Table 3.7). The summed concentration of the 7 principal PBDE congeners in the NFO diet was 5.9 ng/g, with these values reduced to 1.9 and 0.31 ng/g in the DFO and SRO/SO diets, respectively.

Table 3.7. Contents of dioxin and DL-PCB (ng TEQ/kg wet weight) and PBDE (ng/g wet weight) congeners in experimental diets fed to Atlantic salmon for 10 weeks.

Congener/Diet	NFO	DFO	SRO/SO
Sum PCDD	2.17	0.15	0.15
Sum PCDF	6.48	0.11	0.09
Sum mono-ortho PCB	2.27	0.06	0.04
Sum non-ortho PCB	6.40	0.12	0.25
Sum dioxins + PCBs	17.32	0.45	0.53
Sum PBDE	5.94	1.90	0.31

DFO, decontaminated northern fish oil; NFO, northern fish oil; PBDE, polybrominated diphenyl ethers; PCB, polychlorinated biphenyls; PCDD, polychlorinated dibenzo-*p*-dioxins; PCDF, polychlorinated dibenzofurans; SRO/SO, southern fish oil, rapeseed oil and soybean oil (40/30/30).

3.3.5 Growth performance, whole body composition and pigment content

There were no significant differences in growth performance (final weights, SGR and TGC) among the dietary treatments (Table 3.8). There was also no dietary effect on feed efficiency (FCR). The different dietary oils also had no effect on the gross composition of the whole salmon or the concentration of astaxanthin (Table 3.8).

Table 3.8. Growth performance, carcass proximate composition (percentage of wet weight) and pigment content (mg kg^{-1}) of Atlantic salmon flesh after feeding the experimental diets for 10 weeks.

Parameters	NFO	DFO	SRO	SRO/SO	SSO
Growth performance					
Initial wt. (kg)	0.77 ± 0.0	0.78 ± 0.0	0.78 ± 0.0	0.77 ± 0.0	0.79 ± 0.1
Final wt. (kg)	2.19 ± 0.0	2.18 ± 0.1	2.18 ± 0.1	2.19 ± 0.1	2.20 ± 0.0
FCR	0.98 ± 0.00	0.98 ± 0.02	0.98 ± 0.00	0.96 ± 0.02	0.96 ± 0.01
SGR % d^{-1}	1.35 ± 0.05	1.34 ± 0.01	1.34 ± 0.01	1.35 ± 0.03	1.33 ± 0.03
TGC	3.92 ± 0.15	3.86 ± 0.04	3.87 ± 0.07	3.89 ± 0.10	3.87 ± 0.10
Proximate composition (%)					
Protein	16.9 ± 10.3	16.7 ± 0.4	16.7 ± 0.5	17.0 ± 0.7	17.0 ± 0.4
Lipid	19.1 ± 1.4	18.9 ± 1.8	18.8 ± 1.4	19.4 ± 1.9	17.8 ± 2.7
Ash	0.28 ± 0.0	0.31 ± 0.0	0.28 ± 0.1	0.31 ± 0.1	0.29 ± 0.0
Moisture	61.6 ± 1.1	61.1 ± 1.2	61.4 ± 0.8	60.1 ± 0.5	60.6 ± 1.3
Pigment content (mg/kg)					
Astaxanthin	4.7 ± 0.3	4.3 ± 0.3	4.8 ± 0.4	4.8 ± 0.6	4.4 ± 0.6

Results are means ± SD (n = 3). Different superscript letters indicate a significant effect of diet as determined by ANOVA. DFO, decontaminated northern fish oil; FCR, feed conversion ratio; NFO, northern fish oil; SGR, specific growth rate; SRO, southern fish oil and rapeseed oil (40/60); SRO/SO, southern fish oil, rapeseed oil and soybean oil (40/30/30); SSO, southern fish oil and soybean oil (40/60) SGR, specific grwth rate; TGC, thermal growth coefficient.

3.3.6 Apparent digestibility of gross nutrients and fatty acids

The ADC of the DFO diet was significantly lower than all the other diets as evidenced by the lower values for crude protein, crude lipid and dry matter digestibility (Table 3.9). Within the SVO diets, the ADC of the SSO diet was significantly lower than the SRO and SRO/SO diets. The protein digestibilities of the NFO and SVO diets were over 80 % while the protein digestibility of the DFO diet was lower than 80 %. The dry matter digestibilities of the NFO and SVO diets were 67 - 70% while the dry matter digestibility of the fish fed DFO was 62 %. The lipid digestibilities of the NFO and SVO diets were 93 - 95% while the lipid digestibility of the DFO diet was slightly lower at about 91%. Consistent with this, the ADC of virtually every FA, except for

18:0, were generally over 92 %, but were lower in fish fed the DFO diet compared to fish fed the NFO diet (Table 3.9). In general, FA digestibility was highest in fish fed the NFO diet. In addition, the digestibilities of many FAs were often lower in fish fed the DFO diet compared to fish fed the SVO diets, although there were few differences in FA digestibilities between the fish fed the different SVO diets.

3.3.7 Flesh and liver lipid contents and fatty acid compositions

There was a trend for flesh lipid content to be lower in fish fed the SVO diets compared to fish fed the NFO or DFO diets, although this was only significant in the case of fish fed the SRO diet (Table 3.10). There were no significant differences in tissue lipid contents between fish fed the NFO and DFO diets. Similarly, there were no significant differences in flesh lipid content in fish fed the SVO diets (Table 3.10).

Flesh FA compositions of fish fed the DFO diet were generally similar to fish fed the NFO diet. Reflecting the diet, EPA and total n-3PUFA were higher in fish fed DFO than NFO, whereas 18:1n-9 was lower (Table 3.10). Flesh FA compositions were largely changed corresponding to the FA compositions of the diets with fish fed the SVO diets having reduced 14:0, 16:0, EPA, DHA and ARA, but increased 18:2n-6 and 18:3n-3 compared to fish fed the NFO and DFO diets (Table 3.10). The proportions of 18:0 and 18:1n-9 were only increased in fish fed the diets containing SO and RO, respectively. Therefore, as with the diets, flesh 16:0, 18:0 and 18:2n-6 increased with increasing inclusion of SO so that the rank order for their proportions in salmon flesh was in fish fed SSO > SRO/SO > SRO, whereas flesh 18:1n-9 and 18:3n-3 increased with increasing inclusion of RO such that the rank order for their proportions in flesh was fish fed SRO > SRO/SO > SSO. Interestingly, the proportions of EPA and DHA

were significantly higher in fish fed the diet with highest level of SO (SSO diet) compared to the other SVO diets (Table 3.10).

Liver lipid content was significantly lower in fish fed the SVO diets compared to fish fed either the NFO or DFO diets but there was no significant difference between the fish fed the NFO and DFO diets (Table 3.11). This is interesting in that generally liver lipid deposition of fish fed VO diet is higher than that of the fish fed FO diet. The proportions of 18:0, 18:1n-9, 18:2n-6 and 18:3n-3 in liver of fish fed the SVO diets were similar to those described above for flesh, and reflected the dietary FA compositions. However, it was noteworthy that increased proportions of desaturated and elongated products of 18:2n-6 and 18:3n-3 were clearly observed in liver of fish fed the SVO diets. Thus, proportions of 20:2n-6, 20:3n-6 and 20:4n-6 were all increased in liver of fish fed the SVO diets compared to fish fed the NFO and DFO diets. Even more striking was the increased proportions of 20:5n-3 in the livers of fish fed the SVO diets. In contrast, proportions of 22:5n-3 and, to a lesser extent, 22:6n-3 were reduced in fish fed the SVO diets compared to fish fed NFO or DFO (Table 3.11).

Table 3.9. Apparent digestibility of protein, lipid, dry matter and fatty acids in Atlantic salmon after feeding the experimental diets for 10 weeks.

Parameters	NFO	DFO	SRO	SRO/SO	SSO
Protein	83.2 ± 1.3^{ab}	79.5 ± 0.2^{c}	83.4 ± 0.4^{a}	84.2 ± 0.6^{a}	81.5 ± 1.5^{b}
Lipid	95.0 ± 0.6^{a}	91.2 ± 0.4^{d}	94.1 ± 0.1^{b}	93.6 ± 0.1^{bc}	92.8 ± 0.8^{c}
Dry matter	69.3 ± 1.9^{ab}	62.3 ± 0.5^{c}	70.4 ± 0.8^{a}	70.8 ± 1.4^{a}	67.0 ± 2.7^{b}
Fatty acids					
14:00	98.0 ± 0.3^{a}	94.5 ± 1.1^{b}	94.5 ± 0.1^{b}	94.6 ± 1.0^{b}	94.9 ± 1.2^{b}
16:00	97.2 ± 0.2^{a}	92.4 ± 1.2^{c}	93.9 ± 0.2^{b}	94.7 ± 1.2^{b}	96.2 ± 0.5^{a}
18:00	86.2 ± 1.2^{b}	73.3 ± 4.5^{c}	93.2 ± 0.6^{a}	94.3 ± 1.4^{a}	94.6 ± 2.2^{a}
Total saturated	95.7 ± 0.3^{a}	89.0 ± 1.7^{c}	94.0 ± 0.4^{b}	94.7 ± 1.2^{ab}	95.6 ± 0.8^{ab}
16:1n-9,n-7	98.8 ± 0.2^{a}	97.5 ± 0.6^{b}	97.4 ± 0.1^{b}	97.6 ± 0.3^{b}	97.7 ± 0.3^{b}
18:1n-9	99.0 ± 0.1^{a}	97.9 ± 0.5^{c}	98.4 ± 0.2^{b}	98.2 ± 0.3b^{c}	98.3 ± 0.3^{bc}
18:1n-7	98.4 ± 0.3^{a}	96.6 ± 0.6^{c}	97.4 ± 0.2^{b}	97.4 ± 0.4^{b}	97.5 ± 0.4^{b}
20:1n-11,n-9	98.5 ± 0.1^{a}	96.6 ± 0.5^{c}	97.4 ± 0.3^{b}	96.7 ± 0.6^{c}	97.0 ± 0.3^{bc}
22:1n11	97.1 ± 3.0	97.2 ± 1.6	96.5 ± 0.3	95.8 ± 1.0	96.6 ± 0.4
22:1n-9	96.6 ± 2.6	95.5 ± 1.7	95.7 ± 0.5	95.5 ± 1.4	96.7 ± 0.5
Total monoenes	98.7 ± 0.1^{a}	97.3 ± 0.6^{c}	98.2 ± 0.2^{ab}	98.0 ± 0.3^{b}	98.0 ± 0.3^{b}
18:2n-6	99.8 ± 0.1	99.8 ± 0.1	99.8 ± 0.1	99.8 ± 0.1	99.8 ± 0.1
20:4n-6	98.8 ± 0.1	97.4 ± 0.5	99.2 ± 1.5	98.3 ± 1.9	99.2 ± 1.5
22:5n-6	98.5 ± 0.1^{a}	96.9 ± 0.5^{c}	97.7 ± 0.2^{b}	97.6 ± 0.3^{b}	97.5 ± 0.3^{b}
Total n-6 PUFA	98.8 ± 0.1^{b}	97.6 ± 0.5^{c}	98.8 ± 0.1^{b}	99.1 ± 0.3^{ab}	99.3 ± 0.2^{a}
18:3n-3	99.3 ± 0.1^{a}	98.4 ± 0.4^{c}	98.8 ± 0.2^{b}	98.7 ± 0.2^{bc}	98.6 ± 0.3^{bc}
18:4n-3	99.3 ± 0.1^{a}	98.6 ± 0.4^{b}	98.7 ± 0.6^{b}	99.0 ± 0.1^{ab}	98.8 ± 0.2^{b}
20:5n-3	99.2 ± 0.1^{a}	98.2 ± 0.4^{c}	98.7 ± 0.1^{b}	98.7 ± 0.1^{b}	98.5 ± 0.3^{bc}
22:5n-3	98.9 ± 0.1^{a}	97.5 ± 0.5^{a}	96.0 ± 1.8^{b}	97.8 ± 0.2^{a}	97.8 ± 0.3^{a}
22:6n-3	98.8 ± 0.1^{a}	97.2 ± 0.5^{b}	96.9 ± 0.4^{b}	96.8 ± 0.5^{b}	96.7 ± 0.4^{b}
Total n-3 PUFA	99.0 ± 0.1^{a}	97.8 ± 0.4^{c}	98.3 ± 0.2^{b}	98.2 ± 0.2^{b}	98.0 ± 0.3^{b}
Total PUFA	99.0 ± 0.1^{a}	97.8 ± 0.4^{c}	98.4 ± 0.2^{b}	98.4 ± 0.2^{b}	98.4 ± 0.3^{b}

Results are means ± SD (n = 3). Different superscript letters indicate a significant effect of diet as determined by ANOVA DFO, decontaminated northern fish oil; NFO, northern fish oil; PUFA, polyunsaturated fatty acids. SRO, southern fish oil and rapeseed oil (40/60); SRO/SO, southern fish oil, rapeseed oil and soybean oil (40/30/30); SSO, southern fish oil and soybean oil (40/60).

Table 3.10. Lipid content (percentage of wet weight) and fatty acid composition (percentage of total fatty acids) in flesh of Atlantic salmon at the initiation (initial) of the trial and after feeding the experimental diets for 10 weeks.

Parameters	Initial	NFO	DFO	SRO	SRO/SO	SSO
Lipid Content	4.0 ± 0.8	11.3 ± 1.3^{a}	11.2 ± 1.3^{a}	9.8 ± 0.8^{b}	10.9 ± 1.1^{ab}	10.6 ± 1.2^{ab}
Fatty acid						
14:00	4.8 ± 0.2	4.7 ± 0.2^{b}	4.9 ± 0.1^{a}	2.9 ± 0.1^{c}	2.9 ± 0.1^{c}	3.0 ± 0.1^{c}
16:00	16.3 ± 0.4	17.1 ± 0.6^{a}	16.8 ± 0.3^{a}	10.9 ± 0.6^{d}	12.2 ± 0.4^{c}	13.5 ± 0.5^{b}
18:00	3.6 ± 0.1	2.8 ± 0.1^{c}	2.7 ± 0.1^{c}	2.7 ± 0.2^{c}	3.2 ± 0.1^{b}	3.6 ± 0.2^{a}
20:00	0.2 ± 0.0	0.1 ± 0.0^{c}	0.1 ± 0.1^{c}	0.3 ± 0.0^{a}	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}
22:00	0.4 ± 0.0	0.1 ± 0.2^{b}	0.1 ± 0.1^{b}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}
Total saturated	25.7 ± 0.6	25.3 ± 0.8^{a}	25.1 ± 0.5^{a}	17.2 ± 0.8^{d}	19.0 ± 0.6^{c}	21.1 ± 0.8^{b}
16:1n-9	0.1 ± 0.1	0.4 ± 0.1^{b}	0.4 ± 0.1^{b}	0.9 ± 1.4^{a}	0.5 ± 1.1^{b}	0.1 ± 0.1^{c}
16:1n-7	5.6 ± 0.2	6.0 ± 0.2^{a}	5.8 ± 0.1^{a}	2.6 ± 1.4^{c}	2.9 ± 1.1^{bc}	3.4 ± 0.1^{b}
18:1n-9	15.0 ± 0.5	25.1 ± 0.6^{c}	21.9 ± 0.3^{d}	39.0 ± 1.2^{a}	28.9 ± 0.4^{b}	19.9 ± 0.4^{c}
18:1n-7	3.4 ± 0.1	3.1 ± 0.2^{b}	3.1 ± 0.1^{b}	3.3 ± 0.2^{a}	3.0 ± 0.2^{b}	2.6 ± 0.1^{c}
20:1n-11	n.d.	tr	0.2 ± 0.2^{a}	tr	tr	0.1 ± 0.1^{b}
20:1n-9	2.1 ± 0.2	2.5 ± 0.1^{b}	3.0 ± 0.1^{a}	1.9 ± 0.1^{c}	1.5 ± 0.1^{d}	1.1 ± 0.1^{e}
20:1n-7	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
22:1n11	1.6 ± 0.2	1.8 ± 0.1^{b}	3.1 ± 0.2^{a}	0.5 ± 0.1^{d}	0.5 ± 0.1^{d}	0.6 ± 0.1^{c}
22:1n-9	0.4 ± 0.0	0.4 ± 0.0^{b}	0.5 ± 0.0^{a}	0.3 ± 0.0^{c}	0.2 ± 0.0^{d}	0.2 ± 0.0^{d}
24:1n-9	0.7 ± 0.0	1.3 ± 0.2^{a}	1.2 ± 0.1^{b}	0.4 ± 0.0^{c}	0.4 ± 0.0^{c}	0.4 ± 0.1^{c}
Total monoenes	29.1 ± 0.9	40.8 ± 0.8^{b}	39.3 ± 0.6^{c}	49.0 ± 1.1^{a}	38.1 ± 0.3^{d}	28.6 ± 0.5^{e}
18:2n-6	7.7 ± 0.2	5.3 ± 0.2^{d}	5.8 ± 1.0^{d}	11.9 ± 0.3^{c}	20.9 ± 0.6^{b}	28.1 ± 1.1^{a}
18:3n-6	0.2±0.1	0.2 ± 0.1^{b}	0.1 ± 0.1^{b}	0.5 ± 0.3^{a}	0.2 ± 0.1^{b}	0.2 ± 0.1^{b}
20:2n-6	.04 ± 0.0	0.7 ± 0.0^{c}	0.6 ± 0.0^{d}	0.6 ± 0.1^{d}	1.0 ± 0.1^{b}	1.1 ± 0.2^{a}
20:3n-6	0.4 ± 0.1	0.2 ± 0.1^{c}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.4 ± 0.0^{a}
20:4n-6	0.9 ± 0.0	0.6 ± 0.0^{a}	0.6 ± 0.0^{a}	0.5 ± 0.0^{b}	0.5 ± 0.0^{b}	0.5 ± 0.0^{b}
22:5n-6	0.3 ± 0.0	0.3 ± 0.0^{a}	0.3 ± 0.0^{a}	0.1 ± 0.1^{c}	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}
Total n-6 PUFA	10.0 ± 0.3	7.3 ± 0.3^{d}	7.7 ± 1.0^{d}	13.8 ± 0.5^{c}	23.0 ± 0.6^{b}	30.5 ± 1.2^{a}
18:3n-3	1.8 ± 0.1	2.3 ± 0.1^{d}	2.3 ± 0.1^{d}	5.6 ± 0.2^{a}	4.5 ± 0.2^{b}	3.2 ± 0.1^{c}
18:4n-3	1.5 ± 0.1	1.5 ± 0.1^{b}	1.8 ± 0.1^{a}	0.8 ± 0.1^{d}	0.8 ± 0.0^{d}	0.9 ± 0.1^{c}
20:3n-3	0.1 ± 0.0	0.3 ± 0.0^{a}	0.3 ± 0.0^{a}	0.3 ± 0.0^{a}	0.3 ± 0.0^{a}	0.2 ± 0.1^{b}
20:4n-3	1.1 ± 0.0	1.2 ± 0.1^{a}	1.1 ± 0.1^{b}	0.7 ± 0.1^{c}	0.7 ± 0.0^{c}	0.7 ± 0.0^{c}
20:5n-3	11.8 ± 0.5	5.9 ± 0.4^{b}	6.9 ± 0.3^{a}	4.6 ± 0.4^{d}	4.9 ± 0.1^{cd}	5.2 ± 0.3^{c}
22:5n-3	3.4 ± 0.2	2.1 ± 0.2^{a}	2.0 ± 0.1^{ab}	1.7 ± 0.2^{d}	1.8 ± 0.1^{cd}	1.9 ± 0.2^{bc}
22:6n-3	15.0 ± 0.9	13.1 ± 0.6^{a}	13.6 ± 0.6^{a}	6.4 ± 0.5^{c}	6.7 ± 0.2^{c}	7.5 ± 0.5^{b}
Total n-3 PUFA	35.2 ± 1.1	26.6 ± 1.2^{b}	28.0 ± 1.0^{a}	20.0 ± 1.4^{c}	20.0 ± 0.3^{c}	19.8 ± 0.8^{c}
Total PUFA	45.2 ± 1.0	34.0 ± 1.4^{d}	35.7 ± 0.7^{c}	33.8 ± 1.8^{d}	43.0 ± 0.7^{b}	50.4 ± 1.1^{a}

Results are means ± SD (n = 3). Different superscript letters indicate a significant effect of diet as determined by ANOVA. DFO, decontaminated northern fish oil; n.d., not detected; PUFA, polyunsaturated fatty acids; NFO, northern fish oil; SRO, southern fish oil and rapeseed oil (40/60); SRO/SO, southern fish oil, rapeseed oil and soybean oil (40/30/30); SSO, southern fish oil and soybean oil (40/60); tr,< 0.05.

Table 3.11. Lipid content (percentage of wet weight) and fatty acid composition (percentage of total fatty acids) in liver of Atlantic salmon at the initiation (initial) of the trial and after feeding the experimental diets for 10 weeks.

Parameters	Initial	NFO	DFO	SRO	SRO/SO	SSO
Lipid Content	6.0 ±1.0	8.0 ± 2.8 ^{a}	7.0 ± 2.0 ^{a}	5.0 ± 1.0 ^{b}	4.7 ± 0.8 ^{b}	4.4 ± 0.7^{b}
Fatty acid						
14:00	1.9 ± 0.6	2.2 ± 0.2 ^{a}	2.2 ± 0.5 ^{a}	1.3 ± 0.4 ^{b}	1.1 ± 0.2 ^{b}	1.1 ± 0.1^{b}
16:00	13.4 ± 1.0	11.2 ± 1.5 ^{ab}	10.7 ± 1.5 ^{ab}	9.7 ± 2.1 ^{b}	10.7 ± 1.3 ^{ab}	12.1 ± 1.1^{a}
18:00	7.1 ± 0.6	4.1 ± 0.6 ^{b}	4.0 ± 0.4 ^{b}	4.4 ± 0.5 ^{b}	5.5 ± 0.7^{a}	6.0 ± 0.6 ^{a}
20:00	0.3 ± 0.0	0.1 ± 0.1^{b}	0.1 ± 0.1^{b}	0.2 ± 0.1^{a}	0.2 ± 0.2^{a}	0.2 ± 0.0^{a}
22:00	0.8 ± 0.2	0.1 ± 0.1^{a}	0.1 ± 0.2 ^{a}	0.1 ± 0.1 ^{a}	n.d.	n.d.
Total saturated	23.7 ± 1.4	18.0 ± 2.0^{ab}	17.4 ± 2.2^{ab}	15.9 ± 2.8^{b}	17.6 ± 1.4^{ab}	19.4 ± 1.6^{a}
16:1n-9	0.2 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.0	0.4 ± 0.4	0.6 ± 0.7
16:1n-7	2.2 ± 0.6	3.7 ± 0.5 ^{a}	3.4 ± 0.7 ^{a}	1.7 ± 0.4 ^{b}	1.3 ± 0.5 ^{b}	1.0 ± 0.8 ^{c}
18:1n-9	16.2 ± 0.9	23.1 ± 3.3 ^{b}	24.3 ± 3.8 ^{b}	28.3 ± 5.9 ^{a}	20.7 ± 4.0 ^{b}	15.7 ± 1.8 ^{c}
18:1n-7	3.0 ± 0.1	3.8 ± 0.3 ^{a}	3.7 ± 0.3 ^{a}	3.0 ± 0.3 ^{b}	2.6 ± 0.3 ^{c}	2.2 ± 0.2 ^{d}
20:1n-11	n.d.	0.1 ± 0.1^{b}	0.2 ± 0.2 ^{a}	0.1 ± 0.1^{b}	n.d.	n.d.
20:1n-9	1.7 ± 0.4	3.3 ± 0.4^{ab}	3.6 ± 0.4 ^{a}	3.1 ± 0.5 ^{b}	2.1 ± 0.4 ^{c}	1.4 ± 0.2 ^{d}
20:1n-7	0.2 ± 0.0	0.2 ± 0.0 ^{a}	0.2 ± 0.0 ^{a}	0.2 ± 0.0 ^{a}	0.1 ± 0.1^{b}	0.1 ± 0.1^{b}
22:1n11	0.2 ± 0.2	0.9 ± 0.3 ^{a}	1.0 ± 0.5 ^{a}	0.1 ± 0.3 ^{b}	tr	n.d.
22:1n-9	0.3 ± 0.2	0.3 ± 0.1a	0.3 ± 0.1a	0.1 ± 0.0 a	n.d.	tr
24:1n-9	1.1 ± 0.1	0.9 ± 0.1^{ab}	1.0 ± 0.2 ^{a}	0.8 ± 0.2^{ab}	0.7 ± 0.1^{c}	0.7 ± 0.1^{c}
Total monenes	25.0 ± 1.3	36.7 ± 3.9^{a}	38.2 ± 3.3^{a}	37.6 ± 5.5^{a}	27.8 ± 4.1^{b}	21.6 ± 2.5^{c}
18:2n-6	6.4 ± 0.4	3.6 ± 0.4 ^{d}	4.0 ± 1.7^{d}	7.5 ± 2.1^{c}	14.1 ± 2.0 ^{b}	17.7 ± 2.4 ^{a}
18:3n-6	0.1 ± 0.0	0.1 ± 0.1^{a}	tr	0.1 ± 0.1^{a}	tr	0.1 ± 0.1^{a}
20:2n-6	1.2 ± 0.2	1.1 ± 0.1^{c}	1.2 ± 0.3 ^{c}	1.7 ± 0.4 ^{b}	3.2 ± 0.4 ^{a}	3.5 ± 0.4 ^{a}
20:3n-6	0.4 ± 0.0	0.4 ± 0.2 ^{bc}	0.3 ± 0.0 ^{c}	0.4 ± 0.1 ^{bc}	0.5 ± 0.1 ^{a}	0.6 ± 0.1^{a}
20:4n-6	2.8 ± 0.4	1.7 ± 0.4 ^{b}	1.6 ± 0.3 ^{b}	2.0 ± 0.4 ^{a}	2.3 ± 0.3 ^{a}	2.3 ± 0.3 ^{a}
22:5n-6	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Total n-6 PUFA	11.4 ± 0.5	7.3 ± 0.3 ^{c}	7.7 ± 1.9 ^{c}	12.1 ± 2.0 ^{b}	20.5 ± 2.0 ^{a}	24.5 ± 2.2 ^{a}
18:3n-3	1.8 ± 0.2	1.6 ± 0.2^{c}	1.7 ± 0.6 ^{c}	3.0 ± 0.8 ^{a}	2.4 ± 0.4 ^{a}	1.6 ± 0.6 ^{c}
18:4n-3	0.3 ± 0.2	0.4 ± 0.1^{a}	0.4 ± 0.1^{a}	0.2 ± 0.1^{b}	0.2 ± 0.1^{b}	0.2 ± 0.1^{b}
20:3n-3	0.4 ± 0.1	0.5 ± 0.1^{b}	0.6 ± 0.2 ^{b}	0.8 ± 0.2 ^{a}	0.7 ± 0.1^{ab}	0.4 ± 0.0 ^{a}
20:4n-3	0.7 ± 0.1	1.9 ± 0.2 ^{a}	1.7 ± 0.4 ^{a}	0.9 ± 0.2 ^{b}	0.8 ± 0.1^{c}	0.7 ± 0.1 ^{c}
20:5n-3	12.8 ± 0.6	7.7 ± 1.4 ^{b}	7.7 ± 1.1^{b}	9.1 ± 0.9 ^{a}	10.0 ± 1.3 ^{a}	9.7 ± 0.9 ^{a}
22:5n-3	3.7 ± 0.3	4.3 ± 0.8 ^{a}	3.7 ± 0.7^{a}	2.5 ± 0.8 ^{b}	2.7 ± 0.2 ^{b}	2.6 ± 0.2 ^{b}
22:6n-3	20.0 ± 1.5	21.5 ± 1.8 ^{a}	20.7 ± 3.0 ^{ab}	17.7 ± 4.6 ^{b}	17.6 ± 2.1^{b}	19.1 ± 2.5^{ab}
Total n-3 PUFA	39.9 ± 1.7	38.1 ± 2.3 ^{a}	36.7 ± 2.8 ^{ab}	34.5 ± 4.5 ^{b}	34.1 ± 3.0 ^{b}	34.5 ±2.9 ^{b}
Total PUFA	51.3 ± 1.9	45.4 ± 2.5 ^{c}	44.4 ± 2.0 ^{c}	46.6 ± 2.9 ^{c}	54.6 ± 3.1 ^{b}	59.0 ± 1.3 ^{a}

Results are means ± SD (n = 3). Different superscript letters indicate a significant effect of diet as determined by ANOVA. DFO, decontaminated northern fish oil; n.d., not detected; NFO, northern fish oil; PUFA, polyunsaturated fatty acids; SRO, southern fish oil and rapeseed oil (40/60); SRO/SO, southern fish oil, rapeseed oil and soybean oil (40/30/30); SSO, southern fish oil and soybean oil (40/60); tr, < 0.05.

3.3.8 Flesh and liver lipid class compositions

Dietary treatment showed no significant effects on flesh lipid class composition between fish fed the NFO and DFO diets (Table 3.12). In contrast, the proportions of neutral lipids were reduced and polar lipids increased in flesh of salmon fed the SVO diets compared to fish fed the NFO or DFO diets. The lower neutral lipid was primarily due to decreased proportions of TAG. The greater proportions of polar lipids in fish fed the SVO diets were mainly due to increased proportions of the two main phosphoglycerides, PC and PE. One of the few differences between the fish fed the SVO diets was that the flesh of fish fed the diet with highest level of SO (SSO diet) had a lower proportion of TAG and a higher proportion of cholesterol (sterol) than fish fed the other SVO diets. The proportion of FFA in the flesh also increased with increasing inclusion of dietary SO (Table 3.12).

There were no significant effects of dietary treatment on liver lipid class composition between fish fed the NFO and DFO diets other than slightly higher cholesterol in liver of fish fed DFO (Table 3.13). Similar to the effects described above for flesh, the proportions of neutral lipids were reduced and polar lipids increased in liver of salmon fed the SVO diets compared to fish fed the NFO or DFO diets. Lower total neutral lipid reflected to lower level of total liver lipid content. The lower neutral lipid was primarily due to decreased proportions of TAG and steryl esters. The larger proportions of polar lipids in fish fed the SVO diets were also generally due to increased proportions of PC and PE. Within the SVO diets, the liver of fish fed 60 % SO (SSO diet) had a higher sterol level than the fish fed the SRO and SRO/SO diets, as in the case of flesh (Table 3.13).

Table 3.12. Lipid class composition (percentage of total lipid) of flesh of Atlantic salmon at the initiation (initial) of the trial and after feeding the experimental diets for 10 weeks.

Lipid classes	Initial	NFO	DFO	SRO	SRO/SO	SSO
PC	10.0 ± 2.0	4.7 ± 0.3^{c}	5.0 ± 0.6^{bc}	6.5 ± 0.7^{a}	5.6 ± 0.6^{b}	5.4 ± 0.6^{bc}
PE	8.7 ±1.6	3.1 ± 0.4^{b}	3.3 ± 0.4^{b}	4.1 ± 0.5^{a}	4.1 ± 0.6^{a}	4.4 ± 0.6^{a}
PS	1.2 ± 0.4	n.d.	n.d.	n.d.	n.d.	n.d.
PI/PG	1.3 ± 0.5	n.d.	n.d.	n.d.	n.d.	n.d.
SPC	0.8 ± 0.2	n.d.	n.d.	n.d.	n.d.	n.d.
LysoPC	1.5 ± 0.3	n.d.	n.d.	n.d.	n.d.	n.d.
Total PL	23.5 ± 4.5	7.9 ± 0.5^{b}	8.3 ± 1.0^{b}	10.5 ± 1.1^{a}	9.7 ± 1.1^{a}	9.7 ± 1.1^{a}
Total NL	76.5 ± 4.5	92.1 ± 0.5^{a}	91.7 ± 1.0^{a}	89.5 ± 1.1^{b}	90.3 ± 1.1^{b}	90.3 ± 1.1^{b}
TAG	47.7 ± 1.0	82.3 ± 2.5^{a}	83.2 ± 1.7^{a}	80.3 ± 2.5^{b}	79.6 ± 0.6^{b}	75.4 ± 1.9^{c}
Sterol	14.1 ± 2.7	6.2.± 0.8 ^{b}	5.6 ± 0.4^{b}	6.1 ± 0.7^{b}	6.4 ± 0.6^{b}	8.3 ± 2.0^{a}
FFA	14.6 ± 3.0	3.1 ± 0.7^{c}	3.0 ± 0.7^{c}	3.1 ± 0.7^{c}	4.4 ± 0.7^{b}	6.6 ± 1.0^{a}

Results are means ± SD (n = 3). Different superscript letters indicate a significant effect of diet as determined by ANOVA. DFO, decontaminated northern fish oil; FFA, free fatty acid; n.d., not detected; NFO, northern fish oil; NL, neutral lipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI/PG, phosphatidylinositol/ phosphatidylglycerol, PL, polar lipid; PS, phosphatidylserine; SPC, sphingomyelin; SRO, southern fish oil and rapeseed oil (40/60); SRO/SO, southern fish oil, rapeseed oil and soybean oil (40/30/30); SSO, southern fish oil and soybean oil (40/60); TAG, triacylglycerol.

Table 3.13. Lipid class composition (percentage of total lipid) in Atlantic salmon liver at initial and after feeding the experimental diets for 10 weeks.

Lipid classes	Initial	NFO	DFO	SRO	SRO/SO	SSO
PC	22.9 ± 1.5	13.4 ± 0.4^{b}	15.3 ± 2.5^{b}	19.5 ± 0.2^{a}	18.4 ± 1.5^{a}	19.9 ± 2.3^{a}
PE	14.3 ± 0.9	8.7 ± 0.7^{b}	10.8 ± 2.4^{b}	13.5 ± 0.7^{a}	13.9 ± 0.8^{a}	13.4 ± 1.3^{a}
PS	2.4 ± 0.5	2.4 ± 0.7	2.1 ± 0.0	2.4 ± 0.1	2.8 ± 0.6	2.6 ± 0.4
PI/PG	5.6 ± 0.7	5.7 ± 0.2^{a}	5.5 ± 0.3^{a}	5.4 ± 1.3^{a}	4.1 ± 0.5^{b}	4.2 ± 0.3^{b}
SPC	0.6 ± 1.3	2.4 ± 0.3^{a}	2.2 ± 0.1^{a}	2.3 ± 0.2^{a}	1.9 ± 0.3^{b}	1.9 ± 0.3^{b}
Unidentified PL	2.0 ± 1.2	1.5 ± 0.4^{b}	0.6 ± 1.0^{b}	1.3 ± 2.3^{b}	3.8 ± 0.6^{a}	3.4 ± 0.1^{a}
Total PL	47.7 ± 2.4	34.1 ± 1.1^{b}	36.4 ± 4.0^{b}	44.4 ± 1.4^{a}	45.0 ± 3.2^{a}	45.7 ± 4.5^{a}
Total NL	52.3 ± 2.4	65.9 ± 1.1^{a}	63.6 ± 4.0^{a}	55.6 ± 1.4^{b}	56.0 ± 3.2^{b}	54.3 ± 4.5^{b}
TAG	24.9 ± 2.6	39.6 ± 1.6^{a}	37.2 ± 3.8^{ab}	32.4 ± 2.7^{bc}	32.1 ± 3.4^{bc}	28.8 ± 4.5^{c}
Sterol	14.5 ± 1.6	9.1 ± 0.4^{d}	10.7 ± 1.2^{c}	12.7 ± 0.5^{b}	12.3 ± 0.7^{b}	14.5 ± 1.0^{a}
FFA	2.1 ± 0.9	1.6 ± 0.3^{a}	1.6 ± 0.4^{a}	0.5 ± 0.9^{b}	1.6 ± 0.6^{a}	tr.
Sterol ester	10.8 ± 2.7	15.6 ± 0.3^{a}	14.2 ± 1.4^{a}	10.1 ± 1.1^{b}	9.1 ± 1.0^{b}	11.0 ± 1.1^{b}

Results are means ± SD (n = 3). Different superscript letters indicate a significant effect of diet as determined by ANOVA. DFO, decontaminated northern fish oil; FFA, free fatty acid; NFO, northern fish oil; NL, neutral lipid; SRO, southern fish oil and rapeseed oil (40/60); SRO/SO, southern fish oil, rapeseed oil and soybean oil (40/30/30); SSO, southern fish oil and soybean oil (40/60). PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI/PG, phosphatidylinositol/phosphatidylglycerol, PL, polar lipid; PS, phosphatidylserine; SPC, sphingomyelin; TAG, triacylglycerol; tr., < 0.05.

3.3.9 Dioxins, DL-PCBs and PBDEs in flesh

The sum of dioxins + DL-PCBs in the flesh of fish at the initiation of the trial was 1.06 ng TEQ/kg. After feeding the experimental diets for 10 weeks, the value in the flesh of fish fed the NFO diet had increased significantly to 6.42 ng TEQ/kg, while in fish fed the DFO and SRO/SO diets the values were significantly reduced to 0.34 and 0.41 ng TEQ/kg, respectively, compared to the initial fish (Table 3.14).

The sum of the PBDE congeners was 0.26 ng/g wet weight in the flesh of fish at the initiation of the trial. After feeding the experimental diets for 10 weeks the value in flesh of fish fed the NFO diet had increased significantly to 0.94 ng/g, while flesh of

fish fed the DFO diet showed a similar level to the initial fish at 0.25 ng/g and fish fed the SRO/SO diet had a significantly reduced concentration of 0.09 ng/g (Table 3.14).

Table 3.14. Concentrations of dioxin, DL-PCB (ng TEQ/kg wet weight) and PBDE (ng/g wet weight) congeners in flesh of Atlantic salmon at the initiation (initial) of the feeding trial and after feeding the experimental diet for 10 weeks.

Congener/Diet	Initial	NFO	DFO	SRO/SO
Sum PCDD	0.06 ± 0.02 [b]	0.57 ± 0.08 [a]	0.03 ± 0.01 [b]	0.05 ± 0.02 [b]
Sum PCDF	0.12 ± 0.03 [b]	1.48 ± 0.31 [a]	0.03 ± 0.01 [b]	0.04 ± 0.01 [b]
Sum mono-ortho PCB	0.18 ± 0.08 [b]	1.19 ± 0.10 [a]	0.09 ± 0.03 [b]	0.07 ± 0.01 [b]
Sum non ortho PCB	0.73 ± 0.16 [b]	3.21 ± 0.19 [a]	0.20 ± 0.02 [c]	0.24 ± 0.04 [c]
Sum dioxins + PCBs	1.06 ± 0.15 [b]	6.42 ± 0.40 [a]	0.34 ± 0.04 [c]	0.40 ± 0.03 [c]
Sum PBDE	0.26 ± 0.01 [b]	0.94 ± 0.002 [a]	0.25 ± 0.02 [b]	0.09 ± 0.03 [c]

Results are means ± SD (n = 3). Values in the same row assigned a different superscript letter are significantly different (P < 0.05).DFO, decontaminated northern fish oil; NFO, northern fish oil; PBDE, polybrominated diphenyl ethers; PCB, polychlorinated biphenyls; PCDD, polychlorinated dibenzo-*p*-dioxins; PCDF, polychlorinated dibenzofurans; SRO/SO, southern fish oil, rapeseed oil and soybean oil (40/30/30).

3.3.10 Gene expression

The expression of both the Δ6- and Δ5-fatty acyl desaturase (FAD) genes in liver was significantly increased in salmon fed the SVO diets compared to fish fed the diets containing FO (NFO and DFO), with the highest expression observed in liver of fish fed the highest inclusion of SO (SSO diet) (Figure 3.1). The expression of Δ5 and Δ6 FADs of salmon fed the SSO diet was 9-fold and 11-fold higher, respectively, compared to the fish fed NFO diet. The expression of Δ5 FAD was about 6-fold higher in livers of fish fed both SRO and SRO/SO diets compared to fish fed the NFO diet.

The expression of Δ6 desaturase was 6-fold and 5-fold higher in fish fed the in the SRO and SRO/SO diets, respectively, compared to fish fed the NFO diet. In contrast, there was no dietary effect on the hepatic expression of the PUFA elongase gene. There were also no significant differences in expression of any of the genes between fish fed the NFO and DFO diets.

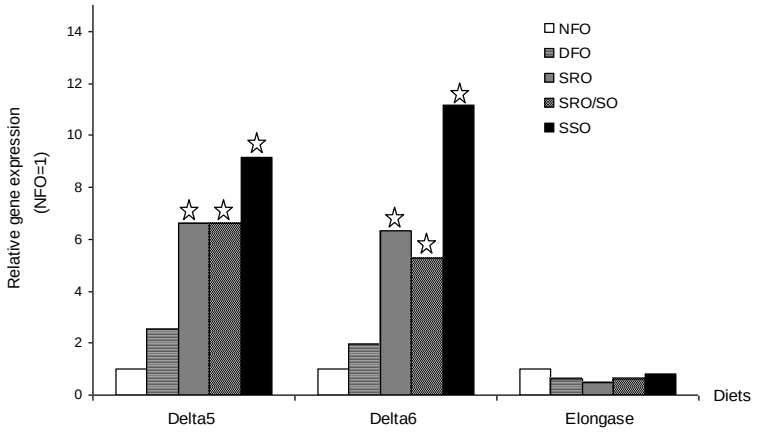


Figure 3.1. Effects of diet on the hepatic expression of Δ5 and Δ6 fatty acyl desaturases, and polyunsaturated fatty acid elongase in Atlantic salmon. Stars indicate the expression of the specific gene in fish fed a particular diet was significantly different ($P<0.05$) compared to its expression in fish fed the NFO diet. DFO, decontaminated northern fish oil; NFO, northern fish oil, SRO, southern fish oil and rapeseed oil (40/60); SRO/SO, southern fish oil, rapeseed oil and soybean oil (40/30/30); SSO, southern fish oil and soybean oil (40/60).

3.4 Discussion and conclusions

3.4.1 Growth performance, apparent digestibility of gross nutrients and fatty acids

The presence of POPs, PCDD/F and DLPCB, levels in flesh was negatively related with the growth rate of farmed Atlantic salmon (Berntssen *et al*. 2005). The use of purification techniques by short path distillation (Breivik & Thorstad 2004) or activating carbon treatment, combined with stripping at low pressure and low

temperature (De Kock *et al.* 2004) has been reported the potential to reduce the level of lipid soluble organic pollutants while maintaining the nutritional status of the marine ingredients used in fish feeds (Berntssen & Lundebye 2007). In the present study, there were no deleterious effects on growth as measured by final weights, SGR or TGC, or on feed efficiency (FCR) of any of fish fed the experimental diets, including the fish fed the SVO diets. This was consistent with the results of several previous trials in which partial replacement of FO by VO, or even 100 % replacement, have had no significant effects on growth performance or feed efficiency in Atlantic salmon when using FM-based diets (Bell & Waagbo 2008).

The digestibilities of the gross dietary nutrients and individual FA were generally high and similar in all the diets with the exception of diet DFO. In the diet formulated with decontaminated FO, the ADC was slightly but significantly lower than that of all the other diets, including the NFO diet, although this had no impact on growth performance. There were some small but significant differences in FA composition between the DFO and NFO diets, and so some differences in FA and, consequently lipid, digestibility may not be entirely unexpected. However, the lower digestibilities of the DFO diet also included protein and dry matter and so it appeared to be a general effect. It is not known if the decontamination process per se was responsible for these effects, but the northern FO and decontaminated FO used in the diet manufacture were exactly the same northern oil, simply decontaminated or not. Therefore, it is likely that some aspect of the decontamination process affected the digestibility, but it cannot be certained without further evidence. Furthermore, it was surprising that the lower digestibilities of the DFO diet did not result in lower growth, as the fish fed the DFO diet attained the same final weight and showed the same SGR, and TGC as fish fed the NFO and the SVO diets. FCR were also unaffected and so the

lower digestibilities with the DFO diet were not reflected in any gross growth performance parameter. This apparent dichotomy between nutrient digestibilities, and growth and feed efficiency has been observed previously in feeding trials in salmon (Torstensen *et al.* 2000). The SGR was higher and FCR was lower in Atlantic salmon held in seawater compared to those in freshwater, despite lower ADC values for dry matter and crude protein (Krogdahl *et al.* 2004). In comparison to the NFO diet, the SVO diets also showed slightly lower lipid digestibility related to lower digestibilities of a number of FAs, but protein digestibility was not affected by feeding VOs. Lipid and FA digestibility in salmon fed VOs appears to be variable, perhaps due to environmental variables, (Torstensen *et al.* 2000; Bendiksen *et al.* 2003; Ng *et al.* 2004), but lower lipid/FA digestibility of diets formulated with VO has been reported previously in trials with salmon (Menoyo *et al.* 2003) and other fish species including Murray cod (*Maccullochella peelii peelii*) (Francis *et al.* 2007). As with the DFO diet, the slightly lower lipid digestibility of the SVO diets did not result in any deleterious effect on growth performance or feed efficiency.

3.4.2 Whole body composition, flesh and liver lipid content

In this study, the proximate composition of whole fish was unaffected by feeding the DFO and SVO diets compared to the NFO diet. Furthermore, feeding the fish the DFO diet did not affect the flesh and liver lipid contents compared to the fish fed the NFO diet. Although the SVO diets also had no effect on the gross composition of the fish, their effect in lowering liver lipid was highly significant. In addition, it appeared that the liver lipid lowering effect was greater with the diets containing SO compared to RO, although this difference was not statistically significant. Differences between VOs in their effects on tissue lipid contents have been observed previously.

Thus, earlier trials on salmon smolts showed that substitution of FO with graded levels of PO had no significant effect on liver lipid (Bell *et al.* 2002), whereas graded substitution with RO tended to increase liver lipid (significantly so at 100 % replacement) (Bell *et al.* 2001). Liver lipid was not increased in salmon fed SO at higher water temperature (Ruyter *et al.* 2006). Furthermore, liver lipid content was lower in salmon fed a VO blend containing RO, PO and LO for 16 months but not at 14 and 22 months of feeding (Jordal A-E.O *et al.* 2007). In that study, the relative proportions of TAG and cholesterol were increased and decreased, respectively, at all stages. Thus, the effects of VO diets on liver lipid levels can vary perhaps related to type of oil, water temperature, season and duration of trial.

Possibly more important in terms of product quality, is the effect that different VOs have on flesh lipid contents in salmon. In the present trial, although there was a trend for flesh lipid content to be lower in fish fed the SVO diets, it was only significantly reduced in fish fed the SRO diet, that is, with 60 % RO substitution. Previously, graded RO substitution in salmon smolts tended to decrease flesh lipid, although this effect was only significant at the 50 % replacement level (Bell *et al.* 2001). With PO substitution in salmon smolts, the flesh lipid lowering effect was very pronounced, with lipid levels decreasing in a graded manner as the level of PO substitution increased (Bell *et al.* 2002). In a study with larger post-smolts, feeding PO reduced flesh lipid content compared to fish fed sunflower oil or a blend of FO and sunflower oil (Torstensen *et al.* 2000). In a trial feeding salmon a VO blend containing RO, PO and LO over the entire 2-year growth cycle, flesh lipid contents were generally lower at all points in both the freshwater and seawater phases (Torstensen *et al.* 2005).

3.4.3 Flesh and liver lipid class compositions

The lipid class compositions of flesh and liver were generally unaffected by feeding the DFO diet compared to the NFO diet but there were some effects of feeding the diets containing VO. In the present study, the lipid class composition of the flesh in fish fed the SVO diets was characterised by decreased proportions of neutral lipid and TAG. Previously, it was reported that neutral lipid and TAG were reduced in white muscle of salmon fed a VO blend compared to fish fed FO (Nanton *et al.* 2007). Overall, it is apparent that the effect that substitution of FO with VO has on tissue lipid contents and compositions is dependent upon a number of factors including the specific VO or VO blends used as well as other factors including the specific tissue itself and possibly growth stage and/or season as well as the genetic origin of the stock (Sargent *et al.* 2002). In the present study, the lower liver lipid level in fish fed the SVO diets was associated with decreased neutral lipids, especially TAG and steryl esters whereas the major membrane lipids, PC, PE and, possibly, cholesterol/sterol were all increased. In Table 3.13, sterols rather than cholesterol are reported as the TLC method is not able to separate cholesterol from many plant sterols. The SVO diets are likely to contain lower levels of cholesterol but, conversely, may contain a range of other plant sterols (Padley *et al.* 1986). Plant sterols are generally not well absorbed by animals and, indeed, are used to block cholesterol uptake in humans. The situation with fish is not known but it is likely similar. Therefore, it is likely that cholesterol is the predominant sterol in fish tissues, but the presence of plant sterols cannot be excluded completely. However, the higher cholesterol level in liver and flesh of fish fed the SVO diets may be a compensatory mechanism, the result of increased cholesterol synthesis in response to reduced dietary cholesterol (Taggart *et al.* 2008).

3.4.4 Liver and flesh fatty acid compositions

The differences in the FA compositions of the NFO and DFO diets cannot be specifically attributed to the decontamination process but it is possibly a factor. For instance, certain FAs could be slightly more adsorbed by the activated carbon than other FAs resulting in lower proportions in DFO compared to NFO. However, the differences observed, lower levels of 18:1n-9 and 18:3n-3, and higher levels of 18:0, 20:1, 22:1, 18:2n-6 and EPA in DFO compared to NFO, did not follow a pattern that could be easily explained by differential adsorption. Some of these differences, particularly the lower level of 18:1n-9 and higher levels of 20:1, 22:1 and EPA were reflected in flesh lipids but not liver lipids, that showed identical compositions in fish fed the NFO and DFO diets. The fact that flesh was more affected than liver by differences in dietary FA composition is predictable based on the lipid content of flesh compared to liver in salmon. The higher lipid content is associated with higher neutral (storage) lipid, specifically TAG, that is more readily influenced by diet than phospholipid (Sargent *et al.* 2002). This simple fact is why changes in dietary lipid have such an impact on salmon flesh composition and why it is, as a consequence, difficult to minimise these effects. This was clearly demonstrated in the present trial where the strategy with VO substitution was to blend them with southern hemisphere FO in an attempt to minimise the impact of the VO inclusion on n-3HUFA in flesh was only partially successful. Thus, despite 60 % of the FO being replaced by VOs, the EPA levels in the salmon flesh were only slightly reduced from about 6 % to 5 %. However, DHA was reduced more severely from around 1 3% to about 6.5 to 7.5 %. This simply reflected the diets that also showed quite significantly reduced DHA but relatively less effect on EPA, which was expected as, although southern hemisphere FOs have higher n-3HUFA than

northern hemisphere FOs, this is due to high percentages of EPA and a high EPA:DHA ratio, but reduced or similar DHA compared to northern FO.

In contrast, the strategy was largely successful in liver as FA compositions of fish fed the SVO diets showed impressive levels of HUFA with ARA and EPA both being significantly increased, and DHA only reduced from around 21 % to about 18-19 %. EPA actually increased from 7.7 % in fish fed the NFO and DFO diets to 9-10 % in fish fed the SVO diets. The only negative impact being increased 18:2n-6 and reduced 18:1n-9 in the diets containing SO (SSO diet). It is important to emphasise that the increased EPA and ARA were not simply the result of increased deposition and retention, but were also the result of conversion of dietary 18:3n-3 and 18:2n-6, respectively, via fatty acyl desaturation activities as seen in previous studies (Bell *et al.* 2001; Tocher *et al.* 2003b). This contention is supported by other evidence. The SVO diets, although formulated with 40 % southern hemisphere FO, which is known to be richer in EPA than northern hemisphere FOs (Sargent *et al.* 1989), were still lower in EPA than the NFO and DFO diets. Similarly, the ARA levels in the SVO diets were lower than in the NFO and DFO diets. Further evidence of active desaturation was provided by the increased level of the intermediate 20:3n-6 in the liver of fish fed the diets containing soybean, although there was also increased production of the dead-end FA 20:2n-6, the direct elongation product of 18:2n-6. Increased proportions of 20:2n-6 and 20:3n-6 have been observed previously in salmon when fed diets containing high levels of 18:2n-6 (Lie *et al.* 1993; Bell *et al.* 2001, 2002; Tocher 2003). Thus, the increased proportions of EPA and ARA in liver were also the result of enhanced desaturation and elongation of dietary 18:3n-3 and 18:2n-6. The increased hepatic expression of FAD genes observed in the present trial is entirely consistent with this assertion. Previously studies demonstrated that increased expression of FAD genes in

liver of salmon fed VOs was associated with increased activity of the HUFA biosynthesis pathway (Zheng *et al.* 2004, 2005). The increased percentages of EPA observed in fish fed the SVO diets was especially noteworthy as it is unusual that dietary VO did not reduce EPA levels, let alone increase them. As mentioned above, it is common to observe increased levels of desaturation products of dietary 18:2n-6 including, most commonly, 20:3n-6, in trials with salmon fed VO (Lie *et al.* 1993; Bell *et al.* 2001, 2002; Tocher 2003). Bell *et al* (1991) also found increased levels of ARA in phospholipids from liver, heart and retina in salmon fed sunflower oil. Increased ARA was also observed in total lipid from liver of salmon fed a RO/PO/LO blend (Zheng *et al.* 2005). As essentially all VO diets will increase dietary 18:2n-6, in comparison to FO diets, this has been a widely reported observation in salmon tissues, especially liver. However, a similar phenomenon has not been generally observed with 18:3n-3. Therefore, increasing dietary 18:3n-3 levels has not been able to maintain tissue EPA or DHA at levels obtained in salmon fed FO. Indeed, even increased levels of 18:4n-3 or 20:4n-3, intermediates in the pathway from 18:3n-3 to EPA, have only rarely been reported. In a trial using graded substitution of FO with LO, 18:4n-3 and 20:4n-3 were increased in salmon liver lipids in a graded manner (Tocher *et al.* 2002). However, EPA, 22:5n-3 and DHA all decreased as the level of LO substitution increased. Direct biochemical measurements of fatty acyl desaturation activities in hepatocytes and enterocytes have conclusively confirmed that HUFA synthesis is up-regulated in salmon fed diets containing VOs, including RO (Bell *et al.* 2001), PO (Bell *et al.* 2002) and LO (Tocher *et al.* 2003b). Molecular studies demonstrated that the mechanism included increased FAD gene expression (Zheng *et al.* 2004, 2005). Thus, in previous trials when dietary FOs were substituted by VOs, the reductions in the levels of dietary EPA and DHA have been too great for the conversion of dietary 18:3n-3 by

the HUFA synthesis pathway to maintain the tissue levels of EPA or DHA. It was very interesting that EPA levels in salmon fed the SVO diets were higher than the levels of EPA in fish fed the NFO and DFO diets in the present trial. The increasing of EPA levels in the SVO diets would be related to low total lipid content and more total polar lipid in the flesh associated with biosynthesis from the 18:3n-3 of fish fed the SVO diets.

Another interesting factor in the observation above is that there was a difference between RO and SO substitution, with the latter, especially the SSO diet with sole replacement by SO, showing the greatest effect. Thus, the percentages of DHA and EPA in both liver and flesh were higher in fish fed the SSO diet compared to fish fed the SRO diet with RO substitution. The difference between fish fed SSO and SRO was highly significant in flesh with total n-3HUFA levels of 15.7 versus 13.6 %, respectively, with reciprocal differences in substrate 18:3n-3 levels of 3.2 versus 5.6 %. Consistent with this, the induction of FAD expression in liver was greatest in the fish fed the SSO diet. It is not clear why this should be. Previous trials on salmonids using substitution of FO with SO have not shown such an effect (Hardy *et al.* 1987; Guillou *et al.* 1995; Ruyter *et al.* 2006). The current paradigm is that it is low dietary HUFA that induces increased FAD expression (Tocher *et al.* 2003b), but there was very little difference between the SRO and SSO diets in terms of HUFA content. In contrast, the SSO diet was characterised by very high levels of 18:2n-6 and lower 18:3n-3 than the SRO diet. In a study comparing different proportions of 18:1n-9, 18:2n-6 and 18:3n-3 (supplied by OO, LO and sunflower oil, respectively) in diets for Atlantic salmon parr, the authors suggested that the desaturation and elongation of 18:2n-6 and 18:3n-3 may be stimulated by substrate availability as they noted the extent of conversion differed despite dietary HUFA levels all being identical (Rollin *et al.* 2003). Although it is known that, like most vertebrate FADs, the salmon FADs are more active towards n-3

than n-6 substrates (Zheng *et al.* 2005), how this particular combination of 18:2n-6 and 18:3n-3 could have such a significant effect is unclear. Certainly, previous trials that used VO blends to produce a better balance of 18:2n-6 and 18:3n-3 including RO/LO (Tocher *et al.* 2003b; Bell *et al.* 2003b) or RO/PO/LO (Zheng *et al.* 2005) have not been effective in maintaining EPA. However, these trials were of longer duration and there may have been a transient increase in HUFA, following change of diet, that wasn't observed due to the frequency of sampling used.

3.4.5 Dioxins, DL-PCBs and PBDEs in flesh

In the present study, the NFO diet contained 17.4 ng TEQ/kg of dioxins + DL-PCBs and, as such, exceeded the EU maximum permitted value of 7.0 ng TEQ/kg for fish feeds (EC Commission Directive 2006). Decontamination of the NFO was very successful with the dietary concentration of dioxin + DL-PCBs falling from 17.4 ng TEQ/kg in the NFO diet to 0.45 TEQ/kg in the DFO diet. It is indicated that using activated carbon, followed by a thin film deodourisation process, removed dioxins and PCBs in the DNFO diet by about 97%, compared with the NFO diet. It has been reported that activated carbon removed PCDD/PCDF in FO but to a lesser extent DL-PCB (Berntssen *et al.* 2006). In the present study, the reduction of the PCDD due to FO decontamination in the DFO was 93% while over 97% of the PCDF and DL-PCB (mono-ortho, non-ortho PCB) were reduced, compared with the NFO diet. The results support the effectiveness of using the activated carbon and thin film deodourisation in the decontamination process for the dioxins and DL-PCB.

Similarly the dietary concentration of dioxin + DL-PCBs was reduced to 0.53 ng TEQ/kg or reduction of 97% in the SRO/SO diet, compared with the NFO diet. Moreover, the reduction of the PCDD was about 93% whereas the reductions of the

PCDF, the DL-PCB and the PBDE were over 95% in the SRO/SO diet compared with the NFO diet. As the current EU limits values for the combined dioxins and DL-PCB are 7 TEQ/kg feed, in the present study the value in the DFO diets would present about 6.3% and in the SRO/SO about 7.6% of the EU limits (EC Commission Directive 2006).

The PBDE value in the DFO diet was higher than that of the SRO/SO diet. The reduction of the PBDE due to using the two step decontamination process for the DFO diet was about 68% compared to the NFO diet whereas the reduction of the PBDE was reduced about 95% due to replacing the NFO with the SRO/SO in the diet indicating that the use of VO in salmon diet significantly reduced POPs loadings which should benefit consumers.

Reflecting the diet, replacing the contaminated northern FO with decontaminated northern FO or the SRO/SO blends significantly reduced the dioxin and DL-PCB concentrations in salmon flesh after feeding diets for 10 weeks. In the present study, the combined dioxins and DL-PCB concentrations in flesh of fish fed the DFO and the SRO/SO diet were lower than those of fish fed the NFO diet by about 94-95%. However, the value of the combined dioxins and DL-PCB in flesh of fish fed all of the experimental diets was lower than the EU limits values. The limits value of the combined dioxins and DL-PCBs in fish products is 8 ngTEQ/kg (EC Commission Directive 2006). From this study, the flesh dioxins and DL-PCB concentration for the fish fed the NFO diet was 6.42 ngTEQ/kg, still below the EU limits of 8 ngTEQ/kg. The flesh concentration of fish fed the DFO (0.34 ngTEQ/kg) and the SRO/SO (0.41 ngTEQ/kg) would represent 3.5% and 3.0% of the EU limit values. This result was in agreement with previous studies substituting FOs with VOs in fish feed reduced the levels of dioxins and DL-PCB in Atlantic salmon flesh (Bell *et al.* 2005a; Berntssen & Lundebye 2007). The concentration of PBDEs in the initial salmon flesh was 0.26 ng/g

wet weight and the PBDE value in the fish fed the NFO diets increased about 73% to 0.94 ng/g wet weight) after culture with the NFO diet for 10 weeks. In contrast, the value of the PBDE in fish fed the DFO diet remained similar to the initial salmon and the value of the PBDE in the fish fed the SRO/SO diet decreased to 0.09 ng/g. This suggests that that PBDE levels in the flesh were significantly reduced by decontamination or replacement with southern FO and VO blends. All these values for flesh PBDEs in the present study, however, were lower than the values previously reported in Atlantic salmon (Jacobs *et al.* 2002a; Hites *et al.* 2004a).

In conclusion, blending VOs with southern hemisphere FO is a strategy that may result in a lower impact upon tissue n-3HUFA levels, and that the decontamination of FO will have no major effect on the nutritional quality of FO as a feed ingredient for Atlantic salmon, although longer trials may be required to confirm this over the whole production cycle. Neither the SVO diets nor the DFO diet had any deleterious effects on growth as measured by final weights, SGR or TGC, or FCR. Therefore, despite lower ADC, decontamination of FO did not significantly impact on its nutritional quality of salmon. The replacement of northern FO with blends of VOs and southern hemisphere FO had minimal impact on HUFA levels in liver, but a greater effect on flesh HUFA levels. SO had significantly less impact than RO. Decontamination of northern FO had no effect on the n-3HUFA content of flesh of salmon fed the oil. Flesh dioxin, DL-PCB and PBDE concentrations were reduced to very low levels in fish fed the DFO and SRO/SO diets and so both strategies were very successful.

Chapter 4 - Influence of genotype/phenotype on the effects of alternative diets in Atlantic salmon (*Salmo salar* L.): Effects of total replacement of fish oil and partial replacement of fishmeal on growth performance, and tissue lipid and fatty acid compositions

4.1 Introduction

Atlantic salmon flesh contains abundant n-3 LC-PUFA, notably EPA and DHA, both of which are well recognised as being beneficial to human health by reducing the risk of inflammatory pathologies including cardiovascular and neurological disease (Brouwer *et al.* 2006; Givens & Gibbs 2006; Eilander *et al.* 2007). The high content of these EFA in salmon flesh is usually derived from their natural prey *e.g.* capelin, herring, sand eels, mackerel, anchovies and sardines (Sargent & Tacon 1999). Many of these marine fish species have traditionally been used for FM and FO production. FM and FO are excellent products for aquaculture feeds in terms of their AA and FA profiles. In addition, both, but particularly FO, are rich sources of energy, for normal growth, development and reproduction of fish (Sargent *et al.* 1999, 2002). In recent years, FM and FO production has been stagnant as the feed grade fisheries are, at best, at their sustainable limit, and an expansion in worldwide aquaculture has created problems for aquafeed production. It has been estimated that in 2006, the aquaculture sector consumed 3,724,000 tonnes of FM and 835,000 tonnes of FO (i.e. 68.2 % of the total global FM and 88.5 % of the total global FO production in 2006); this is equivalent to 16.6 million tonnes of small pelagic forage fish (Tacon & Metian 2008). To conserve and preserve future production from marine fisheries, while simultaneously developing aquaculture production, improvements in fish feed technology are urgently required.

Specifically, this can be achieved by decreasing the utilisation of traditional marine protein and oil sources and by introducing new, alternative raw materials.

Among the many protein sources available, plant proteins (PP) appear to be the most appropriate alternatives to replace FM in fish diets. A number of studies have investigated the utilisation of PPs as substitutes for FM at varying degrees of inclusion. Carnivorous fish depend on lipid and protein rather than on carbohydrates for energy production (Van den Thillart 1986). Thus, partial replacement of FM by PP meals has been accomplished without serious negative effects on the growth of many carnivorous fish species (Nengas *et al.* 1996; Refstie *et al.* 2001; Lozano *et al.* 2007). In order to maintain a balanced AA profile, the application of a mixture of PP sources is more appropriate than the inclusion of a single PP (Gomes *et al.* 1995; Kaushik *et al.* 2004; Torstensen *et al.* 2008).

As lipids not only provide essential PUFA but also are a major source of energy in the diet of salmonids, and given that salmonids have a high capacity to utilise lipid efficiently, high lipid (energy) diets are currently used in salmonid culture to allow dietary protein to be maximally spared for growth (Sargent *et al.* 1989, 2002). A number of studies have investigated VOs as either partial or complete replacements for FO in feeds for a variety of fish species, reporting no major negative effects on the growth of the fish or on feed utilisation as long as the EFA requirements are met by inclusion of FM (Bransden *et al.* 2003; Bell *et al.* 2004; Fonseca-Madrigal *et al.* 2005; Figueiredo-Silva *et al.* 2005; Richard *et al.* 2006; Oo *et al.* 2007; Pettersson *et al.* 2009). The oils from fish species from the northern hemisphere are rich in long-chain monoenes, 20:1n-9 and 22:1n-11, whereas VOs are abundant in18:1n-9 and 18:2n-6 (Bell *et al.* 2003a). Therefore, when selecting potential VOs for substituting FO in fish feeds, the energy availability and the PUFA content in the diet must be considered. RO

and PO have been investigated as promising alternative oils to replace FO in fish feed. Thus, RO contains an n-6/n-3 ratio of only about 2:1, which is suggested as being potentially beneficial to human health (Simopoulos 2000). However, RO that is rich in 18:1n-9 and PUFA, specifically 18:2n-6 and 18:3n-3, is also devoid of n-3 LC-PUFA (Bell *et al.* 2001; Izquierdo *et al.* 2005; Mourente & Bell 2006), and PO contains high levels of 16:0 and 18:1n-9 and lower levels of 18:2n-6, and no 18:3n-3 or LC-PUFA (Ng 2002b). CO from the plant *Camelina sativa*, which is known by many names including, false flax or German sesame, contains high levels of 18:2n-6, 18:3n-3 and 18:1n-9 (Putnam *et al.* 1993; Zubr 2003) and, unusually for a VO, 20:1n-9, which is more commonly found in FOs from species inhabitating high-latitudes and is a rich energy source in fish feeds (Sargent *et al.* 2002). As salmon flesh FA compositions have been shown to be closely correlated with dietary FAs (Rosenlund *et al.* 2001; Lie 2001; Torstensen *et al.* 2004a), using a mixture of different VOs to provide balanced levels of dietary FAs may be better physiologically for salmon health and welfare (Torstensen *et al.* 2005).

Although salmonids are capable of synthesising LC-PUFA, e.g. ARA, EPA and DHA, by desaturation and elongation of C18 precursors (Tocher *et al.* 2002; Lall *et al.* 2002), this capacity might not be efficient to meet HUFA requirements for optimal growth and welfare of fish, and so potential deficiencies must be met from dietary FM and FO (Tocher *et al.* 2003b; Bell *et al.* 2004). When fish are fed a diet containing a blend of VOs as the sole source of lipid, the reduction of n-3 HUFA and the accumulation of n-6 PUFA in flesh tissues can have a major impact on the FA composition of the final fish product (Torstensen *et al.* 2000, 2005; Mourente *et al.* 2005; Mourente & Bell 2006). To maximise nutritional quality in terms of high levels of n-3 HUFA, the application of a "finishing" diet, which contains FO has been reported

to restore n-3 HUFA levels in flesh, to different degrees, in a variety of fish species including salmonids (Robin *et al.* 2003; Bell *et al.* 2004; Caballero *et al.* 2004; Torstensen *et al.* 2005; Mourente *et al.* 2005; Mourente & Bell 2006). Most of the previous studies have investigated replacement of FO with VOs in commercial strains of fish, including Atlantic salmon. In the present study, the effects of phenotype/genotype on VO replacement of FO were investigated in different strains/families of Atlantic salmon.

Thus, the specific aims of the present study were to investigate the effects of the total replacement of FO with a blend of VOs, i.e. RO, PO and CO, in combination with partial replacement of FM in a formulation consisting of 25 % FM and ~45 % PPs, when fed to three genetically different groups of Atlantic salmon post-smolts. The groups of salmon consisted of two families of salmon designated as "FAT" and "LEAN" selected from a breeding programme (Landcatch Natural Selection, Ormsary, Scotland) and showing extremes of flesh lipid content (muscle adiposity), in comparison with a commercial strain used in previous VO studies (Bell *et al.* 2004; Torstensen *et al.* 2005), and obtained from Marine Harvest Scotland Ltd (Caledonian 20, Fort William, Scotland). Fish were fed low FM diets with either 100% FO or VO in duplicate for one year until approaching marketable size, followed by a subsequent washout/finishing period. The growth performance, proximate compositions, lipid class and FA compositions of the flesh, livers and visceral tissues were determined after 55 weeks on the experimental diets. In addition, the lipid and FA composition of flesh during the finishing phase when all fish were fed a FO diet were determined.

4.2 Materials and Methods

4.2.1 Experimental fish

Two groups of Atlantic salmon post smolts were selected as being fat or lean, based on muscle adiposity, by Landcatch Natural Selection (LNS). Each of the FAT and LEAN groups comprised equal numbers of two different genotypic families (Or04 013 & 187, FAT; Or04 107 & 132, LEAN). A third strain was obtained from Marine Harvest Scotland Ltd (Caledonian 20, CAL). Two thousand fish of each strain/family were stocked into 12 x $5m^3$ net pens at the Ardnish Fish Trials Unit (Marine Harvest Scotland, Lochailort, PH38 4LZ, Highland; 500 fish per pen) and the initial mean weights were 52, 88 and 85g for the CAL, FAT and LEAN strains, respectively. The temperature over the experimental period (May 2006 to December 2007) ranged from 5.5 to 17.0^{o}C with a mean temperature of 11.5 ± 2.9^{o}C. Diets were supplied by automatic feeders controlled by an automated feed sensor system (ArvoTec, Sterner AquaTech UK, Scotland). Feed use and mortalities were recorded and removed daily.

4.2.2 Experimental diets

The fish were fed one of two experimental diets, prepared by Skretting ARC (Stavanger, Norway) in 3 different pellet sizes (3, 6 and 9 mm) for a period of 55 weeks until the fish reached a weight of ~3 kg. The diets were formulated to fully satisfy the nutritional requirements of salmonid fish (NRC 1993). Duplicate pens of each of the three groups of salmon were fed a similar basal diet containing either 100 % Northern FO or a VO blend comprising RO/PO/CO in a ratio of 5:3:2. The diets contained 25 – 32 % FM and 40 – 45 % plant meals. After sampling at 55 weeks, 60 fish per pen for each group, weighing between 2.0 and 3.5 kg, were placed in a single pen and the fish that had previously been fed the VO diet were marked by adipose fin clipping. Thus, the

original twelve pens were reduced to three and these pens were fed a finishing diet, having the same basal formulation as the 9 mm ongrowing diet but coated with a decontaminated FO (sand eel/sprat oil, 9:1v/v; Triple Nine Protein, Denmark), for a further 24 weeks. The diet formulations and proximate compositions of the 3, 6 and 9 mm ongrowing feeds and 9 mm a finishing diet are shown in Table 4.1. Dietary FA compositions are shown in Table 4.2. Lipid class and FA compositions of flesh, liver and viscera at the beginning of the trial were given in Tables 4.3-4.4.

Table 4.1. Feed formulations (g kg^{-1}), proximate compositions (g kg^{-1}) and pigment contents (mg kg^{-1}) of the 3, 6, 9 mm ongrowning diets and the 9 mm finishing diet fed to Atlantic salmon.

Components	Diet FO			Diet VO			Finishing
	3 mm	6 mm	9 mm	3 mm	6 mm	9 mm	diet (FO)
Feed ingredients (g/kg)							
LT South Americal fishmeal [a]	320.0	250.0	250.0	320.0	250.0	250.0	250.0
Soybean meal (extract) [b]	125.0	100.0	110.0	125.0	100.0	110.0	110.0
Soya concentrate [c]	50.0	65.6	70.0	50.0	65.6	70.0	70.0
Wheat gluten [d]	149.6	150.0	90.0	150.0	150.0	90.0	90.0
Wheat [e]	70.0	77.0	78.8	72.2	77.0	78.8	78.8
Corn gluten [f]	43.0	57.2	50.0	42.9	50.0	50.0	50.0
Fish oil (Nordic) [g]	227.7	275.4	300.0	-	-	-	300.0
Fish oil [h] (sand eel/sprat oil, 9:1)							
Rapeseed oil [i]	-	-	-	110.0	140.4	150.0	-
Palm oil [b]	-	-	-	70.0	80.0	90.0	-
Camelina oil [j]	-	-	-	45.0	55.0	60.0	-
Astaxanthin 10%	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamins and minerals	15.0	25.2	51.2	15.0	25.2	51.2	51.2
Proximate composition (%)							
Protein	46.8	45.5	41.0	46.7	44.7	40.5	37.7
Lipid	23.5	28.0	32.2	24.0	28.3	32.3	32.6
Moisture	7.4	5.3	4.6	7.2	5.0	5.1	7.6
Ash	7.1	6.9	6.0	6.9	7.5	6.0	8.7
Pigment content (mg/kg)							
Total pigment	41.1	54.9	67.5	66.4	70.9	69.8	55.7
Astaxanthin	35.5	49.8	63.7	36.4	42.0	36.8	46.9
Astacene	2.0	1.9	1.4	2.1	1.6	0.8	1.7
Beta-carotene	-	-	-	22.1	22.6	26.6	4.1
Lutein	1.8	1.6	1.2	4.1	3.3	3.9	1.7
Zeaxanthin	1.7	1.5	1.2	1.8	1.4	1.7	1.5

Values are means of triplicate analysis. [a], Consortio, Peru; [b], Defona, Norway; [c], ADM, Holland,; [d], Cerestar, Scandinavia, AS Denmark; [e], Statkon, Norway; [f], Cargill, USA; [g], Nordsilmed, Norway; [h], Triple Nine Fish Protein, Denmark; [i], Emmelev AS, Denmark; [j], Technology Crop LTD, UK.

4.2.3 Experimental sampling protocol

Ten fish per pen (twenty fish per dietary treatment) were randomly sampled and weighed at the initiation of the trial. After 55 weeks of the growth phase 25 fish per pen were weighed and measured and 20 fish per pen were sampled and pooled for analysis of flesh NQCs. During the washout phase, twenty four fish per dietary treatment pen (12 each previously fed the FO or VO diet) were taken after 8, 16 and 24 weeks on the finishing diet. Fish were anaesthetised by metacaine sulphonate (MS222; 50 mg/L), killed by a blow to the head and the gut cavity opened. Each fish was weighed, measured and gutted weight, viscera weight and liver weight recorded. Livers were dissected and collected individually and several samples produced from each liver. Firstly, approximately 0.5 g of liver were taken into 8 ml glass tubes containing ice-cold chloroform/methanol (2:1, w/v) and stored at -20^{o}C for lipid class composition analysis. A further small piece of each liver, < 0.5 g, was collected into 1.5 ml microcentrifuge tubes and immediately frozen in liquid nitrogen before storage at -80^{o}C for gene expression analysis (see Chapter 5). The remaining liver was placed in10 ml plastic test tubes, and immediately frozen at -20^{o}C for lipid content analysis and FA compositional analysis. The viscera (minus liver) were wrapped in foil, homogenised and stored at -20^{o} C prior to analysis of proximate, lipid class and FA compositions. Flesh samples were taken using the NQC, after removal of skin and bones they were subsequently homogenised and stored at -20^{o} C prior to analysis of pigment content, proximate, lipid class and FA compositions.

4.2.4 Proximate and pigment composition analyses

The moisture, crude protein, crude fat, fibre and ash contents of the diets as well as fish flesh and viscera were determined by standard procedures (AOAC 2000). Crude

fat contents of the experimental diets were determined after acid hydrolysis prior to fat extraction. The analytical methods were thoroughly described in Chapter 2. Pigment analysis of the diets and homogenised fish flesh were performed by HPLC following the method of Barua *et al* (1993). Diet samples were digested with Maxatase enzyme prior to extraction using the same solvent mixtures as used for the flesh samples. All methods are as described in Chapter 2.

4.2.5 Lipid extraction and analysis

Liver, flesh and viscera tissues were homogenates. Total lipids of tissues and diet samples were extracted by homogenising in 20 volumes of ice-cold chloroform/methanol (2:1, by vol.) according to the method of Folch *et al.* (1957). Lipid classes were separated by HPTLC and quantified by densitometry (Henderson & Tocher 1992). The FAME were prepared by acid-catalysed transesterification (Christie 1993), purified as described TLC (Tocher & Harvie 1988), separated and quantified by gas-liquid chromatography and individual FAME identified by comparison to known standards and by reference to published data (Ackman 1980). The methods were described in detail in Chapter 2.

4.2.6 Calculations and statistical analysis

The FCR and SGR were calculated using the formulae presented in Chapter 2. All the data are presented as means ± SD (n as reported) and statistical analyses performed using SPSS 14 (SPSS Inc, 2005). The effects of diet and salmon group (strain/family) on growth performance and nutritional composition were determined by two-way ANOVA. Data that were identified as non-normal distribution (Kolmogorov-

Smirnov test) were subjected to square root, log or arcsin transformation before analysis. Differences were regarded as significant when $P < 0.05$ (Zar, 1999).

4.3 Results

4.3.1 Proximate and fatty acid compositions of the diets

The results of proximate and FA compositions of the diets were provided by the Nutrition Group, Institute of Aquaculture, University of Stirling. The feed formulations, proximate compositions and pigment contents of the experimental diets are given in Table 4.1. The relative protein content decreased and the lipid levels increased as the size of the pellets increased in line with normal commercial practice. Thus, the protein and lipid contents of the 3 mm pellets were 47 % and 24 % respectively; those of the 6 mm pellets were 45 % and 28 %, and, those of the 9 mm pellet diet were 41 % and 32 %. The total pigment contents of the VO diets were higher than the FO diet, at least in the smaller pellet sizes. Astaxanthin was the major carotenoid of all diets. Its composition was lower in the VO diets, particularly in the 6 and 9 mm diets, in comparison to the FO diets. The VO diet contained more beta carotene, lutein and zeaxanthin derived from the plant protein components (Table 4.1).

The FA composition of the FO diets showed higher levels of 14:0, 16:1n-9, 20:1, 22:1, 18:4n-3, 20:4n-3, 22:5n-3 ARA, EPA and DHA, than the VO diet (Table 4.2). The FO diets, however, had lower levels of 20:0, 18:1, 18:2n-6 and 18:3n-3. The percentages of total MUFA in the FO and VO diets were 42 % and 46 % respectively, with 18:1n-9 being the main FA in both diets, although the 18:1n-9 content of the VO diet was approximately double that in the FO diet. In contrast, the 20:1 and 22:1 contents of the FO diet were 2 and 8 times greater than levels found in the VO diet.

Total n-6 PUFA of the VO diet was 3 times greater than that of the FO diet, with 18:2n-6 being the most prominent n-6 FA in both diets (Table 4.2). Total n-3 PUFA content of the FO and VO diets was 25 % and 14 % respectively, with EPA and DHA being the most prevalent n-3 FAs in the FO diet and 18:3n-3 being the predominant n-3 FA in the VO diet. The FA composition of the finishing diet was similar to that of the 9 mm FO diet used in the growth phase (Table 4.2).

4.3.2 Lipid content, lipid class and fatty acid compositions of salmon tissues at the initiation of the trial

The results of lipid and FA compositions of the tissues were provided by the Nutrition Group, Institute of Aquaculture, University of Stirling. The lipid contents and lipid class compositions of the flesh, liver and viscera at the initiation of the trial are given in Table 4.3. The lipid contents of the flesh of the three salmon groups ranged from 3 – 5 %, with the FAT and LEAN groups being similar and higher than that of the CAL strain. The lipid contents of the livers ranged from 3.5 -3.9 % and in rank order CAL > FAT > LEAN. Visceral lipid contents ranged from 36 -45 %, ranked FAT > LEAN > CAL.

All groups of fish showed similar class compositions, with PC and PE were the main polar lipid classes and TAG was the major neutral lipid in all the analysed tissues. Visceral tissue contained the highest proportion of total neutral lipids at over 90%, with about 60 -62 % as TAG. The total neutral and polar lipid contents of the flesh were 75 % - 81 % and 19 % – 25 % respectively, in all groups, with 55 %- 63 % of total lipid being TAG. The total neutral and polar lipid contents of liver ranged from 45 % - 51 % and 45 % - 55 % respectively, with 18 %-21 % consisting of TAG. The flesh and the viscera of both the FAT and LEAN groups had higher levels of neutral lipid and lower

levels of polar lipid compared to the CAL strain, whereas liver of both FAT and LEAN fish had lower neutral lipid contents compared with the CAL strain.

Total SFA of flesh of all fish groups was around 23 %, with about 14 % being 16:0, and total MUFA ranged from 41 - 44 %, with 14 % 18:1n-9 (Table 4.4). The total n-6 PUFA content of the flesh was approximately 6 %, mainly 18:2n-6, and total n-3 PUFA was about 27 %, with 5% EPA and 17% DHA. Total SFA content of the liver of all fish groups was 27 % of total FAs, 20% being 16:0, and MUFA ranged from 20 - 26 %, with 10 % 18:1n-9 (Table 4.5). The liver n-6 PUFA content was about 5 % in all groups with 2 % being 18:2n-6, and n-3 PUFA ranged from 41 – 47 % with 32 - 36 % being DHA. Total SFA of viscera of all fish ranged from 22 % with 13 % being 16:0, and MUFA ranged from 45 – 52 %, predominantly 18:1, 20:1 and 22:1 (Table 4.6). Total n-6 PUFA in viscera was about 7 % with 4 % 18:2n-6, and n-3 PUFA ranged from 20 – 25 %, with 10 – 13 % being DHA (Table 4.6).

Table 4.2. Fatty acid compositions (percentage of total fatty acids) of the experimental diets fed to Atlantic salmon**.**

Parameters	Diet FO			Diet VO			Finishing diet
	3 mm	6 mm	9 mm	3 mm	6 mm	9 mm	
Fatty acid							
14:0	5.7	4.9	5.8	1.2	1.2	0.9	6.8
16:0	16.0	16.5	15.0	17.3	16.8	15.9	17.1
18:0	2.3	2.3	3.0	2.9	2.7	3.5	3.8
20:0	0.3	0.3	0.4	0.7	0.7	0.8	0.4
22:0	0.2	0.3	0.0	0.3	0.3	0.0	1.2
Total saturated[1]	26.3	26.0	26.1	22.7	22.0	21.7	31.1
16:1n-7	5.9	5.3	5.2	1.1	1.2	0.9	6.7
18:1n-9	11.7	17.8	10.1	36.6	37.2	37.6	11.9
18:1n-7	2.4	2.5	1.9	2.0	2.1	1.6	2.6
20:1n-11	0.6	0.6	0.4	0.0	0.2	0.1	0.5
20:1n-9	7.4	5.4	8.5	3.2	3.9	4.0	4.3
22:1n-11	10.6	7.5	13.7	0.4	1.3	1.6	6.3
22:1n-9	0.9	0.8	0.9	0.7	0.9	0.8	0.4
24:1n-9	0.9	1.1	0.9	0.3	0.3	0.4	0.6
Total monoenes[2]	41.6	42.2	42.6	44.6	47.5	47.5	34.5
18:2n-6	4.6	4.7	3.5	16.4	15.0	16.5	3.3
20:2n-6	0.2	0.4	0.3	0.3	0.3	0.4	0.2
20:3n-6	0.2	0.1	0.1	0.2	0.1	0.2	0.1
20:4n-6	0.6	0.6	0.4	0.2	0.1	0.1	0.8
Total n-6 PUFA[3]	6.2	6.2	4.6	17.3	15.5	17.1	5.0
18:3n-3	1.4	2.2	1.6	9.6	9.3	10.0	0.9
18:4n-3	2.5	2.3	3.5	0.3	0.4	0.4	2.2
20:4n-3	0.6	0.5	0.6	0.1	0.1	0.1	0.6
20:5n-3	8.6	7.7	8.5	1.9	1.9	1.2	12.1
22:5n-3	0.9	0.8	0.8	0.3	0.2	0.1	1.4
22:6n-3	10.3	10.8	10.1	2.3	2.3	1.5	8.9
Total n-3 PUFA[4]	24.4	24.4	25.3	14.8	14.5	13.4	26.3
Total PUFA	32.1	31.8	31.3	32.7	30.5	30.7	34.3

[1] included 15:0 ≤ 0.4%, [2] included 16:1n-9 and 20:1n-7 ≤0.3%, [3] included 18:3n-6 and 22:4n-6 and 22:5n-6 ≤0.2%, [4] included 20:3n-3 ≤0.3%.

Table 4.3 Lipid contents (percentage of wet weight), and lipid class compositions (percentage of total lipid) of Atlantic salmon tissues at the start of the trial.

Lipid classes	Group		
	CAL	FAT	LEAN
Flesh			
Lipid contents	3.3 ± 0.5 [b]	4.9 ± 0.4 [a]	4.8 ± 0.5 [a]
Phosphatidylcholine	10.7 ± 0.9 [a]	8.3 ± 0.2 [b]	9.1 ± 0.4 [b]
Phosphatidylethanolamine	8.8 ± 0.8 [a]	7.3 ± 0.5	7.2 ± 0.9
Phosphatidylserine	1.1 ± 0.1	0.8 ± 0.1	0.8 ± 0.3
Phosphatidylinositol	2.3 ± 0.3 [a]	1.7 ± 0.2 [b]	1.8 ± 0.1 [b]
Sphingomyelin	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0
Lysophosphatidylcholine	2.1 ± 0.2 [a]	1.6 ± 0.0 [b]	1.3 ± 0.1 [b]
Total polar lipids	25.3 ± 2.3 [a]	19.4 ± 0.7 [b]	20.5 ± 1.4 [b]
Total neutral lipids	74.7 ± 2.3 [b]	80.6 ± 0.7 [a]	79.5 ± 1.4 [a]
Triacylglycerol	55.0 ± 3.1 [b]	63.0 ± 2.4 [a]	60.2 ± 3.1 [ab]
Sterol	6.9 ± 0.2 [a]	5.8 ± 0.4 [b]	5.8 ± 0.8 [b]
Free fatty acids	10.2 ± 0.6	10.1 ± 1.1	11.3 ± 0.3
Steryl ester	2.4 ± 0.5	1.6 ± 0.5	2.1 ± 1.1
Liver			
Lipid contents	3.9 ± 0.2	3.7 ± 0.4	3.5 ± 0.2
Phosphatidylcholine	20.6 ± 2.1 [b]	27.1 ± 1.9 [a]	27.1 ± 1.9 [a]
Phosphatidylethanolamine	13.6 ± 0.8 [b]	15.8 ± 0.7 [a]	16.4 ± 1.1 [a]
Phosphatidylserine	3.0 ± 0.6	2.9 ± 0.3	2.8 ± 0.3
Phosphatidylinositol	3.4 ± 0.7	3.3 ± 0.3	3.3 ± 1.1
Sphingomyelin	1.7 ± 0.2 [b]	2.4 ± 0.7 [a]	2.2 ± 0.4 [ab]
Lysophosphatidylchlorine	0.1 ± 0.1 [b]	0.4 ± 0.1 [a]	0.4 ± 0.2 [a]
Unidentified polar lipids	2.3 ± 0.3 [b]	2.9 ± 0.4 [a]	3.0 ± 0.4 [a]
Total polar lipids	44.9 ± 4.4 [b]	54.8 ± 3.6 [a]	55.3 ± 2.9 [a]
Total neutral lipids	55.1 ± 4.4 [a]	45.2 ± 3.6 [b]	44.7 ± 2.9 [b]
Triacylglycerol	20.6 ± 2.8	18.6 ± 2.4	17.8 ± 1.4
Sterol	13.0 ± 0.9 [b]	13.9 ± 1.1 [a]	13.8 ± 0.7 [a]
Free fatty acids	2.1 ± 0.5	1.6 ± 1.1	1.7 ± 0.6
Steryl ester	16.8 ± 3.7	8.0 ± 3.7	8.3 ± 3.6
Unidentified neutral lipid	2.6 ± 0.5	3.0 ± 0.5	3.2 ± 0.7
Viscera			
Lipid contents	36.4 ± 11.8	45.4 ± 6.6	39.9 ± 4.6
Phosphatidylcholine	3.4 ± 1.0	2.6 ± 0.5	2.8 ± 0.2
Phosphatidylethanolamine	2.3 ± 0.7	1.9 ± 0.4	1.9 ± 0.2
Phosphatidylserine	0.2 ± 0.0 [ab]	0.4 ± 0.2 [a]	0.1 ± 0.0 [b]
Phosphatidylinositol	0.4 ± 0.1 [a]	0.2 ± 0.0 [ab]	0.1 ± 0.0 [b]
Sphingomyelin	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Unidentified polar lipids	0.8 ± 0.2	0.5 ± 0.3	0.4 ± 0.1
Total polar lipids	7.4 ± 2.1	6.0 ± 0.9	5.7 ± 0.6
Total neutral lipids	92.6 ± 2.1	94.0 ± 0.9	94.3 ± 0.6
Triacylglycerol	59.5 ± 3.4	61.3 ± 4.1	62.7 ± 1.9
Sterol	13.7 ± 2.7 [a]	13.8 ± 0.9 [a]	12.7 ± 1.3 [b]
Free fatty acids	13.4 ± 1.2	14.0 ± 1.1	14.1 ± 0.2
Steryl ester	6.1 ± 0.4	4.8 ± 0.7	4.8 ± 0.2

Results are means ± SD (n = 2). CAL, Caledonian 20 strain; FAT & LEAN, fat and lean families.

Table 4.4. Fatty acid compositions (percentage of total fatty acids) of flesh of 3 groups of Atlantic salmon at the start of the trial.

Parameters	Group		
	CAL	FAT	LEAN
Fatty acid			
14:0	3.7 ± 0.1	4.3 ± 0.2	4.3 ± 0.1
16:0	14.0 ± 0.4	14.8 ± 0.6	14.5 ± 0.4
18:0	3.0 ± 0.2	3.6 ± 0.2	3.2 ± 0.2
20:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Total saturated [1]	22.1 ± 0.4	24.3 ± 1.2	23.3 ± 0.8
16:1n-9	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1
16:1n-7	5.4 ± 0.2 [b]	6.6 ± 0.1 [a]	6.4 ± 0.1 [a]
18:1n-9	14.3 ± 0.4	14.7 ± 0.1	14.4 ± 0.1
18:1n-7	2.9 ± 0.1 [b]	3.3 ± 0.1 [a]	3.2 ± 0.1 [a]
20:1n11	0.9 ± 0.1 [a]	0.5 ± 0.1 [b]	0.6 ± 0.0 [b]
20:1n-9	9.1 ± 0.4 [a]	7.4 ± 0.0 [b]	7.2 ± 0.2 [b]
20:1n-7	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
22:1n11	8.5 ± 0.5 [a]	6.8 ± 0.1 [b]	6.6 ± 0.2 [b]
22:1n-9	0.9 ± 0.1 [a]	0.7 ± 0.0 [b]	0.7 ± 0.0 [b]
24:1n-9	0.7 ± 0.0	0.7 ± 0.1	0.7 ± 0.0
Total monoenes	43.7 ± 1.7 [a]	41.9 ± 0.2 [ab]	41.1 ± 0.5 [b]
18:2n-6	3.7 ± 0.1 [b]	4.3 ± 0.1 [a]	4.3 ± 0.1 [a]
18:3n-6	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:2n-6	0.3 ± 0.0 [b]	0.5 ± 0.0 [a]	0.5 ± 0.0 [a]
20:3n-6	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.0
20:4n-6	0.5 ± 0.1	0.6 ± 0.0	0.6 ± 0.1
22:4n-6	0.4 ± 0.1	0.1 ± 0.1	0.2 ± 0.2
22:5n-6	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Total n-6 PUFA	5.8 ± 0.1 [b]	6.2 ± 0.2 [a]	6.2 ± 0.3 [a]
18:3n-3	0.8 ± 0.0 [b]	1.1 ± 0.1 [a]	1.0 ± 0.0 [a]
18:4n-3	1.4 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
20:3n-3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-3	0.9 ± 0.0	1.0 ± 0.1	1.0 ± 0.1
20:5n-3	4.7 ± 0.2 [b]	5.1 ± 0.2 [a]	5.1 ± 0.1 [a]
22:5n-3	2.0 ± 0.1 [b]	2.2 ± 0.1 [ab]	2.3 ± 0.1 [a]
22:6n-3	17.8 ± 1.2	15.6 ± 0.9	17.2 ± 0.3
Total n-3 PUFA	27.6 ± 1.4	26.5 ± 1.3	28.3 ± 0.3
Total PUFA [2]	34.2 ± 1.3	33.9 ± 1.3	35.6 ± 0.6

Results are means ± SD (n = 2). [1] included 15:0, 17:0, up to 0.3%, [2] included 16:2, 16:3 and 16:4 up to 0.5%, CAL, Caledonian 20 strain; FAT & LEAN, fat and lean families; PUFA, polyunsaturated fatty acids.

Table 4.5. Fatty acid compositions (percentage of total fatty acids) of liver of 3 groups of Atlantic salmon at the start of the trial.

Parameters	Group		
	CAL	FAT	LEAN
Fatty acid			
14:0	2.3 ± 0.3	2.2 ± 0.1	2.2 ± 0.1
16:0	19.6 ± 1.2	19.8 ± 0.7	19.5 ± 0.4
18:0	3.6 ± 0.4 [b]	3.9 ± 0.2 [ab]	4.0 ± 0.1 [b]
20:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Total saturated [1]	26.7 ± 1.4	27.1 ± 1.0	27.0 ± 0.6
16:1n-9	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
16:1n-7	3.2 ± 0.2 [a]	2.8 ± 0.1 [b]	2.7 ± 0.2 [b]
18:1n-9	10.6 ± 0.5 [a]	9.2 ± 0.8 [b]	9.1 ± 0.4 [b]
18:1n-7	2.5 ± 0.1	2.4 ± 0.1	2.4 ± 0.1
20:1n-11	0.5 ± 0.1 [a]	0.3 ± 0.1 [b]	0.3 ± 0.1 [b]
20:1n-9	4.2 ± 0.5 [a]	2.4 ± 0.2 [b]	2.3 ± 0.2 [b]
20:1n-7	0.4 ± 0.3	0.2 ± 0.0	0.2 ± 0.0
22:1n11	2.9 ± 0.7 [a]	1.2 ± 0.1 [b]	1.1 ± 0.2 [b]
22:1n-9	0.4 ± 0.1 [a]	0.2 ± 0.1 [b]	0.1 ± 0.1 [b]
24:1n-9	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
Total monoenes	26.3 ± 1.9 [a]	20.4 ± 1.4 [b]	20.0 ± 1.1 [b]
18:2n-6	2.4 ± 0.1 [a]	1.8 ± 0.1 [b]	1.7 ± 0.1 [b]
18:3n-6	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1
20:2n-6	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
20:3n-6	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
20:4n-6	1.9 ± 0.3 [b]	2.4 ± 0.1 [a]	2.5 ± 0.1 [a]
22:4n-6	0.1 ± 0.1	n.d.	0.1 ± 0.1
22:5n-6	0.2 ± 0.0 [b]	0.4 ± 0.0 [a]	0.4 ± 0.0 [a]
Total n-6 PUFA	5.4 ± 0.3	5.4 ± 0.3	5.5 ± 0.1
18:3n-3	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
18:4n-3	0.6 ± 0.1 [a]	0.4 ± 0.1 [b]	0.4 ± 0.0 [b]
20:3n-3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-3	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
20:5n-3	5.4 ± 0.3 [b]	7.5 ± 0.3 [a]	7.4 ± 0.2 [a]
22:5n-3	1.8 ± 0.1	1.9 ± 0.1	1.8 ± 0.1
22:6n-3	32.1 ± 1.7 [b]	35.7 ± 1.4 [a]	36.3 ± 1.2 [a]
Total n-3 PUFA	41.2 ± 1.9	46.9 ± 1.6	47.2 ± 1.1
Total PUFA [2]	47.0 ± 2.1	52.5 ± 1.7	53.0 ± 1.1

Results are means ± SD (n = 2). [1] included 15:0, 17:0, up to 0.3%, [2] included 16:2, 16:3 and 16:4 up to 0.1%, CAL, Caledonian 20 strain; FAT & LEAN, fat and lean families; PUFA, polyunsaturated fatty acids.

Table 4.6. Fatty acid compositions (percentage of total fatty acids) of viscera of 3 groups of Atlantic salmon at the start of the trial.

Parameters	Group		
	CAL	FAT	LEAN
Fatty acid			
14:0	4.3 ± 0.1 [b]	4.8 ± 0.2 [a]	4.6 ± 0.1 [a]
16:0	12.4 ± 0.1 [b]	13.7 ± 0.2 [a]	13.2 ± 0.3 [a]
18:0	2.4 ± 0.1 [b]	3.0 ± 0.3 [a]	2.8 ± 0.2 [a]
20:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Total saturated [1]	20.3 ± 0.2 [b]	22.9 ± 0.7 [a]	22.2 ± 0.6 [a]
16:1n-9	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
16:1n-7	6.7 ± 0.0 [b]	7.6 ± 0.1 [a]	7.4 ± 0.1 [a]
18:1n-9	16.9 ± 0.5 [a]	16.3 ± 0.3 [ab]	16.0 ± 0.2 [b]
18:1n-7	3.2 ± 0.1 [b]	3.4 ± 0.1 [a]	3.4 ± 0.0 [a]
20:1n-11	0.5 ± 0.2	0.5 ± 0.1	0.6 ± 0.0
20:1n-9	11.7 ± 0.7 [a]	8.0 ± 0.2 [b]	7.8 ± 0.1 [b]
20:1n-7	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.0
22:1n11	10.4 ± 0.4 [a]	7.2 ± 0.3 [b]	7.0 ± 0.1 [b]
22:1n-9	1.1 ± 0.1 [a]	0.7 ± 0.0 [b]	0.7 ± 0.0 [b]
24:1n-9	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
Total monoenes	52.4 ± 1.5 [a]	45.5 ± 1.0 [b]	44.5 ± 0.5 [b]
18:2n-6	4.6 ± 0.1 [b]	4.9 ± 0.1 [a]	5.1 ± 0.1 [a]
18:3n-6	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.1
20:2n-6	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
20:3n-6	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
20:4n-6	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
22:4n-6	0.2 ± 0.2	0.3 ± 0.2	0.1 ± 0.2
22:5n-6	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
Total n-6 PUFA	6.3 ± 0.1 [b]	7.0 ± 0.1 [a]	7.0 ± 0.4 [a]
18:3n-3	0.9 ± 0.0 [b]	1.2 ± 0.0 [a]	1.2 ± 0.1 [a]
18:4n-3	1.7 ± 0.0 [b]	1.8 ± 0.1 [a]	1.9 ± 0.0 [a]
20:3n-3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-3	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.0
20:5n-3	3.9 ± 0.2 [b]	4.9 ± 0.2 [a]	5.2 ± 0.2 [a]
22:5n-3	2.0 ± 0.1 [b]	2.2 ± 0.1 [ab]	2.4 ± 0.1 [a]
22:6n-3	10.3 ± 1.1 [b]	12.0 ± 1.2 [ab]	13.1 ± 0.5 [a]
Total n-3 PUFA	20.0 ± 1.5 [b]	23.2 ± 1.7 [ab]	24.9 ± 0.7 [a]
Total PUFA [2]	27.3 ± 1.4 [b]	31.6 ± 1.6 [a]	33.3 ± 1.0 [a]

Results are means ± SD (n = 2). [1] included 15:0, 17:0, up to 0.3%, [2] included 16:2, 16:3 and 16:4 up to 0.6%, CAL, Caledonian 20 strain; FAT & LEAN, fat and lean families; PUFA, polyunsaturated fatty acids.

4.3.3 Growth performance of salmon during the 55 week ongrowing phase

The sizes (length and weight) of the fish at the initiation of the trial and after 32 and 55 weeks on the experimental diets are given in Table 4.7. The CAL strain fish were significantly smaller than the FAT and LEAN strains at the initiation of the trial. A significant difference in weight and length was found between fish fed the two diets after 32 weeks of the growth period. Significant differences were also observed between fish groups in weight, FCR, SGR, HSI and VSI at week 55. Regardless of diet, the CAL and FAT fish showed significantly lower FCR and HSI than the LEAN fish. In addition, the CAL strain had a significantly greater SGR than the FAT and LEAN fish (Table 4.7). Consistent with this, the LEAN fish had a higher HSI than the FAT fish, which conversely had a greater VSI than the LEAN fish. No significant differences, however, were observed in the HSI between the CAL and the LEAN fish.

4.3.4 Proximate compositions of flesh and viscera and flesh pigment content at week 55 of the growth period

There were significant effects of both diet and group on the protein, lipid and moisture contents of the flesh at week 55 (Table 4.8). There were also significant effects of group on all parameters, but only protein and moisture contents (not lipid and ash) were affected by diet in viscera. All the fish fed the FO diet had a lower protein content in their flesh than the fish fed the VO diet, with the groups ranked LEAN > CAL > FAT in both diets (Table 4.8). Regarding to the diets, the flesh lipid contents of CAL and FAT fish fed the FO diet were higher than the same groups fed the VO diet while, conversely, the LEAN fish fed the VO diet had a higher lipid content than the fish fed the FO diet. For the fish receiving the FO diet, the flesh lipid content can be ranked FAT > CAL $\geq$ LEAN, while for fish consuming the VO diet, the flesh lipid content can be ranked FAT $\geq$ LEAN > CAL (Table 4.8). Similarly, the flesh of CAL and FAT fish

fed the FO diet had lower moisture contents than fish fed the VO diet. The reverse situation was observed for flesh of the LEAN fish fed the FO diet, which had a similar moisture content to LEAN fish fed VO. The proximate composition of the viscera was different from flesh. Within the FO diet, the visceral protein content was ranked CAL > FAT > LEAN, whereas for fish fed the VO diet it was CAL > LEAN > FAT (Table 4.8). Lipid content of the viscera was affected by fish group (strain/family). Thus, the lipid contents of the viscera of the FAT and LEAN fish were higher than the CAL strain irrespective of diet. The viscera of all fish groups fed the FO diet had lower moisture content than those of fish fed the VO diet. The viscera of the CAL and LEAN strains had higher moisture contents than those of the FAT fish irrespective of diet.

Diet and group produced significant differences in the total pigment and the astaxanthin contents of the flesh at week 55 of the growth period (Table 4.8). All fish fed the FO diet had higher pigment levels than those fed the VO diet due to the VO diet had lower levels of pigment than the FO diet. The CAL strain, however, were found to have the highest total pigment and astaxanthin levels which were different from those of FAT and LEAN strains fed either diet (Table 4.8).

Table 4.7. Growth performance of 3 groups of Atlantic salmon.

Parameters	FO			VO			ANOVA		
	MH	FAT	LEAN	MH	FAT	LEAN	Diet	Strain	Diet×strain
Weight									
Initial (g)	52.8 ± 1.5	92.1 ± 4.9	80.6 ± 11.6	51.6 ± 2.1	84.3 ± 8.7	89.5 ± 12.9	0.994	0.001	0.408
32 weeks (kg)	1.44 ± 0.21	1.42 ± 0.22	1.39 ± 0.31	1.43 ± 0.18	1.23 ± 0.25	1.24 ±0.30	0.027	0.058	0.211
55 weeks (kg)	2.75 ± 0.13	3.18 ± 0.08	3.13 ± 0.13	2.84 ± 0.04	2.89 ± 0.04	3.03 ± 0.09	0.118	0.019	0.027
Length (cm)									
Initial	17.4 ± 0.2	20.1 ± 0.4	19.4 ± 0.9	17.3 ± 0.2	19.3 ± 0.6	20.0 ± 0.6	0.801	0.001	0.281
32 weeks	47.1 ± 0.2	46.4 ± 1.0	46.9 ± 0.4	46.2 ± 0.0	44.9 ± 0.7	45.1 ± 0.6	0.006	0.130	0.560
55 weeks	63.1 ± 1.6	63.9 ± 1.0	65.3 ± 0.5	62.8 ± 0.8	62.5 ± 0.7	63.7 ± 1.0	0.100	0.138	0.649
FCR	1.08 ± 0.06	1.08 ± 0.09	1.23 ± 0.03	1.06 ± 0.05	1.19 ± 0.03	1.39 ± 0.22	0.211	0.041	0.495
SGR	1.00 ± 0.01	0.93 ± 0.01	0.92 ± 0.02	1.01 ± 0.01	0.89 ± 0.00	0.91 ± 0.01	0.109	<0.0001	0.065
HSI	0.62 ± 0.04	0.59 ± 0.03	0.71 ± 0.00	0.61 ± 0.01	0.61 ± 0.00	0.70 ± 0.07	0.935	0.010	0.829
VSI	4.97 ± 0.32	5.17 ± 0.09	4.92 ± 0.32	4.82 ± 0.01	5.51 ± 0.18	4.82 ± 0.01	0.706	0.035	0.281

Results are means ± SD (n = 2). CAL, Caledonian 20 strain; FAT & LEAN, fat and lean families; FCR, feed conversion ratio; HSI, hepato-somatic index; SGR, specific growth rate; VSI, viscero- somatic index.

Table 4.8. Proximate compositions (percentage of wet weight) of tissues of 3 groups of Atlantic salmon at week 55.

Parameter	FO			VO			ANOVA		
	CAL	FAT	LEAN	CAL	FAT	LEAN	Diet	Group	Diet ×Group
Proximate compositions (%)									
Flesh									
Protein	19.3 ± 0.1	19.1 ± 0.2	19.4 ± 0.1	19.5 ± 0.1	19.2 ± 0.1	19.9 ± 0.3	0.006	0.001	0.081
Lipid	12.0 ± 0.2	12.8 ± 0.1	11.9 ± 0.1	10.9± 0.1	12.1± 0.1	12.0 ± 0.2	<0.000	<0.000	<0.000
Moisture	67.5 ± 0.3	66.1 ± 0.4	66.5 ± 0.2	68.2 ± 0.1	67.2 ± 0.1	66.0 ± 0.4	0.006	<0.000	0.001
Ash	1.7 ± 0.1	1.7 ± 0.0	1.8 ± 0.2	1.6 ± 0.0	1.6 ± 0.1	1.8 ± 0.0	0.099	0.002	0.124
Viscera									
Protein	13.9 ± 0.1	13.3 ± 0.1	12.7 ± 0.1	14.6 ± 0.1	12.7 ± 0.1	13.9 ± 0.1	<0.000	<0.000	<0.000
Lipid	25.8 ± 0.4	31.7 ± 0.7	28.0 ± 0.4	26.8 ± 0.1	29.4 ± 0.3	29.6 ± 0.3	0.702	<0.000	<0.000
Moisture	56.8 ± 0.4	53.0 ± 0.5	57.1 ± 0.9	57.0 ± 0.1	56.0 ± 0.4	57.2 ± 0.6	0.001	<0.000	0.001
Ash	1.2 ± 0.0	1.1 ± 0.0	1.2 ± 0.0	1.3 ± 0.0	1.2 ± 0.0	1.1 ± 0.1	0.207	<0.000	<0.000
Flesh pigment									
Total pigment	6.08 ± 0.40	5.29 ± 0.19	5.01 ± 0.20	5.24 ± 0.25	5.03 ± 0.14	4.17 ± 0.14	<0.000	<0.000	0.043
Astaxanthin	5.82 ± 0.41	5.11 ± 0.19	4.85 ± 0.21	4.96 ± 0.23	4.80 ± 0.14	3.99 ± 0.15	<0.000	<0.000	0.051

Results are means ± SD (n = 2). CAL, Caledonian 20 strain; FAT & LEAN, fat and lean families; FO, fish oil diet; VO, vegetable oil diet.

4.3.5 Lipid class compositions of salmon tissues at week 55

Neutral lipid accounted for approximately 50-60 %, 87 % and 92 % of total lipid in the liver, flesh and viscera, respectively, of salmon at the end of the ongrowing phase (Tables 4.9–4.11). There were significant effects of diet and fish group on the total polar and neutral lipid composition of the flesh, with polar and neutral lipids being higher and lower, respectively, in fish fed the FO diet compared to salmon fed the VO diet (Table 4.9). Regardless of diet, the levels of flesh total neutral lipid can be ranked LEAN> FAT > CAL , while the reverse situation was observed for the polar lipid content. Flesh PC and PE were also influenced by diet and group, with the CAL strain having a higher PC and PE content than the FAT and LEAN fish irrespective of diet (Table 4.9). The sterol content of the flesh was significantly affected by diet, with FAT and LEAN fish fed the VO diet having higher levels than the same fish fed the FO diet (Table 4.9). Diet and group did not appear to affect flesh levels of TAG and FFA.

The levels of total polar and neutral lipid of liver were affected by both diet and group, with all fish fed the FO diet having higher levels of all polar lipids. The levels of liver total neutral lipid can be ranked LEAN> FAT > CAL and FAT > LEAN > CAL in groups of fish fed the FO and VO diets, respectively. The polar lipid content was the reverse of the neutral lipid. All fish fed the FO diet also had higher PC PE, PI, PS and SM, and lower levels of neutral lipids, particularly TAG and FFA, than fish fed the VO diet (Table 4.10). In addition, the CAL strain had lower levels of liver TAG and FFA, and higher levels of all polar lipids (PC, PE, PI, PS and SM) than the FAT and LEAN fish irrespective of diet. In fish fed the VO diet, the CAL strain was found to have the highest liver sterol level, and LEAN fish showed higher levels of sterol than the FAT

fish irrespective of diet (Table 4.10). All fish fed the VO diet showed higher levels of an unknown neutral lipid than fish fed the FO diet.

There were significant effects of diet and group, and interaction, on TAG and sterol levels in the viscera (Table 4.11). Thus, in fish fed FO, visceral TAG levels were significantly higher in CAL fish compared to FAT and LEAN fish, whereas in fish fed VO the opposite was observed, with CAL fish displaying the lowest level of TAG. In contrast, visceral sterol levels showed the opposite pattern with levels being lowest in CAL fish when fed FO and highest when fed VO (Table 4.11). The pattern observed in TAG levels was reflected in total neutral lipid levels but not to the level of significance. Similarly, the pattern observed in visceral sterol content was mirrored by total polar lipids and individual polar lipid classes, but not reaching statistical significance (Table 4.11). Steryl esters were higher in CAL fish compared to FAT and LEAN fish, irrespective of diet.

Table 4.9. Lipid class composition (percentage of total lipid) of flesh of 3 groups of Atlantic salmon at week 55.

Lipid classes	FO			VO			ANOVA		
	CAL	FAT	LEAN	CAL	FAT	LEAN	Diet	Group	Diet × Group
Phosphatidylcholine	7.0 ± 0.5	6.2 ± 0.5	5.9 ± 0.2	6.3 ± 0.4	6.2 ± 0.4	4.9 ± 0.3	0.002	<0.000	0.091
Phosphatidylethanolamine	6.1 ± 0.4	5.7 ± 0.7	5.5 ± 0.2	5.5 ± 0.4	4.8 ± 0.4	4.6 ± 0.4	<0.000	0.009	0.722
Phosphatidylserine/ Phosphatidylinositol	1.1 ± 0.2	1.5 ± 0.1	1.2 ± 0.3	1.3 ± 0.4	0.8 ± 0.4	1.0 ± 0.4	0.226	0.817	0.035
Sphingomyelin	0.7 ± 0.2	0.7 ± 0.1	0.4 ± 0.0	0.5 ± 0.3	0.4 ± 0.1	0.4 ± 0.1	0.012	0.049	0.293
Lysophosphatidylcholine	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0.4 ± 0.3	0.708	0.196	0.173
Total polar lipids	15.3 ± 0.9	14.4 ± 1.1	13.3 ± 0.2	14.2 ± 0.4	12.4 ± 0.7	11.3 ± 0.9	<0.000	<0.000	0.380
Total neutral lipids	84.7 ± 0.9	85.6 ± 1.1	86.7 ± 0.2	85.8 ± 0.4	87.6 ± 0.7	88.7 ± 0.9	<0.000	<0.000	0.380
Triacylglycerol	72.6 ± 0.5	72.5 ± 2.8	74.1 ± 1.4	72.1 ± 1.5	73.7 ± 0.5	74.6 ± 1.1	0.521	0.046	0.542
Sterol	7.4 ± 0.3	7.4 ± 1.0	7.0 ± 0.5	7.4 ± 0.5	8.2 ± 0.5	9.1 ± 0.5	0.001	0.116	0.011
Free fatty acids	4.8 ± 0.5	5.7 ± 1.0	5.6 ± 1.0	6.3 ± 0.5	5.7 ± 0.5	5.0 ± 0.8	0.493	0.699	0.075
Steryl ester	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-	-	-

Results are means ± SD (n = 2). CAL, Caledonian 20 strain; FAT & LEAN, fat and lean families; FO, fish oil diet; n.d., not detected; VO, vegetable oil diet.

Table 4.10. Lipid class composition (percentage of total lipid) of liver of 3 groups of Atlantic salmon at week 55.

Lipid classes	FO			VO			ANOVA		
	CAL	FAT	LEAN	CAL	FAT	LEAN	Diet	Group	Diet×Group
Phosphatidylcholine	19.9 ± 1.3	18.1 ± 2.7	18.6 ± 0.4	19.0 ± 2.0	13.5 ± 1.2	15.6 ± 0.6	<0.000	0.001	0.085
Phosphatidylethanolamine/ Phosphatidylglycerol/ diposphatidylglycerol	15.2 ± 0.8	14.8 ± 1.1	13.1 ± 0.3	12.2 ± 1.5	12.6 ± 0.9	12.1 ± 1.8	<0.000	0.172	0.264
Phosphatidylserine	3.5 ± 0.4	3.1 ± 0.1	2.7 ± 0.2	2.8 ± 0.3	2.3 ± 0.3	2.5 ± 0.2	<0.000	0.003	0.077
Phosphatidylinositol	4.8 ± 0.7	4.8 ± 0.3	4.3 ± 0.1	4.0 ± 0.8	3.7 ± 0.4	3.4 ± 0.4	<0.000	0.129	0.770
Sphingomyelin	3.1 ± 0.1	2.6 ± 0.1	2.1 ± 0.4	2.5 ± 0.2	1.7 ± 0.3	1.4 ± 0.5	<0.000	<0.000	0.810
Lysophosphatidylcholine	0.5 ± 0.2	0.6 ± 0.2	tr.	tr.	tr.	tr.	-	-	-
Unidentified polar lipid	3.0 ± 0.3	2.1 ± 0.3	3.2 ± 0.3	2.9 ± 0.4	3.0 ± 0.3	3.0 ± 0.8	0.480	0.734	0.989
Total polar lipids	50.0 ± 2.4	46.7 ± 2.0	44.3 ± 0.6	43.9 ± 4.5	36.9 ± 2.9	38.3 ± 0.6	<0.000	0.001	0.260
Total neutral lipids	50.0 ± 2.4	53.3 ± 2.0	55.7 ± 0.6	56.1 ± 4.5	63.1 ± 2.9	61.7 ± 0.6	<0.000	0.001	0.260
Triacylglycerol	24.1 ± 2.8	26.7 ± 3.1	29.8 ± 0.8	30.8 ± 4.7	39.8 ± 3.8	37.6 ± 2.2	<0.000	0.001	0.116
Sterol	14.8 ± 0.5	14.4 ± 0.5	15.3 ± 0.2	15.1 ± 0.3	12.4 ± 1.0	12.8 ± 0.5	<0.000	<0.000	<0.000
Free fatty acids	2.2 ± 0.7	2.9 ± 0.9	2.0 ± 0.3	1.4 ± 0.5	1.7 ± 0.4	1.8 ± 1.0	0.016	0.349	0.365
Steryl ester	8.4 ± 2.9	8.5 ± 1.5	7.1 ± 1.2	6.7 ± 1.4	6.9 ± 2.4	7.2 ± 1.7	0.194	0.863	0.587
Unidentified neutral lipid	0.5 ± 0.1	0.8 ± 0.2	1.4 ± 0.2	2.1 ± 0.5	2.3 ± 0.5	2.3 ± 0.8	<0.000	0.029	0.224

Results are means ± SD (n = 2). CAL, Caledonian 20 strain; FAT & LEAN, fat and lean families; FO, fish oil diet; tr, < 0.5%; VO, vegetable oil diet.

Table 4.11. Lipid class composition (percentage of total lipid) of viscera of 3 groups of Atlantic salmon at week 55.

Lipid classes	FO			VO			ANOVA		
	CAL	FAT	LEAN	CAL	FAT	LEAN	Diet	Group	Diet × Group
Phosphatidylcholine	2.4 ± 0.2	3.0 ± 0.3	2.6 ± 0.4	2.9 ± 0.3	2.8 ± 0.5	2.8 ± 0.2	0.282	0.396	0.265
Phosphatidylethanolamine	2.4 ± 0.4	2.4 ± 0.2	3.1 ± 0.4	3.2 ± 0.7	2.9 ± 0.4	2.5 ± 0.4	0.260	0.865	0.017
Phosphatidylserine	0.5 ± 0.2	0.6 ± 0.1	0.6 ± 0.2	1.0 ± 0.3	0.7 ± 0.1	0.6 ± 0.2	0.105	0.053	0.011
Phosphatidylinositol	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.9 ± 0.2	0.8 ± 0.1	0.6 ± 0.1	0.008	0.054	0.135
Sphingomyelin	tr.	tr.	tr.	tr.	tr.	tr.	-	-	-
Total polar lipids	6.5 ± 0.8	7.3 ± 0.7	7.2 ± 1.3	8.6 ± 1.3	7.8 ± 1.2	6.7 ± 0.6	0.110	0.470	0.059
Total neutral lipids	93.5 ± 0.8	92.7 ± 0.7	92.8 ± 1.3	91.4 ± 1.3	92.2 ± 1.0	93.3 ± 0.6	0.110	0.470	0.059
Triacylglycerol	61.9 ± 3.3	60.8 ± 1.8	59.4 ± 3.8	45.9 ± 2.2	57.7 ± 1.1	57.5 ± 0.6	<0.000	0.001	<0.000
Ssterol	11.9 ± 1.8	13.4 ± 0.9	13.6 ± 0.8	17.9 ± 0.9	13.0 ± 0.5	13.8 ± 0.4	<0.000	0.009	<0.000
Free fatty acids	15.6 ± 1.0	17.9 ± 0.9	16.9 ± 1.7	22.3 ± 1.1	20.1 ± 0.5	20.2 ± 2.0	<0.000	0.772	0.007
Steryl ester	4.2 ± 0.5	1.3 ± 0.4	2.8 ± 0.5	5.2 ± 0.7	1.5 ± 0.5	1.8 ± 1.5	0.431	<0.000	0.0580

Results are means ± SD (n = 2). CAL, Caledonian 20 strain; FAT & LEAN, fat and lean families; FO, fish oil diet; tr, < 0.5 %; VO, vegetable oil diet.

4.3.6 Fatty acid compositions of salmon tissues at week 55

Significant effects of diet and group on the FA profiles of the salmon tissues were clearly observed at week 55 (Tables 4.12-4.14). Diet significantly affected FA composition in all tissues in all groups of fish, but quantitative differences between fish groups, although sometimes significant, were generally much more minor.

Total SFA, 14:0 and 16:0 levels in the flesh were significantly higher but levels of 18:0 and 20:0 were lower in fish fed the FO diet compared with fish fed the VO diet. Total SFA levels in flesh of the CAL and FAT fish were greater than those in LEAN fish irrespective of diet. In addition, the percentage of 16:0 was greater in the CAL and FAT strains fed the VO diet than in LEAN fish. The percentage of 16:0 in the CAL strain was higher than that of the FAT and LEAN fish (Table 4.12). Regardless of diet, flesh 18:0 content in the CAL strain was higher than that found in FAT and LEAN fish (Table 4.12). Flesh 16:1n-7, 18:1n-7, 20:1 and 22:1 were significantly higher in the fish fed the FO diet, but total MUFA and 18:1n-9 levels were higher in fish fed the VO diet (Table 4.12). Total n-6 PUFA, and especially 18:2n-6, levels in the flesh were significantly higher, but ARA significantly lower, in fish fed the VO diet compared to fish fed the FO diet (Table 4.12). In salmon fed the VO diet, flesh 18:2n-6 was significantly higher in LEAN fish than in CAL and FAT fish. Total PUFA and total n-3 PUFA contents of the flesh including the 18:4n-3, 20:4n-3, 22:5n-3, EPA and DHA, were higher in fish fed the FO diet, whereas the 18:3n-3 content of the fish fed the VO diet was about 3 times higher than in fish fed FO (Table 4.12). In fish fed the VO diet, flesh 18:3n-3 was significantly higher in LEAN fish compared to CAL and FAT fish (Table 4.12).

Table 4.12. Fatty acid composition (percentage of total fatty acids) of flesh of 3 groups of Atlantic salmon at week 55.

Parameters	FO			VO			ANOVA		
	CAL	FAT	LEAN	CAL	FAT	LEAN	Diet	Group	Diet × Group
Fatty acid									
14:0	4.5 ± 0.2	4.6 ± 0.1	4.5 ± 0.1	1.5 ± 0.2	1.5 ± 0.1	1.4 ± 0.0	<0.000	0.033	0.186
16:0	15.4 ± 0.3	15.0 ± 0.3	15.0 ± 0.3	13.8 ± 0.2	13.7 ± 0.2	13.1 ± 0.1	<0.000	<0.000	0.040
18:0	2.8 ± 0.0	2.7 ± 0.1	2.6 ± 0.1	3.2 ± 0.1	3.0 ± 0.1	2.9 ± 0.1	<0.000	<0.000	0.136
20:0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	<0.000	0.523	0.467
Total saturated[1]	23.1 ± 0.5	22.8 ± 0.5	22.6 ± 0.4	18.9 ± 0.3	18.8 ± 0.4	17.9 ± 0.3	<0.000	<0.000	0.148
16:1n-7	5.6 ± 0.1	5.6 ± 0.1	5.5 ± 0.1	1.9 ± 0.2	2.0 ± 0.1	1.7 ± 0.1	<0.000	<0.000	0.165
18:1n-9	18.5 ± 0.3	17.8 ± 0.7	17.7 ± 0.5	37.1 ± 0.6	37.3 ± 0.4	37.7 ± 0.3	<0.000	0.345	0.004
18:1n-7	3.5 ± 0.2	3.2 ± 0.1	3.2 ± 0.2	2.7 ± 0.2	2.6 ± 0.5	2.4 ± 0.2	<0.000	0.037	0.699
20:1n-9	7.3 ± 0.1	7.5 ± 0.1	7.6 ± 0.1	5.1 ± 0.1	5.1 ± 0.1	5.2 ± 0.1	<0.000	<0.000	0.005
20:1n-7	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	<0.000	0.311	0.380
22:1n11	7.2± 0.4	7.6 ± 0.2	7.6 ± 0.2	1.3 ± 0.2	1.5 ± 0.1	1.3 ± 0.1	<0.000	0.063	0.499
22:1n-9	0.9 ± 0.4	0.8 ± 0.1	0.9 ± 0.2	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.030	0.843	0.905
Total monoenes	44.3 ± 0.6	43.9 ± 0.3	43.7 ± 0.5	49.6 ± 0.3	49.8 ± 0.4	49.9 ± 0.3	<0.000	0.513	0.040
18:2n-6	5.0 ± 0.1	4.9 ± 0.1	4.9 ± 0.1	13.3 ± 0.3	13.3 ± 0.2	14.0 ± 0.2	<0.000	0.004	<0.000
18:3n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	<0.000	0.054	0.168
20:2n-6	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	1.5 ± 0.0	1.4 ± 0.0	1.6 ± 0.0	<0.000	<0.000	<0.000
20:3n-6	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.6 ± 0.0	0.6 ±0.0	0.6 ± 0.0	<0.000	0.066	0.845
20:4n-6	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.2 ±0.0	0.2 ± 0.0	<0.000	0.093	0.673
Total n-6 PUFA	6.6 ± 0.1	6.4 ± 0.1	6.5 ± 0.2	15.8 ± 0.3	15.7 ± 0.2	16.5 ±0.2	<0.000	<0.000	<0.000
18:3n-3	1.8 ± 0.1	1.9 ± 0.0	1.8 ± 0.1	6.2 ± 0.2	6.3 ± 0.1	6.7 ± 0.1	<0.000	<0.000	<0.000
18:4n-3	1.5 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	0.7 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	<0.000	<0.000	0.012
20:4n-3	1.6 ± 0.1	1.7 ± 0.0	1.7 ± 0.1	0.8 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	<0.000	<0.000	0.598
20:5n-3	5.5 ± 0.2	5.7 ± 0.2	5.8 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	1.7 ± 0.1	<0.000	0.173	0.011
22:5n-3	2.8 ±0.1	2.8 ± 0.1	2.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.0	0.9 ± 0.1	<0.000	0.498	0.123
22:6n-3	12.7 ± 0.6	12.8 ±0.4	13.1 ± 0.4	4.4 ± 0.2	4.2 ± 0.2	4.1 ± 0.2	<0.000	0.904	0.048
Total n-3 PUFA[2]	26.0 ± 1.0	26.9 ± 0.7	27.1 ± 0.7	15.7 ± 0.2	15.7 ± 0.4	15.7 ± 0.3	<0.000	0.075	0.087
Total PUFA	32.6 ± 1.1	33.3 ± 0.8	33.6 ± 0.8	31.5 ± 0.4	31.4 ± 0.6	32.2 ± 0.3	<0.000	0.010	0.350

Results are means ± SD (n = 2). [1] includes 15:0 and 22:0 up to 0.4 %; [2] includes 20:3n-3 up to 0.2 %; CAL, Caledonian 20 strain; FAT & LEAN, fat and lean families; FO, fish oil, PUFA, polyunsaturated fatty acids; VO, vegetable oil.

The effects of diet on the FA compositions of liver were similar to those described above for flesh. Irrespective of group, All FAs were significantly affected by diet except for 18:0, 20:1n-9 and 18:3n-6 (Table 4.13). Total SFA, 14:0 and 16:0 were significantly higher in livers of fish fed the FO diet. In fish fed the FO diet, the percentages of total SFA and 16:0 were significantly higher in CAL and FAT fish than LEAN fish (Table 4.13). In fish fed the VO diet, total SFA and 16:0 was highest in the CAL strain than the other groups (Table 4.13). Levels of 18:1n-9 and total MUFA in liver were highest in fish fed the VO diet, whereas percentages of 16:1n-7, 18:1n-7, 20:1n-7 and 22:1n-11 were higher in fish fed the FO diet (Table 4.13). In fish fed the FO diet, total MUFA and 16:1n-7, 18:1n-9, 18:1n-7, 20:1 and 22:1 were higher in LEAN fish than in CAL and FAT fish. In fish fed the VO diet, total MUFA and 18:1n-9 were higher in the FAT and LEAN fish than in the CAL strain (Table 4.13). The percentages of total n-6PUFA, 18:2n-6, 20:2n-6 and 20:3n-6 were higher but that of ARA lower, in livers of fish fed the VO diet compared to fish fed the FO diet (Table 4.13). Irrespective of diet, the levels of 18:2n-6 were slightly greater in LEAN fish than that of the CAL and FAT fish. In fish fed the FO diet, liver ARA levels were higher in CAL and FAT fish compared to LEAN fish whereas the ARA levels were lower in FAT fish compared to CAL and LEAN fish that were fed VO. The levels of total PUFA, total n-3PUFA, 18:4n-3, 20:4n-3, EPA and DHA were higher in livers of fish fed the FO diet, whereas levels of 18:3n-3 were higher in fish fed the VO diet (Table 4.13). Irrespective of diet, the proportion of 18:3n-3 was higher in LEAN fish than in CAL and FAT fish. In fish fed the FO diet, the levels of 20:4n-3 and 22:5n-3 were higher in the LEAN fish compared to those in the CAL and FAT fish whereas the levels of total PUFA, total n-3 PUFA and DHA were higher in the CAL and FAT fish (Table 4.13). In fish fed the VO diet, the percentages of total PUFA, total n-3 PUFA and DHA, were

higher in the CAL strain compared to the FAT and LEAN fish (Table 4.13).

Diet had a significant effect on the composition of all FAs in viscera (Table 4.14). Total SFA, 14:0 and 16:0 were significantly higher, but 18:0 and 20:0 were lower, in fish fed the FO diet compared to fish fed VO. Fish group, however, did not affect the content of SFAs in the viscera. Total MUFA and 18:1n-9 were higher in viscera of fish fed the VO diet, whereas levels of 16:1n-7, 18:1n-7, 20:1 and 22:1n-11 were higher in the fish fed the FO diet (Table 4.14). In fish fed the FO diet, the proportion of 18:1n-9 was significantly lower in LEAN fish compared to CAL and FAT fish. Total n-6PUFA, 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6 were significantly higher, but ARA was lower, in viscera of fish fed the VO diet compared to fish fed the FO diet (Table 4.14). There were no significant differences in the levels of 18:2n-6 and ARA between the groups of salmon. Total PUFA, total n-3PUFA, 18:4n-3, 20:4n-3, EPA and DHA were higher, but 18:3n-3 was lower, in viscera of salmon fed the FO diet compared to fish fed the VO diet (Table 4.14). Regardless of diet, total PUFA and total n-3 PUFA were higher in LEAN and FAT fish compared to the levels in the CAL strain. In fish fed the FO diet, the amount of EPA and DHA in LEAN and FAT fish was higher than that in the CAL fish, whereas the level of EPA and DHA in FAT fish was slightly higher than those level in CAL and LEAN fish when fed VO (Table 4.14).

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Table 4.13. Fatty acid composition (percentage of total fatty acids) of liver of 3 groups of Atlantic salmon at week 55.

Parameters	FO			VO			ANOVA		
	CAL	FAT	LEAN	CAL	FAT	LEAN	Diet	Group	Diet × Group
Fatty acid									
14:0	2.3 ± 0.1	2.1 ± 0.2	2.3 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	<0.000	0.189	0.010
16:0	19.3 ± 0.4	18.2 ± 0.6	14.9 ± 1.1	14.6 ± 0.8	12.5 ± 0.9	12.6 ± 0.8	<0.000	<0.000	<0.000
18:0	4.9 ± 0.1	5.2 ± 0.3	4.6 ± 0.4	5.0 ± 0.1	5.1 ± 0.2	4.7 ± 0.2	0.470	<0.000	0.282
Total saturated[1]	26.9 ± 0.5	25.8 ± 0.8	22.1 ± 1.5	20.6 ± 0.8	19.1 ± 1.0	18.3 ± 1.0	<0.000	<0.000	0.002
16:1n-7	2.7 ± 0.1	2.7 ± 0.2	3.3 ± 0.4	1.8 ± 0.2	1.8 ± 0.2	1.7 ± 0.1	<0.000	0.026	0.001
18:1n-9	11.1 ± 0.4	12.1 ± 0.8	15.6 ± 1.4	26.6 ± 3.1	31.3 ± 2.2	29.9 ± 1.8	<0.000	<0.000	0.007
18:1n-7	2.5 ± 0.0	2.6 ± 0.1	3.2 ± 0.2	2.2 ± 0.3	2.4 ± 0.3	2.2 ± 0.2	<0.000	<0.000	<0.000
20:1n-9	2.7 ± 0.1	3.2 ± 0.3	5.1 ± 0.3	3.2 ± 0.7	3.9 ± 0.7	3.7 ± 0.4	0.711	<0.000	<0.000
20:1n-7	0.1 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	<0.000	<0.000	0.187
22:1n11	1.4 ± 0.1	1.7 ± 0.2	2.4 ± 0.4	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	<0.000	<0.000	<0.000
22:1n-9	0.1 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	<0.000	<0.000	<0.000
Total monoenes	21.7 ± 0.7	23.8 ± 1.3	31.1 ± 2.5	35.2 ± 4.1	40.8 ± 3.3	39.0 ± 2.5	<0.000	<0.000	0.001
18:2n-6	2.9 ± 0.1	2.9 ± 0.1	3.0 ± 0.3	9.7 ± 0.8	10.1 ± 0.2	10.4 ± 0.2	<0.000	0.011	0.124
18:3n-6	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.262	0.326	0.488
20:2n-6	0.5 ± 0.0	0.5 ± 0.0	0.7 ± 0.0	1.5 ± 0.1	1.6 ± 0.2	1.9 ± 0.1	<0.000	<0.000	0.801
20:3n-6	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	<0.000	0.512	0.732
20:4n-6	2.4 ± 0.3	2.2 ± 0.2	1.9 ± 0.2	1.4 ± 0.2	1.2 ± 0.2	1.4 ± 0.2	<0.000	0.023	0.009
Total n-6 PUFA[2]	6.5 ± 0.3	6.3 ± 0.3	6.3 ± 0.4	14.0 ± 0.8	14.4 ± 0.3	15.3 ± 0.3	<0.000	0.088	0.007
18:3n-3	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.2	4.5 ± 0.2	4.3 ± 0.2	4.7 ± 0.2	<0.000	0.001	0.409
18:4n-3	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	<0.000	0.395	0.377
20:4n-3	1.6 ± 0.0	1.6 ± 0.1	2.3 ± 0.3	1.2 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	<0.000	<0.000	0.001
20:5n-3	8.1 ± 0.1	8.6 ± 0.3	8.2 ± 0.4	5.5 ± 0.7	4.7 ± 0.4	5.2 ± 0.4	<0.000	0.783	0.004
22:5n-3	3.8 ± 0.2	3.6 ± 0.2	4.4 ± 0.3	2.7 ± 0.4	1.9 ± 0.2	2.1 ± 0.1	<0.000	<0.000	<0.000
22:6n-3	29.8 ± 0.9	28.6 ± 1.0	23.7 ± 1.9	15.4 ± 2.6	12.6 ± 1.5	13.1 ± 1.1	<0.000	<0.000	0.001
Total n-3 PUFA[3]	45.0 ± 1.1	44.1 ± 1.1	40.5 ± 1.5	30.2 ± 3.6	25.7 ± 2.4	27.4 ± 1.4	<0.000	<0.000	0.012
Total PUFA	51.4 ± 1.1	50.4 ± 1.2	46.8 ± 1.5	44.2 ± 3.4	40.2 ± 2.6	42.8 ± 1.6	<0.000	0.003	0.004

Results are means ± SD (n = 2). [1] includes15:0 and 20:0 up to 0.4 %; [2] includes 22:5n-6 up to 0.2 %; [3] includes 20:3n-3 up to 0.2 %, CAL, Caledonian 20 strain; FAT & LEAN, fat and lean families; FO, fish oil diet; PUFA; polyunsaturated fatty acids; VO, vegetable oil diet.

Table 4.14. Fatty acid composition (percentage of total fatty acids) of viscera of 3 groups of Atlantic salmon at week 55.

Parameters	FO			VO			ANOVA		
	CAL	FAT	LEAN	CAL	FAT	LEAN	Diet	Group	Diet × Group
Fatty acids									
14:0	4.7 ± 0.2	4.7 ± 0.1	4.8 ± 0.2	1.5 ± 0.1	1.5 ± 0.1	1.3 ± 0.1	<0.000	0.409	0.064
16:0	14.2 ± 0.5	13.8 ± 0.4	13.7 ± 0.3	12.0 ± 0.2	12.0 ± 0.4	11.9 ± 0.2	<0.000	0.168	0.278
18:0	2.7 ± 0.1	2.7 ± 0.1	2.6 ± 0.1	3.0 ± 0.1	3.0 ± 0.1	2.9 ± 0.1	<0.000	0.046	0.346
20:0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	<0.000	0.621	0.106
Total saturated[1]	22.1 ± 0.8	21.6 ± 0.6	21.6 ± 0.5	17.1 ± 0.3	17.1 ± 0.5	16.8 ± 0.3	<0.000	0.162	0.523
16:1n-7	5.8 ± 0.1	5.7 ± 0.1	5.8 ± 0.0	1.7 ± 0.1	1.8 ± 0.1	1.6 ± 0.2	<0.000	0.294	0.007
18:1n-9	19.7 ± 0.4	19.5 ± 0.5	18.5 ± 0.3	38.7 ± 0.4	38.4 ± 0.6	38.7 ± 0.4	<0.000	0.012	0.002
18:1n-7	3.1 ± 0.1	2.9 ± 0.1	3.0 ± 0.1	2.4 ± 0.2	2.4 ± 0.2	2.2 ± 0.1	<0.000	0.103	0.057
20:1n-9	7.8 ± 0.1	7.9 ± 0.0	8.0 ± 0.1	5.4 ± 0.0	5.2 ± 0.1	5.4 ± 0.1	<0.000	0.004	0.005
20:1n-7	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	<0.000	0.440	0.097
22:1n11	7.3 ± 0.2	7.5 ± 0.1	7.7 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	<0.000	0.004	0.004
22:1n-9	0.8 ± 0.0	0.8 ± 0.1	0.7 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.126	0.352	0.718
Total monoenes	45.7 ± 0.3	45.6 ± 0.3	45.0 ± 0.2	51.3 ± 0.2	50.9 ± 0.6	50.8 ± 0.3	<0.000	0.001	0.193
18:2n-6	5.6 ± 0.2	5.6 ± 0.2	5.5 ± 0.2	14.7 ± 0.2	14.5 ± 0.3	14.7 ± 0.2	<0.000	0.540	0.233
20:2n-6	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	1.5 ± 0.0	1.4 ± 0.0	1.6 ± 0.1	<0.000	<0.000	<0.000
20:3n-6	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	<0.000	0.010	0.033
20:4n-6	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	<0.000	0.539	0.814
Total n-6 PUFA[2]	7.3 ± 0.3	7.3 ± 0.3	7.2 ± 0.2	17.2 ± 0.2	16.9 ± 0.3	17.4 ± 0.2	<0.000	0.180	0.059
18:3n-3	1.9 ± 0.1	2.0 ± 0.1	1.9 ± 0.1	6.5 ± 0.1	6.6 ± 0.2	6.7 ± 0.1	<0.000	0.097	0.041
18:4n-3	1.7 ± 0.0	1.8 ± 0.1	1.8 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	<0.000	<0.000	0.176
20:4n-3	1.7 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	0.8 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	<0.000	0.005	0.136
20:5n-3	5.3 ± 0.0	5.5 ± 0.1	5.5 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	<0.000	0.016	0.424
22:5n-3	2.9 ± 0.1	2.8 ± 0.1	3.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	<0.000	0.303	0.145
22:6n-3	11.2 ± 0.3	11.4 ± 0.4	11.8 ± 0.3	3.2 ± 0.2	3.5 ± 0.2	3.3 ± 0.2	<0.000	0.004	0.016
Total n-3 PUFA[3]	24.9 ± 0.7	25.5 ± 0.7	26.1 ± 0.6	14.5 ± 0.4	15.1 ± 0.6	15.0 ± 0.4	<0.000	0.002	0.233
Total PUFA	32.2 ± 0.9	32.8 ± 0.7	32.4 ± 0.5	31.7 ± 0.4	32.0 ± 0.8	32.4 ± 0.5	0.002	0.004	0.668

Results are means ± SD (n = 2). [1] includes 15:0 up to 0.4 %; [2] includes 18:3n-6 and 22:5n-6 up to 0.2 %; [3] includes 20:3n-3 up to 0.2 %; CAL, Caledonian 20 strain; FAT & LEAN, fat and lean families; FO, fish oil diet; PUFA, polyunsaturated fatty acids; VO, vegetable oil diet.

4.3.7 Proximate composition, lipid content and fatty acid composition during the finishing diet phase

After week 55, all fish were transferred onto a finishing diet containing a decontaminated FO as the sole source of lipid for a further 24 weeks. By the end of the 24-week period, there were significant differences in the proximate composition of the flesh as a consequence of the fish group (strain/family) and the diet fed prior to the switch to finishing diet (Table 4.15). The protein content of the flesh was significantly higher in fish previously fed the FO diet than those previously fed the VO diet. Regardless of the diet previously fed to the fish, the flesh protein content of CAL and LEAN fish was significantly higher than that of the FAT fish. In fish previously fed the FO diet, the crude lipid content of flesh was highest in the FAT fish and lowest in the CAL strain, whereas for fish previously fed the VO diet, the crude lipid content was highest in the CAL strain and lowest in LEAN fish (Table 4.15). In order to investigate restoration of HUFA (ARA, EPA and DHA) and PUFA (18:2n-6 and 18:3n-3) levels, flesh samples were taken at weeks 8, 16 and 24 of the wash-out, finishing diet phase. Flesh lipid contents increased throughout the finishing diet period. Thus, the total lipid content of the flesh analysed by Folch method, which was about 8-9 % at week 8, increased to 12-13 % by week 24 (Tables 4.16-4.18). Previous diet did not affect the lipid contents of flesh but regardless of the diet previously fed to the fish, flesh lipid in FAT fish was significantly higher than that of CAL and LEAN fish at weeks 8 and 16 (Tables 4.16-4.17). However, previous diet and fish group had no effects on lipid content of the flesh at week 24 of the finishing diet period (Table 4.18).

Both previous diet and fish group significantly affected the FA compositions as determined at weeks 8 and 16 of the finishing diet phase but, by week 24, only total

SFA, 16:0, total MUFA, 20:1n-9, 20:3n-6, ARA, EPA and DHA were affected by both previous diet and fish group (Table 4.18). For fish previously fed the VO diet, the percentages of 18:2n-6 and 18:3n-3 in flesh decreased while the levels of ARA, EPA and DHA increased over the finishing diet period (Figure 4.1). In all groups during the finishing diet phase, the levels of 18:2n-6, 18:3n-3 ARA, EPA and DHA in fish previously fed VO approached those of fish fed FO throughout (Figure 4.1). The magnitude of these changes were greatest in the initial 8 weeks of the finishing diet and, in this period, the changes were greater in the LEAN and FAT fish than in the CAL strain (Figure 4.1). For fish previously fed FO, the finishing diet phase had few effects on the levels of 18:2n-6, 18:3n-3, ARA, EPA and DHA between the groups of fish. The levels of 18:2n-6, 18:3n-3 and ARA in fish previously fed VO, however, were slightly changed between weeks 16 and 24, particularly in the FAT and LEAN fish. At 24 weeks, irrespective of diet previously fed, the level of EPA in the CAL strain was higher than that of FAT and LEAN fish, and the level of DHA in LEAN fish was higher than that of CAL strain and FAT fish.

Table 4.15. Proximate composition (percentage of wet weight) of flesh of 3 groups of Atlantic salmon after 24 weeks feeding on the fish oil finishing diet.

Compositions	FO			VO			ANOVA		
	CAL	FAT	LEAN	CAL	FAT	LEAN	Diet	Group	Diet × Group
Protein	18.7 ± 0.0	18.4 ± 0.1	18.8 ± 0.1	18.6 ± 0.1	18.3 ± 0.2	18.7 ± 0.1	0.022	<0.000	0.454
Lipid	13.5 ± 0.1	14.7 ± 0.1	14.5 ± 0.0	15.4 ± 0.1	15.1 ± 0.2	14.1 ± 0.2	<0.000	<0.000	<0.000
Moisture	66.4 ± 0.1	65.9 ± 0.2	65.7 ± 0.1	64.7 ± 0.1	65.3 ± 0.2	65.9 ± 0.1	<0.000	0.043	<0.000
Ash	1.7 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	0.211	0.831	0.201

Results are means ± SD (n = 2). CAL, Caledonian 20 strain; FAT & LEAN, fat and lean families; FO, fish oil diet; VO, vegetable oil diet.

Table 4.16. Lipid content (percentage of wet weight) and fatty acid composition (percentage of total fatty acids) of flesh of 3 groups of Atlantic salmon after 8 weeks feeding on the fish oil finishing diet.

Parameters	FO			VO			ANOVA		
	CAL	FAT	LEAN	CAL	FAT	LEAN	Diet	Group	Diet × Group
Lipid content	8.7 ± 0.6	8.9 ± 0.4	8.4 ± 0.7	8.7 ± 1.0	9.2 ± 0.4	7.6 ± 04	0.478	0.001	0.130
Fatty acid									
14:0	5.4 ± 0.3	5.3 ± 0.2	5.4 ± 0.2	3.5 ± 0.1	4.9 ± 0.6	4.3 ± 0.7	<0.000	0.001	<0.000
16:0	16.6 ± 0.7	16.2 ± 0.2	16.1 ± 0.5	15.1 ± 0.4	16.3 ± 0.5	15.2 ± 0.8	<0.000	0.052	0.009
18:0	3.4 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.5 ± 0.2	3.4 ± 0.3	3.2 ± 0.1	0.016	<0.000	0.580
20:0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	<0.000	<0.000	<0.000
Total saturated	26.0 ± 1.1	25.3 ± 0.3	25.2 ± 0.7	22.7 ± 0.5	25.2 ± 0.9	23.3 ± 1.6	<0.000	0.071	0.005
16:1n-7	6.6 ± 0.1	6.5 ± 0.1	6.6 ± 0.1	4.3 ± 0.2	5.9 ± 0.6	5.3 ± 0.7	<0.000	<0.000	<0.000
18:1n-9	16.0 ± 0.3	16.2 ± 1.3	16.0 ± 0.3	27.8 ± 1.0	20.8 ± 3.9	22.7 ± 1.8	<0.000	<0.000	<0.000
18:1n-7	3.3 ± 0.1	3.3 ± 0.1	3.3 ± 0.1	2.9 ± 0.1	3.3 ± 0.2	3.1 ± 0.3	0.002	0.025	0.014
20:1n-9	5.5 ± 0.3	5.7 ± 0.2	5.7 ± 0.1	4.6 ± 0.1	5.3 ± 1.0	5.1 ± 0.4	<0.000	0.009	0.187
20:1n-7	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.003	<0.000	0.132
22:1n11	5.4 ± 0.4	5.5 ± 0.4	5.6 ± 0.1	2.2 ± 0.1	4.4 ± 1.7	3.7 ± 0.4	<0.000	<0.000	0.001
22:1n-9	0.6 ± 0.1	0.7 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.7 ± 0.1	0.6 ± 0.1	0.080	0.017	0.694
Total monoenes	38.6 ± 1.0	39.1 ± 0.8	38.9 ± 0.4	43.2 ± 0.8	41.4 ± 1.0	41.4 ± 1.2	<0.000	0.096	0.011
18:2n-6	4.5 ± 0.1	4.7 ± 0.5	4.5 ± 0.1	9.7 ± 0.3	6.4 ± 1.8	7.5 ± 1.0	<0.000	0.002	<0.000
18:3n-6	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	<0.000	0.360	0.388
20:2n-6	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	1.1 ± 0.0	0.7 ± 0.2	0.8 ± 0.1	<0.000	<0.000	0.001
20:3n-6	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	<0.000	0.011	0.086
20:4n-6	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	<0.000	0.276	0.002
Total n-6 PUFA[1]	6.8 ± 0.3	6.8 ± 0.5	6.6 ± 0.1	12.2 ± 0.3	8.5 ± 2.0	9.9 ± 1.1	<0.000	<0.000	<0.000
18:3n-3	1.5 ± 0.0	1.6 ± 0.2	1.5 ± 0.0	4.1 ± 0.1	2.6 ± 0.9	3.3 ± 0.5	<0.000	<0.000	<0.000
18:4n-3	1.4 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	0.9 ± 0.0	1.3 ± 0.3	1.2 ± 0.4	<0.000	<0.000	0.007
20:4n-3	1.5 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.1 ± 0.0	1.3 ± 0.2	1.3 ± 0.0	<0.000	0.001	0.035
20:5n-3	7.4 ± 0.4	7.4 ± 0.3	7.3 ± 0.1	5.1 ± 0.3	6.3 ± 0.7	5.9 ± 0.5	<0.000	0.006	0.002
22:5n-3	3.7 ± 0.3	3.7 ± 0.4	3.7 ± 0.1	2.5 ± 0.1	3.0 ± 0.3	3.0 ± 0.2	<0.000	0.067	0.026
22:6n-3	12.8 ± 1.0	12.7 ± 0.9	13.4 ± 0.4	7.6 ± 0.7	10.1 ± 1.6	10.5 ± 0.4	<0.000	<0.000	0.002
Total n-3 PUFA[2]	28.6 ± 1.7	28.8 ± 1.5	29.3 ± 0.6	21.8 ± 1.1	24.8 ± 2.1	25.4 ± 0.6	<0.000	0.001	0.015
Total PUFA	35.3 ± 1.9	35.6 ± 1.0	35.8 ± 0.6	34.0 ± 0.9	33.4 ± 1.6	35.3 ± 0.6	0.022	0.074	0.305

Results are means ± SD (n = 2); [1] includes 22:5n-6 up to 0.2 %; [2] includes 20:3n-3 up to 0.3 %; CAL, Caledonian 20 strain; FAT & LEAN, fat and lean families; FO, fish oil diet; PUFA, polyunsaturated fatty acids; VO, vegetable oil diet.

Table 4.17. Lipid content (percentage of wet weight) and fatty acid composition (percentage of total fatty acids) of flesh of 3 groups of Atlantic salmon after 16 weeks feeding on the fish oil finishing diet.

Parameters	FO			VO			ANOVA		
	CAL	FAT	LEAN	CAL	FAT	LEAN	Diet	Group	Diet × Group
Lipid content	11.5 ± 0.6	13.5 ± 0.9	11.2 ± 1.1	11.0 ± 0.7	12.2 ± 1.0	11.5 ± 0.4	0.089	<0.000	0.084
Fatty acid									
14:0	5.4 ± 0.1	5.4 ± 0.1	5.2 ± 0.3	4.0 ± 0.1	5.2 ± 0.5	4.9 ± 0.3	<0.000	0.004	0.001
16:0	16.2 ± 0.4	16.0 ± 0.4	15.6 ± 0.5	15.3 ± 0.2	15.7 ± 0.4	15.7 ± 0.4	0.005	0.518	0.015
18:0	3.3 ± 0.1	3.3 ± 0.1	3.3 ± 0.2	3.4 ± 0.1	3.4 ± 0.1	3.3 ± 0.1	0.008	0.386	0.564
20:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	<0.000	<0.000	<0.000
Total saturated	25.5 ± 0.6	25.2 ± 0.6	24.7 ± 1.0	23.3 ± 0.2	24.5 ± 0.1	24.5 ± 0.6	<0.000	0.350	0.004
16:1n-7	6.7 ± 0.1	6.5 ± 0.1	6.5 ± 0.3	4.9 ± 0.1	5.6 ± 0.3	6.0 ± 0.2	<0.000	<0.000	<0.000
18:1n-9	15.7 ± 0.3	16.5 ± 0.8	15.6 ± 0.6	24.4 ± 0.3	20.9 ± 1.1	19.1 ± 0.9	<0.000	<0.000	<0.000
18:1n-7	3.3 ± 0.1	3.3 ± 0.1	3.3 ± 0.1	3.0 ± 0.1	3.3 ± 0.3	3.3 ± 0.1	0.096	0.225	0.023
20:1n-9	5.2 ± 0.1	5.4 ± 0.1	5.4 ± 0.4	4.5 ± 0.1	4.5 ± 0.1	4.7 ± 0.3	<0.000	0.055	0.361
20:1n-7	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.002	0.461	0.893
22:1n11	5.2 ± 0.1	5.3 ± 0.3	5.5 ± 0.3	3.0 ± 0.1	3.7 ± 0.4	4.1 ± 0.3	<0.000	<0.000	0.002
22:1n-9	0.5 ± 0.0	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.0	0.112	0.015	0.064
Total monoenes	37.7 ± 0.7	38.6 ± 0.3	38.0 ± 1.0	41.3 ± 0.3	39.5 ± 0.7	38.8 ± 1.1	<0.000	0.004	<0.000
18:2n-6	4.4 ± 0.0	4.7 ± 0.5	4.4 ± 0.1	8.2 ± 0.3	6.7 ± 0.7	6.1 ± 0.3	<0.000	<0.000	<0.000
18:3n-6	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.015	0.230	0.010
20:2n-6	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.9 ± 0.0	0.7 ± 0.1	0.6 ± 0.0	<0.000	0.014	<0.000
20:3n-6	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	<0.000	0.048	0.162
20:4n-6	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	<0.000	<0.000	<0.000
Total n-6 PUFA[1]	5.6 ± 0.1	6.8 ± 0.5	6.7 ± 0.1	10.6 ± 0.3	9.2 ± 0.8	8.5 ± 0.4	<0.000	<0.000	<0.000
18:3n-3	1.4 ± 0.0	1.5 ± 0.2	1.4 ± 0.1	3.2 ± 0.1	2.6 ± 0.3	2.2 ± 0.2	<0.000	<0.000	<0.000
18:4n-3	1.5 ± 0.0	1.6 ± 0.0	1.5 ± 0.1	1.1 ± 0.0	1.3 ± 0.1	1.3 ± 0.0	<0.000	<0.000	0.015
20:4n-3	1.5 ± 0.1	1.6 ± 0.0	1.6 ± 0.1	1.2 ± 0.0	1.3 ± 0.0	1.4 ± 0.1	<0.000	<0.000	0.001
20:5n-3	8.5 ± 0.4	7.9 ± 0.2	7.9 ± 0.3	6.5 ± 0.2	7.1 ± 0.4	7.3 ± 0.4	<0.000	0.769	<0.000
22:5n-3	4.3 ± 0.2	4.0 ± 0.1	4.2 ± 0.2	3.2 ± 0.1	3.6 ± 0.1	3.8 ± 0.2	<0.000	0.005	<0.000
22:6n-3	12.8 ± 0.6	12.6 ± 0.2	13.8 ± 0.4	9.2 ± 0.2	10.6 ± 0.5	11.9 ± 0.5	<0.000	<0.000	<0.000
Total n-3 PUFA[2]	30.2 ± 1.1	29.4 ± 0.5	30.6 ± 0.5	24.8 ± 0.4	26.8 ± 1.0	28.2 ± 1.1	<0.000	<0.000	<0.000
Total PUFA	36.8 ± 1.2	36.2 ± 0.6	37.3 ± 0.5	35.4 ± 0.5	36.1 ± 0.9	36.7 ± 1.2	0.025	0.022	0.202

Results are means ± SD (n = 2); [1] includes 22:5n-6 up to 0.2 %; [2] includes 20:3n-3 up to 0.3 %; CAL, Caledonian 20 strain; FAT & LEAN, fat and lean families; FO, fish oil diet; PUFA, polyunsaturated fatty acids; VO, vegetable oil diet.

Table 4.18. Lipid content (percentage of wet weight) and fatty acid composition (percentage of total fatty acids) of flesh of 3 groups of Atlantic salmon after 24 weeks feeding on the fish oil finishing diet.

Parameters	FO			VO			ANOVA		
	CAL	FAT	LEAN	CAL	FAT	LEAN	Diet	Group	Diet × Group
Lipid content	11.9 ± 0.4	12.9 ± 1.1	12.7 ± 0.7	13.1 ± 0.6	12.7 ± 1.0	12.1 ± 0.5	0.585	0.423	0.018
Fatty acids									
14:0	5.6 ± 0.2	5.5 ± 0.2	5.3 ± 0.3	4.8 ± 0.2	4.7 ± 0.1	4.6 ± 0.5	<0.000	0.119	0.825
16:0	16.3 ± 0.5	16.0 ± 0.5	15.5 ± 0.3	15.5 ± 0.2	15.4 ± 0.5	15.3 ± 0.5	0.001	0.039	0.257
18:0	3.4 ± 0.1	3.4 ± 0.2	3.2 ± 0.1	3.4 ± 0.0	3.3 ± 0.1	3.3 ± 0.1	0.351	0.064	0.351
20:0	0.1 ± 0.0	0.10 ± .0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	<0.000	0.714	0.471
Total saturated	25.9 ± 0.7	25.5 ± 0.6	24.7 ± 0.8	24.3 ± 0.5	24.0 ± 0.8	23.9 ± 1.0	<0.000	0.028	0.340
16:1n-7	6.9 ± 0.1	6.6 ± 0.3	6.7 ± 0.3	5.8 ± 0.2	5.7 ± 0.1	6.0 ± 0.4	<0.000	0.211	0.371
18:1n-9	14.9 ± 0.4	16.0 ± 1.7	15.9 ± 0.9	20.1 ± 0.5	20.8 ± 0.7	19.7 ± 2.6	<0.000	0.315	0.433
18:1n-7	3.5 ± 0.2	3.4 ± 0.1	3.4 ± 0.2	3.3 ± 0.1	3.2 ± 0.2	3.2 ± 0.2	0.003	0.238	0.871
20:1n-9	4.7 ± 0.2	4.9 ± 0.2	5.0 ± 0.2	4.2 ± 0.0	4.6 ± 0.3	4.4 ± 0.2	<0.000	<0.000	0.201
20:1n-7	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.015	0.071	0.186
22:1n11	4.9 ± 0.1	5.0 ± 0.7	5.0 ± 0.1	3.5 ± 0.0	3.8 ± 0.4	3.9 ± 0.7	<0.000	0.345	0.440
22:1n-9	0.6 ± 0.1	0.5 ± 0.0	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	0.5 ± 0.1	0.910	0.126	0.233
Total monoenes	36.5 ± 0.5	37.4 ± 0.8	37.6 ± 0.7	38.4 ± 0.3	39.7 ± 0.3	38.6 ± 1.2	<0.000	0.002	0.123
18:2n-6	4.4 ± 0.1	4.5 ± 0.3	4.7 ± 0.3	6.6 ± 0.2	6.7 ± 0.4	6.5 ± 1.2	<0.000	0.808	0.193
18:3n-6	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.502	0.240	0.671
20:2n-6	0.4 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	0.7 ± 0.0	0.6 ± 0.0	0.7 ± 0.1	<0.000	0.472	0.370
20:3n-6	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.001	0.032	0.589
20:4n-6	0.8 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	<0.000	<0.000	0.028
Total n-6 PUFA[1]	6.6 ± 0.1	6.8 ± 0.4	7.0 ± 0.3	9.0 ± 0.2	8.9 ± 0.4	8.8 ± 1.2	<0.000	0.762	0.242
18:3n-3	1.2 ± 0.0	1.4 ± 0.2	1.5 ± 0.2	2.4 ± 0.1	2.5 ± 0.2	2.3 ± 0.6	<0.000	0.301	0.068
18:4n-3	1.4 ± 0.1	1.5 ± 0.1	1.5 ± 0.0	1.2 ± 0.0	1.3 ± 0.1	1.3 ± 0.1	<0.000	0.298	0.923
20:4n-3	1.6 ± 0.0	1.5 ± 0.1	1.5 ± 0.1	1.4 ± 0.0	1.4 ± 0.1	1.4 ± 0.1	<0.000	0.066	0.825
20:5n-3	8.9 ± 0.1	8.3 ± 0.4	8.3 ± 0.3	7.9 ± 0.2	7.2 ± 0.3	7.5 ± 0.5	<0.000	<0.000	0.472
22:5n-3	4.5 ± 0.1	4.4 ± 0.4	4.5 ± 0.2	4.1 ± 0.1	3.8 ± 0.2	4.0 ± 0.4	<0.000	0.051	0.663
22:6n-3	13.2 ± 0.2	12.9 ± 0.8	13.4 ± 0.6	11.0 ± 0.1	10.9 ± 0.6	11.9 ± 1.1	<0.000	0.041	0.339
Total n-3 PUFA[2]	31.0 ± 0.4	30.2 ± 1.6	30. ± 0.8	28.3 ± 0.3	27.4 ± 1.0	28.6 ± 1.6	<0.000	0.069	0.696
Total PUFA	37.6 ± 0.4	37.0 ± 1.3	37.8 ± 0.7	37.3 ± 0.4	36.3 ± 0.9	37.5 ± 0.6	0.116	0.01	0.718

Results are means ± SD (n = 2); [1] includes 22:5n-6 up to 0.2 %; [2] includes 20:3n-3 up to 0.3 %; CAL, Caledonian 20 strain; FAT & LEAN, fat and lean families; FO, fish oil diet; PUFA, polyunsaturated fatty acids; VO, vegetable oil diet.

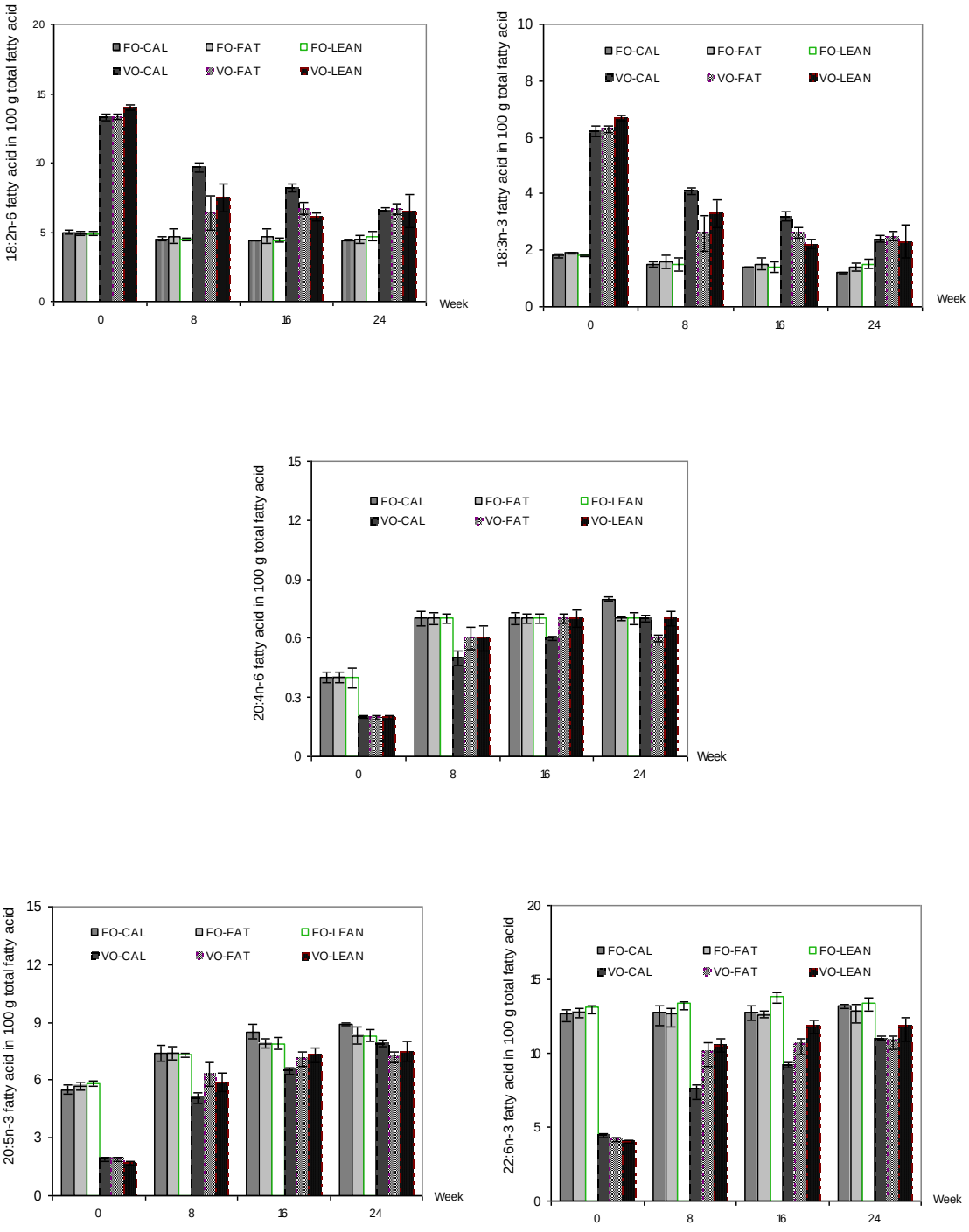

Figure 4.1. Trend of the 18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3 and 22:6n-3 contents in flesh of 3 groups of Atlantic salmon at week 0 (week 55 of growth phase) and weeks 8, 16 and 24 of the finishing diet period.

4.4 Discussion and conclusion

4.4.1 Growth performance and proximate composition during the 55 week growth phase

The aim of this trial was to investigate the effects of dual substitution of FM with 45 % PPs, 25% FM in diet and 100 % replacement of FO with VO on the growth and tissue composition of three groups (one strain and two families) of large Atlantic salmon. Plant proteins were included at ~45% of the formulation with the major components being maize and wheat glutens and soy meal concentrate. These plant proteins have been shown to promote good growth in previous FM replacement studies (Kaushik 2004; Espe *et al.* 2006). The oil component added to the base diet was either 100% northern FO or a blend of VOs (RO/PO/CO; (5/3/2 by weight). RO and PO are commonly used in commercial fish feed without detrimental effect to fish growth (Torstensen *et al.* 2000; Ng *et al.* 2003; Bell *et al.* 2003b; Mourente & Bell 2006; Karalazos 2007). The oil from *Camelina sativa* is low in saturates, and has high levels of EFA 18:2n-6, 18:3n-3 and 18:1n-9 and 20:1n-9 (Putnam *et al.* 1993; Zubr 2003). The results from present study have shown that neither diet had any detrimental effects on the growth of the fish, with diet having no significant effects on FCR, SGR, HSI and VSI. Significant differences in fish final weight were attributable to fish strain and there was also an interaction between strain and diet. After 55 weeks and irrespective of diet, the FAT and LEAN groups had slightly higher weight and length than the CAL strain but this was likely related to these fish being larger at the initiation of the trial. Specifically, the FAT and LEAN fish fed the FO diet were larger than the same fish fed VO diet while the CAL strain fed the VO diet was larger than the same strain fed the FO diet. This result suggested that the CAL strain may have performed more effectively with the high VO diet. In addition, the CAL strain actually showed higher SGR and

lower FCR than either of the FAT or LEAN families showing that, although they attained smaller size, growth and feed efficiency were better in the commercial CAL strain than in the FAT and LEAN families that represented extremes of flesh adiposity. The results from the present study implied that growth rate of the three groups of salmon was at least partially under genetic control. The SGR value in the present study was slightly lower and the FCR value was higher than the those of previous study with Atlantic salmon fed diets containing high energy/lipid formulations from start feeding until harvest size (Torstensen *et al.* 2005; Berntssen *et al.* 2005), the levels of FM and VOs inclusion in the present study, however, were slightly lower and higher than those in the study of those previous studies. The SGR and FCR values in the present study were in the same range as those of studies which used high energy/lipid diets in Atlantic salmon for 40 weeks (Bell *et al.* 2004; Torstensen *et al.* 2004a) Hence, in the present trial, the diet FA contents and compositions in the VO diet appeared to supply sufficient energy requirement, EFA and FAs for catabolism and protein sparing and, therefore, there were no measurable detrimental effects on fish growth. Although the dietary lipid levels used in the present study (32%) were higher than those used previously (Bell *et al.* 2004; Torstensen *et al.* 2005) the findings are in agreement with the earlier studies in that the complete replacement of FO with VO from different sources, in both short and long duration feeding trials, had no effect on the growth and FCR of several species of fish (Bell *et al.* 2003a; Regost *et al.* 2004; Torstensen *et al.* 2004b, 2005; Fonseca-Madrigal *et al.* 2005; Fountoulaki *et al.* 2009). In addition, the diet formulations used here, consisting of ~45% PPs, were in the same range as those used in studies that demonstrated inclusion of single PPs up to 40 % had no negative effects on fish growth (Nengas *et al.* 1996; Refstie *et al.* 2001; Lozano *et al.* 2007). Other studies, exploring the use of PP blends replacing up to 70 % of dietary FM, also had no detrimental effects

on growth of salmonids but replacement of FO with PP blends more than 70% showed growth reduction (Gomes *et al.* 1995; Espe *et al.* 2006; Oo *et al.* 2007; Torstensen *et al.* 2008). The VO diet used in the present study had much lower levels of DHA and EPA (1.5 and 1.2% of total FAs) compared to the diets used by Torstensen *et al* (2008) that only had 70% FO replacement and percentages of DHA and EPA of 3.0 and 2.5, respectively. This 50% reduction in dietary n-3 LC-PUFA resulted in no apparent reduction in growth compared to the salmon produced by Torstensen *et al* (2008). Little information has so far been available in the literature concerning the effects of dietary lipid and genetic factors on fish growth and body composition e.g. catfish (*Clarias gariepinus*) (Hoffman *et al.* 1995), rainbow trout (Quillet *et al.* 2005) and anadromous and landlocked Atlantic salmon (Rollin *et al.* 2003; Peng *et al.* 2003). In a study of dietary lipid and strain on growth in two strains of Atlantic salmon parr (landlocked or anadromous) fed 5 different diets varying in relative ratios of OO, LO and sunflower oil for 8 weeks, the authors found that growth rate was higher in the landlocked strain (Rollin *et al.* 2003). Similarly, landlocked fry fed on commercial diet revealed higher growth compared to anadromous fry (Peng *et al.* 2003). These findings imply the genetic influences to growth performances of fish.

Dietary lipid influences the deposition of lipid in fish tissues, and the positive correlation between dietary lipid and the deposition of lipid in the flesh has been reported (Bell *et al.* 1998). In the present study, the lipid contents of the flesh and viscera increased as the size of the fish increased. In addition, the lipid contents of the three fish groups were not only reliant on the dietary lipid content but also depend on the strain/families as seen in the present study. These findings are supported by a number of studies with Atlantic salmon where the deposition of fat was seen to increase as the fish grew larger, which coincided with an increasing lipid content in the feed as

pellet size of feed increased as seen in the present study (Jobling & Johansen 2003; Torstensen *et al.* 2005). The inclusion of VOs in the diet has affected flesh composition in different ways in different studies. Replacement of FO with PO or RO decreased fat deposition in the flesh of Atlantic salmon (Bell *et al.* 2001, 2002) whereas substitution of FO with LO, RO or a blend of both oils (Bell *et al.* 2003b, 2004; Menoyo *et al.* 2005) or SO (Ruyter *et al.* 2006), had no significant effect on flesh lipid content. Other studies have also reported that the composition of muscle was unaffected by the source of dietary oils (Bendiksen *et al.* 2003; Regost *et al.* 2004). However, a number of authors have reported that protein and moisture contents increased with decreasing dietary lipid content that was influenced by the replacement of FO with VO (Bell *et al.* 2001; Torstensen *et al.* 2004a; Karalozos 2007). Genotype also has been stated as influencing body composition of four African sharptooth catfish strains of larvae; domestic, netherlands, wild and golden, fed on commercial diet. The authors summarised that strain strongly influenced body total lipid content, but not body protein content, and the body total lipid content was associated with growth rate (Hoffman *et al.* 1995). Similar to rainbow trout, there were high genetic correlations between differently aged fish for lipid traits (lipid body weight, percent muscle and body lipid) but not for the protein traits (protein body weight, percent muscle and body protein) (Tobin *et al.* 2006). In a study where rainbow trout had been selected on carcass adiposity using a non-invasive fat meter, the strains (Fat line, control line and Lean line) were all different from each other, based on muscle lipid content analysed by Soxhlet, in the order Fat line > Control line > Lean line (Quillet *et al.* 2005). No details of diet were provided except that it was a commercial diet containing 22% lipid, which would likely contain predominantly FO. However, in comparison to salmon in present study, where differences were observed in VSI and HSI % lipid, due both to strain and interaction of

strain and diet, no differences in those VSI and HSI % lipid between the strains was observed in rainbow trout (Quillet *et al.* 2005). The present study has indicated that strain/family of fish is also a factor in tissue lipid deposition that can be affected by replacement of FO by VO. By the end of the growth phase in the present study, the lipid contents of flesh of CAL and FAT fish were lower when fed the VO diet than when fed the FO diet. Diet did not appear to affect the lipid content of the viscera but the values for VSI and HSI were lower and higher, respectively, in LEAN fish compared to FAT and CAL fish fed either diet. The values of VSI and HSI were related to the TAG contents in these tissues reflecting increased fat deposition in liver of the LEAN family and in viscera of the FAT family. In addition, the protein contents of the flesh and viscera of the CAL and LEAN fish, and moisture contents of flesh and viscera of the CAL and FAT fish were greater in fish fed the VO diet compared to those fed the FO diet. These results suggest that use of different dietary oils may have different effects on fat deposition and resultant tissue compositions in different strains/families of salmon.

Pigmentation of flesh is an important factor in salmon flesh quality (Regost *et al.* 2004), which is affected by several factors including fish size and age, sexual maturation, diet composition including the type of oil (Bjerkeng *et al.* 1999) and melting point of oils (Hardy *et al.* 1987), dietary concentration of carotenoids, and genetic factors (Storebakken & No 1992; Lie 2001). The studies of Bell *et al*, (2001) and Torstensen *et al* (2005), however, reported that replacement of FO with VO in Atlantic salmon diets did not affect the flesh pigment or astaxanthin content. In contrast to this, the previous studies observed significant differences in the pigment content of salmon fillets fed different oils (Hardy *et al.* 1987; Bjerkeng *et al.* 1999). In the present study, the levels of astaxanthin and total pigment in the flesh were higher in fish fed the FO diet, but this was due to an error in formulation that resulted in lower levels of

astaxanthin in the VO diet, notably the 9 mm pellet feeds. However, the CAL strain showed a higher pigment content than the FAT and, especially, the LEAN fish indicating that pigment deposition may also be affected by strain/family. The pigment content did not appear to be directly related to flesh lipid levels.

4.4.2 Tissue lipid class and fatty acid compositions during the growth phase

In the present study, significant differences effects of both diet and strain/family on lipid class compositions of fish tissues were observed. Flesh and liver total neutral lipids being higher in fish fed the VO diet compared to salmon fed the FO diet. Moreover, TAG composition in flesh was unaffected by feeding VO diet. The results was in contrast to results in Chapter 3 and a previous study by Nanton *et al* (2007) showing that salmon fed a VO blend diet decreased total neutral and TAG. The duration of the present study was over one year while the trial 1 (Chapter 3) was carried out for 10 weeks. Possibly the different of duration of the study, environment, growth stage and strain of fish can explain these dissimilar findings. Apart from the dietary VO, specific tissue and other factors including growth stage, season as well as genetics also affects tissue lipid content (Sargent *et al.* 2002). Thus, the LEAN and FAT fish showed greater total neutral lipid in both flesh and liver tissues than the CAL strain, regardless of diet. The CAL strain had a high PC and PE which corresponded to that of the CAL strain that contained high total polar lipid content in flesh. TAG contents of flesh were affected insignificantly but the pattern observed in TAG levels was reflected in total neutral lipid levels. However, TAG content in liver and viscera tissues were affected by diet and groups of fish. Irrespective of diet, the FAT and LEAN fish had liver TAG contents higher than that of the CAL fish. In viscera tissue, the TAG content of the CAL fish was highest and lowest when fed the FO and VO, respectively. The sterol content of the

flesh, liver and viscera tissues of the fish fed the VO diet was also higher than that of the same fish fed the FO diet. Hence, the FAT and LEAN fish were genetically selected based on fat adiposity which corresponded to the finding of the present study in that those two fish showed higher total neutral lipid and lower polar lipid content, including individual polar lipid classes, than that of the CAL fish in specific tissues.

In the present study, significant differences in both diet and strain/family on tissue FA compositions were observed. The flesh and viscera FA composition mainly was influenced by diet while liver FA composition was affected by both diet and strain/family. The results were in accordance with a study on FA compositions in landlocked and anadromous Atlantic salmon parr fed diet containing blends of OO, LO and sunflower oil and the authors stated that different diets had a more pronounced effect on carcass FA composition than that of the different strains (Rollin *et al.* 2003). It is well known that the FA compositions of fish tissues are a reflection of the dietary FA composition (Bell *et al.* 2001; Fonseca-Madrigal *et al.* 2005; Ruyter *et al.* 2006; Hansen *et al.* 2008; Pettersson *et al.* 2009). In the present study, all flesh FAs were significantly affected by diet, which is expected, and was seen in similar studies with salmon fed high levels of VO (Tocher *et al.* 2003c; Bell *et al.* 2004; Torstensen *et al.* 2005). Specifically, increasing levels of dietary VO resulted in elevated levels of 18:1n-9, 18:2n-6 and 18:3n-3 and decreased n-3HUFA in tissues (Bell *et al.* 2003b; Izquierdo *et al.* 2005; Mourente *et al.* 2005). Similarly, in the present study, the relative proportions of 18:1n-9, 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6 and 18:3n-3 were higher, and the percentages of 14:0, 16:0, 16:1n-7, 18:1n-7, 22:1n-11, ARA, 18:4n-3, 20:4n-3, EPA, 22:5n-3 and DHA lower, in the liver, flesh and viscera of fish fed the VO diet compared to fish fed the FO diet. The higher proportions of 18:1n-9, 18:2n-6 and 18::3 n-3 coincided with higher levels of total neutral lipid and TAG in the flesh and liver of fish

fed the VO diet. In contrast, 16:0, EPA and DHA are FAs that tend to dominate in polar lipids (Jobling *et al.* 2008), and so higher levels of 16:0, 20:4n-3, EPA, 22:5n-3 and DHA in the flesh and liver of fish fed the FO diet coincided with higher levels of total polar lipids. In the present study, however, there were no dietary effects on the relative proportions of polar and neutral lipids in visceral tissue. Different tissues, however, respond to changes in dietary FA composition in different ways or to different extents. Atlantic salmon store large amounts of lipid in their flesh and, therefore, dietary FA compositions can drastically affect flesh FA compositions in salmon (Bell *et al.* 2004). Torstensen *et al* (2000, 2004b) concluded that both red and white muscle were markedly more influenced by the FA composition of the diet compared to the liver. Similarly, in the present study, the FA profiles of the flesh and viscera reflected dietary FA composition more than the FA profiles of liver. The percentages of total SFA in all the tissues were similar, but the proportions of total MUFA (especially 18:1n-9), 18:2n-6 and 18:3n-3 in the flesh and the viscera were higher than those in the liver. In contrast to this, the levels of total PUFA, total n-3PUFA and the HUFA, ARA, EPA and DHA, were higher in the liver than in flesh and viscera. Therefore, the differences in the FA compositions between the tissues can be partially explained by the distribution of neutral and polar lipids in the different tissues. Hence, the higher proportion of total MUFA and the lower proportion of n-3 HUFA in the flesh and viscera reflected the higher proportion of TAG. In particular, the levels of DHA reflected the lower proportion of polar lipids and the lipid class composition of the flesh and the viscera. Previously, the belly flap, myosepta and visceral fat (adipose rich, lipid storage tissues), containing high proportions of TAG and lower proportions of polar lipid, had lower levels of n-3 HUFA compared to the levels found in lean muscle tissue (Nanton *et al.* 2007).

Differences in FA composition between tissues and the diet suggest selective utilisation/retention of FA in the tissues (Torstensen *et al.* 2004a; Karalazos 2007). Moreover, the selective utilisation or retention of FAs for energy production via beta-oxidation depends upon the relative abundance of individual FAs in the diet (Henderson 1996; Bell *et al.* 2003b, 2004). A number of authors have reported that SFA, MUFA, 18:2n-6 and 18:3n-3 are the preferred FA sources for energy production via β-oxidation in salmonids (Henderson & Tocher 1987; Kiessling & Kiessling 1993; Henderson 1996). In the present study, the 16:0 and total SFA were significantly affected by both group (strain/family) and diet in flesh and liver tissues but no effects of group on those FA were observed in viscera tissue. Fish group was less affected on viscera FA composition as the distributions of individual FAs of fish fed either FO or VO diet showed a similar pattern in all fish. Moreover, no significant differences in total SFA was found which corresponded to the total neutral lipid were similar in all fish groups. In flesh and liver tissues, the CAL strain having higher saturates and lower monoenes than the LEAN and FAT fish, irrespective of diet. The higher levels of total SFA and monoenes indicate higher deposition of theses FAs in tissues for energy (Sargent *et al.* 1989). Generally this was balanced by the LEAN fish having higher n-3 LC-HUFA levels, although the levels of the n-3 HUFA were not significantly different from the CAL strain in flesh but lower than the CAL strain in liver tissues. Tissues with less neutral and more polar lipid would tend to accumulate more LC-PUFA and less saturates and monoenes (Sargent *et al.* 2002).

Total neutral lipid in flesh and liver tissues of the LEAN fish was not significantly different from those of the CAL and FAT at week 32 (data not shown) but the LEAN fish were higher in total neutral and lower in total polar lipids at the end of week 55, and this corresponds to the lipid content of flesh extracted by the soxhlet

method. However, the TAG composition of the LEAN fish was not higher than that of the CAL and FAT fish. This result probably indicates the differences between fish groups in lipid composition.

All fish groups utilised 18:1n-9 and 22:1n-11 from VO diet but selectively utilised 22:1n-11 and retained the 18:1n-9 when fed a FO diet, compared to the levels of these FAs in diet. The levels of 18:1n-9 in liver tissue of the CAL strain were lower than the levels of the FAT and LEAN fish fed either diet, and the levels of 22:1n-11 in the CAL strain fed FO diet were also lower than that of the two groups fed the same diet suggesting the CAL strain had high efficiency to utilise these FAs for energy production to support growth, as better SGR and FCR was seen in the CAL strain. It has been reported that increasing the level of VO inclusion in the diet stimulates the beta-oxidation capacity of Atlantic salmon (Stubhaug *et al.* 2005b). Moreover, the lower levels of 18:1n-9 in the flesh and the viscera of fish fed the VO diet, and in the livers of the fish fed both diets, compared to the levels of 18:1n-9 found in the diet suggests that 18:1n-9 is selected for catabolism. The lower levels of 22:1n-11 in all the tissues of fish fed FO compared to dietary levels also indicates this FA is a preferred substrate for beta-oxidation as previously suggested (Henderson & Sargent 1981). The findings from this study support those of earlier studies as the percentages of 22:1n-11 were lower than those of 18:1n-9 in the flesh and the viscera of FO-fed fish although they were present in the FO diet in similar quantities. The level of flesh and liver 18:2n-6 and 18:3n-3 of the LEAN fish fed VO diet were higher than that of the CAL and FAT fish. Moreover, the quantities of 18:2n-6 and 18:3n-3 present in all the tissues were lower than those in the diets, except for the levels of 18:3n-3 in the flesh and the viscera of the fish fed the FO diet, which suggests the selective utilisation of these FAs, especially in fish fed VO. These results were similar to the findings of previous studies showing that

the levels of 18:2n-6 and 18:3n-3 in the muscle or liver were less than those in the diet because these FAs were selectively utilised and metabolised for energy (Henderson & Sargent 1985, Caballero *et al*. 2002). The percentages of DHA in the tissues were greater than those found in the diet in the present study, with the liver showing the highest percentage, indicating that DHA was selectively deposited. Similarly, higher levels of EPA were found in the livers of fish fed both diets and in the flesh and viscera of the fish fed the VO diet than the levels of EPA and DHA found in the diet. Previous studies have reported that when FAs were provided at low concentrations in the diets, they tended to be preferentially retained or deposited in tissue (Henderson 1996; Caballero *et al.* 2002). In the present study, EPA was found to be preferentially deposited in the tissues of fish fed the VO diet and preferentially utilised in the fish fed the FO diet.

The FA compositions of salmon tissues are determined by both the type of dietary lipid and by the ability of the individual fish species to modify that dietary input via both catabolism and biosynthesis pathways (Torstensen *et al.* 2000; Bell *et al.* 2001, 2002). In addition to this, a number of previous studies have reported that fatty acyl desaturation and elongation activities in the liver are stimulated by the inclusion of VO in the diet of salmonids (Tocher *et al.* 2000, 2001; Bell *et al.* 2001, 2002). In the present study, tissue EPA and DHA may be obtained by their direct absorption from the diet and partially supplemented by synthesis by desaturation and elongation of 18:3n-3 (Henderson 1996; Caballero *et al.* 2002). The effects of dietary lipid and strain on the PUFA composition and conversion of C18 to ARA, EPA and DHA have been investigated previously (Hoffman *et al.* 1995; Pickova *et al.* 1999; Rollin *et al.* 2003; Peng *et al.* 2003). The LEAN fish had high levels of 18:2n-6 (LA) and 18:3n-3 (ALA), and higher levels of biosynthesis products 20:2n-6 and 20:4n-3 than the CAL and FAT

fish in both diets suggesting higher capacity of elongase in the LEAN fish. In contrast, the CAL strain had lower levels of flesh n-3 LC-PUFA than the LEAN fish fed FO diet but higher than the LEAN fish in VO diet. Again, the higher levels of LC n-3 HUFA indicate higher capacity for ALA and LA conversion (Sargent *et al.* 2002; Tocher 2003).Therefore, the above results strongly indicate that the conversions of dietary ALA to n-3 LC-PUFA and LA to n-6 LC-PUFA were carried out in both CAL and LEAN fish greater than in the FAT fish and thus the genetics influenced tissue FA composition in the present study.

4.4.3 Effect of a FO finishing diet and washout phase on flesh lipid and fatty acid composition

Salmon is a species with oily flesh that can deliver significant levels of n-3 LC-PUFA to consumers and it is important to maintain a positive image for the species in this respect as the health benefits of increased dietary n-3 LC-PUFA, EPA and DHA, intake in humans are well documented (Calder 2007; Park *et al.* 2009; Hibbeln & Davis 2009). One method of n-3 LC-PUFA restoration is to dilute or wash out the VO-derived FAs using a FO finishing diet and this has been shown to be an effective process (Bell *et al.* 2003b, 2004, 2005b; Torstensen *et al.* 2005). However, the effect of different fish strains, that have proven differences in flesh lipid deposition, may affect the efficacy of the wash out process. Regarding the EPA + DHA in fish previously fed VO diet at the end of week 24, the LEAN fish showed better levels of EPA + DHA, and more rapid restoration of LC-PUFA, than those of the FAT and CAL fish, with the FAT having lowest levels of EPA + DHA. The LEAN fish showed greater levels of EPA + DHA (19%) than that of those FAT and CAL fish (18% and 16%) after feeding the finishing diet for 16 weeks. This suggests that the LEAN strain were able to accumulate n-3 LC-PUFA more rapidly than the other two strains. This study does suggest that LC-

PUFA uptake and deposition may be under genetic control and could be exploited to maintain or restore LC-PUFA, following a period of dietary restriction, by selecting for this trait.

Regarding the restoration of EFA including LA, ALA, ARA, EPA and DHA of the fish previously fed VO diet compared to the same fish fed a FO diet throughout. In the present study, after feeding the VO diet for 55 weeks, the 18:2n-6 and 18:3n-3 contents of the flesh were approximately 2.5- and 3-fold higher, respectively, than the flesh of fish fed the FO diet. After consuming the finishing diet for 24 weeks, the concentrations of 18:2n-6 and 18:3n-3 in the fish fed the VO diet were still higher than those of fish fed the FO diet throughout, although the levels of 18:2n-6 and 18:3n-3 had declined by around 70 % and 60 %, respectively. The results were in accordance with the findings of earlier studies where the levels of 18:2n-6 and 18:3n-3 after feeding VO to Atlantic salmon (Bell *et al.* 2004), red sea bream, (Glencross *et al.* 2003), gilthead sea bream (Izquierdo *et al.* 2005) and European sea bass (Montero *et al.* 2005) were substantially reduced, although not completely, by feeding a finishing diet. After consuming the finishing diet for 24 weeks, the ARA levels in fish previously fed the VO diet had recovered to about 86 -100 % of the values found in the fish fed the FO diet with the LEAN and CAL fish showing better recovery than the FAT fish. After feeding the VO diet for 55 weeks, the EPA concentration in flesh was significantly lower, approximately one third, of that in flesh of fish fed the FO diet. After feeding the finishing diet for 24 weeks, the levels of EPA were still lower than the fish that had been fed the FO diet throughout, but recovery was almost 90 %, with the LEAN and CAL fish showing a higher recovery than the FAT fish. A similar result was observed in the concentration of DHA which was found to decrease by two thirds after consuming the VO diet for 55 weeks, compared with the fish fed the FO diet. After feeding the

finishing diet for 24 weeks, the level of DHA in the tissues remained lower than the fish fed the FO diet throughout, although the percentage recovery of the DHA was approximately 83 – 90 %, with the LEAN and CAL fish showing a greater recovery than the FAT fish. An earlier study by Bell *et al* (2003a) working with Atlantic salmon found complete restoration of EPA in 4 weeks and that DHA was restored within 12 weeks after feeding the finishing diet. This experiment, however, was performed over a shorter period, and used smaller fish, than the present study. Other, longer, experiments conducted in Atlantic salmon have demonstrated that about 80 % of EPA and DHA were restored (Bell *et al.* 2003b; 2004; 2005a). In European sea bass, complete restoration of EPA and DHA was found when a partial replacement of FO with VO in diet was used before switching to a FO finishing diet (Montero *et al.* 2005). However, the complete restoration of EPA and DHA was not found in gilthead seabream when fed a partial replacement diet with VO prior to feeding the finishing diet (Fountoulaki *et al.* 2009). The fish species, fish age and the time when the finishing diet is given, as well as the concentrations of EPA and DHA in the finishing diet, may explain the different degrees of EFA restoration in tissues.

In conclusion, the results from this study suggest that the total replacement of FO with VO and the partial substitution of FM with PPs in the diets had no negative effects on the growth performance of the experimental fish, including FCR, SGR, HSI and VSI. Switching the diet to one that contained FO as the sole source of lipid as a means of improving the nutritional quality of the fillet when given to the fish for 24 weeks resulted in a 67 - 72 % decrease of 18:2n-6 and a 52 - 63 % reduction in the level of 18:3n-3. The level of ARA in the LEAN strain was successfully restored up to 100 %. The levels of EPA and DHA were restored approximately 90 % in all the three groups of fish. A novel aspect of this study was the use of different strains/families to

investigate differences in growth and FA deposition/retention. Differences were seen between strains due to diet as well as interactions between strain and diet. The differences observed suggested that genetics had an influence on lipid deposition and metabolism and that selective breeding programmes could be used to select salmon families that retained more n-3 LC-PUFA in their flesh, particularly when fed diets low in these FAs.

Chapter 5 - Influence of genotype/phenotype on the effects of alternative diets in Atlantic salmon (*Salmo salar* L.): Effects of total replacement of fish oil and partial replacement of fishmeal on gene expression in liver

5.1 Introduction

Expansion of the aquaculture industry coupled with overfishing of wild marine stocks has placed increasing pressure on the supply of FM and FO, which has now become unsustainable. Farmed-fish feed, however, still relies heavily on marine fishery products for its main ingredients. It has been estimated that in 2006, the aquaculture sector consumed 3.7 million tonnes of FM and 0.85 million tonnes of FO, this represents 68 % and 89 % of global FM and FO, respectively, production in 2006 (Tacon & Metian 2008). A number of studies have investigated the replacement of FM and FO with products derived from plant sources and from these studies, it is clear that a substantial reduction in the FM inclusion can be achieved without compromising fish growth. Complete replacement of FM by plant protein ingredients, however, can reduce growth (Gomes *et al.* 1995; Kaushik *et al.* 1995, 2004; Hardy 1996; Watanabe *et al.* 1998). In addition, replacing FO with VO has been used successfully without compromising growth in a variety of fish species including Atlantic salmon (Bell *et al.* 2004; Torstensen *et al.* 2005; Petropoulos *et al.* 2008; Fountoulaki *et al.* 2009; Pettersson *et al.* 2009), although flesh n-3 HUFA content and liver FA metabolism are both affected (Torstensen *et al.* 2000; Stubhaug *et al.* 2005a).

Recently, the influence of substituting FO with VO in the diet of salmonids and the subsequent expression of genes has been examined in liver (Jordal *et al.* 2005, 2006; Leaver *et al.* 2008; Panserat *et al.* 2009), muscle (Johansen & Overturf 2006) and intestine (Froystad *et al.* 2008). FO replacement with VO has been reported to increase cholesterol and HUFA biosynthesis (Leaver *et al.* 2008; Taggart *et al.* 2008; Panserat *et al.* 2009). In addition, the removal of FO from the diet of rainbow trout was found to increase expression of genes of lipid biosynthesis and decrease expression of lipid catabolism genes (Panserat *et al.* 2008).

In the present study, the effects of feeding a diet with 100 % of FO substituted with VO on liver gene expression were investigated in two families of Atlantic salmon, the FAT and LEAN groups, which were fully described in the previous chapter. The aims of this study were to investigate VO replacement and subsequent effects of phenotype/genotype on hepatic gene expression, particularly those related to lipid metabolism. Liver was specifically selected for study as the main organ involved in nutrient utilisation and intermediary metabolism. Atlantic salmon were fed a diet containing either 100 % FO or a 100 % of blend of RO, LO and CO (5:3:2) for a period of 55 weeks. Livers were collected, RNA extracted and global gene expression determined by analysis of the transcriptome using the SGR/TRAITS 17K cDNA salmon microarray (Taggart *et al.* 2008). The effects of VO inclusion on the expression of key genes associated with lipid metabolism including FA oxidation and HUFA synthesis were investigated alongside key genes involved in cholesterol metabolism.

5.2 Materials and methods

5.2.1 Experimental fish and diets

Three different groups of Atlantic salmon were used in the present study. The LEAN and FAT families were supplied by Landcatch Natural Selection (LNS). The commercial CAL strain (Caledonian 20) was supplied by Marine Harvest Ltd, (Fort William, Scotland, UK). The experiment was carried out from May 2006 to July 2007 at the Marine Harvest FTU, Ardnish, Lochailort, Scotland. The fish, trial and feeds were as described in detail in sections 4.2.1. and 4.2.2 in Chapter 4. Details of feed formulations (Skretting ARC, Norway), proximate and FA compositions are presented in Tables 4.1-4.3 in the previous chapter.

5.2.2 Experimental samples

The general sampling protocol used for the fish was fully described in section 4.2.3 of Chapter 4. Thus, samples of approximately 0.5 g of liver were collected into microcentrifuge tubes from ten fish per pen (20 per dietary treatment) for molecular analysis and rapidly frozen in liquid nitrogen prior to storage at -80^{o}C.

5.2.3 Gene expression (microarray and quantitative PCR)

Total RNA was extracted from individual liver samples by homogenising each in TriReagent as described in Chapter 2. For the microarray analysis, first strand and second strand cDNA synthesis of the total RNA from 6 biological replicates were subjected to aRNA synthesis and amplification prior to microarray hybridisation according to the instruction manual of the kit for RNA amplification for microarray analysis (Amino Allyl messageAmp II aRNA amplification kit, Ambion, UK.). Differential gene expression was verified by qPCR (Quantica thermocycler, Techne) as

described in Chapter 2, using first strand cDNA synthesis of total RNA from the above 6 biological replicates. Microarray analysis was confined to a comparison between the LEAN and FAT families, whereas gene expression by qPCR was investigated in all three groups of fish. The PCR primer sequences for the target genes and the housekeeping genes using for the qPCR analysis are given in Table 5.1.

Table 5.1. Primer sequences for target and housekeeping (normalisation) genes used in real-time qPCR.

Accession no	Genes	primers	Sequence 5′ -3′
AY458652	Δ6 fatty acyl desaturase	F	CCCCAGACGTTTGTGTCAG
		R	CCTGGATTGTTGCTTTGGAT
AF478472	Δ5 fatty acyl desaturase	F	GTGAATGGGGATCCATAGCA
		R	AAACGAACGGACAACCAGA
FJ237532	Elongase 2	F	CGGGTACAAAATGTGCTGGT
		R	TCTGTTTGCCGATAGCCATT
FJ237531	Elongase 1 (elovl5b)	F	ACAAAAAGCCATGTTTATCTGAAAGA
		R	AAGTGGGTCTCTGGGGCTGTG
DY708590	Mevalonate kinase	F	CCCTTAATCAGGGTCCCAAT
		R	GGTGCTGGTTGATGTCAATG
DW561983	7-dehydrocholesterol	F	CTTCTGGAATGAGGCATGGT
		R	ACAGGTCCTTCTGGTGGTTG
DY733476	SREBP2[1]	F	GACAGGCACAACACAAGGTG
		R	CAGCAGGGGTAAGGGTAGGT
BE518590	HMG-CoA reductase[2]	F	CCTTCAGCCATGAACTGGAT
		R	TCCTGTCCACAGGCAATGTA
AM230810	Carnitine palmitoyl transferase	F	CCTGTACCGTGGAGACCTGT
		R	CAGCACCTCTTTGAGGAAGG
AF321836	Elongation factor-1α (housekeeping)	F	CTGCCCCTCCAGGACGTTTACAA
		R	CACCGGGCATAGCCGATTCC
AF012125	β-actin (housekeeping)	F	ACATCAAGGAGAAGCTGTC
		R	GACAACGGAACCTCTCGTTA
AJ425111	Unresponsive EST (housekeeping)	F	AGCCTATGACCAACCCACTG
		R	TGTTCACAGCTCGTTTACCG

[1]Sterol regulatory element binding protein, [2] 3-hydroxy-methyl-glutaryl-CoA reductase

5.2.4 Statistical analysis

The microarray data were analysed by both two-way ANOVA and one-way ANOVA with GeneSpring GX version 10.0.2 (Agilent Technologies, Cheshire, UK). The fold change of the expression of genes that were verified by qPCR were analysed by REST 2005 (Pfaffl 2001).

5.3 Results

5.3.1 Analysis of gene expression by microarray

Liver gene expression in two families of Atlantic salmon, LEAN and FAT, selected on the basis of flesh adiposity was investigated by transcriptomics using the SGP/TRAITS 17k cDNA microarray. To determine the effects of diet and family on gene expression, a two way ANOVA without multiple testing correction of the cDNA array data was performed. The analyses identified 712 gene features that were differentially expressed between the two families, and 539 gene features that were significantly different ($P < 0.05$) due to the effects of diet. In addition, a significant interaction between the effects of family and diet was observed for 787 of these gene features. All the Atlantic salmon gene clones were identified by BLASTN and BLASTX homolog searching against Tentative Consensus (TC) contigs from the Atlantic salmon gene index (ASGI) (http://compbio.dfci.harvard.edu/tgi/tgipage.html) as described in Chapter 2. For each factor (i.e. family, diet, and their interaction), the top 100 clones (i.e. based on statistical significance, the most highly significant) that were identified as being significantly different were categorised according to function and the results presented as pie charts in Figure 5.1. Note that the pie charts do not include gene features in the top 100 that were of unknown (i.e. unannoted clones).

The functional categories most affected by family and diet were those associated with metabolism. Over a third (35 %) of the gene features affected by diet were associated with metabolic processes, with the majority of these (23 %) linked to lipid metabolism, with 5 % involved in carbohydrate metabolism, 5 % associated with protein and AA metabolism and 3 % related to vitamin metabolism. Family had less of an effect on metabolism than diet, with just over a quarter (27 %) of gene features affected being related to metabolic processes of which 9 % were linked to lipid metabolism, 6 % to metabolic energy pathways and 12 % to protein and AA metabolism. Most of the observed interactions between diet and family were in genes associated with metabolic functions, with almost a third (32 %) of the top 100 genes affected being associated with metabolism. Of these, 18 % of the genes were linked with lipid metabolism, 4 % with carbohydrate metabolism and 10 % with protein and AA metabolism. In addition to metabolic genes, groups of genes related to immune / stress responses, and thus fish health and welfare, were also affected. Therefore, approximately 18 % of the top 100 responding genes affected by strain/family 14 % of the genes affected by diet and 8% of genes affected by their interaction were associated with immune/stress responses (Figure 5.1).

Given that the two-way ANOVA found significant effects associated with both family and diet but that there was significant interaction between them, a series of two-by-two comparisons were performed fixing one of the variables and using t-test to analyse the statistical significance of the differences. Therefore, to examine the specific effects of diet, that is, feeding either a FO or VO, on liver gene expression was separately investigated in each individual family, FAT and LEAN. Furthermore, the effect of family on liver gene expression was investigated when fed either a FO or a VO diet. Therefore, four comparisons were performed; 1) comparison of dietary FO vs VO

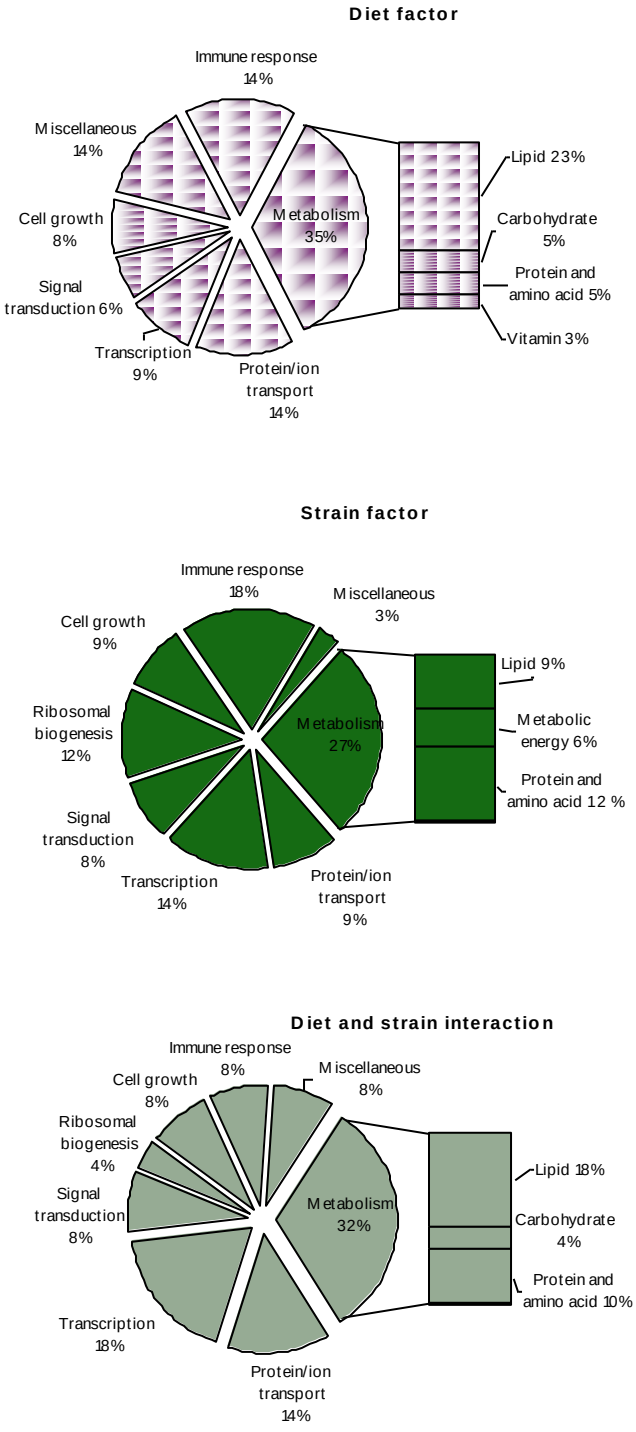

Figure 5.1. The functional categories of the top 100 significant cloned genes that were differentially expressed by the FO and VO diets, the strain and the interaction between diet and strain. Non-annotated and unknown cloned genes are not included.

in the LEAN family, 2) comparison of dietary FO vs VO in the FAT family, 3) comparison of LEAN and FAT families fed a FO diet and, 4) comparison of LEAN and FAT families fed a VO diet. Diet FO and FAT fish were used as control. The results from the t-tests, without multiple testing correction, found that there were 272 gene clones whose expression was significantly different ($P < 0.05$) in LEAN fish fed either a FO or a VO diet or, in other words, diet significantly affected the expression of 272 gene features in LEAN fish. Similarly, diet significantly affected the expression of 441 gene features in FAT fish. Family significantly affected the expression of 563 gene features in fish fed the VO diet, and 246 genes in fish fed the FO diet. As with the two-way ANOVA, the top 100 significantly different gene clones (based on statistical significance) in each of the four comparisons were classified according to biological function and are presented in Tables 5.2-5.5. Beside the top 100 responding genes, other lipid metabolism/transport-related and cholesterol/steroid biosynthesis-related genes that were found to be significantly different following the t-test analysis of the four comparisons were also categorised and reported in Table 5.6.

The results of the two-by-two comparisons indicated that the levels of expression of metabolism-related genes in the liver differed between the two families of salmon when fed either a FO or a VO diet. Effects of diet on the expression of the HUFA biosynthesis genes, Δ6 and Δ5 fatty acyl desaturases, were observed in the LEAN family (Table 5.2) whereas effects on Δ5 desaturase were not observed in the FAT family (Table 5.3). Thus, the expression of Δ6 desaturase was approximately 1.5 to 2.8 fold higher and that of Δ5 desaturase was about 2.0 fold higher in the LEAN family fed the VO diet than fish fed the FO diet (Table 5.2). In the FAT family, the expression of the Δ6 desaturase was around 1.9 fold higher in fish fed the VO diet compared to fish fed the FO diet (Table 5.3). Differences between families in the expression of FA and

HUFA biosynthesis genes were observed in fish fed the FO diet (Table 5.4) whereas there were no significant differences in those genes in fish fed the VO diet (Table 5.5). Thus, the liver transcript levels of the Δ5 desaturase and FA synthase genes were approximately -1.7 and -1.2 fold lower, respectively, in the LEAN family than in the FAT family fed the FO diet (Table 5.4). Therefore, the difference in expression of Δ5 desaturase between the two families observed in the diet comparisons in Tables 5.2 and 5.3 was reflected in the family comparisons of fish fed FO (Table 5.4) and not of fish fed VO (Tabel 5.5) indicating that it was the different expression level in fish fed FO that led to the differential expression between the families.

Other differences in liver gene expression were observed between the families. The expression levels of acyl-CoA binding protein, isopentenyl-diphosphate, angiopoitin-like 3 (ANGPTL3) and squalene monooxygenase in the LEAN family were up-regulated in fish fed the VO diet compared to fish fed the FO diet (Tables 5.2 and 5.6). The expression levels of ANGPTL3 and lipoprotein lipase (LPL) were also up-regulated in the FAT family in fish fed VO compared to fish fed FO, whereas no significant effects of diet on the expression of acyl-CoA binding protein, isopentenyl-diphosphate and squalene monooxygenase were observed in the FAT family (Tables 5.3 and 5.6). Furthermore, in fish fed the FO diet, the expression of LPL was higher in the LEAN family than in the FAT family, whereas the expression levels of isopentenyl-diphosphate, ANGPTL4 and squalene monooxygenase were lower in the LEAN family compared to the levels in the FAT family (Tables 5.4 and 5.6). In fish fed the VO diet, the levels of expression of LPL, ANGPTL3 and ANGPTL4 were higher in the LEAN family compared to the levels found in the FAT family (Tables 5.5 and 5.6).

Other dietary differences in liver gene expression also showed differential effects between the families. Thus, many of the immune response-related genes in the

LEAN family showed lower levels of expression in liver of fish fed the VO diet than in liver of fish fed the FO diet (Table 5.2). The only exception was the heat shock protein (hsp90), whose expression was 1.3-fold greater in the LEAN family in fish fed VO compared to fish fed FO. In contrast, the levels of expression of the immune response-related genes in the FAT family were higher in fish fed the VO diet than in fish fed the FO diet (Table 5.3). It should be noted that the dietary effects on the expression of hsp90 were not found in the FAT family. Consistent with this difference, the liver expression of hsp 90 in the LEAN family was 1.5 fold higher than that of the FAT family in fish fed the FO diet (Table 5.4) while the expression of hsp70 was 1.2 fold greater in the LEAN family than that in the FAT family in fish fed the VO diet (Table 5.5).

5.3.2 Analysis of gene expression by qPCR

To validate the microarray results, specific genes related to FA and HUFA synthesis, cholesterol biosynthesis, lipid transport and beta-oxidation were selected for qPCR analyses. These analyses showed similar trends in the differential expression of the genes with respect to dietary and family/strain effects, and so the qPCR data largely confirmed the results obtained from the microarray analyses. In the LEAN family of salmon, the expression of both Δ6 and Δ5 fatty acyl desaturases and elongase (elongase2, a gene not present in the cDNA array) were 4.0, 8.3 and 2.4 fold higher in fish fed the VO diet than in fish fed the FO diet (Table 5.7). In the FAT family, the expression of Δ6 desaturase was about 3.6 fold higher in fish fed the VO diet than in fish fed the FO diet, whereas the increased expression of Δ5 desaturase was not significant, as with the microarray results. In the CAL strain, the expression of another fatty acyl elongase (elongase1, not present in the cDNA array) was about 2.9 fold lower (reported as 0.34, down-regulation) in fish fed VO than fish fed FO (Table 5.7).

Furthermore, significant differences ($P < 0.05$) in the expression of the genes of HUFA biosynthesis were observed between the different families/strains of fish fed the same diet. For fish fed the FO diet, the expression levels of the Δ5 and Δ6 desaturases in the LEAN family were about 2.2 and 2.8 fold lower (0.45 and 0.36 down-regulation, respectively) than the levels determined in the FAT family (Table 5.8). It should be noted that, in contrast, the expression levels of the Δ6 desaturase between the LEAN and FAT family fed the FO diet were not significantly different as determined by the microarray. For fish fed the VO diet, the level of expression of elongase 2 in the LEAN family was 1.67 fold greater than that of the FAT family (Table 5.9). There were no significant differences in the gene expression between the CAL strain and the other two families of salmon fed either diet (Tables 5.8 and 5.9). No significant differences were found in the expression levels of mevalonate kinase, 7-dehydrocholesterol reductase, sterol regulatory element binding protein, HMG-CoA reductase or carnitine palmitoyl transferase-I genes between fish fed either of the diets or between the different fish groups fed the same diet (Tables 5.7-5.9).

Table 5.2. Effects of diet on liver gene expression in the LEAN salmon family.

Accession no	Clone gene	VO/FO ratio	p-value
Metabolic areas/pathways			
HUFA biosynthesis			
No accession no.	Delta-6 fatty acyl desaturase	2.1	<0.0000
No accession no.	Delta-6 fatty acyl desaturase	2.0	<0.0000
No accession no.	Delta-6 fatty acyl desaturase	2.8	0.0001
EG647320	Delta-6 fatty acyl desaturase	1.8	0.0026
No accession no.	Delta-6 fatty acyl desaturase	1.5	0.0143
No accession no.	Delta-5 fatty acyl desaturase	2.1	0.0014
No accession no.	Delta-5 fatty acyl desaturase	2.3	0.0016
CK887422	Delta-6 fatty acyl desaturase	2.0	0.0002
CK876943	Similar to fatty acid synthase	1.4	0.0054
Lipid metabolism and transport			
BM413891	Angiopoietin-like 4	1.4	0.0021
CK884265	Ganglioside GM2 activator	1.3	0.0083
EG648040	Acyl-coenzyme A-binding protein	1.7	0.0087
BI468158	ATP-binding cassette sub-family A member 1	2.0	0.0114
Cholesterol and isoprenoid biosynthesis			
CK879648	Squalene monooxygenase	1.9	0.0030
CK875291	Isopentenyl-diphosphate	2.0	0.0086
Carbohydrate metabolism			
CK886572	GSK-3-binding protein putative (glycogen synthase kinase)	1.9	0.0002
AJ425598	Glycogenin-1 putative mRNA	1.2	0.0039
No accession no.	Alpha-enolase	1.2	0.0006
Protein metabolism			
CK886932	Probable O-sialoglycoprotein endopeptidase	1.5	0.0031
CK885604	C-1-tetrahydrofolate synthase	-1.5	0.0071
CO469782	Lysyl-tRNA synthetase	1.3	0.0096
EG355385	Arginase-1	1.7	0.0138
Immune response / stress response / inflammatory response			
No accession no.	Leukotriene B4 receptor 1 putative	-1.4	0.0151
DW589721	SAPK substrate protein 1	-1.4	0.0093
CK877325	Interleukin-1 receptor-associated kinase 4	-1.2	0.0053
CK893821	Sequestosome 1or ubiquitin-binding protein P62	-1.3	0.0043
No accession no.	HIG1 domain family member 1A	-1.3	0.0142
AM083396	Heat shock protein hsp90	1.3	0.0139
Apoptosis			
AM042428	Tumor necrosis factor receptor number 11b	-1.2	0.0043
CK880960	PREDICTED: similar to B-cell CLL/ lymphoma 10	-1.4	0.0110

Table 5.2 (continued)

Accession no	Clone gene	VO/FO ratio	p-value
Regulation of transcription			
CK874058	Retinoic acid receptor gamma a (Rarga), coiled-coil	-1.5	0.0101
CK894063	Zinc finger protein 183	-1.5	0.0105
CK894959	Transcription factor AP-1	-1.5	0.0046
CK878904	PREDICTED: zinc finger protein 423 (ZNF423), mRI	1.3	0.0048
CK874146	Immediate early response 2	1.4	0.0049
CK897760	Retinoic acid receptor gamma a (Rarga)	-1.2	0.0049
CK879361	Thioredoxin interacting protein (TXNIP)	1.4	0.0064
CK875839	Retinoic acid receptor gamma a (Rarga)	-1.2	0.0078
CK887913	Transcriptional regulator	1.4	0.0148
CK883165	Hypoxia-inducible factor 1 alpha	1.3	0.0162
CK890454	Zinc finger protein XlCOF22	1.3	0.0154
CK888548	Rev protein - Human immunodeficiency virus 1	1.2	0.0130
Translation / ribosome biogenesis and assembly			
CN181210	40S ribosomal protein S27	1.3	0.0134
CO472436	Mitochondrial 60S ribosomal protein	1.4	0.0047
Protein transport / extracellular and intracellular transport / ion transport			
CK886667	Na/K ATPase	1.5	<0.0000
CK896189	Mitochondrial solute carrier family 25 member 25	-1.7	0.0062
CK890974	Calcium-binding mitochondrial carrier protein SCaMC	1.7	0.0007
Signal transduction / signal cascade			
No accession no.	S100-A1 calcium binding	1.4	0.0026
CK884745	Ras GTPase-activating protein1	-1.3	0.0083
CK892148	Growth factor receptor-bound protein 7	-1.5	0.0098
AJ424743	Fibroleukin precursor	1.4	0.0176
CO470718	Serine/threonine kinase	1.3	0.0143
Cell growth / adhesion / proliferation and differentiation / cytoskeleton			
CK882232	Stomatin-like protein 2	1.3	0.0035
EG647828	Amyloid beta A4 precursor	1.4	0.0067
AJ424583	Filamin-A putative	1.4	0.0132
Miscellaneous / unknown			
DW590010	Histone H2A	1.2	0.0053
CK894173	EFHD2 (EF hand domain containing2)	1.2	0.0038
EG649410	D-dopachrome tautomerase	1.4	0.0053
No accession no.	Retinoblastoma-binding protein 9	-1.5	0.0065

The top 100 liver transcripts (59 annotated and arranged by categories of biological function, 41 unknown) exhibiting differential expression between LEAN fish fed diets containing either FO or VO, identified by t-test. FO is a control diet.

Table 5.3. Effects of diet on liver gene expression in the FAT salmon family.

Accession no.	Clone gene	VO/FO ratio	p-value
Metabolic areas/pathways			
HUFA biosynthesis			
No accession no.	Delta-6 fatty acyl desaturase	1.9	0.0054
CK887422	Delta-6 fatty acyl desaturase	1.8	0.0029
Lipid metabolism and transport			
CK883097	5'-AMP-activated protein kinase subunit gamma-3	1.8	0.0005
CK894344	Phospholipase D3	-1.2	0.0027
No accession no.	Angiopoietin-like 3	1.4	0.0054
CK894278	Carnitine O-acetyltransferase	-1.2	0.0061
CK881643	Peroxiredoxin-6 (EC 1.11.1.15) (Antioxidant protein 2)	1.4	0.0073
CO472476	Lipoprotein lipase	1.7	0.0081
No accession no.	Phosphoinositide 3-protein kinase	-1.3	0.0109
CK891619	Endothelial lipase precursor	1.2	0.0117
BM414066	Endothelial lipase precursor	1.2	0.0121
Steroids / cholesterol and isoprenoid biosynthesis			
DW589347	3-beta-hydroxysteroid dehydrogenase	1.3	0.009
Carbohydrate metabolism			
No accession no.	Glutamine-fructose-6-phosphate transaminase 1	1.8	0.0119
Generation of metabolic energy			
CK888359	Dihydrodipicolinate synthase-like, mitochondrial	-1.4	0.0053
CK885069	Obg-like ATPase 1 (GTP-binding protein 9)	1.3	0.0062
BI468169	Cytochrome c oxidase	1.3	0.0076
AM402530	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitocondrial	1.3	0.009
Protein and amino acid metabolism			
CK897836	Betaine-homocysteine S-Methyltransferase (EC 2.1.1.5)	1.6	0.0044
EG648795	AMP deaminase 1 (EC 3.5.4.6) (Adenine monophosphate deaminase 1)	-1.4	0.0086
CK897702	Gamma-glutamyl hydrolase (conjugase folylpolygammaglutamyl hydrolase)	1.2	0.0131
DW592075	Proteasome (Prosome, macropain) subunit beta type-7	-1.3	0.0115
AM402452	Phenylalanyl-tRNA synthetase, alpha subunit	1.9	0.0093
EG648518	Proteasome subunit beta type 5 precursor	-1.3	0.0084
CK885098	Proteasome subunit alpha type	1.2	0.0011
CK883277	Ubiquitin-conjugating enzyme E2 D2	1.2	0.0041
AM083373	Serine/threonine-protein kinase	1.6	0.0013
CK879021	Selenoprotein X	-1.3	0.0124
Vitamin metabolism			
CK897269	Biotinidase precursor (EC 3.5.1.12)	1.7	0.0028
AM402518	Biotinidase	1.9	0.0115

Table 5.3 (continued)

Accession no	Clone gene	VO/FO ratio	p-value
Immune response / stress response / inflammatory response			
CK887922	Tumor necrosis factor, alpha-induced protein 8-like protein 2	-1.4	0.0018
CK880083	Interleukin-15 precursor	1.3	0.0025
CK883643	Interferon-gamma receptor alpha chain	1.3	0.0034
AM402574	Peptidoglycan recognition protein	1.4	0.0047
CK888813	Complement component C3-4	1.6	0.0081
AM402762	Complement component C8 alpha chain	1.7	0.0091
CK895744	Properdin P factor	1.5	0.0096
No accession no.	Scavenger receptor cysteine-rich gene	1.4	0.0127
AJ424648	Macrophage mannose receptor 1	-1.4	0.0113
AJ425750	Non-histone chromosomal protein H6	1.3	0.0005
CK896665	Interferon-induced protein kinase-interacting protein (P52rIPK)	-1.3	0.0029
Apoptosis			
No accession no.	Phosducin-like protein 3	-1.3	0.0079
Regulation of transcription			
EG648112	Retrovirus-related Pol polyprotein	1.3	0.0099
DW588532	Polybromo 1(PBRM1)	-1.6	0.0089
CK898402	Zinc finger protein 391 putative	-1.3	0.0013
BI468167	Glucocorticoid-induced leucine zipper protein	1.3	0.0026
CK876689	Class B basic helix-loop-helix	1.3	0.0111
AM041780	Butyrate response factor 1	1.5	0.0034
CK884728	Butyrate response factor 1	1.5	0.0034
Ribosome biogenesis and assembly			
No accession no.	60S ribosomal protein	-1.2	0.0086
Protein transport / extracellular and intracellular transport / ion transport			
EG648737	Golgi associated, gamma adaptinear containing, ARF binding protein 1	-1.3	0.0085
No accession no.	Excitatory amino acid transporter	-1.2	0.0051
EG649484	Plasma retinol-binding protein II (PRBP-II)	1.2	0.0068
CK892246	Importin beta-3 (Karyopherin beta-3)	1.3	0.013
Signal transduction / signal cascade			
CK881430	N-arachidonyl glycine receptor (Alt nane G-protein coupled receptor 18)	-1.2	0.0112
AM397509	Tensin-like C1	1.3	0.004
No accession no.	Guanine nucleotide-binding protein G(T) gamma-T1	1.5	0.0092
CK885828	Chemokine receptor-like 1	1.3	0.0106
Cell growth / adhesion / proliferation and differentiation / cytoskeleton			
CK894523	Galectin like protein 3	-1.2	0.0023
No accession no.	CD9 antigen	1.3	0.0086
DW590534	Collagen alpha-2 chain type 1	1.3	0.0093
CK893640	Cyclin L1	1.4	0.0094

Table 5.3 (continued)

Accession no	Clone gene	VO/FO ratio	p-value
Blood coagulation			
BI468137	Plasma protease C1 inhibitor	1.4	0.0072
AM402942	Plasminogen precursor (EC 3.4.21.7)	1.3	0.0116
Miscellaneous			
CK885096	UDP-glucuronosyltransferase 1-2 precursor microsomal	1.4	0.0063
CK878707	RNA-binding protein with multiple splicing (RBP-MS)	-1.3	0.0084
CO469710	Transmembrane protein 30A	1.2	0.0129
AM402582	Surfeit locus protein 4	1.3	0.0032
AM397500	Brain protein 44-like protein	1.3	0.0100
DW588813	Ring finger protein 122 putative	1.3	0.0126

The top 100 liver transcripts (69 annotated and arranged by categories of biological function, 31 unknown) exhibiting differential expression between FAT fish fed diets containing either VO or FO, identified by t-test. FO is a control diet.

Table 5.4. Effects of family on liver gene expression in salmon fed diets containing FO.

Accession no.	Clone gene	FO-fed lean/fat ratio	p-value
Metabolic areas/pathways			
Fatty acid biosynthesis			
No accession no.	Delta-5 fatty acyl desaturase	-1.7	0.0094
CK876943	Similar to fatty acid synthase	-1.2	0.0053
Lipid metabolism and transport			
CK884265	Ganglioside GM2 activator	-1.3	0.0038
CO472011	Peroxisomal biogenesis factor 3 (Pex3)	1.2	0.0136
CK889835	N-acylethanolamine-hydrolyzing acid amidase precursor	-1.4	0.0136
BM414094	Phosphatidylethanolamine N-methyltransferase	-1.5	0.0162
CK880279	Delta 3, 5-delta 2, 4-dienoyl-CoA isomerase	-1.3	0.0152
CO470568	Lipoprotein lipase	1.2	0.0145
No accession no.	Apolipoprotein B	-1.4	0.0068
Cholesterol and isoprenoid biosynthesis			
CK879648	Squalene monooxygenase (Squalene epoxidase) (SE)	-1.9	0.0038
CK875291	Isopentenyl-diphosphate	-1.9	0.0197
Carbohydrate metabolism			
BI468173	Lysosome-associated membrane glycoprotein 2	1.3	0.0145
Protein metabolism			
CO469782	Lysyl-tRNA synthetase	-1.2	0.0149
EG648459	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase	-1.4	0.0221
Immune response/stress response/inflammatory response			
BM414402	Heat shock 90 kDa	1.5	0.0211
CK896665	Interferon-induced protein kinase-interacting protein (P52rIPK)	-1.3	0.0212
AM041615	Barrier-to-autointegration factor	1.3	0.0222
CK890485	Interleukin-4 receptor alpha chain	1.3	0.0222
AJ424124	MHC class I (Major Histocompatibility complex)	-1.2	0.0147
Apoptosis			
AM042454	Induced myeloid leukemia cell differentiation protein Mcl-1	1.3	0.0225
EG648732	Phosducin-like protein 3	-1.3	0.0088
Regulation of transcription			
DW589311	TFG (TRK- fused gene)	-1.3	0.0096
DW589427	Cullin-associated and neddylation-dissociated 1 (CAND1	1.4	0.0019
CK898005	Homeobox protein HoxB13ab (HoxB13ab)	-1.3	0.0068
CK888834	BTEB (basic transcription element binding protein) transcription factor alt name Kruppel-like factor 9	-1.4	0.0085
CK888955	Retinoic acid receptor gamma b	-1.3	0.0086
EG647480	Retinoblastoma-binding protein 7	-1.3	0.0015
CK888548	Rev protein - Human immunodeficiency virus 1	-1.5	0.0028

Table 5.4 (continued)

Accession no.	Clone gene	FO-fed lean/fat ratio	p-value
Translation / ribosome biogenesis and assembly			
EG649414	Eukaryotic translation initiation factor 4E [Capsicum annuum]	-1.5	0.0022
CK887633	Ribosome biogenesis protein NSA2 (TGF-beta-inducible nuclear protein 1)	-1.3	0.0150
CO470133	Pescadillo (PES1)	-1.2	0.0185
Protein transport / extracellular and intracellular transport / ion transport			
CK894787	Importin 7	-1.2	0.014
CK880160	Vacuolar protein sorting-associated protein 26B-B	-1.2	0.0158
CK893304	ABC-type amino acid transport/signal transduction system, periplasmic component/domain precursor	1.4	0.0108
CK890533	Translocon-associated protein subunit delta precursor	1.3	0.0019
CK884068	Mannose binding-like lectin precursor	-1.2	0.0072
CO469716	ER lumen protein retaining receptor 2	-1.3	0.0132
CK880187	ATP-binding cassette sub-family B member 8, mitochondrial precursor	-1.3	0.0006
Protein processing and turnover			
EG648399	PTC7 protein phosphatase homolog	-1.2	0.0112
CK885053	Disulfide-isomerase A3	1.4	0.0119
No accession no.	Dual specificity protein kinase CLK2	-1.6	0.0166
Signal transduction / signal cascade			
AJ424743	Fibroleukin precursor (Fibrinogen-like protein 2)	-1.6	0.0028
CK895984	Progestin membrane receptor component 1	1.2	0.0156
CO470718	Serine/threonine kinase	-1.3	0.0167
Cell growth /adhesion / proliferation and differentiation / cytoskeleton			
EG648449	Transmembrane protein 97 (Protein MAC30)	1.3	0.0155
AJ424345	Integral membrane protein 2B	-1.4	0.0213
EG647863	N-myc downstream regulated family member 3a	1.2	0.0206
AJ425853	Cofilin-2	-1.4	0.0085
Chromosome / RNA and DNA processing			
DW590843	Origin recognition complex subunit 3-like	-1.3	0.0016
BM414303	Deoxyribonuclease gamma (Deoxyribonuclease I-like 3)	-1.3	0.0098
AM402622	Non-POU domain containing, octamer-binding	-1.7	0.0117
Blood coagulation			
CK878961	Annexin A5	1.4	0.0061
AM402675	Vitamin K-dependent gamma-glutamyl carboxylase	1.3	0.0098
CK893526	Annexin A5	1.3	0.0126
Miscellaneous / unknown			
CK892585	Nebulin-like	1.4	0.0064
No accession no.	Collagen type X alpha 1	1.5	0.0170
EG649410	D-dopachrome tautomerase	-1.2	0.0174
DW588788	Myelin P0 protein	-1.7	0.0189
CO470414	N-acetyltransferase 9	-1.2	0.0217
AM402582	Surfeit locus protein 4	1.4	0.0090
AM397500	Brain protein 44-like protein	1.4	0.0132

The top 100 liver transcripts (61 annotated and arranged by categories of biological function, 39 unknown) exhibiting differential expression between FAT and LEAN fish fed FO, identified by t-test. FAT strain is a control.

Table 5.5. Effects of family on liver gene expression in salmon fed diets containing VO.

Accession no.	Clone gene	VO-fed lean / fat ratio	p-value
Metabolic areas/pathways			
Lipid metabolism and transport			
BI468158	ATP-binding cassette sub-family A member 1	-2.2	0.0001
CK899433	ATP binding cassette transporter	-1.5	0.0001
AM397514	Apolipoprotein B	-1.4	0.0019
Cholesterol and isoprenoid biosynthesis			
EG647463	Cytochrome P450 reductase	-1.3	0.0017
Carbohydrate metabolism			
BI468162	Glucose-6-phosphatase	-1.7	0.0012
CK886572	GSK-3-binding protein putative (glycogen synthase kinase)	1.8	0.0003
Protein metabolism			
CK877143	Calpain 1 catalytic subunit	-1.4	0.0029
EG648604	Serine protease-like protein	1.4	0.0030
No accession no.	Ubiquitin fusion degradation 1-like protein	-1.8	<0.0000
EG647643	Purine nucleoside phosphorelase	-2	0.0001
Immune response / stress response / inflammatory response			
EG648409	Heat shock 70 kDa protein 4	1.2	0.0036
CK877922	High affinity immunoglobulin	1.3	0.0037
CK886548	T cell receptor (TCR)-alpha/delta locus	-1.2	0.0001
CK897993	Extracellular matrix protein 1	-1.4	0.0023
Apoptosis			
EG648888	Tumor protein p53 inducible nuclear protein 2	-1.3	0.0035
Regulation of transcription			
DW588967	Cellular nucleic acid-binding protein	1.2	0.0005
BM414226	DNA-directed RNA polymerase III	1.4	0.0035
CK885437	Echinoderm microtubule-associated protein-like 1	-1.4	<0.0000
AJ425468	Retinoic acid receptor gamma a (Rarga)	-1.7	0.0001
CK876689	Class B basic helix-loop-helix	-1.5	0.0004
CK874419	Hepatic leukemia factor	-1.4	0.0004
CK881770	Hematopoietically-expressed homeobox protein	-1.6	0.0001
BI468167	Glucocorticoid-induced leucine zipper protein	-1.3	0.0012
EG648323	Zinc finger DHHC domain-containing protein 4	1.2	0.0014
Translation / ribosome biogenesis and assembly			
DW591332	60S ribosomal protein L39	1.2	<0.0000
EG649017	60S ribosomal protein L24	1.3	0.0014
EG648836	60S ribosomal protein L22	1.3	0.0021
CK895350	60S ribosomal protein L36	1.3	0.0035
No accession no.	40S ribosomal protein S8	1.3	0.0011
EG648238	40S ribosomal protein S6	1.3	0.0012
No accession no.	40S ribosomal protein S23	1.3	0.0012
EG648835	40S Ribosomal protein S6	1.3	0.0015
AM412040	18S ribosomal RNA	1.8	0.0010
AJ424434	Ribosome production factor 1	1.2	0.0005
AJ425467	Eukaryotic translation initiation factor 6	-1.2	0.0005
EG649336	Eukaryotic translation elongation factor 1	1.3	0.0011

Table 5.5 (continued)

Accession no.	Clone gene	VO-fed lean / fat ratio	p-value
Protein transport / extracellular-intracellular transport / ion transport			
DW589920	Ras-related protein Rab-35	1.3	0.0010
CK884617	Ras-related protein Rab-25	-1.8	0.0006
CK886667	Na/K ATPase	1.6	<0.0000
EG647764	ADP-ribosylation factor 3	1.3	0.0038
CK896189	Solute carrier family 25 member 25	-2.3	0.0012
CK890974	Solute carrier family 25 member 25	-2.2	0.0007
Protein processing / folding			
BI468154	FK506-binding protein 5 putative	-1.5	0.0001
DW592207	Deoxyhypusine synthase	-1.2	0.0012
Signal transduction / signal cascade			
CK892148	Growth factor receptor-bound protein 7 (GRB7)	-1.4	0.0015
CK873407	Regulator of G-protein signaling 3	-1.5	0.0024
EG647738	Transducer of ERBB2 (Tob1)	-1.8	0.0031
AJ425351	Tensin-like C1 domain-containing phosphate	-1.6	0.0001
CK891988	Transducer of ERBB2 (Tob1)	-1.7	0.0012
Cell cycle / growth / adhesion / proliferation and differentiation / cytoskeleton			
CK888542	Insulin-like growth factor binding protein1	-2.5	0.0001
No accession no.	Nuclear protein 1 putative	-2.1	0.0002
CN181394	Nuclear lamin A	-1.8	0.0007
CK879713	Zonadhesin-like gene	-1.2	0.0013
AM397509	Tensin-like C1 domain-containing	-1.3	0.0014
No accession no.	Cyclin B2	-1.4	0.0014
CK898014	Similar to Protein fuzzy homolog	-1.2	0.0002
No accession no.	Ankyrin 1	-1.9	0.0027
Miscellaneous / unknown			
EG647739	Ferritin, heavy subunit	-1.4	0.0024
No accession no.	Iron-responsive element-binding	-1.2	0.0001
CK893078	Hemoglobin subunit alpha	1.5	0.0012
CK880112	Tropomyosin-1 alpha chain	1.4	0.0015
DW590534	Collagen alpha-2 chain type 1	-1.3	0.0028
EG647787	Transmembrane protein 107	1.2	0.0035
No accession no.	PQ-loop repeat-containing protein 2	-1.2	0.0033
No accession no.	Retinoblastoma-binding protein 9	-1.5	0.0026

The top 100 liver transcripts (65 annotated and arranged by categories of biological function, 35 unknown) exhibiting differential expression between FAT and LEAN fish fed VO, identified by t-test. FAT strain is a control.

Table 5.6. Annotated liver transcripts related to lipid metabolism/transport that were not listed in the top 100 genes but whose expression was significantly affected by family or diet as identified by t-test.

Accession no.	**Clone gene**	**LEAN (VO/FO ratio)**	**P-value**
No accession no.	Acyl-CoA-binding protein	1.6474	0.0179
Accession no.	**Clone gene**	**FAT (VO/FO-ratio)**	**P-value**
CO470568	Lipoprotein lipase	1.3185	0.0141
No accession no.	Angiopoietin-like 3	1.6473	0.0177
CK890036	Lipoprotein lipase	1.6427	0.0251
Accession no.	**Clone gene**	**FO (LEAN/FAT ratio)**	**P-value**
EG648040	Acyl-coenzyme A-binding protein	-1.4909	0.0333
BM413891	Angiopoietin-like 4	-1.2307	0.0394
CK890036	Lipoprotein lipase	1.3952	0.0417
Accession no.	**Clone gene**	**VO (LEAN/FAT ratio)**	**P-value**
BM413891	Angiopoietin-like 4	1.2581	0.0108
No accession no.	Angiopoietin-like 3	1.4144	0.0207
CK890036	Lipoprotein lipase	1.5215	0.0418

Table 5.7. Effects of diet on the expression of selected genes in liver of the three groups (CAL, LEAN and FAT families) of Atlantic salmon as determined by qPCR.

Gene	**CAL**		**LEAN**		**FAT**	
	Expression	**P value**	**Expression**	**P value**	**Expression**	**P value**
Delta-6 fatty acyl desaturase	3.170	0.226	**8.275 (up)**	**<0.000**	**3.606 (up)**	**0.004**
Delta-5 fatty acyl desaturase	1.427	0.912	**3.956 (up)**	**<0.000**	1.624	0.106
Elongase 2	0.588	0.865	**2.359 (up)**	**0.002**	0.768	0.376
Elongase1	**0.341 (down)**	**0.045**	1.179	0.50	0.774	0.359
Mevalonate kinase	0.580	0.721	1.882	0.051	0.730	0.367
7-dehydrocholesterol reductase	0.908	0.982	1.536	0.09	0.690	0.228
SREBP [1]	1.80	0.628	2.061	0.073	1.087	0.854
HMG-CoA reductase [2]	1.033	0.989	0.954	0.889	0.874	0.715
Carnitine palmitoyl transferase I	0.795	0.955	0.862	0.505	0.599	0.064

Results are the normalised expression ratio (with reference genes, *elf* 1α, β-actin, and an unresponsive EST) and P value of the target transcripts in the CAL, LEAN and FAT fish fed VO diet in relation to the control FO diet (analysed by REST2005). Values are means (n = 6).

Table 5.8. Effects of family on the expression of selected genes in liver of the Atlantic salmon fed FO as determined by qPCR.

Gene	CAL vs FAT		LEAN vs FAT		LEAN vs CAL	
	Expression	P value	Expression	P value	Expression	P value
Delta-6 fatty acyl desaturase	0.90	0.946	**0.452 (down)**	**0.046**	0.401	0.805
Delta-5 fatty acyl desaturase	0.875	0.967	**0.361 (down)**	**0.002**	0.329	0.545
Elongase 2	1.514	0.804	0.542	0.116	0.285	0.318
Elongase1	1.765	0.546	0.716	0.477	0.324	0.52
Mevalonate kinase	1.234	0.846	0.446	0.088	0.288	0.399
7-dehydrocholesterol reductase	0.749	0.794	0.475	0.058	0.505	0.905
SREBP 2 [1]	0.401	0.340	0.550	0.315	1.095	0.974
HMG-CoA reductase [2]	0.781	0.801	1.051	0.929	1.073	0.992
Carnitine palmitoyl transferase I	0.881	0.926	0.861	0.815	0.779	0.993

Results are the normalised expression ratio (with reference genes, *elf* 1α, β-actin, and an unresponsive EST) and P value of the target transcripts in fish fed the FO diet, between CAL vs FAT; LEAN vs FAT; and LEAN vs CAL (analysed by REST2005). Values are means (n = 6).

Table 5.9. Effects of family on the expression of selected genes in liver of the Atlantic salmon fed VO diet in comparison to FO diet as determined by qPCR.

Gene	CAL vs FAT		LEAN vs FAT		LEAN vs CAL	
	Expression	P value	Expression	P value	Expression	P value
Delta-6 fatty acyl desaturase	0.992	0.984	1.038	0.839	1.046	0.908
Delta-5 fatty acyl desaturase	0.965	0.944	0.879	0.323	0.911	0.876
Elongase 2	1.455	0.264	**1.665 (up)**	**0.006**	1.145	0.728
Elongase1	0.974	0.978	1.090	0.52	1.119	0.819
Mevalonate kinase	1.231	0.590	1.149	0.462	0.934	0.857
7-dehydrocholesterol reductase	1.236	0.585	1.057	0.733	0.855	0.686
SREBP 2 [1]	0.832	0.638	1.043	0.855	1.254	0.542
HMG-CoA reductase [2]	1.158	0.718	1.147	0.448	0.990	0.980
Carnitine palmitoyl transferase I	1.468	0.206	1.240	0.196	0.845	0.694

Results are the normalised expression ratio (with reference genes, *elf* 1α, β-actin, and an unresponsive EST) and P value of the target transcripts in fish fed the VO diet between CAL vs FAT; LEAN vs FAT; and LEAN vs CAL (analysed by REST2005). Values are means (n = 6).

5.4 Discussion and conclusions

In the present study in Atlantic salmon, liver was selected to investigate the effects of family/strain on the expression of metabolic genes, largely focusing on genes related to lipid metabolism. Liver is the primary tissue involved in the metabolic processing of nutrients obtained from the diet and has particular roles in distributing dietary lipid, and the *de novo* synthesis of lipids as well as participating in detoxification and excretion pathways (Leaver *et al.* 2008). The present study demonstrated that the expression levels of Δ5 and Δ6 fatty acyl desaturases and elongases in the liver were greater in salmon fed the VO diet than in fish fed the FO diet. Of particular interest in the present study, this effect was most pronounced in the LEAN family than in the FAT family. The dietary effects were in agreement with those reported in previous studies, which showed that either the partial or total substitution of dietary FO with VO increased the expression of hepatic Δ5 and Δ6 desaturases as well as elongase in Atlantic salmon (Tocher *et al.* 2003c; Zheng *et al.* 2004; Leaver *et al.* 2008). Jump *et al* (2005) reported reported that dietary FAs such as DHA and EPA regulate hepatic gene expression by controlling the activities of key transcription factors involved in the regulation of the hepatic metabolic enzymes. The authors stated that, for example, in vitro studies established that PPARα and SREBP–1c-regulated genes are key targets for PUFA control of hepatic gene expression. PUFA activate PPARα by direct binding, leading to the induction of hepatic FA oxidation. PUFA inhibit hepatic FA synthesis by suppressing SREBP – 1c nuclear abundance through several mechanisms, including suppression of SREBP–1c gene transcription and enhancement of proteasomal degradation and mRNA SREBP1-c decay. Moreover, Bell *et al* (2001, 2002) suggested that the stimulation of hepatic desaturation by VO substitution of FO presumably reflects increased availability of 18:2n-6 and 18:3n-3 substrates coupled with a reduced

tissue levels of end-product HUFA such as EPA and DHA. The concentrations of the long chain n-3 PUFA, particularly 22:6n-3, in diet are known to suppress the pathway conversions of 18:3n-3 to 22:6n-3 in trout as found by Buzzi (1996). This author stated that the ability of trout hepatocytes to synthesise the 22:6n-3 from both 18:3n-3 and 20:5n-3 can be markedly stimulated by a diet deficient in long chain n-3 PUFA, particularly 22:6n-3.

In the current study, the replacement of FO with VO in the diet was shown to influence the expression of lipid metabolism and transport genes, and also that these effects varied between the families. Therefore, in the FAT family, the results from the microarray analysis revealed that the expression levels of LPL and ANGPTL3 were greater in fish fed the VO diet than in fish fed the FO diet. In contrast, in the LEAN family, no significant effects of diet on the expression levels of these two genes were observed, but the expression levels of ANGPTL4 and acyl-CoA binding protein were observed to be higher in the LEAN family in fish fed VO compared to fish fed FO. In addition, the levels of expression of those genes were greater in the LEAN family than in the FAT family, irrespective of diet, illustrating the influence of fish family/strain on the expression of lipid metabolism genes. ANGPTL3 and ANGPTL4, members of the family of angiopoietin genes, are known to regulate lipid metabolism in mice where both proteins increase plasma TAG levels (inducing hyperlipidemia) (Yoshida *et al.* 2002). Lipoprotein lipase is another key enzyme in lipid metabolism, hydrolysing plasma TAG obtained from circulating chylomicrons and VLDL and promoting uptake of lipids into tissues (Stryer 1988). The expression of the LPL gene has been shown to be affected by the composition of dietary FAs in red sea bream (Liang *et al.* 2002a) and rainbow trout (Richard *et al.* 2006). In the present study, an increase in liver LPL expression may also result in increased liver TAG levels. The increased expression (and

presumably) activity of the ANGPTLs, LPL and the acyl-COA binding protein in the LEAN family fed VO compared to FO, would increase lipid transport from the liver to tissues and lipid uptake in the tissues and in the liver. The higher concentration of LPL mRNA in fish fed VO in the present study is in agreement with the previous study of Liang *et al* (2002b) which reported that LPL gene expression was increased in red sea bream fed a VO diet. However, later studies have contradicted this, reporting that rainbow trout fed a FO diet had increased levels of LPL mRNA in liver (Richard *et al.* 2006), and that ANGPTL3 was down regulated in Atlantic salmon fed a VO diet compared to fish fed a FO diet (Leaver *et al.* 2008). Carnitine palmitoyl transferase I (CPT-I) and CPT-II are considered to be key enzymes for mitochondrial beta-oxidation regulation (Power & Newsholme 1997; Froyland *et al.* 1998; Torstensen *et al.* 2000, 2009). In the present study, no significant effects of diet on the expression of CPT-I were observed. The results from the present study are consistent with those of previous studies which showed that there were no significant differences in the hepatic expression of CPT-I (Leaver *et al.* 2008) or CPT-II (Torstensen *et al.* 2009) between Atlantic salmon fed VO or FO diets.

Previous studies have demonstrated that the complete substitution of dietary FO with VO increased the expression of genes related to cholesterol biosynthesis, including mevalonate kinase, HMG-CoA reductase, 7-dehydrocholesterol reductase and the main transcription factor regulating cholesterol biosynthesis, sterol regulatory element binding protein 2 (SREBP2) (Leaver *et al.* 2008). The results of the microarray analyses revealed that there were also differences between the LEAN and FAT salmon families in the response of the cholesterol metabolism genes to diet. Thus, in the LEAN family, the cholesterol/steroid biosynthesis genes, squalene monooxygenase and isopentenyl-diphosphate, were up-regulated in fish fed the VO diet compared to fish fed the FO diet.

In contrast however, there were no significant effects of dietary treatment in the expression of these genes in the FAT family. Squalene is the biochemical precursor to the whole family of steroids and choleserol derivatives (Stryer 1988). The results from the current microarray study showed that neither diet nor family had any effect on the expressions of HMG-CoA reductase, 7α- cholesterol hydroxylase and SREBP2 were not significantly different and this was also confirmed by the qPCR analysis. As suggested above, SREBPs play a major role in lipid and cholesterol metabolism, regulating the transcription of genes that encode enzymes in the biosynthetic pathways of cholesterol and FAs. The SREBPs are encoded by two genes, SREBP1 and SREBP2. SREBP1a and SREBP1c are associated with both cholesterol and FA homeostasis, whereas SREBP2 is preferentially involved in regulating cholesterol metabolism (Magana & Osborne 1996; Field *et al.* 2001; Desvergne *et al.* 2006). HMG-CoA reductase is involved in the synthesis of cholesterol where it is reputedly the rate-limiting enzyme in the cholesterol biosynthesis pathway (Gornati *et al.* 2005), and 7α-cholesterol hydroxylase is the first and rate-limiting enzyme in the bile acid synthesis pathway that occurs exclusively in the liver (Russell & Setchell 1992). It is known that in VOs, cholesterol is very low whereas the sterol is present as plant or phytosterols including sitosterol, stigmasterol, campesterol or blassicasterol depending on source (Phillips *et al.* 2002). The phytosterols are not taken up by mammals and compete with intestinal cholesterol uptake and thereby reduce the uptake of dietary cholesterol (Orzechowski *et al.* 2002). From the present study, the levels of cholesterol in the liver were not reduced by the VO diet (Table 4.10, Chapter 4) and the expression of key enzymes of cholesterol biosynthesis were not generally increased suggesting that the levels of cholesterol in the VO diet appear to be sufficient for metabolic processes.

When altering the formulation and dietary ingredients in fish feed by replacing FM and FO with plant proteins and oils, the health and welfare of fish should also be taken into consideration. In the present study, it appeared that there were differences between the families in this aspect of metabolism and adaptation to dietary change. Whereas, in the LEAN family the expression of heat shock response protein 90 (hsp90) was significantly different between fish fed the VO diet compared to fish fed the FO diet, there were however no significant effects of diet in the expression of these genes in the FAT family. In addition, the expression levels of hsp90 and hsp70 were higher in the LEAN family compared to the FAT family, irrespective of diet. This suggested that both diet and genetic variation, at least partly, influence immune function and responses in salmon. It is well known that nutrients influence the immune system and resistance to disease (Blazer 1992; Carder 1999, 2001). The replacement of FO with VO could impact on the immune system of fish as well as their resistance to infectious disease, especially if the VOs are rich in n-6 FAs. In fish, the introduction of n-6 FA-enriched diets altered the dietary n-3/n-6 PUFA ratio and can influence the composition of fish immune cells (Thompson *et al.* 1996; Mourente *et al.* 2005). The effects of dietary replacement of FO with VO on some immune parameters in the FAT and LEAN families, and CAL strains of Atlantic salmon have been reported previously (Petropoulos *et al.* 2008). Thus, there is evidence that immune function, in terms of haematocrit, total number of circulating leucocytes, granulocytes and serum lysozyme activity may be influenced by the complete substitution of FO with a blend of VOs (Petropoulos *et al*. 2008), and that these effects were most prominently displayed in the LEAN family in the present study. Other studies have shown that increasing replacement of plant-based ingredients in the diet also induced a physiological stress response in rainbow trout (Panserat *et al.* 2008). Furthermore, feeding rainbow trout

diets containing 30 % of the diet as soybean-protein altered the liver protein profile and the levels of several stress proteins (*e.g.* heat shock protein), possibly related to the presence of increased anti-nutritional factors (Martin *et al.* 2003). Heat shock protein 90 is active in supporting various components of the cytoskeleton and steroid hormone receptors (Iwama *et al.* 1998; Young *et al.* 2001), whereas hsp70 assists the folding of nascent polypeptide chains, acts as a molecular chaperone, and mediates the repair and degradation of altered or denatured proteins (Iwama *et al.* 1998; Kiang & Tsokos 1998). Thus, the significant differences in expression of the heat shock proteins found in the present study may signal an early warning indicator of impaired fish performance and health in Atlantic salmon fed diets with high levels of replacement of marine FM and FO with plant-based ingredients.

In conclusion, the results showed that liver gene expression was significantly affected in Atlantic salmon by the dietary oil composition and that the effects varied between different groups of fish. These results clearly showed that the FAT and LEAN families, and CAL strain, adapt differently, in terms of the expression of genes involved in metabolic pathways in the liver, to the complete substitution of dietary FO with a blend of VOs. Specifically, the LEAN family generally showed the higher levels of response to substitution of FO with VO. Clearly, the relationship between phenotype/genotype and biochemical mechanisms of regulation is an area that requires further study.

Chapter 6 - Dual replacement of dietary fish oil and fish meal in feeds for large Atlantic salmon (*Salmo salar* L.). I- Effects of replacement of dietary fishmeal with increasing levels of plant protein sources

6.1 Introduction

Atlantic salmon are an important high value, carnivorous fish species that are generally raised in intensive systems and fed high-energy extruded feeds containing high quality protein. Traditionally, the protein content of the feed for farmed salmon has been supplied by marine FM derived from the industrial, reduction fisheries (Hardy 1996; Sargent & Tacon 1999; Pike 2005). It is clear that FM (and FO) supplies from these finite fisheries are strictly limited and, if aquaculture continues to expand worldwide, the requirements for FM and FO will soon exceed global supplies (FAO 2006). The constraints that utilisation of these marine products impose has resulted in increasing investigation of the use of alternative protein and oil sources in aquafeeds to sustain aquaculture development.

Many studies have investigated replacement of FM in feeds with a variety of plant proteins (PPs) at different levels of inclusion for a range of fish including Atlantic salmon (Storebakken *et al.* 1998a, 1998b; Refstie *et al.* 2000; Carter & Hauler 2000; Refstie *et al.* 2001; Opstvedt *et al.* 2003; Mundheim *et al.* 2004; Dias *et al.* 2005), gilthead sea bream (Sitja-Bobadilla *et al.* 2005; Lozano *et al.* 2007), Atlantic cod (Olsen *et al.* 2007; Hansen *et al.* 2007b), European sea bass (Dias *et al.* 2005), Atlantic halibut *Hippoglossus hippoglossus* (Berge *et al.* 1999) and sunshine bass (*Morone chrysops* × *M. saxatilis*) (Lewis & Kohler 2008). Partial substitution of FM with

soybean meal in diets at levels up to 30 - 40 % showed no reduction in growth of Atlantic salmon, rainbow trout, European sea bass and gilthead sea bream (Smith *et al.* 1995; Nengas *et al.* 1996; Robaina *et al.* 1997; Opstvedt *et al.* 2003; Kaushik *et al.* 2004; Dias *et al.* 2005). Substitution of FM with soybean protein concentrate up to 80 % or 100 % in diets for Atlantic halibut (Berge *et al.* 1999) and rainbow trout (Kaushik *et al.* 1995) respectively, showed no adverse effects on growth performance or nutrient utilisation. Wheat gluten can be utilised to substitute up to 40 % of FM in diets for salmon and trout (Hardy 1996). Furthermore, addition of pea protein concentrate, corn gluten, sunflower meal, or dehulled peas at levels up to 30 % of total protein showed no adverse effect on growth performance or carcass composition in salmonids and sea bream (Mente *et al.* 2003; Thiessen *et al.* 2003; Gill *et al.* 2006; Lozano *et al.* 2007; Overland *et al.* 2008; Overland *et al.* 2009).

The above studies demonstrated the potential of replacing FM with PP in fish feeds without growth retardation, providing the substitution of PP is not too high and nutritional balance is maintained. Furthermore, blends of PP are able to be used as replacements for FM at high inclusion levels. Thus, a mixture of soybean meal and corn gluten meal could be used at up to 69 % of total protein replacement without any negative effect on growth and feed intake in Atlantic cod (Albrektsen *et al.* 2006). Total (100 %) replacement of FM with PP, however, affected growth performance of rainbow trout (Gomes *et al.* 1995) and Atlantic salmon (Espe *et al.* 2006). However, using a blend of PP, substitution of FM in feeds close to 100% was possible in Atlantic salmon with no negative effect on growth if the AA profile in the feed was well balanced and if feed intake was comparable to a high FM diet (Espe *et al.* 2007). The decreased global availability and high utilisation of FO in feeds for farmed fish, particularly salmonids, has been criticised resulting in fish nutritionists searching for alternative oil sources.

Currently, VOs are considered the prime sustainable oil sources for FO replacement in aquafeeds due to the steadily increasing production, high availability and economic value (Fountoulaki *et al.* 2009).

There are differences, however, in FA composition between VO and FO. Feeds for farmed Atlantic salmon have traditionally contained high levels of n-3 HUFA, such as EPA and DHA, due to the inclusion of marine FO. The FA compositions of VOs show high levels of predominantly C18 FAs such as 18:1n-9, 18:2n-6 and sometimes 18:3n-3, but are devoid of the long chain n-3 HUFA, EPA and DHA, found in marine products (Bell *et al.* 2003a; Caballero *et al.* 2002; Sargent *et al.* 2002). For instance, RO has a very high level of 18:1n-9, moderate 18:2n-6 and some 18::3n-3 (Bell *et al.* 2001, 2003a). Several studies have shown that the use of single VO to replace FO in aquafeeds at levels of > 50% replacement for all species, or indeed complete replacement in the case of salmon, is now feasible in practical feeds without affecting growth of fish, but does significantly impact on tissue FA composition and metabolism (Bell *et al.* 2001; Caballero *et al.* 2002; Montero *et al.* 2003; Bransden *et al.* 2003; Izquierdo *et al.* 2005; Fountoulaki *et al.* 2009). Therefore, replacing FM and FO with alternative non-marine ingredients affects not only production parameters but also the FA composition of fillet (Torstensen *et al.* 2004b; Lewis & Kohler 2008). Although Atlantic salmon is capable of desaturating and elongating the C18 PUFA (18:2n-6 and 18:3n-3) found in plant ingredients into the essential HUFA, ARA (20:4n-6), EPA (20:5n-3) and DHA (22:6n-3), the capacity of endogenous production of these HUFA may not fulfill requirements for optimal growth and well being of the fish when feeding diets containing low inclusion of marine raw materials (Bell *et al.* 2001).

In the present study, the effects of dual substitution of FM and FO were investigated in adult Atlantic salmon of initial weight of 1.3 Kg that were grown to

market size (> 3 Kg) over a period of 19 weeks on diets with 60 % of dietary FO replaced by RO, and increasing proportions of FM substituted by PPs (a mixture of sunflower meal, corn gluten meal, soybean meal, wheat gluten and field pea). The level of FO substitution represented the upper level of FO replacement currently used in commercial ongrowing diets. The control diet contained 25 % FM and 45 % PP, which also represented the current commercial level of FM inclusion in Norway. Three further diets had FM inclusion reduced to 18, 11 and 5 %, with PP inclusion increased to 50, 55 and 60 %, of the diet. Effects on growth performance, feed utilisation efficiency, lipid class and FA compositions of flesh and liver were investigated.

6.2 Materials and Methods

6.2.1 Experimental fish

One thousand eight hundred Atlantic salmon of initial mean weight 1.3 ± 0.1 kg were randomly distributed among 12 cages of 125 m^3 (5 × 5 × 5 m) with 150 fish/cage at the Marine Harvest Fish Trials Unit (FTU), Ardnish, Scotland, and fed one of four diets in triplicate cages. The experiment was conducted over 19 weeks from October 2007 to February 2008 under natural photoperiod. The mean temperature, salinity and turbidity measured by secchi dish of seawater at the first phase of the experiment were 11.0 ± 1.0^{o}C, 26.5 ± 6.8 and 5.2 ± 1.7 m while those parameters at the second phase were 7.1 ± 1.4^{o}C, 26.4 ± 5.5 and 5.8 ± 1.4 m respectively. Fish were fed to apparent satiation using automatic feeders (Arvo-tec, Sterner Arvo-tec UK, Inverness, Scotland). Mortalities, feed consumption and waste feed were recorded daily.

6.2.2 Sampling protocol

All the experimental fish were bulk weighed at the initiation, at the end of week 8 and at the termination of the trial, week 19. At the end of the trial, 2 fish per pen (6 fish per dietary treatment) were anaesthetised with metacaine sulphonate (MS222; 50 mg/L) and killed by a blow to the head. Flesh samples were taken from the NQC and were homogenised in a food processor after removal of skin and bones and stored at -20 ^{o}C prior to lipid analysis. Livers were also collected from the six fish and a 1-2 g sample placed into glass vials containing chloroform/methanol (2:1, by vol.) for analysis of lipid class and FA composition, and the remaining portion immediately frozen on dry ice (for lipid content). Both liver samples were then stored at -20 ^{o}C prior to analysis.

6.2.3 Experimental diets

Four diets were formulated to satisfy the nutritional requirements of salmonid fish (NRC. 1993) and manufactured at the BioMar TechCentre, Brande, Denmark. All diets contained 35 % crude protein and 28 % crude lipid. The control diet (25F) was formulated to represent the maximum level of PP inclusion currently in commercial use and contained 45 % PP (a blend of sunflower meal, corn gluten, soybean protein concentrate and field bean) and 25 % FM (Table 6.1). The remaining three diets followed a regression with FM inclusion reduced to 18 % (18F), 11 % (11F) and 5 % (5F) and PP inclusion increased to 50 %, 55 % and 60 % of total diet, respectively. All diets were coated with a 60:40 blend of RO and northern hemisphere FO. All diets were supplemented with crystalline AAs, lecithin and carophyll pink as sources of specific AAs, phospholipids and pigments (Table 6.1).

Table 6.1. Feed formulation (g kg-[1]) and analysed feed composition (percentage) of the experimental diets.

Feed ingredients	25F	18F	11F	5F
Fishmeals (67% protein/10% lipid)[1]	250	180	110	50
Sunflower meal (37% protein/10 lipid)	115	77	40	-
Corn gluten (62% protein/2 lipid)	85	135	175	215
Soy concentrate (60% protein/2% lipid) [2]	85	135	175	225
Wheat gluten (77% protein/3% lipid)	-	2	18	20
Binders	160	160	160	160
Rapeseed oil[3]	173	175	178	180
Fish oil [4]	116	117	118	120
Vitamins and minerals [5]	12.0	17.6	23.6	29.0
L-lysine [6]	0.62	1.72	3.44	4.26
L-threonine[6]	-	-	0.43	0.67
DL-methionine [6]	0.57	1.03	1.56	2.01
Lecithin	5.0	5.0	5.0	5.0
Astaxanthin[7]	0.4	0.4	0.4	0.4
Antioxidant [8]	4.25	4.25	4.25	4.25
Proximate composition (%)				
Protein	34.3	35.1	35.0	34.7
Lipid	29.8	29.5	27.9	27.3
Moisture	6.7	6.0	6.2	6.9
Ash	6.0	5.6	5.2	4.8
Fibre	3.5	3.3	2.6	3.0
NFE	19.7	20.5	23.1	23.3

Values are means of triplicate analysis.
[1] Blend of Peruvian super prime and standard fishmeals produced from Anchoveta
[2] SPC produced from defatted soy flakes
[3] Non-GM, double-low rapeseed oil
[4] North-Atlantic standard fish oil
[5] Vitamin and mineral premixes (Premier Nutrition Products Ltd., Staffordshire, UK) with limestone carrier added according to the commercial standards of BioMar AS
[6] Highly purified (99%) crystalline amino acids
[7] Carophyll Pink CWS 10% product from F. Hoffman-La Roche Ltd., Basel, Switzerland
[8] Blend of antioxidants and starch carrier added according to the commercial standards of BioMar AS

6.2.4 Proximate composition analysis

Diets were ground prior to determination of proximate composition including moisture, crude protein, crude fat and ash contents according to standard procedures (AOAC 2000). Crude lipid contents of the experimental diets were determined after acid hydrolysis followed by Soxhlet lipid extraction. The methods followed for the above extractions and analyses are thoroughly described in Chapter 2.

6.2.5 Pigment composition analysis

Pigment contents and compositions of the diets and flesh samples were determined according to the method of Barua (Barua *et al*. 1993). The extraction and measurement of individual carotenoids, beta-carotene, astaxanthin, canthaxanthin, astacene and lutein was carried using HPLC as described in Chapter 2.

6.2.6 Lipid extraction and analysis

Total lipids of tissue and diet samples were extracted and prepared according to the method of (Folch *et al*. 1957). Separation of lipid classes was performed by HPTLC, with quantification by densitometry according to the method of Henderson and Tocher (Henderson & Tocher 1992). FAMEs were prepared by acid-catalysed transesterification as described by Christie (Christie 1993), extracted and purified as described by Tocher & Harvie (Tocher & Harvie 1988), and separated and quantified by gas-liquid chromatography. The detailed methods are thoroughly described in Chapter 2.

6.2.7 Calculations and statistical analysis

Growth and feed efficiency parameters, SGR, TGC, FCR, VSI and HSI were calculated according to the formulae presented in Chapter 2. ADC data were obtained

from the BioMar AS. All data are presented as means ± SD (n value as stated). The effects of dietary treatments on growth performance were analysed by one way ANOVA. The significance of differences were determined by one-way ANOVA with $P < 0.05$ (Zar 1999). Statistical analyses were performed using SPSS version 14 (SPSS inc, 2005) as described in Chapter 2.

6.3 Results

6.3.1 Proximate and pigment composition of the diets

The experimental diets contained protein in the range 34 – 35 % and lipid in the range 27-30 %. The lipid and fibre compositions of the diets were slightly decreased while the nitrogen-free extracts (NFE) was slightly increased when FM in diets was replaced with high level of PP inclusion (Table 6.1). There were some differences in pigment content of the diets. Generally, total pigment, beta-carotene and lutein content of the diets tended to increase whereas astacene content tended to decrease as PP inclusion in the diets increased. Astaxanthin content of the experimental diets was higher in the 18F diet than in the 11F and 5F diets (Table 6.2).

6.3.2 Lipid and fatty acid composition of the diets

Total polar and neutral lipid compositions of the experimental diets were about 8-9% and 91-92%, respectively. The majority of lipid supplied by the feeds was neutral lipid, predominantly TAG about 73-75% of total lipid (Table 6.2). The main polar lipids in the feeds were PC, PE, and PI / PS, and PC decreasing and PI / PS increasing as PP inclusion increased. All diets contained approximately 54 % total MUFA, predominantly 18:1n-9 (oleic acid), with around 16 % total SFA, mainly 16:0, and 30 % PUFA, with half of that being 18:2n-6 and the remainder being n-3 PUFA, 18:3n-3,

EPA and DHA (Table 6.3). However, there were some small differences in proportions of specific FAs among the dietary treatments. Thus, the proportions of 14:0, 16:1, 20:1, 22:1 and DHA decreased, and percentages of 18:1n-9 and 18:3n-3 increased as PP inclusion increased in the diets.

Table 6.2. Lipid class composition (percentage of total lipid) and pigment content (g kg^{-1}) of the experimental diets.

Parameters	25F	18F	11F	5F
Lipid classes				
PC	2.8	2.7	2.1	1.9
PE	3.5	3.6	3.9	3.2
PI/PS	1.5	2.0	3.1	2.8
SM	0.2	0.2	0.1	n.d.
LPC	0.1	0.1	tr.	n.d.
Total polar lipid	8.2	8.6	9.3	7.9
Total neutral lipid	91.8	91.4	90.7	92.1
Triacylglycerol	74.2	72.7	73.9	75.6
Sterol	8.5	8.6	6.9	6.9
Free fatty acid	9.1	10.1	9.9	9.6
Steryl ester	tr.	tr.	tr.	tr.
Pigments content (mg/kg)				
6 mm				
Total pigment	47.2	65.9	66.8	75.2
Beta-carotene	4.2	5.5	7.0	7.7
Astaxanthin	27.3	34.7	25.4	26.9
Astacene	1.3	1.4	1.0	0.9
Lutein	14.4	24.3	33.4	39.7
9 mm				
Total pigment	32.2	43.6	43.1	43.4
Beta-carotene	3.9	5.1	6.0	5.7
Astaxanthin	16.3	18.3	12.8	12.1
Astacene	0.5	0.5	0.4	0.4
Lutein	11.5	19.7	23.9	25.2

Results are means of tripicate analysis. LPC, lysophosphatidylcholine; NL, neutral lipids. n.d., not detected; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol, PL, polar lipids; PS, phosphatidylserine; SM, sphingomyelin; tr = < 0.05.

Table 6.3. Fatty acid compositions (percentage of total fatty acids) of the experimental diets fed to salmon for 19 weeks.

Parameters	25F	18F	11F	5F
Fatty acids				
14:0	2.6	2.6	2.5	2.3
16:0	8.7	9.1	9.1	8.6
18:0	2.7	2.8	2.6	3.1
20:0	0.5	0.5	0.5	0.6
22:0	0.9	1.0	0.8	1.3
Total saturated[1]	15.5	16.1	15.7	16.1
16:1n-9	0.1	0.1	0.1	0.1
16:1n-7	3.0	3.0	2.8	2.6
18:1n-9	38.4	38.8	41.1	41.4
18:1n-7	2.7	2.7	2.7	2.7
20:1n-9	4.5	3.8	3.6	3.3
20:1n-7	0.2	0.2	0.2	0.2
22:1n-11	4.6	3.7	3.4	3.0
22:1n-9	0.7	0.7	0.6	0.6
24:1n-9	0.3	0.3	0.3	0.3
Total monoenes	54.6	53.4	54.8	54.3
18:2n-6	15.0	15.4	15.1	15.1
18:3n-6	0.1	0.1	0.1	0.1
20:2n-6	0.1	0.1	0.1	0.1
20:3n-6	0.2	0.2	0.3	0.3
20:4n-6	0.2	0.2	0.2	0.2
22:5n-6	0.1	0.1	0.1	0.1
Total n-6 PUFA	15.7	16.1	15.8	15.8
18:3n-3	5.6	5.7	5.8	6.2
18:4n-3	1.0	0.9	0.8	0.8
20:4n-3	0.2	0.2	0.2	0.2
20:5n-3	4.1	4.3	3.9	3.9
22:5n-3	0.4	0.4	0.4	0.4
22:6n-3	3.0	2.9	2.5	2.3
Total n-3 PUFA	14.2	14.4	13.7	13.8
Total PUFA	29.9	30.5	29.4	29.6

Results are means of tripicate replicates analysis. [1]Totals includes 15:0 present at up to 0.2%.

6.3.3 Growth performance

There were no significant differences in survival rate of fish on any of the dietary treatments (Table 6.4). There were also no significant differences in initial weight of fish. After 19 weeks, the overall growth performance of fish showed that final body weight and weight gain were significantly reduced by FM replacement, resulting in reduced SGR and TGC. There was a significant linear reduction in TGC when FM inclusion reduced from 25 to 5 % at all stages of the trial, while there was a significant linear reduction in SGR in the first phase of the trial and the overall trial (Table 6.4). Hence, the group of fish fed the 25F (control) diet with the highest FM inclusion had highest TGC and SGR of all the dietary treatments, and the group of fish fed the lowest FM/highest PP inclusion rate, 5F, had the lowest TGC and SGR (Table 6.4). This growth reduction corresponded to a significant reduction in body weight and weight gain associated with the reduction of FM or the increasing PP inclusion in the diets. Therefore, the body weight of fish was in the order 25F > 18F >11F >5F. The body weight and weight gain of fish fed the control diet 25F were 3.8 kg and 2.5 kg $fish^{-1}$ at the end of the trial while group of fish fed the 5F diet had body weight and weight gain 3.0 kg and 1.7 kg $fish^{-1}$, respectively (Table 6.4).

The decreased growth was associated with decreased feed consumption, as the level of PP inclusion increased (Table 6.4). Feed consumption was significantly decreased when the FM inclusion decreased from 25 % to 5 % throughout the experiment, and was correlated with final fish weight. The fish fed the 25F diet showed the highest feed intake throughout the trial and this decreased significantly with reduction in FM inclusion. Consequently, FCR significantly increased with decreasing FM inclusion but only in the first phase of the trial, with the fish fed the control (highest FM) diet showing the lowest FCR.

Protein utilisation measured by PER showed a significant reduction with decreasing FM inclusion in the first phase and the overall trial, but it was not difference at the second phase. Thus, the group of fish fed the control diet had highest PER and the group of fish fed the 5F had lowest PER, 2.8 and 2.6, respectively (Table 6.4). There was no significant effect on the hepato-somatic index (HSI) of increasing PP inclusion in diets. However, the viscero-somatic index (VSI) showed a significantly increase as the level of FM inclusion decreased and PP inclusion increased (Table 6.4).

The ADC of protein and fat were significantly affected by the levels of dietary FM and PP ((Figure 6.1). The ADC of protein significantly increased from 82.6 % to 85.3 % while the ADC of fat significantly decreased from 94.6 % to 90.5 % with decreasing dietary FM and increasing dietary PP inclusion (Figure 6.1).

Table 6.4. Growth performance, feed efficiency and biometric parameters of salmon fed the experimental diets for 19 weeks.

Parameters	25F	18F	11F	5F
Survival(%)				
0-8 weeks	100 ± 0.00	99.33 ± 0.67	98.89 ± 1.02	99.56 ± 0.76
8-19 weeks	100 ± 0.00	99.77 ± 0.39	100 ± 0.00	99.78 ± 0.37
0-19 weeks	100 ± 0.00	99.10 ± 1.02	98.89 ± 1.02	99.34 ± 0.66
Body weight (kg)				
Initial	1.31 ± 0.01	1.30 ± 0.02	1.30 ± 0.01	1.32 ± 0.01
8 weeks	2.47 ± 0.03 ^{a}	2.37 ± 0.04 ^{b}	2.16 ± 0.02 ^{c}	1.98 ± 0.05 ^{d}
19 weeks	3.84 ± 0.08 ^{a}	3.64 ± 0.04 ^{b}	3.35 ± 0.05 ^{c}	3.00 ± 0.10 ^{d}
Weight gain (kg fish$^{-1)}$				
0-8 weeks	1.16 ± 0.02 ^{a}	1.05 ± 0.05 ^{a}	0.85 ± 0.05 ^{b}	0.66 ± 0.05 ^{c}
8-19 weeks	1.37 ± 0.11 ^{a}	1.26 ± 0.07 ^{a}	1.23 ± 0.11^{a}	1.01 ± 0.07 ^{b}
0-19 weeks	2.53 ± 0.09 ^{a}	2.34 ± 0.05 ^{a}	2.08 ± 0.14 ^{b}	1.68 ± 0.10 ^{c}
SGR % d^{-1}				
0-8 weeks	1.27 ± 0.01^{a}	1.19 ± 0.04^{a}	1.00 ± 0.04^{b}	0.81 ± 0.03 ^{c}
8-19 weeks	0.57 ± 0.03	0.55 ± 0.03	0.56 ± 0.01	0.53 ± 0.01
0-19 weeks	0.84 ± 0.01^{a}	0.80 ± 0.02 ^{ab}	0.75 ± 0.04 ^{b}	0.64 ± 0.01 ^{c}
TGC				
0-8 weeks	4.69 ± 0.06 ^{a}	4.35 ± 0.17 ^{b}	3.60 ± 0.13 ^{c}	2.88 ± 0.11^{d}
8-19 weeks	3.97 ± 0.23 ^{a}	3.81 ± 0.18 ^{ab}	3.72 ± 0.06 ^{ab}	3.45 ± 0.10 ^{b}
0-19 weeks	4.32 ± 0.08 ^{a}	4.07 ± 0.09 ^{b}	3.65 ± 0.09 ^{c}	3.15 ± 0.10 ^{d}
FCR				
0-8 weeks	1.04 ± 0.01 ^{c}	1.06 ± 0.02 ^{c}	1.17 ± 0.07 ^{b}	1.29 ± 0.01 ^{a}
8-19 weeks	1.03 ± 0.06	1.02 ± 0.07	1.00 ± 0.05	1.03 ± 0.06
0-19 weeks	1.03 ± 0.02	1.04 ± 0.04	1.03 ± 0.06	1.13 ± 0.04
Feed consumption (g fish^{-1} day^{-1})				
0-8 weeks	24.15 ± 0.20 ^{a}	22.37 ± 0.91 ^{b}	19.55 ± 0.43 ^{c}	16.90 ± 0.93 ^{d}
8-19 weeks	18.05 ± 0.21 ^{a}	16.52 ± 0.93 ^{b}	15.03 ± 0.28 ^{c}	13.30 ± 0.05 ^{d}
0-19 weeks	20.44 ± 0.04 ^{a}	18.84 ± 0.81 ^{b}	16.80 ± 0.24 ^{c}	14.72 ± 0.35 ^{d}
PER				
0-8 weeks	2.81 ± 0.04 ^{a}	2.68 ± 0.06 ^{a}	2.44 ± 0.14 ^{b}	2.23 ± 0.02 ^{c}
8-19 weeks	2.84 ± 0.16	2.81 ± 0.20	2.97 ± 0.16	2.81 ± 0.17
0-19 weeks	2.83 ± 0.07 ^{a}	2.75 ± 0.11^{ab}	2.73 ± 0.12 ^{ab}	2.55 ± 0.09 ^{b}
HSI (%)				
0-9 weeks	1.1 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
VSI (%)				
0-9 weeks	11.7 ± 0.7 ^{b}	12.3 ± 0.7 ^{ab}	13.2 ± 0.4 ^{a}	13.6 ± 0.2 ^{a}

Results are means ± SD (n = 3). Values within a row with different superscript letters are significantly difference as determined by ANOVA. FCR, feed conversion ratio; HSI, hepatosomatic index; PER, protein efficiency ratio; TGC, thermal growth coefficient; VSI, viscerosomatic index.

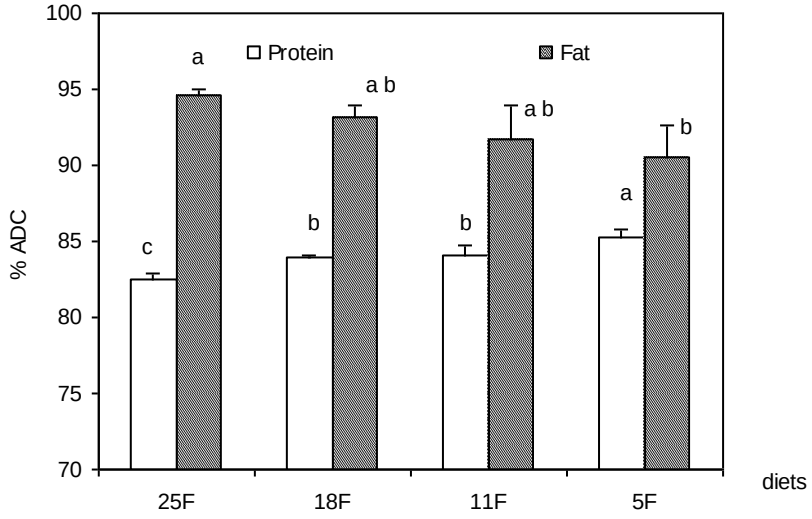

Figure 6.1. Apparent digestibility coefficients (%ADC) for total protein and fat in Atlantic salmon fed the 25F, 18F, 11F and 5F diets. Values are means ± SD (n = 3).

6.3.4 Lipid content, lipid class composition of flesh and liver and pigment content of flesh

Lipid content of flesh varied between 11.6 and 13.2 % but was not significantly different between fish fed the different dietary treatments (Table 6.5). Similarly there was no statistically significant difference between lipid contents of livers from salmon fed the different diets, but there was a clear trend for liver lipid to decrease as the FM inclusion decreased and PP inclusion increased, reducing from 7.1 % in fish fed the highest level of FM down to 5.2 % in fish fed the lowest FM inclusion (Table 6.6). The dietary treatments had no significant effects on the proportions of total polar and neutral lipids in flesh (Table 6.5) and liver (Table 6.6). Some small differences in individual polar lipid classes were observed in flesh, with PC and LPC of fish fed the18F diet being lower than the other groups of fish. In contrast, flesh PI/PS of fish fed the 18F diet were higher than the other 25F and 11F diets (Table 6.5). There were no significant differences in the relative percentages of any individual lipid classes in liver (Table 6.6).

The total pigment and astaxanthin content of the flesh was unaffected by dietary treatment although there were slight but biologically insignificant differences in lutein content (Table 6.5).

Table 6.5. Lipid content (percentage of wet weight), lipid class composition (percentage of total lipid) and pigment content (mg kg^{-1}) of flesh from salmon fed the diets for 19 weeks.

Parameters	25F	18F	11F	5F
Lipid content	11.7 ± 1.2	13.2 ± 1.8	11.7 ± 1.8	11.6 ± 1.1
Lipid class				
PC	4.4 ± 0.3 ^{ab}	3.8 ± 0.6 ^{b}	4.7 ± 0.7 ^{a}	4.5 ± 0.4 ^{ab}
PE	4.4 ± 0.9	4.2 ± 0.7	4.6 ± 0.9	3.7 ± 0.2
PI/PS	0.9 ± 0.2 ^{a}	1.1 ± 0.2 ^{a}	1.0 ± 0.1 ^{a}	0.6 ± 0.1 ^{b}
SM	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0
LPC	0.1 ± 0.1 ^{b}	tr.	0.2 ± 0.1 ^{a}	0.3 ± 0.1 ^{a}
Total PL	10.0 ± 1.1	9.3 ± 1.1	10.8 ± 1.4	9.3 ± 0.5
Total NL	90.0 ± 1.1	90.7 ± 1.1	89.2 ± 1.4	90.7 ± 0.5
Triacylglycerol	78.9 ± 1.1	80.1 ± 2.0	78.7 ± 2.6	78.8 ± 0.8
Sterol	5.8 ± 0.6	5.7 ± 0.8	5.8 ± 0.7	6.4 ± 0.4
Free fatty acids	5.4 ± 0.6	5.0 ± 0.5	4.7 ± 1.0	5.5 ± 0.5
Steryl ester	tr.	tr.	tr.	tr.
Pigment content				
Total pigment	5.3 ± 0.5	5.1 ± 0.9	5.2 ± 0.8	5.4 ± 0.8
Astaxanthin	4.3 ± 0.5	4.0 ± 0.7	4.1 ± 0.7	4.0 ± 0.5
Canthaxanthin	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.7 ± 0.2
Lutein	0.5 ± 0.1 ^{b}	0.6 ± 0.2 ^{ab}	0.5 ± 0.0 ^{b}	0.7 ± 0.0 ^{a}

Results are means ± SD (n = 3). Values within a row with different superscript letters are significantly different as determined by ANOVA. LPC, lysophosphatidylcholine; NL, neutral lipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, polar lipids; PS, phosphatidylserine; SM, sphingomyelin.

Table 6.6. Lipid content (percentage of wet weight) and lipid class composition (percentage of total lipid) of liver from salmon fed the experimental diets for 19 weeks.

Parameters	25F	18F	11F	5F
Lipid content	7.1 ± 2.3	6.4 ± 2.8	5.7 ± 1.5	5.2 ± 1.2
Lipid class				
PC	16.0 ± 3.9	16.6 ± 2.6	16.5 ± 3.1	16.4 ± 1.8
PE	10.2 ± 2.5	10.4 ± 1.3	10.7 ± 2.1	10.6 ± 0.8
PI	3.6 ± 1.0	3.6 ± 0.6	3.4 ± 1.1	3.5 ± 0.8
PS	2.3 ± 0.9	2.5 ± 0.6	2.4 ± 0.9	2.7 ± 1.1
SM	1.8 ± 0.6	2.0 ± 0.3	1.8 ± 0.5	1.6 ± 0.2
LPC	0.2 ± 0.1	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1
UPL	2.3 ± 1.4	2.2 ± 0.5	2.4 ± 0.9	2.9 ± 0.7
Total PL	36.4 ± 9.5	37.4 ± 4.4	37.4 ± 8.3	37.8 ± 4.9
Total NL	63.6 ± 9.5	62.6 ± 4.4	62.6 ± 8.3	62.2 ± 4.9
Triacylglycerol	49.0 ± 12.7	46.9 ± 7.7	46.0 ± 11.7	46.6 ± 5.9
Sterol	10.4 ± 2.1	11.6 ± 2.7	12.7 ± 2.2	11.0 ± 0.9
Free fatty acid	0.9 ± 0.9	0.6 ± 1.5	1.8 ± 0.2	1.8 ± 0.5
Steryl ester	tr	tr	tr	tr
UNL	3.3 ± 0.8	3.5 ± 1.3	3.4 ± 1.3	2.8 ± 0.6

Results are means ± SD (n = 3). Values within a row with different superscript letters are significantly different as determined by ANOVA. LPC, lysophosphatidylcholine; NL, neutral lipids; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PL, polar lipids; PS, phosphatidylserine; SM, sphingomyelin; tr., trace; UNL, unidentified neutral lipid, UPL, unidentified polar lipids.

6.3.5 Fatty acid composition of flesh and liver tissues

The gross FA composition of flesh reflected the diet compositions. Flesh FAs contained over 50 % total MUFA, predominantly18:1n-9, and around 17 % SFA, mainly 16:0. PUFA represented over 30 % of total FAs with 18:2n-6 being the most abundant followed by DHA, EPA and 18:3n-3 (Table 6.7). It was clear that DHA was retained at a higher concentration in the flesh than provided in the diet. The EPA retention showed a reciprocal trend with dietary levels, with the level in flesh of fish fed 25F lower than in the diet, while EPA in flesh of fish fed the diets with lower FM

inclusions being higher than the diet levels (Table 6.7). The levels of 20:1 decreased from 5.3 % to 3.5 % and 22:1 decreased from 4.4 % to 3.3 % in the flesh with decreasing FM inclusion similar to the dietary trend, but other differences in FA composition were not related directly to dietary levels. Thus, 16:0 increased from 10.2 % to 11.2 % and 16:1 increased from 3.4 % to 3.8 % as FM inclusion decreased. Similarly, the levels of 18:2n-6 tended to decrease from 12.3 % to 10.9 % and 18:3n-3 decrease from 4.0 % to 3.7 % while the levels of desaturated and elongated products including 20:4n-6, 22:5n-3 and 22:6n-3 increased with decreasing FM inclusion (Table 6.7). This may suggests some endogenous synthesis of LC-PUFA was occurring with reduction in FM and consequent increase of 18:3n-3.

The FA composition of liver showed more variability between treatments, but was generally similar to the diet compositions, with MUFA, mainly 18:1n-9, predominating at between 47 and 52 %, and around 15 % SFA, mainly 16:0, and 33-38 % PUFA (Table 6.8). There were no significant differences in total SFA, total MUFA, total n-3 PUFA and total PUFA in liver. However, total n-6 PUFA tended to increase in fish fed the diets containing higher PP inclusion. As with flesh, the proportion of DHA was higher in the liver lipids than in the diets, and was the predominant PUFA followed by 18:2n-6, EPA and 18:3n-3. Decreasing FM inclusion resulted in slightly reduced 14:0 and, particularly, reduced proportions of 20:1 and 22:1 in liver total lipid. In contrast to flesh, dietary treatments had no major effect on liver 18:2n-6 or 18:3n-3 levels, but 20:4n-6 and 22:5n-3 were significantly increased and there were trends of increasing EPA and DHA in response to decreasing dietary FM inclusion (Table 6.8).

Table 6.7. Fatty acid compositions (percentage of total fatty acids) of flesh from salmon fed the experimental diets for 19 weeks.

Parameters	25F	18F	11F	5F
Fatty acids				
14:0	2.9 ± 0.1	3.0 ± 0.1	3.0 ± 0.3	3.1 ± 0.2
16:0	10.5 ± 0.4 ^{ab}	10.2 ± 0.1 ^{b}	11.2 ± 0.5 ^{a}	11.2 ± 0.4 ^{a}
18:0	2.8 ± 0.1	2.8 ± 0.1	2.8 ± 0.2	2.8 ± 0.1
20:0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
Total saturated[1]	16.8 ± 0.5	17.3 ± 0.2	17.6 ± 0.8	17.8 ± 0.7
16:1n-7	3.4 ± 0.1^{b}	3.5 ± 0.3^{ab}	3.6 ± 0.3^{ab}	3.8 ± 0.2^{a}
18:1n-9	35.4 ± 1.0	34.6 ± 1.4	34.4 ± 1.8	34.9 ± 2.4
18:1n-7	3.1 ± 0.2	3.1 ± 0.2	3.2 ± 0.2	3.2 ± 0.1
20:1n-9	5.3 ± 0.2 ^{a}	4.6 ± 0.4 ^{b}	4.5 ± 0.3 ^{b}	4.5 ± 0.1 ^{b}
22:1n-11	3.7 ± 0.3 ^{a}	3.0 ± 0.2 ^{b}	2.6 ± 0.1 ^{c}	2.6 ± 0.1 ^{c}
22:1n-9	0.7 ± 0.1	0.7 ± 0.6	0.6 ± 0.0	0.7 ± 0.0
24:1n-9	0.6 ± 0.2	0.5 ± 0.3	0.5 ± 0.0	0.5 ± 0.0
Total monoenes[2]	52.6 ± 1.5	50.5 ± 1.7	49.9 ± 1.9	50.7 ± 2.3
18:2n-6	12.1 ± 0.5 ^{ab}	12.3 ± 0.5 ^{a}	11.4 ± 0.6^{bc}	10.9 ± 0.4 ^{c}
20:2n-6	1.0 ± 0.1 ^{b}	1.0 ± 0.1 ^{b}	1.2 ± 0.1 ^{a}	1.2 ± 0.0 ^{a}
20:3n-6	0.4 ± 0.0 ^{b}	0.5 ± 0.0 ^{a}	0.5 ± 0.1 ^{a}	0.5 ± 0.1^{a}
20:4n-6	0.3 ± 0.1 ^{b}	0.4 ± 0.1 ^{ab}	0.4 ± 0.1 ^{ab}	0.5 ± 0.1 ^{a}
Total n-6 PUFA[3]	14.1 ± 0.4 ^{ab}	14.5 ± 0.4 ^{a}	13.9 ± 0.6 ^{ab}	13.4 ± 0.4 ^{b}
18:3n-3	4.0 ± 0.2 ^{a}	4.1 ± 0.1 ^{a}	3.9 ± 0.3 ^{ab}	3.7 ± 0.2 ^{b}
18:4n-3	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
20:3n-3	0.4 ± 0.0 ^{b}	0.4 ± 0.1 ^{ab}	0.4 ± 0.1^{ab}	0.5 ± 0.4 ^{a}
20:4n-3	0.7 ± 0.1^{b}	0.8 ± 0.1^{a}	0.8 ± 0.0^{a}	0.8 ± 0.1^{a}
20:5n-3	3.8 ± 0.3 ^{b}	4.5 ± 0.6 ^{ab}	4.8 ± 0.7 ^{a}	4.7 ± 0.7 ^{ab}
22:5n-3	1.6 ± 0.2 ^{b}	2.0 ± 0.2 ^{ab}	2.2 ± 0.3 ^{a}	2.3 ± 0.4 ^{a}
22:6n-3	5.2 ± 0.9	5.3 ± 1.1	5.7 ± 0.8	5.4 ± 1.0
Total n-3 PUFA	16.4 ± 1.3	17.7 ± 1.9	18.6 ± 1.7	18.1 ± 2.2
Total PUFA	30.5 ± 1.4	32.2 ± 1.7	32.5 ± 1.5	31.5 ± 2.1

Results are means ± SD (n = 3). Values within a row with different superscript letters are significantly different as determined by ANOVA. [1]Totals includes 15:0 and 22:0 present at up to 0.2 %; [2]Totals includes 16:1n-9 and 20:1n-7 present at up to 0.3 %; [3]Totals includes 18:3n-6, 20:2n-6, 22:4n-6 and 22:5n-6 present at up to 0.2 %. PUFA, polyunsaturated fatty acid.

Table 6.8. Fatty acid compositions (percentage of total fatty acids) of liver from salmon fed the experimental diets for 19 weeks.

Parameters	25F	18F	11F	5F
Fatty acids				
14:0	1.6 ± 0.1[a]	1.6 ± 0.1[a]	1.5 ± 0.1[ab]	1.4 ± 0.1[b]
16:0	8.7 ± 1.7	9.1 ± 1.6	9.5 ± 1.9	9.0 ± 1.5
18:0	4.0 ± 0.8	4.6 ± 0.5	4.1 ± 0.3	4.0 ± 0.6
Total saturated[1]	14.6 ± 2.4	15.6 ± 2.0	15.5 ± 2.3	14.6 ± 2.0
16:1n-7	2.5 ± 0.6	2.2 ± 0.4	2.3 ± 0.5	2.1 ± 0.2
18:1n-9	35.8 ± 5.5	32.5 ± 4.7	33.1 ± 4.9	33.5 ± 2.3
18:1n-7	3.4 ± 0.3	3.2 ± 0.3	3.3 ± 0.2	3.2 ± 0.2
20:1n-9	7.5 ± 1.2 [a]	6.2 ± 0.6 [ab]	5.7 ± 1.0 [b]	5.6 ± 0.6 [b]
22:1n-11	1.6 ± 0.2 [a]	1.1 ± 0.2 [b]	1.1 ± 0.1 [b]	1.0 ± 0.1 [b]
24:1n-9	0.6 ± 0.2	0.5 ± 0.1	0.6 ± 0.2	0.6 ± 0.1
Total monoenes[2]	52.4 ± 7.5	46.7 ± 6.0	47.1 ± 6.6	47.1 ± 3.2
18:2n-6	8.7 ± 0.6 [ab]	9.5 ± 0.8 [a]	8.4 ± 0.5 [b]	8.5 ± 0.6 [b]
20:2n-6	2.1 ± 0.3	2.4 ± 0.2	2.1 ± 0.3	2.2 ± 0.2
20:3n-6	0.7 ± 0.1 [ab]	0.6 ± 0.1 [b]	0.7 ± 0.1 [ab]	0.8 ± 0.1 [a]
20:4n-6	0.9 ± 0.4 [b]	1.2 ± 0.4 [ab]	1.3 ± 0.4 [ab]	1.4 ± 0.2 [a]
Total n-6PUFA[3]	12.5 ± 0.6 [b]	13.9 ± 0.6 [a]	12.8 ± 0.5 [b]	13.1 ± 0.6 [ab]
18:3n-3	2.5 ± 0.3	2.5 ± 0.4	2.6 ± 0.3	2.6 ± 0.3
20:3n-3	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
20:4n-3	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1
20:5n-3	5.5 ± 1.6	7.0 ± 1.5	7.3 ± 1.6	7.4 ± 0.6
22:5n-3	1.4 ± 0.2 [b]	2.0 ± 0.4 [a]	2.2 ± 0.5 [a]	2.2 ± 0.2 [a]
22:6n-3	9.7 ± 4.2	10.7 ± 3.2	10.9 ± 2.9	11.3 ± 2.0
Total n-3PUFA[4]	20.5 ± 5.5	23.8 ± 4.6	24.7 ± 4.8	25.2 ± 2.2
Total PUFA	33.0 ± 5.4	37.7 ± 4.3	37.4 ± 4.8	38.3 ± 2.1

Results are means ± SD (n = 3). Values within a row with different superscript letters are significantly different as determined by ANOVA. [1]Totals includes 15:0 and 20:0 present at up to 0.2 %; [2]Totals includes 16:1n-9, 20:1n-7 and 24:1n-9 present at up to 0.4%; [3]Totals includes18:3n-6, 20:2n-6 and 22:5n-6 present at up to 0.2 %; [4]Totals includes 18:4n-3 presents at up to 0.2 %. PUFA, polyunsaturated fatty acid.

6.4 Discussion and conclusion

The reduction of FM from 25 % to 5 % in the diets by progressively increasing replacement with the mixed PP sources (sunflower meal, soybean protein concentrate,

corn gluten, wheat gluten and field beans) did not affect the survival of Atlantic salmon (mortality less than 1 %) indicating that the experimental diets did not have any major negative effects on fish health. However, the dietary treatments significantly affected growth performance of salmon in the present study. As FM inclusion decreased from 25 % to 5 % there was a progressive reduction in growth resulting in final body weights being reduced by 5 %, 13 % and 22 % in fish fed 18, 11 and 5 % FM, respectively, compared to fish fed 25 % FM. Moreover, SGR for the fish fed the 18F, 11F and 5F diets was reduced by 5 %, 11 % and 23 %, and TGC by 5 %, 16 % and 27 %, respectively, compared to fish fed the control 25F diet. Despite the lower growth performance compared to the control diet, the fish fed the 18F and 11F diets showed weight gains, TGCs and SGRs in a similar range to salmon of similar size fed high FM diets (Karalazos 2007; Torstensen *et al.* 2008). The level of each PP used in the present study was within the range used in several previous studies showing their support of adequate fish growth (Kaushik *et al.* 1995, 2004; Robaina *et al.* 1997; Mente *et al.* 2003; Thiessen *et al.* 2003; Opstvedt *et al.* 2003; Dias *et al.* 2005; Lozano *et al.* 2007; Hansen *et al.* 2007b; Overland *et al.* 2008). However, in general, these studies showed that replacement of high quality FM with PP sources could cause a reduction in growth depending on the sources and levels of replacement. It has been reported that replacing FM with PP higher than 75% resulted in dramatic reduction in growth rate in Atlantic salmon (Sveier *et al.* 2001), gilthead sea bream (Sitja-Bobadilla *et al.* 2005) and Atlantic cod (Hansen *et al.* 2007b). The levels of dietary FO used in previous studies were between 22 and 30 % of the total diet (Opstvedt *et al.* 2003; Espe *et al.* 2006), which was much higher than the level of FO used in the present study, with FO being around 12 % and VO 18%, of the total diet. Therefore, the effect of FM replacement on growth is dependent not only upon the level of FM replacement but also

on the level of FO in the diet. Therefore, in another study investigating dual replacement of FM and FO, Atlantic salmon fed diets with 80 % of the FM replaced by blends of PPs and krill meal, along with 70 % FO replaced by VOs, (LO/RO/PO) showed significant growth reduction, whereas diets with substitution of 40 % FM and 70 % VO, or 80 % FM and 35 % VO showed no negative effects (Torstensen *et al.* 2008). This study was supported by studies showing that gilthead sea bream had no growth retardation when fed diets containing 15% FM and high levels of blends of PPs inclusion associated with VO up to 66% replacement, but a growth reduction was found when dietary FO substitution was 100% (Benedito-Palos *et al.* 2008, 2009).

Reduction in growth in salmonids at high dietary inclusion levels of PP has been explained by various factors including decreased digestible and indigestible carbohydrate levels (starch/fibre levels) (Opstvedt *et al.* 2003; Hemre *et al.* 2003), reduced feed palatability and presence of anti-nutrients (Krogdahl *et al.* 1994; Francis *et al.* 2001), and imbalanced dietary AA concentration (Espe *et al.* 2006, 2007). In the present study, the NFE (N-free extract plus fiber) level increased as the level of PP inclusion increased, but also the feed intake was reduced by feeding the diets with increasing levels of PP inclusion. This reduced consumption of diets containing high PPs and contributed to the lower growth observed in fish fed these diets. This result was consistent with previous studies, which reported that increasing replacement of FM by PPs in diets for salmonids resulted in reduced growth performance that was caused by reduced feed intake (Kaushik *et al.* 2004; Espe *et al.* 2006). Moderate reductions in feed intake in fish can severely affect cumulative nutrient absorption and consequently correlate with decreased growth (Refstie *et al.* 1998, 2001; Carter & Hauler 2000). Previous studies suggested that feed intake was not affected by low inclusion of PP sources, but the quality and quantity of FM inclusion affects feed intake, and

digestibility of protein and energy (Mundheim *et al.* 2004). In the present study, high and low qualities of FM were utilised in a ratio of 1:1 together with low levels of FM inclusion and high PP inclusion that, together, may alter the physiology, texture and flavour and, consequently, reduce the palatability of the diets. It was shown in previous studies that both the quality and levels of FM inclusion enhanced the palatability and feed efficacy and so reducing FM and changing the composition of ingredients likely influences the flavour, reducing palatability of the diets and diminishing the appetite of the fish (Webster *et al.* 1999). The palatability of diets and feed acceptance can be improved and enhanced by inclusion of relatively minor amounts of specific feed attractants including krill meal, fish protein or squid hydrolysates into diets (Espe *et al.* 1999; Refstie *et al.* 2004; Hevroy *et al.* 2005; Olsen *et al.* 2006).

Although reduced feed intake was the most obvious consequence of the reduction in dietary FM inclusion in the present study, there were also significant effects on nutrient utilisation between salmon fed high and low FM inclusion. FCR was increased and PER decreased by decreasing dietary FM inclusion in the first phase of the trial while there were no significant differences between any of the dietary treatments in these parameters in the second phase of the trial. FCR and PER in the present study were comparable to previous studies. This may indicate that there was metabolic adaptation to the diets in salmon that improved nutrient utilisation of the high PP diets in the later stages of the study. Previous studies indicated that PER was high in European eel (*Anguilla anguilla*) fed high dietary carbohydrate (high PPs), resulting in a large amount of fat being deposited in the fish (De Silva & Anderson 1995). Plant meals contain significant amounts of carbohydrate that may have detrimental effects on Atlantic salmon performance (Waagbo *et al.* 1994; Hemre *et al.* 1995), as observed in the present study. High energy (fat and digestible carbohydrate) levels leads to better

utilisation of ingested protein because of an increased contribution of the non-protein energy sources to energy expenditure (Cho & Kaushik 1985, 1990). The level of dietary lipid (32 %) used in the present study was sufficient to meet both energy and EFA requirements of salmon and allowed protein sparing, resulting in increasing ADC of protein and PER and consequently increased perivisceral lipid deposition as evidenced by the increased VSI in fish fed the lower levels of FM. However, the salmon showed a negative relation between ADC of fat and inclusion level of PPs in diets in the present study. The diets with higher PP inclusion levels had higher NFE contents, especially the 11F and 5F diets. Generally, PP ingredients contain relatively high contents of carbohydrates, which are not well digested in carnivorous fish, including salmonids (Singh & Nose 1967; Hansen *et al.* 2007b) and that may have detrimental effects on Atlantic salmon performance (Waagbo *et al.* 1994; Hemre *et al.* 1995).

The ADC of protein (90 – 95 %) and fat (80 – 83 %) measured in the present study were comparable to those reported in previous studies in Atlantic salmon (Opstvedt *et al.* 2003; Mundheim *et al.* 2004; Aslaksen *et al.* 2007) but lower than digestibilities reported in rainbow trout (Cho & Kaushik 1990). This is likely due to differences in the sources and levels of ingredients, and of course, fish species. In previous studies on smaller Atlantic salmon, increasing dietary PPs as replacement for up to 50 % of FM reduced the ADCs of protein and fat, and protein retention, and the authors concluded that protease inhibitors and interaction between fat and carbohydrate fractions reduced protein and lipid digestibility (Aksnes 1995; Opstvedt *et al.* 2003; Mundheim *et al.* 2004). The interaction between carbohydrate and lipid in that high carbohydrate/fibre in diet has been reported limiting the metabolic capacity of the lipid in distal intestine epithelium (Storebakken 2002). Thus, the decreased ADC fat in the present study possibly related to the high PPs diets containing high non starch

carbohydrates such as pectin and acidic polysaccharide in legumes (Storebakken 2002), which cause increasing viscosity of ingested diet possibly resulting reduced fat digestibility.

As discussed above, the main reason that fish growth was reduced was likely related to reduce feed consumption. This would result in less lipid intake and so less energy available and less protein sparing. In addition, the AA provision would also being inadequate to meet the requirements to support maximum growth. Lewis & Kohler (2008) suggested that dietary AA imbalance resulted in elevated FCR of sunshine bass fed diets with decreasing dietary FM inclusion from 24 to 8 %. Generally, another major consequence of diets containing high PPs, in addition to the high carbohydrate contents, is the unbalanced AA profile of PPs compared to FM. Protein utilisation was reduced when dietary lysine was limited, but it was not observed when dietary methionine was limited (Berge *et al.* 1998; Espe *et al.* 1999). However, dietary AA balance can be improved by the addition of crystalline AAs to diets (Espe *et al.* 2008). Thus, several studies have demonstrated that high PP inclusion in diets did not effect protein utilisation as long as dietary AAs were balanced and feed intake was not significantly reduced (Espe *et al.* 2006, 2007). Dietary AA composition in the present study was formulated to mimic the AA composition of FM, and to meet protein and AA requirements of Atlantic salmon (NRC 1993), and so crystalline AAs (lysine, threonine, methionine) were added as supplements. All groups of salmon improved similarly with respect to nutrient utilisation (FCR and PER) during the second phase and at end of the trial indicating that the effect on FCR and PER are more related to feed intake reduction than to imbalanced AA composition in the diets.

All diets contained 28 – 30 % total lipid, primarily supplied by FO and RO in the same proportions, and all were supplemented with equal amounts of lecithin. The

reducing FM content had only small and largely non-significant effects on lipid composition of the fish. Flesh lipid content was not affected by the dietary treatment but the liver lipid content showed a clear downward trend from 7.1 % in fish fed the highest level of FM down to 5.2 % as FM inclusion decreased and PP inclusion increased. Contrastingly, the dietary treatments in the present study did not affect liver lipid class composition but did affect flesh lipid class composition. Total polar lipid in both flesh and liver tissues were predominantly PC and PE that are generally the major membrane phospholipids in fish tissues, while PS and PI are present in smaller quantities (Torstensen *et al.* 2000; Rosenlund *et al.* 2001; Bell *et al.* 2002, 2003a, 2004). There are biochemical and molecular mechanisms for remodeling phospholipid classes, and phospholipid remodeling is certainly involved in adaptation of biological membranes to different environmental conditions (Tocher *et al.* 2008). So, it is perhaps possible that the interaction between dietary protein sources and lipid sources resulted in effects on the flesh lipid class composition, but the changes in composition were so small that it was unlikely to have been physiologically relevant.

The nutritional quality of fish products is important with respect to human consumption, particularly in terms of flesh FA composition, specifically the n-3 HUFA, EPA and DHA. The strong relationship between dietary lipid and tissue FA composition is well documented (Bell *et al.* 2003b, 2004). In the present study, it was noteworthy that substituting FM up to very high levels did not reduce levels of EPA and DHA in the flesh below those observed in salmon fed the 25FM diet. In fact EPA and ARA both tended to increase in both flesh and liver with increasing FM replacement by PPs, and DHA also showed an increasing trend in liver with increasing dietary PP inclusion. There were also indications of decreasing 18:2n-6 and 18:3n-3 in flesh as PP inclusion increased. These changes in tissue PUFA composition cannot be adequately explained

by dietary FA compositions as ARA and EPA were constant in the diets and DHA decreased with increasing PP inclusion. Therefore, the effects observed are likely due to changes in metabolism with increased desaturation of 18:2n-6 and 18:3n-3 and/or increased retention of HUFA as PP inclusion increased. However, this may be partly related to reduced lipid levels in liver where TAG decreased and phospholipid increased. When FAs were provided at low concentrations in diets, they tend to be preferentially retained or deposited in tissue (Henderson & Sargent 1985b; Henderson 1996; Caballero *et al.* 2002). Levels of ARA, EPA and, especially, DHA were higher in flesh, and especially liver, than in the diets suggesting retention, whereas levels of 18:2n-6 and 18:3n-3 in tissues were less than in the diet suggesting that these FAs were selectively utilised for energy or for synthesis of longer chain and more unsaturated products. The FA composition of flesh may also reflect another metabolic effect as the increasing proportions of 16:0 and 16:1 with increasing PP inclusion may suggest increased lipogenesis in the fish with decreased feed intake. In contrast, the tissue levels of some FAs did reflect dietary levels, with 20:1 and 22:1 both decreasing in liver and flesh as FM inclusion decreased.

In conclusion, high inclusion of PP and reduced levels of FM in dual-substituted diets affected growth and nutrient digestibility in Atlantic salmon. ADC of fat was decreased with increasing PP inclusion in the diets probably associated with increased carbohydrate/fiber, whereas ADC of protein was increased as PP inclusion in diet increased. However, the fish consumed less feed as FM inclusion in the diets decreased and this effect was observed throughout the trial although it appeared greater in the first growth phase. Changing feed ingredients may have affected the physical texture of the feeds and/or chemical olfactory attractants that reduced the palatability of the diets. Enhancing the palatability of the diets by adding additional feed attractants may help to

minimise detrimental effects on feed intake. The FCR and PER were improved at the second and at the end of the trial suggesting that there was more related to metabolic adaptation to the diets than AA limitation in diets. The inclusion of PPs appeared to decrease liver lipid content but increased visceral lipid deposition. EFA composition, n-3 PUFA, EPA, DHA and ARA in both flesh and liver tissues were not reduced by reduced FM inclusion and, indeed, were slightly increased in the fish fed the lower FM inclusions. Therefore, reduction of FM inclusion from 25 % down to 5 % did not significantly affect nutritional quality of the fish product (flesh) in fish fed dual-substituted feeds with FM content reduced to just 5 % and FO content at 12 %.

Chapter 7 - Dual replacement of dietary fish oil and fish meal in feeds for large Atlantic salmon (*Salmo salar* L.). II- Effects of different blends of alternative protein sources as replacers for dietary fishmeal

7.1 Introduction

The supply of major fish feed ingredients such as FM and FO, the predominant sources of protein and lipid for carnivorous fish has become a limiting factor for expansion of aquaculture due to the fishing pressure on feed-grade fisheries (Sargent & Tacon 1999; Pike 2005). The production of these marine raw materials is not able to increase above current levels and, coupled with the increasing demand for feeds for both land animal and fish farming, this is resulting in market forces driving the price upwards. As a result it is no longer feasible to use FM and FO at the current high level of use either now and, especially, in the future (FAO 2006). Therefore, the growth in aquaculture production will not be sustainable if aquafeeds continue to depend substantially on these marine products. This has led fish nutritionists to investigate new sustainable protein and oil sources as alternatives to FM and FO in aquafeeds to ensure healthy and cost-effective production (Greene & Selivonchick 1990; Guillou *et al.* 1995; Turchini *et al.* 2003). Previous studies indicated that replacement of FM and FO with unconventional protein and lipid sources from plant and animal sources would be possible provided the AA and EFA requirements are met (Steffens 1997; Sargent & Tacon 1999).

Studies have been conducted using land animal products including poultry by-products, meat, bone and blood meals to substitute FM in fish diets (Murai 1992; Smith *et al.* 1995; Robaina *et al.* 1997; Webster *et al.* 2000). In addition, PP sources seem to offer great potential as truly sustainable protein sources for aquafeeds although

they often may contain ANFs, such as those found in soybean meal, which affect growth performance and health of fish (Francis *et al.* 2001). Thus, rapeseed, soybean, sunflower, beans and peas are inexpensive and readily available in high quantities, although they have their own desirable and undesirable (e.g. antinutrients, fibre) characteristics that both support and limit their use (Krogdahl *et al.* 1994; Francis *et al.* 2001; Krogdahl *et al.* 2005). Corn gluten meal has been shown to be a good alternative for FM replacement in salmon diets, being low in ANFs (Mente *et al.* 2003; Pereira & Oliva-Teles 2003). Wheat gluten has high digestibility and palatability but is expensive (Hardy 1996). Soy protein concentrates (SPC) have been reported as excellent sources for partial substitution of FM for many species of fish without reducing growth (Nengas *et al.* 1996; Refstie *et al.* 2001; Kaushik *et al.* 2004), and field peas and pea protein have been shown to be good protein sources for Atlantic salmon (Aslaksen *et al.* 2007). Partial replacement of FM with plant meals at a variety of different levels of substitution in fish diets has been studied in several fish species including rainbow trout (Smith *et al.* 1995; Kaushik *et al.* 1995), European sea bass (Kaushik *et al.* 2004), gilhead sea bream (Benedito-Palos *et al.* 2008), Atlantic cod (Hansen *et al.* 2007b) and Atlantic salmon (Espe *et al.* 2006; Torstensen *et al.* 2008). Generally, the replacement of up to 30 - 40 % FM with single plant meals does not compromise growth of fish (Nengas *et al.* 1996; Refstie *et al.* 2001; Lozano *et al.* 2007). However, replacement of greater than 70 % of dietary FM by blends of plant meal resulted in negative effect on growth performance of various fish including salmonids (Gomes *et al.* 1995; Espe *et al.* 2006; Torstensen *et al.* 2008). Although generally complete replacement of FM by plant meals has not been very successful, substitution of close to 100 % of dietary FM by blends of PP was possible in Atlantic salmon with no major negative effects on growth if the AA profile in the feed was well balanced, and if feed intake was

comparable to a high FM diet (Espe *et al.* 2007). This demonstrated the potential of replacing dietary FM with mixtures of alternative protein sources in Atlantic salmon.

Plant (vegetable) oils have been suggested as the primary sustainable alternatives to marine FO in aquafeeds. However, replacing FO in aquaculture diets presents difficulties due to the important differences in FA compositions of FO and VO. Capelin oil, a northern hemisphere FO frequently used in salmon feeds, is generally rich in long-chain MUFAs and n-3 HUFAs (Torstensen *et al.* 2005). In contrast, most VOs are rich in C18 FAs and only a few contain much n-3 PUFA. For example, RO is rich in 18:1n-9, has moderate levels of 18:2n-6 and some 18:3n-3 but, as with all VOs, is devoid of the n-3 HUFA EPA and DHA, found in marine FO (Bell *et al.* 2003a; Caballero *et al.* 2002; Sargent *et al.* 2002). In Atlantic salmon, a number of studies have reported that there was no growth reduction when dietary FO was substituted by single or blended VOs (Bell *et al.* 2001, 2002; Torstensen *et al.* 2004a, 2005), but tissue FA composition was altered, with reduced levels of EPA and DHA (Bell *et al.* 2002, 2003b). As a result, several studies have reported the use of a high FO "finishing" diet to restore the n-3 HUFA levels in flesh prior to harvesting (Bell *et al.* 2003a, 2004; Torstensen *et al.* 2005). The study described in the previous chapter reported the effects of progressive reduction in dietary FM in dual-substituted feeds for Atlantic salmon. Compared to a control diet, formulated to represent the current upper levels of substitution of FM and FO, growth of Atlantic salmon was progressively reduced as the FM content of the diet was similarly reduced from 25 % down to 5 % (Chapter 6). The aim of present study was to examine the effects of different alternative protein blends (mixtures of sunflower meal, corn gluten meal, soybean meal, wheat gluten, field pea and land animal products) on growth of Atlantic salmon fed dual-substituted feeds with almost 90 % of FM replaced and 60 % of dietary FO replaced by RO. Large Atlantic

salmon of initial weight of 1.3 Kg were grown to market size (> 3 Kg) on the different feeds over a period of 19 weeks. All diets were supplemented with crystalline AA and soybean lecithin. The growth performance, feed utilisation, lipid class composition and FA profiles of flesh and liver were investigated.

7.2 Materials and Methods

7.2.1 Experimental fish

Two thousand two hundred and fifty Atlantic salmon of initial mean weight 1.3 ± 0.1 kg were randomly distributed among 15 cages of 125 m^3 (5 × 5 × 5 m) with 150 fish/cage at the Marine Harvest Fish Trials Unit (FTU), Ardnish, Scotland. The fish were fed one of five diets in triplicate cages. The experiment was conducted over 19 weeks from October 2007 to February 2008 under natural photoperiod. The mean temperature, salinity and turbidity of seawater throughout the experiment were 8.6 ± 2.3, 26.4 ±6.0 and 5.6 ± 1.6 m, respectively. Fish were fed by automatic feeders (Arvo-tec, Sterner Arvo-tec UK, Inverness, Scotland) to apparent satiation. Mortalities, feed consumption and waste feed were recorded daily.

7.2.2 Sampling protocol

All the experimental fish were bulk weighed at the initiation, at the end of week 8 and at the termination of the trial, week 19. At the end of the trial, 2 fish per pen (6 fish per dietary treatment) were anaesthetised with metacaine sulphonate (MS222; 50 mg/L) and killed by a blow to the head. Flesh samples were taken using the NQC and were homogenised in a food processor after removal of skin and bones and stored at -20 ^oC prior to lipid analysis. Livers were also collected from the six fish and a 1 - 2 g sample placed into glass vials containing chloroform/methanol (2:1, by vol.) for

analysis of lipid class and FA composition, and the remaining portion immediately frozen on dry ice (for lipid content) subsequently to stored at -20 ^{o}C.

7.2.3 Experimental diets

Five diets were formulated to satisfy the nutritional requirements of salmonid fish (NRC. 1993) and manufactured at Biomar TechCentre, Brande, Denmark. All diets contained ~35 % crude protein and ~28 % crude lipid. The control diet was formulated to contain 25 % FM, the minimum level of FM inclusion currently in commercial use, and the remaining experimental diets contained 11 % FM. The protein ingredients of the control diet (25F) contained 25 % FM and 45 % alternative proteins (a blend of PPs including sunflower meal, corn gluten, soybean protein concentrate and field beans). The basal ingredients of the remaining four experimental diets were 11% FM and 55 % alternative proeins that were, diet 11FW (qualitatively similar blend to 25F plus some wheat gluten), 11FP (qualitatively similar blend to 11FW plus pea protein), 11FB (qualitatively similar blend to 11FW plus blood meal) and 11FK (qualitatively similar blend to 11FW plus kidney beans) (Table 7.1). All diets were coated with a 60:40 blend of RO and northern hemisphere FO and all the experimental diets were supplemented with crystalline AAs, lecithin and carophyll pink as sources of AAs, phospholipid and pigments (Table 7.1).

7.2.4 Proximate composition analysis

Diets were ground prior to determination of proximate composition (AOAC 2000). Crude lipid contents of the experimental diets were determined by acid hydrolysis followed by Soxhlet lipid extraction. The analyse methods are thoroughly described in Chapter 2.

Table 7.1. Feed formulation (mg kg^{-1}) and proximate composition (percentage) of the experimental diets.

Feed ingredients	25F	11FW	11FP	11FB	11FK
Fishmeals (67% protein/10% lipid)[1]	250	110	110	110	110
Sunflower meal (37% protein/10 lipid)	115	40	4	108	40
Corn gluten (62% protein/2 lipid)	85	175	130	100	175
Soy concentrate (60% protein/2% lipid) [2]	85	175	130	100	175
Wheat gluten (77% protein/3% lipid)	-	18	17	11	18
Binders	160	160	160	160	-
Kidney beans	-	-	-	-	160
Pea protein	-	-	130	-	-
Bloodmeal	-	-	-	84	-
Rapeseed oil[3]	173	178	176	180	178
Fish oil[4]	116	118	117	120	118
Vitamin and mineral[5]	11.95	23.59	25.48	21.49	23.59
L-Lysine6	0.62	3.44	1.96	0.02	3.44
L-Threonine[6]		0.43	0.59	0.08	0.43
DL-Methionine[6]	0.57	1.56	2.33	2.22	1.56
Lecithin	5.0	5.0	5.0	5.0	5.0
Astaxanthin[7]	0.4	0.4	0.4	0.4	0.4
Antioxidant[8]	4.25	4.25	4.25	4.25	4.25
Proximate composition (%)					
Protein	34.3	35	34.5	36.1	35.3
Lipid	29.8	27.9	27.5	28.8	28.3
Moisture	6.7	6.2	6.2	6.5	5.5
Ash	6.0	5.2	5.4	5.0	5.3
NFE	23.2	25.7	26.4	23.6	25.6

[1] Blend of Peruvian super prime and standard fishmeals produced from Anchoveta
[2] Soy protein concentrate produced from defatted soy flakes
[3] Non-GM low erucic acid and glycosinolates
[4] North-Atlantic standard fish oil
[5] Vitamin and mineral premixes (Premier Nutrition Products Ltd., Staffordshire, UK) with limestone carrier added according to the commercial standards of BioMar AS
[6] Highly purified (99%) crystalline amino acids
[7] Carophyll Pink CWS 10% product (F. Hoffman-La Roche Ltd., Basel, Switzerland)
[8] Blend of antioxidants and starch carrier added according to the commercial standards of BioMar
[9] NFE (nitrogen free extract) calculated by subtraction, 100 - (crude protein + crude fat + moisture + ash)

7.2.5 Lipid extraction and analysis

Total lipids of tissues and diet samples were extracted and prepared according to the method of Folch *et al.* (1957). Separation of lipid classes was performed by HPTLC, and quantification was by densitometry according to the method of Henderson & Tocher (1992). FAMEss were prepared by acid-catalysed transesterification as described by Christie (Christie 1993), extracted and purified by TLC (Tocher & Harvie 1988), and separated and quantified by gas-liquid chromatography (Ackman 1980). The methods and analyses are described in detail in Chapter 2.

7.2.6 Calculations and statistical analysis

The FCR, SGR and TGC were calculated using the formulae presented in Chapter 2. The effects of dietary treatment and growth performance were analysed by ANOVA. Statistical analyses were performed using a SPSS version 14 (SPSS inc, 2005). Significant differences were regarded as significant when $P < 0.05$ (Zar, 1999) as described in Chapter 2.

7.3 Results

7.3.1 Proximate, lipid and fatty acid composition of the diets

The experimental diets contained protein in the range 34 – 36 % and lipid in the range 27-30 %. The lipid, moisture and ash contents of the 11F diets were slightly lower than those of the 25F diet. The nitrogen-free extract (NFE) content of the11FP diet was higher than the 11F and 25F diets (Table 7.1). The polar lipid contents of the diets ranged from 8 - 10 % whilst the neutral lipid contents ranged from 90 - 92 % (Table 7.2). The main lipid supplied in the dietary treatments was neutral lipid, predominantly TAG about 74% ot toal lipid. The major polar lipid classes in the dietary lipids were

PC, PE, and PI /PS, with PE generally the predominant polar lipid in all diets. The control (25F) diet tended to have higher PC and lower PE and PI/PS in comparison to the diets containing the lower (11 %) FM contents (hereafter collectively termed the 11F diets). Of the 11F diets, the 11FW diet had lowest PE content and highest PI/PS content. Regarding to neutral lipid, the 25F and 11FK diets contained slightly higher sterol than the other three 11F diets. Among the 11F diets, the 11FW contained highest FFA composition (Table 7.2).

There were a few quantitatively minor differences in the FA compositions among the diets. The proportion of total SFAs tended to be higher in the diets with FM inclusion of 11 % compared to the control diet with 25 % FM (Table 7.3). Of the 11F groups, the 11FB and 11FK diets had higher SFA values than the 11FW and 11FP diets. Dietary SFA changed mainly due to the proportions of 16:0, with the 25F diet containing 8.7 % compared to 9.1 - 9.4 % in the diets with FM inclusion decreased to 11 %. Within the 11F diets, the 11FK diet had highest 16:0 content (Table 7.3). Total MUFA ranged from 52 to 55 %, with the 25F, 11FW and 11FP diets having higher MUFA than the 11FB and 11FP diets. The principal MUFA was 18:1n-9 representing 38 – 41 % of total FAs. The 25F diet generally contained higher 16:1, 20:1 and 22:1, but lower 18:1 than the 11F diets. Within the 11F diets, the 11FW diet had higher proportions of 18:1, 20:1 and 22:1 than the other 11F diets (Table 7.3). The dietary treatments contained approximately 16 - 17 % total n-6 PUFA of which 18:2n-6 was the major FA. Of the 11F diets, the 18:2n-6 content was highest in the 11FB diet. The total n-3 PUFA content of the dietary treatments ranged from 14 to 15 %. The total n-3 PUFA content of the 11FW diet was lowest in comparison to the other four diets. The 18:3n-3 content was lowest in the 25F diet which was opposited to the 22:6n-3 composition. In contrast, the 20:5n-3 composition was high in the 11F diets, particularly

the 11FB and 11FK. Total PUFA content of the diets ranged from 30 – 32 % with the the 11FB had highest total PUFA.

Table 7.2. Lipid class composition (percentage of total lipid) of the experimental diets.

Parameters	25F	11FW	11FP	11FB	11FK
Lipid classes					
PC	2.8	2.1	2.7	2.4	1.9
PE	3.5	3.9	5	5.4	4.5
PI/PS	1.5	3.2	1.9	1.7	2.3
SM	0.2	0.1	0.1	0.2	0.1
LPC	0.1	tr.	0.2	0.2	0.2
Total PL	8.2	9.3	9.9	10.1	9.1
Total NL	91.8	90.4	90.1	89.9	90.9
Triacylglycerol	74.2	73.9	74.6	73.2	73.8
Sterol	8.5	6.9	7.2	7.1	8.6
Free fatty acid	9.1	9.9	8.5	9.6	8.6
Steryl ester	tr.	tr.	tr.	tr.	tr.

Results are means of tripicate analysis. LPC, lysophosphatidylcholine; NL, neutral lipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, polar lipids; PS, phosphatidylserine; SM, sphingomyelin.

Table 7.3. Fatty acid compositions (percentage of total fatty acids) of the diets.

Parameters	25F	11FW	11FP	11FB	11FK
Fatty acids					
14:0	2.6	2.5	2.5	2.6	2.7
16:0	8.7	9.1	9.1	9.3	9.4
18:0	2.7	2.6	3.0	3.2	3.0
20:0	0.5	0.5	0.6	0.6	0.6
22:0	0.9	0.8	1.1	1.1	1.1
Total saturated[1]	15.5	15.7	16.3	16.9	16.9
16:1n-9	0.1	0.1	0.1	0.1	0.1
16:1n-7	3.0	2.8	2.8	2.8	2.9
18:1n-9	38.4	41.1	40.5	39.1	39.2
18:1n-7	2.7	2.7	2.7	2.9	2.9
20:1n-9	4.5	3.6	3.3	2.8	2.9
20:1n-7	0.2	0.2	0.2	0.2	0.2
22:1n-11	4.6	3.4	3.0	2.3	2.3
22:1n-9	0.7	0.6	0.6	0.6	0.7
24:1n-9	0.3	0.3	0.3	0.3	0.3
Total monoenes	54.6	54.8	53.5	51.1	51.5
18:2n-6	15.0	15.1	14.5	15.9	15.5
18:3n-6	0.1	0.1	0.1	0.1	0.1
20:2n-6	0.1	0.1	0.1	0.1	0.1
20:3n-6	0.2	0.3	0.2	0.3	0.2
20:4n-6	0.2	0.2	0.2	0.3	0.3
22:5n-6	0.1	0.1	0.1	0.1	0.1
Total n-6 PUFA	15.7	15.8	15.2	16.6	16.3
18:3n-3	5.6	5.8	6.1	6.1	6.3
18:4n-3	1.0	0.8	0.9	0.9	0.8
20:4n-3	0.2	0.2	0.2	0.2	0.2
20:5n-3	4.1	3.9	4.5	4.9	5.0
22:5n-3	0.4	0.4	0.5	0.5	0.5
22:6n-3	3.0	2.5	2.8	2.8	2.6
Total n-3 PUFA	14.2	13.7	15.0	15.3	15.4
Total PUFA	29.9	29.4	30.2	31.9	31.7

Values are means of tripicate analysis. [1]Totals includes 15:0 present at up to 0.2 %. PUFA, polyunsaturated fatty acid.

7.3.2 Growth performance

No significant difference in survival rate was observd between the fish fed the different dietary treatments throughout the trial (table 7.4). There were, however, significant differences between dietary treatments in body weights at both week 8 and week 19. The fish fed the control 25F diet had the highest body weights of 2.5 and 3.8 kg respectively at the 8th week and 19th week, significantly higher than the weights of fish fed the 11F diets, that ranged from 2.2 - 2.3 kg and 3.3 - 3.5 kg at week 8 and 19, respectively. Although the fish fed 11FP diet, the diet containing pea protein, had a slightly higher average body weight at the end of the experiment, there were no significant differences in body weight between fish fed the 11F diets (Table 7.4).

The fish fed the 25F diet had the highest weight gains that were generally significantly higher than the fish receiving the 11F diets. Over the duration of the entire experiment, the control group displayed the significantly highest weight gain (> 2.5 kg), whilst there were no statistical differences between the 11F groups (2.0 - 2.2 kg) (Table 7.4). Within the fish fed 11F diets, the fish fed the 11FB diet had the highest weight gain at 8 weeks, which was significantly different from the 11FW group. Between weeks 8 and 19, there were no significant differences in weight gain between the dietary treatments (1.2 - 1.4 kg) other than the 11FB group now had the lowest weight gain (1.0 kg).

The fish fed the 25F diet had the highest SGR and TGC values, which were significantly different from the 11F groups at the week 8 assessment. Comparison within the 11F group at 8 weeks showed the 11FW group had lowest SGR and TGC. From 8 to 19 weeks though, other than the 11FB group showing significantly lower SGR and TGC compared to fish fed all other dietary treatments, there were no significant differences in SGR and TGC between the 25F and the other three 11F

groups. Over the whole experiment, SGR and TGC of fish fed the 25F diet were significantly higher than fish fed the 11F diets. Within the 11F groups, there were no significant differences in SGR while there was a significant difference in the TGC value only between the 11FP and 11FB groups (Table 7.4).

Feed consumption of the control 25F group was significantly higher than the 11F groups throughout the experiment. At week 8, there was no significant difference in feed consumption between the 11F groups, but between week 8 and 19, the 11FP group showed significantly higher feed consumption than the 11FW and 11FB groups. Over the whole trial, the 11FB group displayed the lowest feed consumption, which was significantly different from the 11FP and 11FK groups (Table 7.4). At week 8, fish fed the 25F diet had the lowest FCR and highest PER values, although it was only statistically different between the 25F and 11FW groups. By week 19 and over the entire experiment, there were no significant differences in FCR and PER between fish fed the different dietary treatments (Table 7.4).

No significant differences in HSI were observed between fish fed the different dietary treatments in this experiment. In addition, there were no significant differences in VSI between the fish fed the control 25F diet and fish fed any of the 11F diets. Within the 11F diets, fish fed the 11FW diet had the highest VSI that was significantly different to fish fed the 11FP and 11FK diets (Table 7.4).

Table 7.4. Growth performance of Atlantic salmon fed the experimental diets for 19 weeks.

Parameters	25F	11FW	11FP	11FB	11FK
Survival(%)					
0-8 weeks	100 ± 0.0	98.9 ± 1.0	99.3 ± 0.0	99.6 ± 0.4	99.8 ± 0.4
8-19 weeks	100 ± 0.0	100 ± 0.0	100 ± 0.0	99.8 ± 0.4	99.8 ± 0.4
0-19 weeks	100 ± 0.0	98.9 ± 1.0	99.3 ± 0.0	99.3 ± 0.7	99.6 ± 0.6
Body weight(kg)					
Initial	1.3 ± 0.0	1.3 ± 0.0	1.3 ± 0.0	1.3 ± 0.0	1.3 ± 0.0
8 weeks	2.5 ± 0.0 ^{a}	2.2 ± 0.0 ^{b}	2.3 ± 0.0 ^{b}	2.3 ± 0.0 ^{b}	2.2 ± 0.1 ^{b}
19 weeks	3.8 ± 0.1 ^{a}	3.4 ± 0.1 ^{b}	3.5 ± 0.1 ^{b}	3.3 ± 0.1 ^{b}	3.4± 0.1 ^{b}
Weight gain (kg fish^{-1})					
0-8 weeks	1.2 ± 0.0 ^{a}	0.9 ± 0.1 ^{c}	0.9 ± 0.0 ^{bc}	1.1 ± 0.0 ^{b}	0.9 ± 0.1 ^{bc}
8-19 weeks	1.4 ± 0.1 ^{a}	1.2 ± 0.1 ^{a}	1.3 ± 0.1 ^{a}	1.0 ± 0.1 ^{b}	1.2 ± 0.0 ^{ab}
0-19 weeks	2.5 ± 0.1 ^{a}	2.1 ± 0.1 ^{b}	2.2 ± 0.1 ^{b}	2.0 ± 0.1 ^{b}	2.1 ± 0.1 ^{b}
SGR (%)					
0-8 weeks	1.3 ± 0.0 ^{a}	1.0 ± 0.0 ^{c}	1.1 ± 0.0 ^{bc}	1.1 ± 0.0 ^{b}	1.1 ± 0.1 ^{bc}
8-19 weeks	0.6 ± 0.0 ^{a}	0.6 ± 0.0 ^{a}	0.6 ± 0.0 ^{a}	0.5 ± 0.0 ^{b}	0.6 ± 0.0 ^{a}
0-19 weeks	0.8 ± 0.0 ^{a}	0.7 ± 0.0 ^{b}	0.7 ± 0.0 ^{b}	0.7 ± 0.0 ^{b}	0.7 ± 0.0 ^{b}
TGC					
0-8 weeks	4.7 ± 0.1 ^{a}	3.6 ± 0.1 ^{c}	3.9 ± 0.1 ^{bc}	4.0 ± 0.1 ^{b}	3.8 ± 0.2 ^{bc}
8-19 weeks	4.0 ± 0.2 ^{a}	3.7 ± 0.1 ^{a}	3.9 ± 0.2 ^{a}	3.1 ± 0.1 ^{b}	3.7 ± 0.1 ^{a}
0-19 weeks	4.3 ± 0.1 ^{a}	3.7 ± 0.1 ^{bc}	3.9 ± 0.1 ^{b}	3.6 ± 0.0 ^{c}	3.7 ± 0.1 ^{bc}
FCR					
0-8 weeks	1.0 ± 0.0^{b}	1.2 ± 0.1^{a}	1.1 ± 0.0^{ab}	1.1 ± 0.0^{ab}	1.1 ± 0.1^{ab}
8-19 weeks	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.0
0-19 weeks	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.0	1.1 ± 0.0
Feed Consumption (g fish^{-1}day^{-1})					
0-8 weeks	24.2 ± 0.2 ^{a}	19.6 ± 0.4 ^{b}	20.2 ± 0.2 ^{b}	20.7 ± 0.8 ^{b}	19.8 ± 0.7 ^{b}
8-19 weeks	18.1 ± 0.2 ^{a}	15.0 ± 0.3 ^{c}	15.8 ± 0.2 ^{b}	13.7 ± 0.1 ^{d}	15.7 ± 0.4 ^{bc}
0-19 weeks	20.4 ± 0.0 ^{a}	16.8 ± 0.2 ^{bc}	17.5 ± 0.1 ^{b}	16.5 ± 0.3 ^{c}	17.3 ± 0.6 ^{b}
PER					
0-8 weeks	2.8 ± 0.0 ^{a}	2.4 ± 0.1 ^{b}	2.6 ± 0.1 ^{ab}	2.6 ± 0.0 ^{ab}	2.6 ± 0.1 ^{ab}
8-19 weeks	2.8 ± 0.2	2.9 ± 0.2	2.9 ± 0.2	2.6 ± 0.2	2.7 ± 0.1
0-19 weeks	2.8 ± 0.1	2.7 ± 0.1	2.8 ± 0.1	2.6 ± 0.1	2.7 ± 0.0
HSI (%)					
0-9 weeks	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.0	1.1 ± 0.1
VSI (%)					
0-9 weeks	11.7 ± 0.7 ^{ab}	13.2 ± 0.4 ^{a}	11.4 ± 0.4 ^{b}	12.2 ± 0.7 ^{ab}	11.5 ± 0.6 ^{b}

Results are means ± SD (n = 3). Values within a row with different superscript letters are significantly difference as determined by ANOVA. FCR, feed conversion ratio; HSI, hepatosomatic index; PER, protein efficiency ratio; TGC, thermal growth coefficient; VSI, viscerosomatic index.

7.3.3 Lipid contents and lipid class compositions of flesh and liver

There were no significant differences in flesh lipid contents between the 25F and any of the 11F groups (Table 7.5). Within fish fed the 11F diets, the only significant difference was the lipid content of the flesh of fish fed diet 11FP that was higher than that of fish fed diet 11FK. Similarly, there were no significant differences in the proportions of total polar and neutral lipids in the flesh between fish fed the 25F diet and those fed any of the 11F diets (Table 7.5). Within fish fed the 11F diets, the flesh of fish fed the 11FB diet had significantly lower polar, and higher neutral, lipid compared to fish fed the 11FW and 11FK diets. The predominant neutral lipid in the flesh was TAG, with PC and PE being the major polar lipids. There were no significant differences in individual neutral lipid classes in flesh and the only significant differences in polar lipid classes were in PI/PS content with flesh of fish fed the 25F, 11FW and 11FK diets having slightly higher levels than flesh of fish fed the 11FP and 11FB diets (Table 7.5).

Liver lipid content was lower in fish fed the 11F diets compared to fish fed the 25F diet, significantly so in the case of the 11FB and 11FK groups (Table 7.5). The fish fed the 11FB diet also showed a lower proportion of neutral lipids (and higher polar lipids) in liver lipid than fish fed the 25F and 11FW diets. As with flesh, the predominant neutral lipid in liver was TAG with the major polar lipids being PC, PE, PI and PS. The higher polar lipids in liver of fish fed diet 11FB compared to fish fed diet 25F was due to higher proportions of PE, PI and PS. The proportions of PI and PS were also significantly higher in the 11FB group than the 11FW group. There were few differences in neutral lipid classes in liver other than the 11FB and 11FP groups had higher FFA compared to the 25F and 11FW groups.

Table 7.5. Lipid contents (Percentage of wet weight) and lipid class compositions (percentage of total lipid) of flesh and liver of Atlantic salmon fed the experimental diets for 19 weeks.

Parameters	25F	11FW	11FP	11FB	11FK
Flesh					
Lipid contents	11.7 ± 1.2 ^{ab}	11.7 ± 1.8 ^{ab}	13.2 ± 1.1 ^{a}	12.2 ± 1.2 ^{ab}	10.5 ± 0.6 ^{b}
Lipid class					
PC	4.4 ± 0.3	4.7 ± 0.7	4.4 ± 0.7	4.3 ± 0.7	5.0 ± 0.5
PE	4.4 ± 0.9	4.6 ± 0.9	3.9 ± 0.7	3.7 ± 0.7	4.1 ± 0.6
PI/PS	0.9 ± 0.2 ^{ab}	1.0 ± 0.1^{ab}	0.4 ± 0.4^{c}	0.4 ± 0.4^{c}	1.2 ± 0.4 ^{a}
SM	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ±0.1	0.3 ± 0.2
LPC	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
Total PL	10.0 ± 1.1^{ab}	10.8 ± 1.4 ^{a}	8.9 ± 0.9 ^{ab}	8.7 ± 1.5 ^{b}	10.9 ± 1.1 ^{a}
TotalNL	90.0 ± 1.1^{ab}	89.2 ± 1.4 ^{b}	91.0 ± 0.9 ^{ab}	91.3 ± 1.5 ^{a}	89.1 ± 1.1 ^{b}
TAG	78.9 ± 1.1	78.7 ± 2.6	80.0 ± 2.3	80.6 ± 1.5	78.4 ± 1.9
Sterol	5.8 ± 0.6	5.8 ± 0.7	6.3 ± 0.8	5.8 ± 0.4	5.7 ± 0.7
FFA	5.4 ± 0.6	4.7 ± 1.0	4.7 ± 0.9	4.8 ± 0.6	4.9 ± 0.9
SE	tr.	tr.	tr.	tr.	tr.
Liver					
Lipid contents	7.1 ± 2.3^{a}	5.7 ± 1.5^{ab}	4.7 ± 1.4^{ab}	4.2 ± 0.6^{b}	4.3 ± 0.7^{b}
Lipid class					
PC	16.0 ± 3.9	16.5 ± 3.1	19.4 ± 2.9	19.4 ± 2.7	17.5 ± 1.7
PE	10.2 ± 2.5 ^{b}	10.7 ± 2.1 ^{ab}	12.9 ± 2.1^{ab}	14.0 ± 2.0 ^{a}	11.1 ± 1.3^{ab}
PI	3.6 ± 1.0 ^{b}	3.4 ± 1.1 ^{b}	4.1 ± 1.0 ^{ab}	5.6 ± 1.6 ^{a}	4.5 ± 0.6^{ab}
PS	2.3 ± 0.9 ^{b}	2.4 ± 0.9 ^{b}	2.9 ± 0.9 ^{ab}	4.6 ± 1.7 ^{a}	3.4 ± 0.6^{ab}
SM	1.8 ± 0.6	1.8 ± 0.5	2.2 ± 0.3	3.3 ± 0.7	2.0 ± 0.3
LPC	0.2 ± 0.1	0.1 ± 0.1	0.3 ± 0.2	0.3 ± 0.2	0.2 ± 0.1
UPL	2.3 ± 1.4	2.4 ± 0.9	2.7 ± 0.6	3.1 ± 1.3	3.0 ± 0.6
TotalPL	36.4 ± 9.5 ^{b}	37.4 ± 8.3 ^{b}	44.5 ± 7.6 ^{ab}	49.6 ± 8.7 ^{a}	41.9 ± 4.8^{ab}
TotalNL	63.6 ± 9.5 ^{a}	62.6 ± 8.3 ^{a}	55.5 ± 7.6 ^{ab}	50.4 ± 8.7 ^{b}	58.1 ± 4.8^{ab}
TAG	49.0 ± 12.7	46.0 ± 11.7	36.9 ± 8.9	33.2 ± 11.1	41.1 ± 5.2
Sterol	10.4 ± 2.1	12.7 ± 2.2	13.1 ± 2.2	13.4 ± 1.6	12.1 ± 1.3
FFA	0.9 ± 0.9 ^{d}	1.8 ± 0.2 ^{cd}	2.5 ± 0.9 ^{abc}	3.4 ± 1.0 ^{a}	2.2 ± 0.4 ^{bcd}
Steryl ester	tr.	tr.	tr.	tr.	tr.
UNL	3.3 ± 0.8^{a}	3.4 ± 1.3^{a}	2.9 ± 1.7 ^{a}	0.6 ± 0.3^{b}	2.7 ± 1.2 ^{a}

Results are means ± SD (n = 3). Values within a row with different superscript letters are significantly different as determined by ANOVA. FFA, free fatty acid; LPC, lysophosphatidylcholine; NL, neutral lipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, polar lipids; PS, phosphatidylserine; SM, sphingomyelin; TAG, triacylglycerol; tr., trace; UNL, unidentified neutral lipid; UPL, unidentified polar lipid.

7.3.4 Fatty acid compositions of flesh and liver

Changes in flesh FA compositions largely corresponded to differences in the FA compositions of the diets (Table 7.6). Dietary treatment had no affect on flesh total SFA, or on total n-6 PUFA other than this being lower in fish fed diet 11FP compared to fish fed the other 11F diets. Total MUFA was significantly higher in flesh of fish fed the 25F diet compared to fish fed the 11F diets, while total n-3 PUFA and total PUFA were significantly lower in flesh of fish fed diet 25F compared to fish fed diets 11FW, 11FB and 11FK. There were no significant differences in flesh total MUFA and total n-3 PUFA between fish fed any of the 11F diets. The major SFA, 16:0, was lower in flesh of fish fed diet 25F compared to fish fed the 11F diets, significantly so in the case of diets 11FP and 11FB. In contrast, flesh 20:1 and 22:1 were significantly lower in fish fed the 11F diets compared to fish fed diet 25F. There were no significant differences in proportions of 18:2n-6 and 18:3n-3 in flesh of fish fed diet 25F compared to fish fed the 11F diets. Desaturated and elongated products of 18:2n-6 and 18:3n-3 especially 18:3n-6, 20:3n-6 and ARA, and EPA and 22:5n-3 were generally all significantly higher in flesh of fish fed the 11F diets compared to fish fed the 25F diet. Moreover, DHA tended to be higher in flesh of fish fed the 11F diets compared to fish fed the 25F diet.

Liver FA compositions were more variable than flesh, but there were clear trends for higher SFA, total n-6, total n-3 and total PUFA and lower MUFA in fish fed the 11F diets compared to fish fed the 25F diet, significantly so in the cases of total MUFA, total n-3 PUFA and total PUFA (Table 7.7). Similar to flesh, liver FA compositions reflected the dietary profile. Thus, liver 16:0 increased while the 18:1, 20:1 and 22:1 decreased in line with decreased dietary FM inclusion. There were no significant differences in 18:2n-6 and 18:3n-3 in liver between fish fed the 25F and 11F diets but, as with flesh, the liver of fish fed the 11F diets showed significantly higher

levels of ARA, EPA and DHA than fish fed the 25F diet. The retention levels of desaturated and elongated products at a higher concentration than provided in the diets were clearly observed in liver.

Table 7.6. Fatty acid compositions (percentage of total fatty acids) of flesh of Atlantic salmon fed the experimental diets for 19 weeks.

Parameters	25F	11FW	11FP	11FB	11FK
14:0	2.9 ± 0.1	3.0 ± 0.3	3.1 ± 0.3	3.0 ± 0.2	2.9 ± 0.2
16:0	10.5 ± 0.4 ^{b}	11.2 ± 0.5 ^{ab}	11.4 ± 0.6 ^{a}	11.3 ± 0.4 ^{a}	11.1 ± 0.3 ^{ab}
18:0	2.8 ± 0.1	2.8 ± 0.2	2.9 ± 0.2	2.9 ± 0.1	2.8 ± 0.1
20:0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
22:0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
Total saturated[1]	16.8 ± 0.5	17.6 ± 0.8	18.0 ± 1.1	17.8 ± 0.6	17.4 ± 0.6
16:1n-9	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
16:1n-7	3.4 ± 0.1	3.6 ± 0.3	3.7 ± 0.3	3.6 ± 0.2	3.5 ± 0.3
18:1n-9	35.4 ± 1.0	34.4 ± 1.8	35.1 ± 1.3	34.8 ± 1.0	34.2 ± 1.2
18:1n-7	3.1 ± 0.2	3.2 ± 0.2	3.2 ± 0.2	3.1 ± 0.2	3.3 ± 0.2
20:1n-9	5.3 ± 0.2 ^{a}	4.5 ± 0.3 ^{b}	4.1 ± 0.2 ^{bc}	3.5 ± 0.1 ^{d}	3.8 ± 0.2 ^{cd}
20:1n-7	0.3 ± 0.0 ^{a}	0.3 ± 0.0 ^{a}	0.3 ± 0.0 ^{a}	0.2 ± 0.0 ^{b}	0.3 ± 0.0 ^{a}
22:1n-11	3.7 ± 0.3 ^{a}	2.6 ± 0.1 ^{b}	2.4 ± 0.2 ^{b}	1.9 ± 0.1 ^{c}	2.0 ± 0.1 ^{c}
22:1n-9	0.7 ± 0.1 ^{a}	0.6 ± 0.0 ^{b}	0.6 ± 0.0 ^{b}	0.6 ± 0.0 ^{b}	0.6 ± 0.0 ^{b}
24:1n-9	0.6 ± 0.2 ^{a}	0.5 ± 0.0 ^{ab}	0.4 ± 0.0 ^{b}	0.5 ± 0.1 ^{ab}	0.4 ± 0.0 ^{b}
Total monoenes	52.6 ± 1.5 ^{a}	49.9 ± 1.9 ^{b}	50.1 ± 1.4 ^{b}	48.4 ± 0.7 ^{b}	48.3 ± 1.2 ^{b}
18:2n-6	12.1 ± 0.5 ^{ab}	11.4 ± 0.6 ^{b}	11.3 ± 0.8 ^{b}	12.6 ± 0.5 ^{a}	12.5 ± 0.5 ^{a}
18:3n-6	0.1 ± 0.0 ^{b}	0.2 ± 0.0 ^{a}	0.2 ± 0.0 ^{a}	0.2 ± 0.0 ^{a}	0.2 ± 0.0 ^{a}
20:2n-6	1.0 ± 0.1 ^{b}	1.2 ± 0.1 ^{a}	1.0 ± 0.1 ^{b}	1.0 ± 0.1 ^{b}	1.1 ± 0.1 ^{ab}
20:3n-6	0.4 ± 0.0 ^{b}	0.5 ± 0.1 ^{a}	0.5 ± 0.0 ^{a}	0.5 ± 0.0 ^{a}	0.5 ± 0.1 ^{a}
20:4n-6	0.3 ± 0.1 ^{b}	0.4 ± 0.1 ^{a}	0.4 ± 0.0 ^{a}	0.5 ± 0.0 ^{a}	0.5 ± 0.1 ^{a}
22:5n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Total n-6 PUFA[2]	14.1 ± 0.4 ^{ab}	13.9 ± 0.6 ^{ab}	13.5 ± 0.9 ^{b}	14.9 ± 0.5 ^{a}	14.9 ± 0.5 ^{a}
18:3n-3	4.0 ± 0.2 ^{ab}	3.9 ± 0.3 ^{b}	4.0 ± 0.3 ^{ab}	4.1 ± 0.2 ^{ab}	4.3 ± 0.3 ^{a}
18:4n-3	0.7 ± 0.1 ^{b}	0.8 ± 0.1 ^{ab}	0.8 ± 0.1 ^{ab}	0.9 ± 0.0 ^{a}	0.7 ± 0.0 ^{b}
20:3n-3	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0
20:4n-3	0.7 ± 0.1	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.1	0.8 ± 0.1
20:5n-3	3.8 ± 0.3 ^{b}	4.8 ± 0.7 ^{a}	4.6 ± 0.6 ^{a}	4.8 ± 0.3 ^{a}	4.9 ± 0.5 ^{a}
22:5n-3	1.6 ± 0.2 ^{b}	2.2 ± 0.3 ^{a}	2.1 ± 0.2 ^{a}	2.1 ± 0.1 ^{a}	2.4 ± 0.4 ^{a}
22:6n-3	5.2 ± 0.9	5.7 ± 0.8	5.7 ± 0.5	5.9 ± 0.5	5.8 ± 0.5
Total n-3 PUFA	16.4 ± 1.3 ^{b}	18.6 ± 1.7 ^{a}	18.4 ± 1.4 ^{ab}	19.0 ± 0.7 ^{a}	19.4 ± 1.1 ^{a}
Total PUFA	30.5 ± 1.4 ^{c}	32.5 ± 1.5 ^{abc}	31.8 ± 1.9 ^{bc}	33.9 ± 0.7 ^{ab}	34.3 ± 0.8 ^{a}

Results are means ± SD (n = 3). Values within a row with different superscript letters are significantly different as determined by ANOVA. [1]Totals includes 15:0 present at up to 0.1 %; [2]Totals include 22:4n-6 presents at up to 0.1 %. PUFA, polyunsaturated fatty acid.

Table 7.7. Fatty acid compositions (percentage of total fatty acids) of liver of Atlantic salmon fed the experimental diets for 19 weeks.

Parameters	25F	11FW	11FP	11FB	11FK
14:0	1.6 ± 0.1	1.5 ± 0.1	1.6 ± 0.3	1.4 ± 0.1	1.4 ± 0.1
16:0	8.7 ± 1.7	9.5 ± 1.9	12.0 ± 3.0	11.7 ± 2.7	11.3 ± 2.7
18:0	4.0 ± 0.8	4.1 ± 0.3	4.7 ± 0.8	4.9 ± 0.3	4.9 ± 0.4
20:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Total saturated[1]	14.6 ± 2.4	15.5 ± 2.3	18.6 ± 3.8	18.4 ± 2.9	18.0 ± 2.8
16:1n-9	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
16:1n-7	2.5 ± 0.6	2.3 ± 0.5	2.0 ± 0.4	1.9 ± 0.2	1.8 ± 0.2
18:1n-9	35.8 ± 5.5 [a]	33.1 ± 4.9 [ab]	27.3 ± 4.6 [ab]	26.1 ± 4.[9 b]	27.1 ± 4.7 [b]
18:1n-7	3.4 ± 0.3 [a]	3.3 ± 0.2 [a]	2.9 ± 0.2 [b]	3.1 ± 0.2 [ab]	3.1 ± 0.2 [ab]
20:1n-9	7.5 ± 1.2 [a]	5.7 ± 1.0 [ab]	4.0 ± 1.1 [bc]	3.5 ± 1.1 [c]	3.7 ± 1.2 [c]
20:1n-7	0.4 ± 0.1 [a]	0.3 ± 0.0 [ab]	0.2 ± 0.0 [b]	0.2 ± 0.0 [b]	0.2 ± 0.1 [b]
22:1n-11	1.6 ± 0.2 [a]	1.1 ± 0.1 [b]	0.8 ± 0.2 [c]	0.5 ± 0.1 [d]	0.6 ± 0.1 [cd]
22:1n-9	0.4 ± 0.1 [a]	0.4 ± 0.1 [a]	0.3 ± 0.1 [b]	0.3 ± 0.1 [b]	0.3 ± 0.1 [b]
24:1n-9	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Total monoenes	52.4 ± 7.5 [a]	47.1 ± 6.6 [a]	38.5 ± 6.2 [b]	36.5 ± 6.5 [b]	37.9 ± 6.2 [b]
18:2n-6	8.7 ± 0.6	8.4 ± 0.5	7.9 ± 0.9	8.4 ± 0.9	8.5 ± 0.9
18:3n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:2n-6	2.1 ± 0.3	2.1 ± 0.3	1.6 ± 0.3	1.9 ± 0.5	1.8 ± 0.5
20:3n-6	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
20:4n-6	0.9 ± 0.4 [b]	1.3 ± 0.4 [ab]	1.8 ± 0.4 [a]	1.9 ± 0.4 [a]	2.0 ± 0.4 [a]
22:5n-6	0.1 ± 0.0 [b]	0.2 ± 0.0 [ab]	0.2 ± 0.1 [ab]	0.3 ± 0.0 [a]	0.2 ± 0.0 [b]
Total n-6PUFA	12.5 ± 0.6	12.8 ± 0.5	12.2 ± 0.7	13.3 ± 1.0	13.3 ± 1.0
18:3n-3	2.5 ± 0.3	2.6 ± 0.3	2.5 ± 0.4	2.5 ± 0.2	2.8 ± 0.3
18:4n-3	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
20:3n-3	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
20:4n-3	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
20:5n-3	5.5 ± 1.6 [b]	7.3 ± 1.6 [ab]	9.0 ± 2.0 [a]	8.5 ± 0.6 [a]	8.7 ± 1.0 [a]
22:5n-3	1.4 ± 0.2 [b]	2.2 ± 0.5 [ab]	2.8 ± 0.6 [a]	2.9 ± 0.9 [a]	2.9 ± 1.0 [a]
22:6n-3	9.7 ± 4.2 [b]	10.9 ± 2.9 [ab]	14.8 ± 2.5 [ab]	16.5 ± 3.5 [a]	14.8 ± 3.1 [ab]
Total n-3PUFA	20.5 ± 5.5 [b]	24.7 ± 4.8 [ab]	30.7 ± 4.4 [a]	31.9 ± 4.6 [a]	30.8 ± 4.5 [a]
Total PUFA	33.0 ± 5.4 [c]	37.4 ± 4.8 [bc]	42.9 ± 4.0 [ab]	45.1 ± 3.7 [a]	44.1 ± 3.7 [ab]

Results are means ± SD (n = 3). Values within a row with different superscript letters are significantly different as determined by ANOVA. [1]Totals includes 15:0 present at up to 0.2 %. PUFA, polyunsaturated fatty acid.

7.4 Discussion and conclusion

There were no major effects of dietary treatments on survival rate, or biometric parameters such as HSI and VSI of salmon in present study. However, reducing inclusion of FM from 25 % to 11 % by substitution with a blends PP or PP and land animal protein significantly reduced growth of the fish, irrespective of the particular blend of alternative proteins used. Thus, one of the main aims of this study was not achieved as the growth reduction observed in the previous trial by increasing substitution of FM with PPs was not prevented by any of the different formulations. Thus, at the end of trial, final body weights of fish fed the 11FW, 11FP, 11FB, and 11FK diets were decreased 11 %, 8 %, 13 % and 11 %, respectively, compared to the 25F group. The reduced weight gain of fish fed all the 11F diets, except 11FB, was mainly in the first phase of the trial, with no statistical differences between weight gains of fish fed the 11FW, 11FP and 11FK diets and fish fed the 25F group observed in the second phase (8-19 weeks). However, weight gain of all the 11F groups was still not comparable to weight of the 25F group at the end of trial. The weight gain data were supported by SGR and TGC data. During the first phase of trial, SGR and TGC of all the 11F groups were significantly lower than the 25F group, while in the second phase SGR and TGC in the 11FW, 11FP and 11FK groups were comparable to the 25F group. However, at the end of trial fish fed the 25F diet had higher SGR and TGC values than the 11F groups. The TGC and SGR of the Atlantic salmon in the present trial were largely similar to those reported in previous similar studies (Menoyo *et al.* 2003; Karalazos 2007; Torstensen *et al.* 2008). Although weight gain of the fish fed diet 11FP was the best and 11FB the poorest by the end of the second phase (8-9 weeks), there were no significant differences between fish fed the 11F diets by the end of the overall of the trial. The growth retardation observed in the present study was related to

reduced feed consumption in fish fed the low inclusion of FM (11F groups), although this was observed throughout the trial (i.e. in both phases). The results were consistent with previous studies that reported that increasing replacement of FM by PP or animal protein meals in salmonids resulted in reduced growth performance that was caused by reduced feed intake (Fowler 1991; Carter & Hauler 2000; Kaushik *et al.* 2004; Sitja-Bobadilla *et al.* 2005; Espe *et al.* 2006).

The above effects of diet on growth parameters were supported by FCR and PER values. Significant differences in FCR and PER between fish fed the 25F and 11F diets were observed during the first period of trial while these differences largely disappeared in the second phase of the trial. This supports the position that the physiology of the fish adapted to the changed feed ingredients enabling them to utilise the nutrients more efficiently in the later stages of the study than they were able to do in the first period of the experiment. Several studies had previously demonstrated that high PP inclusion in diets did not effect protein utilisation as long as dietary AAs were balanced and feed intake was not significantly reduced (Espe *et al.* 2006, 2007, 2008). Thus, in the present study, crystalline AAs (lysine, threonine, methionine) were supplemented in diets to meet AA requirements of Atlantic salmon (NRC. 1993). As all groups of salmon performed similarly with respect to nutrient utilisation (based on PER and FCR) at the end of the trial, this suggests that supplementation was successful and that AA limitation was not a factor in growth reduction.

The present study represented dual-replacement, with FM replaced by blends of PPs or PPs and animal proteins together with replacement of 60 % of the dietary FO by RO. Growth retardation was observed in all groups of fish fed the 11F diets compared to fish fed control diet. This result was in accordance with several studies showing that replacement of FM in diet with PPs above 80% caused growth reduction in Atlantic

salmon (Kaushik *et al.* 1995; Opstvedt *et al.* 2003; Espe *et al.* 2006), Atlantic halibut (Berge *et al.* 1999), sunshine bass (Lewis & Kohler 2008) and rainbow trout (Gomes *et al.* 1995). These previous studies did not substitute FO, which was included at 22 to 30 %, higher than the level of FO (12 %) used in the present study. The level of FO substitution used in the trial previously showed no detrimental effects on salmon growth (Torstensen *et al.* 2000; Bell *et al.* 2001, 2003a, 2004; Montero *et al.* 2003). Recently, Torstensen *et al* (2008) reported that salmon fed diets containing PPs and VOs at replacement levels of 80/35 or 80/70 showed growth reduction while a replacement combination of 40/70 showed no negative effect on fish growth supporting that replacement of FO by VO up to 70 % in diets did not reduce growth of fish.

The inclusion levels of individual PPs or animal protein sources used in the diets in the present study were in the range used in previous studies that showed no growth retardation e.g. field pea or pea protein around 20 % to 33 % in salmonid diets (Carter & Hauler 2000; Thiessen *et al.* 2003; Overland *et al.* 2009), dehulled pea seed up to 30 % in European sea bass diets (Gouveia & Davies 2000), blends of soybean protein concentrate and corn gluten at 21 % or corn gluten at 24 % in diets for Atlantic cod (Hansen *et al.* 2007a), and animal by-products at 20 % in diets for Pacific salmon (Fowler 1991). However, low nutrient digestibility and energy digestibility were found when Atlantic salmon were fed soy bean meal and pea protein concentrate at 20 % inclusion, respectively (Overland *et al.* 2009). In the present study, growth retardation in all groups of fish fed the 11F diets may be explained partially by low digestibility of the high NFE (carbohydrate and fibre) contents in the 11FW, 11FP and 11FK diets. Generally, Atlantic salmon has a low ability to utilise carbohydrate in PPs (Krogdahl *et al.* 2004; Froystad *et al.* 2006) and consequently PPs may reduce nutrient digestibility (Krogdahl *et al.* 2005) and have detrimental effects on growth performance (Waagbo *et*

al. 1994; Hemre *et al.* 1995). In contrast, the level of NFE in diet 11FB was not higher than in diet 25F, but feed consumption was lowest in fish fed the 11FB diet and so growth reduction in that group may be more related to lower palatability (Fowler 1991) and/or digestibility of the animal by-products (Robaina *et al.* 1997). The suitability of animal by-products for FM replacement has variable effects on growth performance among fish species. For instance, there was no effect on feed consumption and growth performance in sunshine bass and gilthead seabream fed diets with partial replacement of FM by poultry by-product meal or meat and bone meal (Robaina *et al.* 1997; Webster *et al.* 2000).

Although effects of NFE on nutrient digestibility may be a factor in reducing growth in fish fed some of the diets as described above, feed consumption was lower in fish fed all of the 11F diets compared to the fish fed the 25F diet. Reduction of fish growth caused by reduced feed intake has been reported in a number of studies (Refstie *et al.* 1998, 2001; Storebakken *et al.* 1998a; Carter & Hauler 2000; Kaushik *et al.* 2004; Espe *et al.* 2006), and those authors suggested this was due to lower palatability and decreased appetite. It was known that quality and levels of FM inclusion influence palatability (Webster *et al.* 1999), and so reducing dietary FM and changing the composition of ingredients likely influences the flavour, reducing palatability of the diets and diminishing appetite of the fish. Replacing FM by PPs in salmon disturbed the digestion and absorption efficiency of nutrients (Refstie *et al.* 2000) and they require time to adapt before accepting diets high in PPs (Torstensen *et al.* 2008). Feed palatability can be enhanced by application of feed attractants such as fish protein or squid hydrolysates (Espe *et al.* 1999, 2006; Dias *et al.* 2005; Olsen *et al.* 2006).

It is well documented that tissue FA composition in fish reflects dietary composition (Torstensen *et al.* 2000; Bell *et al.* 2001; Rosenlund *et al.* 2001; Xue *et*

al. 2006; Karalazos 2007). Furthermore, previous studies showed that reduced dietary FM inclusion affected lipid metabolism and FA profile in sunshine bass, altering n-3 FA composition, especially reducing EPA (Lewis & Kohler 2008). In the present study, reducing dietary FM inclusion from 25 % to 11 % reduced total MUFA and increased total n-3 PUFA in salmon flesh and liver. However, it was unexpected and highly noteworthy that reducing dietary FM did not reduce levels of ARA, EPA and DHA in the flesh and liver of fish fed any of the 11F diets compared to fish fed the 25F diet. In fact, fish fed the 11FP, 11FB and 11FK diets showed the highest proportions of EPA and DHA resulting in higher n-3/n-6 ratios compared to the fish fed the control and 11FW diets. That reducing dietary FM could actually result in increased levels of EPA and DHA without any changes in the actual lipid components of the diet changing was very surprising and an important finding in the present trial. This was something that was hinted at in the previous trial reported in Chapter 6, but the effect is much clearer in the three further 11F diets investigated in the present trial, 11FP, 11FB and 11FK.

These changes in tissue HUFA composition cannot be explained totally by dietary FA compositions as their levels were all higher in tissues than in the diets suggesting they were selectively retained and deposited. Previous studies reported that DHA was preferentially deposited and retained in flesh lipids, regardless of the concentration in the diet, while EPA was progressively favored for energy production if it was presented in high concentration in diet (Kiessling & Kiessling 1993; Torstensen *et al.* 2000). However, more specifically, EPA was significantly higher in diets 11FP, 11FB and 11FK, compared to the 25F and 11FW diets, whereas DHA levels were lower in all the 11F diets compared to diet 25F. Therefore it is possible that DHA was produced as a result of increased desaturation and elongation in fish fed the lower FM diets. Increased levels of 22:5n-3 in fish fed the 11F diets support this suggestion. The

increasing of tissue HUFA composition is partly due to reduction of lipid content and increment of polar lipid as obviously seen in the liver of fish fed the 11FB diet. Therefore, the increased HUFA in the tissues of fish fed the 11F diets could be due to greater retention and/or biosynthesis.

In conclusion, decreasing dietary FM from 25 % to 11 % and correspondingly increasing substitution by alternative protein sources to 55 % of total diet, reduced growth of large Atlantic salmon irrespective of the combination of PP and animal proteins used as substitutes, and despite dietary supplementation with crystalline AAs and lecithin. This may be partly due to increased NFE in some of the 11F diets reducing nutrient digestibilities but probably mainly due to reduced feed consumption. This was likely due to reduced palatibilty and consequently reduced appetite as the reduced feed consumption was largely evident in the first phase of the dietary trial and not so apparent in the second phase suggesting adaptation to the feeds. The increased substitution of dietary FM also tended to reduce liver lipid content but there were few effects on flesh or liver lipid class compositions in either tissue. Perhaps the most surprising and interesting result was that increasing the substitution of dietary FM did not reduce tissue HUFA levels and, in particular, both EPA and DHA levels were significantly higher in flesh and liver of fish fed the 11F diets compared to fish fed the 25F diet. Of the 11F diets, the 11FP diet, containing pea protein, has potential to be a good alternative feed component for Atlantic salmon as it revealed higher final body weight than than the other 11F diets. Enhancing the palatability of the feed and a longer-term study starting with smaller fish should be investigated.

Chapter 8 - General summary, conclusions and future perspectives

8.1 General summary

FM and FO have been traditionally used in aquafeeds due to their excellent nutritional qualities, with FM providing EAA, while FO is an excellent energy source and is a unique source of n-3 HUFA, such as EPA and DHA (NRC 1993; Sargent *et al.* 2002). Nevertheless, the presence of persistent organic pollutants (POPs) in FM and FO and thus in farmed-fish products has given rise to media scares that can affect aquaculture producers (Hites *et al.* 2004a, 2004b; Berntssen *et al.* 2005; Bell *et al.* 2005a). Considering that global FM and FO supplies are static and of limited availability, the ecological concern of using non-sustainable marine resources, along with issues of POP contaminant levels in these feed ingredients, further development and expansion in aquaculture dictates that substitution of dietary FO and FM must be investigated. The overall aim of the present study was to develop alternative, sustainable diets for Atlantic salmon. Specific objectives were to investigate alternative feed ingredients as replacements / substitutes for both FO and FM in Atlantic salmon diets. In addition, different strategies for their application and use, to ensure optimal growth, feed efficiency and health of the fish and at the same time maintaining the nutritional quality, especially flesh levels of n-3 HUFA, EPA and DHA, for the human consumer. In trials 1 and 2, the focus was primarily on FO replacement while in trials 3 and 4, the focus was on the substitution of FM. In both trials 1 and 2, FM was also partially replaced as the diets were formulated to be practical and thus reflect current feed formulation practices. In line with this ethos, in trials 3 and 4, the maximum level

of FM used was 25 % and the oils added were FO:VO (40:60%). Thus, all the trials in this study were examples of dual FM and FO substitution.

The results trial 1 and 2 in the present study revealed that the replacement of FOs with VOs do not have negative effects on the growth metrics of Atlantic salmon including SGR, TGC, FCR, VSI and HSI. These findings suggest that the lipid levels in the diets supply sufficient quantities of EFA and FAs for catabolism and protein sparing resulting in no measurable detrimental effects on fish growth. The findings from this study are in agreement with those of earlier studies in that the complete replacement of FO with VO from a variety of different sources, in both short and long duration feeding trials, had no effect on the growth and FCR of several species of fish (Bell *et al*. 2001, 2003, 2004; Caballero *et al.* 2002; Torstensen *et al*. 2004, 2005; Regost *et al*. 2004; Fountoulaki *et al*. 2009). Modification in feed formulation may alter the physiology, texture and flavour and, consequently, reduce the palatability of the diet. In trials 3 and 4, however, it was found that when the levels of FM included in the diet were reduced to lower than 25% of the diet, 60% VO substitution and combined with a high proportion of blended plant proteins or plant and blood meal, were found to affect the growth performance of fish. Reduced growth was the result of a reduced intake of feed possibly caused by the low palatability of the diet. The palatability of diets and the acceptance of feed, therefore, can be improved by the inclusion of specific feed attractants such as krill meal, fish protein or squid hydrolysates into the diets (Espe *et al.* 1999; Refstie *et al.* 2004; Hevroy *et al.* 2005; Olsen *et al.* 2006). The experimental findings of trials 3 and 4 follow those of a number of previous studies in that fish fed diets containing lower than 20% FM and 70% VOs displayed low growth performance (Benedito-Palos *et al*. 2008, 2009; Torstensen *et al*. 2009). Of the alternative 11% FM diets tested in trial 4, the fish fed formulations containing plant proteins sources (*i.e.*

pea, wheat and kidney bean), displayed better growth than those fish fed a diet containing blood meal. The diet containing pea protein (11FP) has the potential to be a good alternative source of plant protein as the fish consuming this diet had a higher weight gain than the fish groups fed the other 11% FM diets (i.e. wheat, kidney bean and blood). The fish groups fed the wheat (11FW), pea (11FP) and kidney bean (11FK) diets showed greater growth during the second phase of the experimental trial (weeks 8 – 19) indicating that the fish had had time to adapt to the new diets. Evidence for this was shown by the improvements in FCR and PER in the second phase of the feed trial.

In addition to the effects of dietary lipid, the genetic background of the fish has also been reported to affect fish growth and body composition (Peng *et al.* 2003; Rollin *et al.* 2003; Quillet *et al.* 2005), including pigment composition (Storebakken & No 1992). The results from the present study showed that the CAL strain fed a dual replacement of FO and FM had greater growth and a higher flesh pigment content than the FAT and LEAN strains of fish. The LEAN strain, however, appeared to adapt to the new diets more readily than the CAL and FAT. Moreover, the microarray analysis clearly illustrated that the FAT and LEAN strains adapt differently in terms of the level of gene expression each display in their livers when presented with diets where the entire FO content was substituted with a blend of VOs. The qPCR results confirmed the microarray findings that the LEAN fish respond differently to the FAT and CAL fish.

Replacement of FO with VOs sources did not affect nutrient and FA digestibilities in large Atlantic salmon in the present study. Substituting FM with high levels of PPs (plant proteins) in experimental diets were found to increase the level of protein digestibility but decreased that of fat. In addition, the ADC of protein appeared to increase, together with improvements of PER in the second phase of experimental measurement, suggesting that fish increase their utilisation of protein as dietary PPs

increased. The apparent digestibility of fat decreased with increasing PP inclusion in the diets may result from high PP content diets which contain high levels of non starch carbohydrates *e.g.* pectin and acidic polysaccharides as found in legumes (Jobling 1993). These non-starch carbohydrates increase the viscosity of ingested materials, limiting the metabolic capacity of lipid in distal intestine epithelium subsequently reducing the digestibility of fat.

In the present study, the consequences of altering the composition of the diet by reducing the utilisation of FO and / or FM on the nutrient composition of fish tissues were observed. In trial 2, the percentages of n-3 LC-PUFA, ARA, EPA and DHA in the flesh, liver and the visceral tissues of Atlantic salmon were seen to decrease by over 50% when the fish were fed a diet containing 100% VO when compared to fish fed a FO diet. The replacement of FO with VO in the Atlantic salmon diets which decreased the n-3 LC-PUFA content of fish tissues in this study confirms the experimental findings of other researchers (Bell *et al.* 2003; 2004). The low levels of these EFAs in the flesh, however, were minimised when Atlantic salmon were fed a diet where the entire content of northern FO was substituted with combinations of southern FO and VOs as seen in trial 1. In addition, the levels of n-3 LC-PUFA content, particularly EPA and DHA, in the flesh and the liver were not affected by the replacement of FM with alternative PPs or with a combination of PPs and animal proteins as seen in trials 3 and 4. A key finding from trials 3 and 4 was that when a diet containing 5% FM, 30% lipid level consisting of 12% FO and 18% VO (40:60%) did not reduce the levels of tissue EPA and DHA, but were found to slightly increase in the fish fed the diet with the lower FM inclusion than those fish fed the 25FM inclusion diet. The increasing levels of ARA, EPA and DHA observed are a consequence of the fish obtaining these EFAs directly from diet and / or from changes in metabolism with resultant increased

desaturation / elongation of 18:2n-6 and 18:3n-3 as the inclusion level of alternative PPs in the diet are increased.

8.2 Summary of results and conclusions

Trial 1 – Investigating alternative strategies, including dual replacement of FO and FM, for reducing contaminant levels and maintain n-3 HUFA simultaneously in farmed salmon. The objectives were to investigate the effects of the total replacement of NFO with decontaminated NFO and to evaluate the effects of the total replacement of NFO with SFO/VO blends on growth, tissue proximate composition, nutrients and FA digestibility, tissue FA compositions and contaminant levels in Atlantic salmon.

1. The alternative strategies to reduce POPs of using decontaminated FO or blending VOs with southern hemisphere FO both had no deleterious effects on growth of salmon as measured by final weights, SGR or TGC, or on feed efficiency (FCR).

2. Both strategies, decontaminated FO and blended VOs with southern hemisphere FO, were successful in very effectively reducing the dioxin, DL-PCB and PBDE concentrations to very low levels in salmon flesh.

3. Using decontaminated FO had no major effects on either the nutritional qualities of FO as a feed ingredient for Atlantic salmon or on the nutritional quality of the salmon fillet in terms of the n-3 HUFA. The strategy of using blended VOs with southern hemisphere FO had more impact upon tissue n-3 HUFA levels, reducing them by a moderate amount.

Trial 2 – Investigating the effects of genetic background of salmon on responses to alternative, dual-substituted feeds. The effects of long-term (> 1 yr) dual replacement of 100 % of dietary NFO with blended VOs in dual-substituted diets (45% PPs and 25% FM inclusions) followed by a subsequent FO "finishing" diet in three groups (strain/family) of Atlantic salmon was investigated on growth, tissue proximate and FA composition, and liver gene expression.

1. The three groups (strain/family) of Atlantic salmon showed no significant differences in growth performance, including SGR, or FCR, HSI and VSI when fed the dual-substituted diet with total replacement of NFO with VO blends along with partial substitution of FM with PPs.

2. Irrespective of diet, the CAL fish showed higher growth than the FAT and LEAN fish, and also showed better growth when fed the VO diet compared with the same fish fed the FO diet.

3. Long-term feeding with the dual-substituted feed significantly reduced the n-3 HUFA content of the salmon fillet in all strains/families compared with the fish fed the control diet. The ARA level was completely restored and the EPA and DHA levels were about 90 % restored after feeding the finishing diet containing FO as the sole source of lipid for 24 weeks.

4. There were few significant differences between the fish of different genetic backgrounds in FA composition but the LEAN and CAL fish tended to adapt to the alternative diets better than the FAT fish.

5. Long-term feeding of the three groups of Atlantic salmon with the dual-substituted diet significantly affected gene expression in liver. The expression of several genes related to lipid metabolism, particularly fatty acyl desaturase and elongase genes and some genes related to cholesterol biosynthesis, were significantly up-regulated in fish fed the substituted diet compared with fish fed the FO diet.

6. The effects on gene expression varied between fish of different genetic background, showing that the FAT and LEAN families, and CAL strain, adapted differently to the complete substitution of dietary FO with a blend of VO. The LEAN family generally showed higher levels of response to substitution of FO with VO.

Trial 3 – Investigating effects of further reduction of FM to very low levels in salmon fed dual-substituted feeds. In trial 3, a regression design was employed to investigate the effect of graded substitution of FM with plant products in dual-substituted diets on growth, feed efficiency, nutrient digestibility and FA composition in large Atlantic salmon. The primary hypothesis failed as salmon fed diets containing FM inclusion lower than 25 % showed growth retardation, mainly due to feed intake reduction. No dietary treatment affected tissue FA composition.

1. High inclusion of PP and reduced levels of FM in dual-substituted diets affected growth most probably due to reduced feed intake and nutrient digestibility. The FCR and PER were improved during the latter (second) phase of the trial suggesting that there was metabolic adaptation to the diets and no AA limitation.

Changing feed ingredients may have affected the physical texture of the feeds and/or chemical olfactory attractants that reduced the palatability of the diets.

1. The inclusion of graded increased levels of PPs appeared to decrease liver lipid content but increased visceral lipid deposition. The reduction of FM inclusion from 25 % to 5 %, FO contents at 12%, did not significantly affect nutritional quality of the fish as the n-3 HUFA (EPA and DHA) contents in the flesh (and liver) were not lowered by the reduced FM inclusion but, indeed, were slightly increased in the fish fed the lower FM inclusions.

Trial 4 – Investigating effects of alternative FM replacers. The effects of different blends of replacement proteins on growth, feed efficiency and tissue FA composition were determined in large Atlantic salmon fed 11 % FM, that induced growth retardation in trial 3. None of the alternative blends was able to restore growth to that observed in fish fed 25 % FM. Atlantic salmon had better growth when fed diet containing plant protein compared with the fish fed diet containing animal protein. The pea protein appeared to be a good alternative source of plant protein for Atlantic salmon.

1. Decreasing dietary FM inclusion to 11 % and correspondingly increasing substitution by a variety of alternative protein sources to 55 % of total diet, reduced growth of large Atlantic salmon irrespective of the combination of PPs and animal proteins used as substitutes mainly due to a reduction in feed consumption.

2. Comparison among the alternative feed ingredients of the 11F diets suggested that pea protein has potential to be a good alternative feed component for Atlantic salmon as it revealed higher final body weight than that the other 11F diets.

8.3 Future perspectives

Considering the aforementioned effects of increasing PPs and VO levels associated with decreasing FM and FO inclusion on growth performance and nutritional quality of Atlantic salmon, several areas of research require further scientific investigation to ensure future aquaculture development and the sustainability of fish farming.

1. **Increased duration of nutritional trials.** The beneficial effects of n-3 LC-PUFA to human health are in the prevention and attenuation of a number of inflammatory disorders, including cardiovascular disease, immune dysfunction and neurological conditions. The adverse effects of persistent organic pollutions (POPs), dioxins, DL-PCBs and PBDEs can cause a range of diseases in humans including the development of cancers, disruption of the immune system *etc*. It is for these reasons, that fish nutritionists and fish farmers ensure that the farmed fish products maintain high n-3HUFA contents and remain low POP contamination. Thus, modifying feed formulation and ingredients can alter dietary composition which subsequently affects the final farmed Atlantic salmon product. Although the results from trial 1 showed that Atlantic salmon fed either a decontaminated FO diet or diets containing a combination of southern FO and VO blends (SVOs) had lower levels of dioxins, DL-PCBs and PBDEs in their flesh below EU limits. Interestingly, fish fed SVO diets remained high in n-3 HUFA, EPA and DHA which was unexpected but this positive observation requires further trials to confirm this result. This trial, however, was only 11 weeks long and thus the length of future trials ideally should be a minimum of 6-months duration or cover the whole production cycle to reflect commercial conditions.

1. **Alternative finishing diets.** The partial or total replacement of FO in salmon diets with VO resulted in a reduced n-3 LC-PUFA composition of the tissues. A solution to the restoration of the reduced n-3 HUFA, EPA and DHA levels in the fillets of market sized fish was to feed fish a finishing diet, a diet containing FO as the sole oil source for a certain period. Adding traditional FO back to fish feed diets that previously contained VOs, however, may subsequently increase the levels of POP contaminants in the fillet. Trial 1 showed that both strategies of feeding fish with decontaminated FO and SVOs diets were very effective in reducing contaminant levels in the flesh without serious reductions in the levels of EPA and DHA. Therefore, further studies should consider using decontaminated FO or carefully selected blends of southern FO and VO as alternative finishing diets for Atlantic salmon.

2. **The addition of feed attractants.** The formulation of future salmon feeds should consider decreasing the proportion of FM and FO and replacing them with increasing contributions of plant ingredients. The key to this in finding the appropriate ingredients to serve as replacements and implementing strategies to maintain the palatability of the feed and the appetite of the fish. The current study found that Atlantic salmon fed diets containing a very low FM inclusion (< 25 %) showed growth retardation, a consequence of the reduced intake of feed. The fish, however, showed greater growth after been fed on the diet for 2 months suggesting that the fish required time to adapt to the new diet. Future studies, therefore, should investigate enhancing the palatability of diets by adding appropriate feed attractants and by conducting longer-term studies to clarify the palatability of the test feeds and the period of adaptation required by the fish.

3. **Parallel histopathology and immunology studies.** An inappropriate alteration to the feed ingredients of salmon diets by, for example, increasing the replacement of plant and land animal proteins and oils in the salmon diet may induce a physiological stress response in the fish with potential consequential cell damage to various metabolic organs, the gills, liver and the intestine *etc*, compromising the health of the fish. Lesions on the livers of Atlantic salmon fed high PPs / plant and blood meal proteins were observed during the collection and preparation samples. In addition, the gene expression studies conducted in the present study showed that genes of inflammatory and immune responses, including the heat shock protein (hsp) 70kda and 90kda, were found to be differentially expressed in fish fed the control, FO and VO diets. The significant differences in the expression of the heat shock proteins genes found in the present study may change in response to pathogen challenge and immune status that may represent an early warning indicator of impaired fish performance and health in Atlantic salmon fed diets with high levels of replacement of marine FM and FO with plant-based ingredients. Thus, to ensure that altering the feed ingredients of Atlantic salmon diets does not impact deleteriously on fish health and status, future nutrition studies should consider conducting detailed parallel studies to investigate gene expression, histopathology and immunology of key metabolic tissues to provide a comprehensive assessment of the trial diets.

4. **Further molecular analysis.** As commented on above, substitution of marine FM and FO products with plants or land animal ingredients may affect fish health that may include inducing a physiological stress response in fish. Further scientific investigations at the molecular level are therefore required to understand how cells function and how their function changes in response to

varying environmental conditions. The altered expressions of genes in diseased tissues, for example, represent useful diagnostic markers. The patterns of gene expression vary depending on a range of factors including cell type (*e.g.* brain, liver *etc*), environmental factors (*e.g.* temperature, nutrient availability) *etc*. Transcriptomics (gene expressions), therefore, provides a global and quantitative analysis of differential gene expression under defined conditions and currently is one of tools used to study the differences in the transcription of genes in normal and diseased tissues. The use of modern techniques such as a transcriptomics (microarray) approach for determining the gene expression of nutrition induced changes that result in disease, therefore, should be considered as a vital component of future investigations. Such studies should also be expanded to consider and analyse other key metabolic tissues.

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Appendix I

Preparation of astaxanthin standard solution:

Approximately 3 mg of standard of all-E-astaxanthin is weighed into 100 ml volumetric flask. Ten ml of chloroform were added together with ultrasonic treatment until completely dissolved. The solution was made up to 100 ml with n-hexane. 5 ml of this solution are transferred into a 100 ml volumetric flask and 4 ml of chloroform was added subsequent made up to the volume with iso-hexane, resulting in an astaxanthin concentration of about 1.5 mg per litre of n-hexane/chloroform (95.5**:**4.5; v/v).

Preparation of buffer stock solution:

10 X TBE (tris/borate/EDTA) electrophoresis buffer (stock solution/per litre)

108g Tris base (BioRad laboratory, Hercules, CA, U.S.A)

55g Boric acid (BioRad laboratory, Hercules, CA, U.S.A)

20 ml of 0.5 M Ethylenediaminetetraacetic acid ,EDTA (BDH, poole, U.K.).

Adjust with deionised water up to 1000 ml.

50 X TAE electrophoresis buffer (stock solution/per litre)

242 g tris base (2-amino-2-hydroxymethyl-propane-1,3-diol) (= 2 mole)

57.1 ml glacial acetate acid (=100% acetic acid) (57.19 ml = 1mole)

100 ml 0.5 M EDTA (pH8.0)

Adjust with deionised water up to 1000 ml.

To prepare 0.5 M EDTA (pH8)

Add 186.1 g of disodium ethylenediaminetetraacetate .$2H_2O$. Stir vigorously. Adjust the pH to 8.0 with NaOH (ca. 20 g of NaOH). Sterilise by autoclaving. (the disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to ca. 8.0 by the addition of NaOH).

Appendix ii

Publications and presentations

Publications in peer-reviewed journals

Pratoomyot, J., Bendiksen, E. Å., Bell, J. G. And Tocher, D. R. 2008. Comparison of effects of vegetable oils blended with southern hemisphere fish oil and decontaminated northern hemisphere fish oil on growth performance, composition and gene expression in Atlantic salmon (*Salmo salar* L.). *Aquaculture*, 280, 170-178.

Bell, J. G., Sprague, M., Bendiksen, E. Å , Dick, J. R., Strachan, F., Pratoomyot, J., Berntssen, M. H. G. and Tocher, D. R. 2008. Using decontaminanted fish oil or a vegetable/ fish oil blend to reduce organic contaminant concentrations in diets and flesh of farmed Atlantic salmon (*Salmo salar*). *Organohalogen Compounds*, 70, 894-897.

Sprague, M., Bendiksen, E. Å , Dick, J. R., Strachan, F., Pratoomyot, J., Berntssen, M. H. G. Tocher, D. R. and Bell. J. G. 2010. Effects of decontaminated fish oil or a fish and vegetable oil blend on persistent organic pollutant and fatty acid compositions in diet and flesh of Atlantic salmon (*Salmo salar*). *British Journal of Nutrition,* 103, 1442-1451.

Pratoomyot, J., Bendiksen, E. Å., Bell, J. G. And Tocher, D. R. 2010**.** Effects of increasing replacement of dietary fishmeal with plant protein sources on growth performance and body lipid composition of Atlantic salmon (*Salmo salar* L.). *Aquaculture*, 305, 124-132

Bell, J. G., Pratoomyot, J., Strachan, F., Henderson, J. R., Fontanillas, R., Hebard, A., Guy, D. R., Hunter, D., Tocher, D. R. 2010. Influence of genotype/phenotype on effects of replacement of dietary fish oil with vegetable oils in Atlantic salmon (*Salmo salar)* families/strains selected on the basis of flesh adiposity: growth, flesh proximate and fatty acid compositions. *Aquaculture*, 306, 225-232

Pratoomyot, J., Bendiksen, E. Å., Bell, J. G. And Tocher, D. R. Effects of different blends of alternative protein sources as replacers for dietary fishmeal on growth performance and body lipid composition of Atlantic salmon (*Salmo salar* L.). Manuscript in preparation.

Posters presentations in conferences

Pratoomyot, J., Bendiksen, E. Å. Bell, J. G. and Tocher, D. R. 2007. Comparison of dietary vegetable oils and contaminant-stripped fish oil on growth performance and composition of Atlantic salmon (*Salmo salar* L.). European Aquaculture Society Conference 2007 (EAS 07), 24-27 October 2007, Istanbul, Turkey.

Pratoomyot, J., Bendiksen, E. Å. Bell, J. G. and Tocher, D. R. 2008. Comparison of dietary vegetable oils and contaminant-stripped fish oil on growth performance and composition of Atlantic salmon (*Salmo salar* L.). 1st PhD. Aquaculture Conference, 28 October 2008, Institute of Aquaculture, University of Stirling.