The assessment of omega 3 oil sources for use in aquaculture – alternatives to the unsustainable harvest of wild fish stocks

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DECLARATION

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ABSTRACT

Worldwide harvest of wild marine fisheries for fish oil cannot increase. However, the demand for fish oil is increasing due to a rapidly expanding aquaculture industry and is further increased by nutraceutical/biomedical and agricultural companies. Aquaculture uses fish oil as a source for essential fatty acids in particular omega-3 long chain-polyunsaturated fatty acids (ω3 LC-PUFA) and for energy. Other novel sources of renewable, environmentally sustainable oil that provide these nutritional requirements for Atlantic salmon (*Salmo salar* L.) are needed. This research looked at alternate sources of oil containing the ω3 LC-PUFA that are associated with the many health benefits of eating Atlantic salmon. This thesis also contributed to the development of three techniques for use in aquaculture lipid nutrition research: 1) advanced chromatography and mass spectroscopy to examine intact molecular membrane lipids; 2) nuclear magnetic resonance (^{13}C NMR) to assess the regiospecific distribution of ω3 LC-PUFA in oil, and 3) molecular RT-PCR to investigate endogenous ω3 LC-PUFA production.

Two ways of supplying the nutritional requirement for ω3 LC-PUFA in aquafeeds for Atlantic salmon were studied in a series of feeding trials. A biosynthetic precursor of ω3 LC-PUFA from Patterson's curse (*Echium plantagineum* L.) was fed in two trials to Atlantic salmon parr and to smolt. It was shown that feeding oil rich in the biosynthetic precursor, stearidonic acid (18:4ω3 SDA), maintained concentrations of ω3 LC-PUFA in the flesh of salmon parr comparable to fish fed a traditional fish oil diet. In smolt, it was demonstrated that dietary SDA elevated the expression of the genes encoding the enzymes responsible for the desaturation and elongation steps involved in the ω3 LC-PUFA biosynthetic pathway. However, with increased expression and bypassing the Δ^6 desaturation step through the provision of SDA, the smolt stage, unlike parr, did not

maintain concentrations of ω3 LC-PUFA. The high concentrations of ω3 LC-PUFA found in traditional fish oil fed adult salmon will likely not be provided by diets rich in SDA.

Single cell organisms such as microalgae, including thraustochytrids, diatoms and other micro-organisms de novo synthesis ω3 LC-PUFA and are the original sources in the marine food web. Thraustochytrids are heterotrophic protists, commonly found in the marine environment and produce high levels of ω3 LC-PUFA rich oils. Thraustochytrid oil was fed to Atlantic salmon parr to investigate the effect of feed containing high concentrations of ω3 LC-PUFA, in particular docosahexaenoic acid (22:6ω3, DHA), on performance and how this important fatty acid is incorporated into cell membranes and stored in the fish. The thraustochytrid oil in the diet significantly increased the amount of DHA in Atlantic salmon muscle and therefore is a candidate for use in oil blends for salmon diets. Thraustochytrid oil also significantly increased the ability of salmon parr to undergo smoltification. Regiospecificity analyses of intact lipids can indicate how diet, in particular high dietary DHA, can affect the membrane structure of muscle tissues. However, in the gill and liver, adaptive changes due to smoltification were the major factors that contributed to differences in membrane structure. The incorporation of high concentrations of dietary DHA into the membrane structure and storage molecules is achieved by adaptation of molecular species. Regiospecific analysis of the storage lipid demonstrated that increased dietary DHA increased its bioavailability to the consumer.

Other factors involved in oil replacement were examined. These included the effect and accumulation of minor components, such as phytosterols in vegetable oil and the effect rising ocean temperature has on the membrane structure and lipid storage in salmon. Phytosterols have a beneficial effect in humans by reducing low density lipoprotein (LDL) cholesterol. The digestibility of natural abundances of phytosterols by

Atlantic salmon was poor compared to cholesterol. However, significantly increased concentrations of the phytosterols were observed in both the liver and white muscle of Atlantic salmon fed vegetable oils which ultimately may provide health benefits to the consumer. Salmon adapt their membrane structures due to an elevated water temperature of 19^oC. This temperature now often occurs in Tasmanian waters in summer and autumn and is approaching the upper limit for Atlantic salmon to maintain health and performance. Adaptation of structural and storage lipids at elevated temperatures was shown by a reduction in PUFA, especially eicosapentaenoic acid (EPA $20:5\omega3$), and an increase of saturated fatty acids in the gill and white muscle. Salmon altered their membrane structure to compensate for elevated water temperature, which could affect dietary FA requirements.

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Rock n roll

CO-AUTHOURSHIP

The following people and institutions contributed to the publication of the work undertaken as part of this thesis

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The following people have contributed to the following chapters

- Nichols, P.D. and Carter, C.G assisted with the general supervision of all aspects of this thesis. These included experimental design, interpretation of data and proof reading manuscripts (10% of chapters)
- Bridle, A. R. assisted in the gene expression sample preparation and data collection and contributed to the proofing of chapter 3. (10% of chapter)
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Barnes, J. (PhD candidate) with Carter, C.G. designed and performed the elevated temperature Atlantic salmon trial in chapter 6. Other than fish weight data no other data from this trial is used in this thesis.

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis.

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LIST OF ABBREVIATIONS

The following abbreviations are used in this thesis:

^{13}C NMR, ^{13}C nuclear magnetic resonance
ARA, arachidonic acid
ADC, apparent digestibility coefficients
ALA, α-linolenic acid
ANOVA, 1-way analysis of variance
BSFTA, N,O-bis(trimethylsilyl)-trifloroacetamide
CDCl$_3$, deuterated chloroform
CHD, coronary heart disease
CMC, carboxymethyl cellulose
DHA, docosahexaenoic acid
DM, dry matter
DPA, docosapentaenoic acid
EPA, eicosapentaenoic acid
ESI-RP-LCMS, electrospray ionization reversed-phase liquid chromatography-mass spectrometry
ETA, eicosatetraenoic acid
FA, fatty acid(s)
FAD5, fatty acid Δ^5 desaturase
FAD6, fatty acid Δ^6 desaturase
FAE, fatty acid elongase
FAME, fatty acid(s) methyl ester
FC, total feed consumption
FER, feed efficiency ratio
FFA, free fatty acids
GLA, γ-linolenic acid
GC, gas chromatography
GC-MS, gas chromatography mass spectroscopy
HPLC, high pressure liquid chromatography
HIS, hepatosomatic index
HNF, hepatic nuclear factors
LA, linolenic acid
LC, long chain ($\geq C_{20}$)
LDL, low density lipoprotein
LXR, liver X receptor
mRNA, messenger ribonucleic acid
MUFA, monounsaturated fatty acid(s)
NOE, nuclear overhauser effect
NRQ, normalised relative quantities
OA, oleic acid
PCB, polychlorinated biphenyls
PC, phosphatidylcholine
PCA, principal components analysis
PE, phosphatidylethanolamine
PG, phosphatidylglycerol
PI, phophatidylinositol

PKS, polyketide synthases
PL, polar lipid
PLFA, polar lipid fatty acid
PPAR, peroxisome proliferators-activated receptors
PS, phosphatidylserine
PUFA, polyunsaturated fatty acid(s)
RT-PCR, real-time quantitative polymerase chain reaction
RXR, retinoid X receptor
SCO, single cell oils
SDA, stearidonic acid
S.E. standard error
SFA, saturated fatty acid(s)
SGP, salmon genome project
SGR, specific growth rate
SREP-1c, sterol regulatory element protein-1c
ST, sterol(s)
TAG, triacylglycerol
TLC-FID, thin layer chromatography-flame ionisation detection
TLE, total lipid extract
tr, trace amounts
UPL undetermined polar lipid
UFA, unsaturated fatty acid(s)
ω3, omega 3
ω3 LC-PUFA, omega 3 long chain ($\geq C_{20}$)-polyunsaturated fatty acid(s)
ω6, omega 6
WW, wet weight

CHAPTER 1

General Introduction

GENERAL INTRODUCTION

1.1 *Introduction*

This thesis addresses the problem of identifying suitable renewable sources of oil, which contain omega 3 long chain ($\geq C_{20}$) polyunsaturated fatty acids (ω3 LC-PUFA), to replace fish oil in aquafeeds. Due to increased demand and price of wild caught marine sources of ω3 LC-PUFA rich oil, their replacement in aquafeeds has become an industry priority. This introductory chapter explains the rational for research on replacement oils, the benefits and challenges involved in changing dietary oil and then examines three major potential sources, other than fish oil, of ω3 LC-PUFA for aquafeeds. This study examined Atlantic salmon (*Salmo salar*) as it is the major marine finfish species that is intensively cultured and has the ability to both utilise and store large amounts of oil, in particular ω3 LC-PUFA, in their flesh (Bell, 2000; Hardy and Tacon, 2002). Additionally, advanced analytical and molecular techniques were utilised to examine the underlying nutritional physiology and biochemistry, with respect to ω3 LC-PUFA, of Atlantic salmon fed alternate dietary sources of lipid.

1.2 *Factors driving replacement oil research*

1.2.1 Sustainability of wild fish stocks

Historically, the intensive culture of Atlantic salmon has relied on natural fisheries to supply fishmeal and oil as ingredients for aquafeeds. Therefore the stability and sustainability of the wild fishery are of vital importance to the security of ingredients for aquafeeds. Worldwide, capture fisheries have plateaued at around 85-95 million tonnes per annum even though fishing effort has intensified (Naylor, et al., 2000; Black, 2001). There is a growing concern about the health of ocean fisheries stocks and the ecological effects of industrial fishing, with evidence that most fisheries are over or fully fished (Pauly and Christensen, 1995; Tegner and Dayton,

1999; Naylor, et al., 2000; Pauly, et al., 2002; Worm and Myers, 2004). One recent estimation

predicted that by 2048 all commercial fish and seafood species may collapse due to loss of

biodiversity through selective overfishing (Worm, et al., 2006). Fish oil/meal production is

strongly dependent on the availability of wild fisheries and the mismatch between demand and

expected supply of fish oil is expected to reach 40 million tonnes by 2030 (Tacon, 2003).

Dramatic changes, even collapse, can occur in fish populations of the species involved in fish

oil production (Schwartzlose, et al., 1999; Naylor, et al., 2000). Problems facing most fisheries

are complex and changes can be caused by numerous factors such as environmental, biological

and ecological, and not necessary fishing impacts (Schwartzlose, et al., 1999). It has been

demonstrated that climatic events such as El Niño can also significantly decrease fish

populations and therefore increase pressure on fish oil/meal supply (Barlow, 2000; Lehodey, et

al., 2006; Gutierrez, et al., 2007). One quarter of the world's fish oil supply and one third of the

fish meal for aquaculture diets come from one anchoveta (*Engraulis ringens*) fishery off the

coast of Peru (Schwartzlose, et al., 1999; Sanchez, et al., 2000). Collapse of this fishery alone

would increase the pressure on world fish meal and oil supply and threaten global aquaculture

production security (Schwartzlose, et al., 1999; Sanchez, et al., 2000).

1.2.2 Sustainable Atlantic salmon farming

Aquaculture is the fastest growing food producing sector in the world (Tacon, 2003). The

Atlantic salmon aquaculture production has grown from 55,000 tonnes in 1985 to more than 1.2

million tonnes in 2006 (Bell, et al., 2002; FAO/Golbefish, 2007). By 2010 it is estimated that

85% of global fish oil will be needed for salmon and trout production (Sargent and Tacon,

1999). However, fish oils are increasingly being used in the nutraceutical and agricultural

industries and thus, both demand and price have substantially increased, stimulating the need

for replacement oils. Replacement of oil with renewable land based products such as vegetable

oils has been extensively researched and incorporated into commercial aquafeeds (Bell, et al., 1993; Bransden, et al., 2003; Torstensen, et al., 2005; Ng, et al., 2007).

Salmon are carnivorous and require dietary protein and lipid which have traditionally been supplied from wild caught marine sources. Feed conversion ratios (FRC) for Atlantic salmon are continuously improving with tailored aquafeeds and are around 1:1 (one kilogram of fish produced per kilogram of feed). An estimated 3.2 kg of wild fish stock is required to produce 1 kg of aquafeed for salmon aquaculture (Naylor, et al., 2000; Black, 2001). However, aquaculture has a significant ecological advantage over wild salmon capture with 1 kg of growth in the wild relates to 10-15 kg of fish stock eaten or captured as by-catch (Forster, 1999; Tidwell and Allen, 2001). The efficiency of the aquaculture industry is continually improving as nutritional requirements are better understood. However further research into sustainability and security of feed ingredients is vital for the growth of the industry.

1.2.3 Lipid content and nutrition of aquafeeds

Lipids, or oil, provide the main source of metabolic energy in aquafeeds for many carnivorous fish, particularly salmonoids. Current extrusion technologies allow aquafeeds to contain up to 40% oil. The natural marine diet of Atlantic salmon contains high concentrations of ω3 LC-PUFA in particular eicosapentaenoic acid (20:5ω3, EPA) and docosahexaenoic acid (22:6ω3, DHA), low concentrations of ω6 PUFA, as well as moderate levels of monounsaturated FA (MUFA) and saturated FA (SFA) (Table 1.1).

The lipid component of aquafeeds requires the supply of essential fatty acids (EFA) which are necessary for cellular metabolism (synthesis of prostaglandins, eicosanoids, leukotrienes and other EFA metabolites) and maintaining cell membrane structure and integrity (Sargent, et al., 1995; Corraze, 1999). EFA include ω3 and ω6 PUFA (Sargent, et al., 2002). Digestion, absorption, transport, accumulation, biosynthesis and metabolism of lipids in particular EFA

and ω3 LC-PUFA have been closely studied in Atlantic salmon and reported in reviews (Sargent, et al., 1995; Sargent, et al., 1999; Bell, 2000; Sargent, et al., 2002; Tocher, 2003).

In Atlantic salmon a multitude of nutritional "diseases" or pathologies due to lipid imbalances have been observed (Bell, et al., 1991; Tacon, 1996; Seierstad, et al., 2005). Atlantic salmon can display reduced growth, poor feed efficiency, evacuolated pyloric caeca tissue and increased incidence of pancreatic disease with EFA deficient diets (Tacon, 1996). It is therefore a requirement of aquafeed to supply ω3 LC-PUFA as a part of the oil component of the diet (Sargent, et al., 1995). It has been demonstrated that a diet consisting of 100% vegetable oil lacking ω3 LC-PUFA causes severe heart lesions, thinning of ventricular wall, muscle necrosis and can influence the devolvement of arteriosclerotic changes in Atlantic salmon (Bell, et al., 1991; Seierstad, et al., 2005).

Currently, ω3 LC-PUFA for aquafeeds are sourced from marine oil obtained from wild fisheries. The intensive aquaculture industry has been using fish oil with blends of vegetable and animal oil in the manufacture of aquafeeds to reduce cost and decrease the pressure on this finite resource. Many trials with replacement oils have demonstrated that the FA profile of the salmon closely reflects that of its diet (Polvi and Ackman, 1992; Tocher, et al., 2000; Torstensen, et al., 2000; Bell, et al., 2001; Rosenlund, 2001; Bell, et al., 2002; Grisdale-Helland, et al., 2002; Bell, et al., 2003; Bendiksen, et al., 2003; Bransden, et al., 2003; Carter, et al., 2003; Bell, et al., 2004; Ng, et al., 2004; Torstensen, et al., 2004). It is suggested that in Atlantic salmon, 75% of dietary fish oil can be replaced with vegetable oil without compromising growth and performance or significantly affecting fish health or welfare (Rosenlund, 2001; Tocher, et al., 2003; Torstensen, et al., 2005). Vegetable oils do not contain any ω3 LC-PUFA, but contain higher concentrations of SFA, MUFA and ω6 PUFA (Beardsell,

et al., 2002). Salmon fed replacement vegetable oils have a reduced ω3 LC-PUFA content

which is a reflection of their diet.

Recently, there has been an increased interest in the quality and toxicological properties of

fish oil as an ingredient in aquafeeds for salmon aquaculture (Jacobs, et al., 2002; Pereira, 2004;

Bell, et al., 2005; Hamilton, et al., 2005; Bethune, et al., 2006; Berntssen, et al., 2007). There

has been considerable health concerns associated with the presence of dioxins and dioxin like

PCB (poly-chlorinated bi-phenyls) residues in fish oil (Jacobs, et al., 2002; Bell, et al., 2005).

Concentrations of such residues vary greatly in fish oil sources from around the world with

seasonal and/or spatial variations common (Jacobs, et al., 2002). Dioxins and PCB are fat

soluble xenobiotics that are believed to be carcinogenic to humans (Pereira, 2004; Schwarz and

Appel, 2005) and are known to cause skin aliments, liver disease, reproductive disorders and

neurological problems (Mozaffarian and Rimm, 2006). Furthermore, dioxins and PCB are

lipophilic and resistant to degradation, therefore can accumulate in significant concentrations in

fish oil. Lastly, dioxins and PCB can persist in the environment for many years bio-

accumulating up the food chain thereby further highlighting the potential harmful effects to the

human consumer. Alternative oils such as vegetable or single cells oils do not contain any

dioxins and PCB, making them favourable as replacement oils in aquafeeds.

1.2.4 Omega 3/omega 6 PUFA

Over the last 20 years there has been increased evidence that ω3 LC-PUFA have unique

nutritional and health benefits to the consumer. As salmon have the ability to store considerable

amounts of ω3 LC-PUFA in their flesh, they are considered a good source of this key

ingredient. Both ω3 and ω6 LC-PUFA are nutritionally and biologically important lipids that

warrant understanding when considering replacement oils in aquafeeds for health benefits of

both the fish and human consumers.

It is generally acknowledged that dietary consumption of ω3 LC-PUFA, in particular EPA and DHA, has many benefits in human health (Tapiero, et al., 2002). Fish and shark liver oils have been used in many traditional medicines "He also drank a cup of shark liver oil each day…..it was very good against all colds and grippes and it was good for the eyes" (Hemingway, 1952). The first significant scientific evidence of the benefits of fish oil came from nutritional studies of Eskimo populations and the correlation between their high fish consumption and low plasma levels of cholesterol and low incidence of heart disease (Bang, et al., 1971). Recent studies have shown that the consumption of ω3 LC-PUFA helps prevent the excess production of inflammatory eicosanoids, a group of hormone like substances that contribute to the increase of heart disease, arthritis, asthma, stroke, and other diseases in the human body (Simopoulos, 2002a; Tapiero, et al., 2002). As ω3 LC-PUFA are necessary constitutes of cell membranes and of many cell-signalling systems, deficiencies in PUFA can be associated with defects in cellular function, which may lead to disease (Lands, 2001; Stillwell and Wassall, 2003). The ω3 LC-PUFA, in particular DHA, are predominantly utilised in cell membranes and have been shown to favourably alter numerous properties of membranes including fluidity, phase behaviour, elastic compressibility, permeability, fusion, flip-flop and protein activity (Stillwell and Wassall, 2003). Other suggested benefits of increased dietary ω3 LC-PUFA are cardiovascular health, brain development, joint health, visual acuity, reduced symptoms of depression, cancer prevention, protection against type 2 diabetes, alleviating symptoms of skin disorders, relieving asthma and relieving symptoms of irritable bowl syndrome (Takahata, et al., 1998; Horrocks and Yeo, 1999; Kris-Etherton, et al., 2004; Shahidi and Miraliakbari, 2004; Brouwer, et al., 2006; MacLean, et al., 2006).

Most human nutrition studies indicate that excessive amounts of ω6 PUFA are unhealthy as they promote inflammation. It is thought that ω6 PUFA, in particular linoleic acid (18:2ω6,

LA), are excessive in most western diets as which are dominated by vegetable oils and processed foods. Excess ω6 PUFA, in particular LA, has been associated with many disorders including cardiovascular disease, cancer, and inflammatory and autoimmune disease in humans (Simopoulos, 2002b). Arachidonic acid (20:4ω6, ARA) is of particular importance as it is a precursor of eicosanoids in particular prostaglandins, leukotrienes and lipoxins (Tapiero, et al., 2002). ARA also plays a major role in fish as it is the primary eicosanoid precursor and is important in the production of leukotrienes which control over many biological systems, especially inflammation, immunity and act as messengers in the central nervous system (Bell and Sargent, 2003).

It has been increasingly recognised that the ratio of ω3 to ω6 (PUFA) plays a vital role in human health (Gibson, et al., 1994; Simopoulos, 2002b; Goodstine, et al., 2003) and is important in aquafeeds, as it best represents the natural diets of salmon (Bell, 2000). An optimal ω3/ω6 ratio of 1 to 0.25 is suggested for a healthy diet in humans (Simopoulos, 2002b). In evolutionary terms, humans descended from a estimated dietary ω3/ω6 ratio of 1.29 in paleolithic hunter-gatherers (Eaton, et al., 1998). It has been estimated that the modern Australian diet has a very low ω3/ω6 ratio (0.13) and that this is constant over age group and gender (Meyer, et al., 2003). To improve their dietary ω3/ω6 ratio, Australians need to increase their ω3 intake and reduce their ω6 intake, and eating oily, ω3 LC-PUFA rich fish such as salmon is thought to be a good way to address this issue (Meyer, et al., 2003).

1.2.5 Biosynthetic pathway of omega 3 and omega 6 PUFA

Atlantic salmon have an endogenous ability to biosynthesise ω3 LC-PUFA from dietary precursors (Figure 1.1). Understanding and utilising their biosynthetic capacity through the provision of different precursors may offer possible ways of ensuring salmon meet their requirement for ω3 LC-PUFA. Salmon lack Δ12 and Δ15 fatty acid desaturases and cannot

produce LA and α-linolenic acid (18:3ω3, ALA) from the precursor oleic acid (18:1ω9, OA)

(Tocher, 2003). However, salmon have the capacity to biosynthesise dietary ALA through to

ω3 LC-PUFA (Tocher, 2003). The conversion of ALA to EPA and DHA is inefficient in

marine fish, such as Atlantic salmon, which have high concentrations of LC-PUFA in their

natural diet (Henderson and Tocher, 1987).
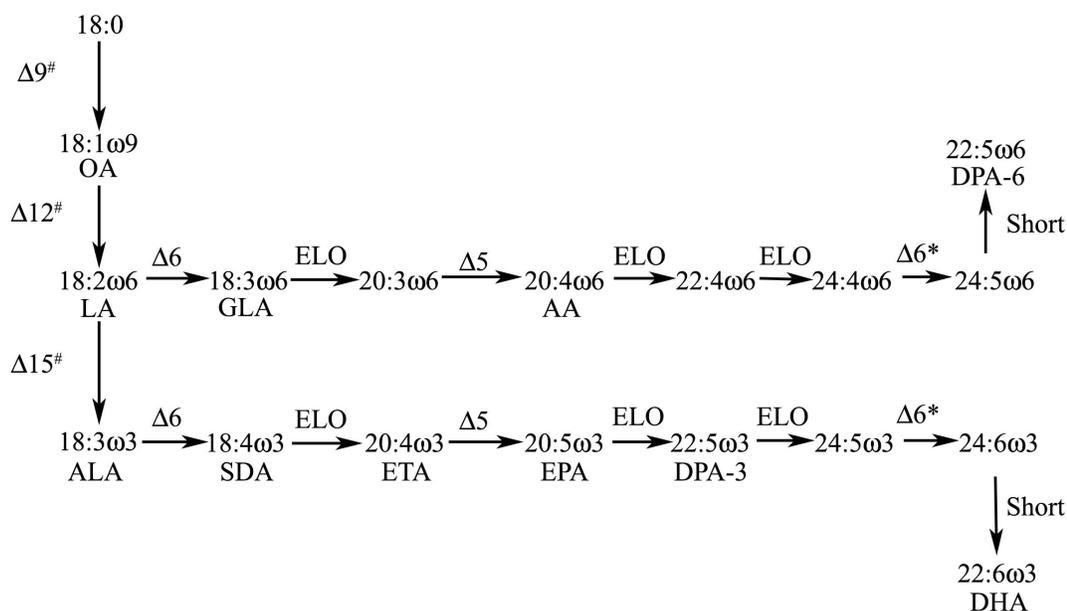
Figure 1.1: Adapted from Tocher 2003. Representation of the ω3 and ω6 LC-PUFA biosynthetic pathway from their C$_{18}$ FA precursors in Atlantic salmon.
Δ5, Δ6, and Δ6* Fatty acyl desaturases; ELO, Fatty acyl elongases; Short, Fatty acyl Peroxisomal chain shortening; Δ6* may or may not be the same desaturase enzyme as Δ6. Δ9$^{\#}$, Δ12$^{\#}$ and Δ15$^{\#}$ are not present in Atlantic salmon. LC-PUFA, Long chain-polyunsaturated fatty acids; DHA, Docosahexaenoic acid; DPA-3, Docosapentaenoic acid (ω3); DPA-6, Docosapentaenoic acid (ω6); EPA, Eicosapentaenoic acid; ETA, Eicosatetraenoic acid; SDA, Stearidonic acid; LA, Linoleic acid; ALA, α-Linolenic acid; ARA, Arachidonic acid; GLA, γ- Linolenic acid; OA, Oleic acid.

The conversion of ALA to ω3 LC-PUFA is greater in freshwater fish, which have high

concentrations of ALA and limited DHA in their natural diet (Sargent, et al., 2002). Therefore

the evolutionary pressure of FA availability has affected the ability of fish to biosynthesise ω3

LC-PUFA. Atlantic salmon are anadromous, where the adult fish live in the sea, but breed and

have their early development stages in fresh water. Therefore the ability of Atlantic salmon to

biosynthesise ω3 LC-PUFA from precursors changes throughout a life cycle (Zheng, et al.,

2005). It is of particular interest to understand the capacity of salmon to biosynthesize ω3 LC-

PUFA from different FA precursors, especially in their different life stages, in regards to

replacement oil diets.

1.3 *Challenges and benefits of replacement oils*

1.3.1 *Advantages/disadvantages with vegetable/plant based oils*

The major advantages that vegetable oil have over fish oil as an ingredient in aquafeeds is

that they are cheap, renewable and reliable sources of oil. Terrestrial sources of oil can be

produced in the quantities required to meet growing aquaculture demand. However, vegetable

oils do not contain the ω3 LC-PUFA or ω6 LC-PUFA and generally have high concentrations

of OA, LA and in some instances ALA. The major sources of replacement plant oils that been

extensively researched for Atlantic salmon include sunflower (Torstensen, et al., 2000;

Bransden, et al., 2003), linseed (Tocher, et al., 2000; Rosenlund, 2001; Bell, et al., 2003; Bell,

et al., 2004), canola (rapeseed) (Bell, et al., 2001; Bell, et al., 2003; Bendiksen, et al., 2003; Ng,

et al., 2004; Torstensen, et al., 2004), soybean (Rosenlund, 2001; Grisdale-Helland, et al.,

2002), olive (Torstensen, et al., 2000; Torstensen, et al., 2004), and palm oil (Torstensen, et al.,

2000; Rosenlund, 2001; Bell, et al., 2002; Ng, et al., 2004). The fatty acid profiles of the major

replacement oils for aquaculture diets differ greatly (Table 1.1).

Table 1.1: Fatty acid profiles (g/100g) of possible plant/vegetable replacement oils for fish oil

	Palm oil[a]	Canola oil[b]	Linseed oil[c]	Sunflower oil[d]	Olive oil[e]	Soy oil[f]	Echium oil[g]	Thrausto-chryid oil[h]	Fish oil[i]
14:0	1.2	0.0	0.0	0.0	0.0	0.0	0.0	8.9	6.2
16:0	46.7	4.7	6.3	6.4	10.8	9.7	7.5	26.1	16.4
18:0	5.2	2.1	5.1	4.1	3.3	3.5	3.6	0.8	3.5
Other SFA	0.3	0.6	0.0	1.5	0.6	0.2	0.0	1.0	1.2
Total SFA	53.5	7.4	11.4	12.0	14.6	13.4	11.2	36.7	27.3
16:1ω7c	0.0	0.0	0.0	0.0	0.7	0.7	0.0	0.6	8.2
18:1ω9c	33.8	58.3	18.3	25.3	75.4	22.5	17.2	1.3	21.1
18:1ω7c	1.3	4.3	1.3	2.0	2.5	1.8	1.0	0.4	3.6
20:1ω9	0.0	1.1	0.0	0.0	0.0	0.0	0.8	0.1	3.8
Other MUFA	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.2	6.2
Total MUFA	35.1	63.7	19.6	27.2	78.6	25.0	19.9	2.5	42.9
18:3ω3	0.0	7.3	53.3	0.0	0.0	6.4	28.1	0.1	0.6
18:4ω3	0.0	0.0	0.0	0.0	0.0	0.0	**11.4**	0.4	1.5
20:4ω3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0
20:5ω3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.2	**12.7**
22:5ω3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	1.7
22:6ω3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**36.7**	**7.3**
Other ω3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Total ω3	0.0	**7.3**	**53.3**	0.0	0.0	**6.4**	**39.6**	**41.1**	**25.3**
18:2ω6	11.4	21.6	15.7	60.7	6.8	55.2	19.5	0.6	3.4
18:3ω6	0.0	0.0	0.0	0.0	0.0	0.0	9.8	0.3	0.0
20:3ω6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0
20:4ω6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.4	1.0
22:5ω6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	15.8	0.0
Other ω6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
Total ω6	11.4	21.6	15.7	60.7	6.8	55.2	29.3	19.6	4.5
ω3/ω6	0.0	0.3	3.4	0.0	0.0	0.1	1.3	2.1	5.6
Oil price ($US/Tonne) [j]	720	844	795	820	3,430	731	4,000-8,000[k]	20,000-100,000[k]	890

[a] Aoroma, Hallam, Victoria, Australia
[b] Steric Trading Pty Ltd, Villawood, NSW, Australia
[c] Melrose Laboratories, Mitcham, Vic, Australia
[d] Meadowlea foods, Mascot, NSW, Australia
[e] Island Olive Grove, Cambridge Tasmania, Australia
[f] Carolina Soy Product, Warsaw, North Carolina, USA
[g] Croda Chemicals, East Yorkshire, UK,
[h] Martek, Columbia, Maryland, USA
[i] From jack mackerel, *Trachurus symmetricus* L, Skretting Australia, Cambridge, Tasmania, Australia
[j] Prices taken on 14/6/07 from Hamburg market prices, Oil World ISTA Mielke GmbH Hamburg, Germany
[k] Estimated present costs, however costs can reduce when the volume of production increases

The FA profile of vegetable oils can vary due to location, season and species. Alongside FA profile, cost and production capabilities of these oils are considerable factors in their selection as ingredients in aquafeeds. Globally, soy and palm are the most abundant oil crops and have the lowest market price. The cheapest choice of oil for aquafeeds in non European Union countries are poultry and animal fats (lard, $US 532 per tonnes, Hamburg 17/6/2007) in which the FA profiles are dominated by SFA in particular palmitic acid (16:0) and stearic acid (18:0). Regulations in Europe, post the outbreaks of bovine spongiform encephalopathy (BSE) commonly known as "mad cow disease", forbids the use of animal lard in aquafeeds.

Aquafeed producers are increasingly using blends of vegetable oils and, in non European countries animal fats in their formulations of diets, but need to provide the ω3 LC-PUFA content in particular EPA and DHA, present in fish oil. Blending oils gives the feed producers flexibility to meet dietary nutritional requirements and also allows these companies to seasonally adjust diets due to factors such as cost and availability. Since vegetable oils do not contain any ω3 LC-PUFA or ω6 LC-PUFA they are as a sole oil source, unable to meet the nutritional requirements for Atlantic salmon.

1.3.2 Phytosterols

A benefit of increasing replacement of fish oil and meal with plant based products in salmon aquafeeds is increased amounts of phytosterols in the diet. Minor components of vegetable oil which are not present in natural diets of fish need to be investigated and monitored. Phytosterols are naturally occurring molecules found in plant oil and meal which are structurally related to cholesterol (Ostlund, 2002). Phytosterols are known to affect cholesterol metabolism and have been shown to reduce the low density lipoprotein (LDL) cholesterol levels in humans, reducing the risk of coronary heart disease (CHD) (Ostlund, 2002; Trautwein, et al., 2003; Earnest, et al., 2007). Phytosterols are lipophilic and have been introduced to

margarines, butters, spreads and breakfast cereals and promoted as "functional foods" to reduce CHD (Law, 2000; Engel and Schubert, 2005; Kuhlmann, et al., 2005). Phytosterols in combination with ω3 LC-PUFA in Atlantic salmon fed replacement oil diets could provide increased coronary protection. Presently there is no literature on the digestion, accumulation and metabolism of phytosterols in Atlantic salmon when fed replacement diets containing plant-based oils.

Phytosterols in pulp and paper mill effluent have been shown to affect behaviour, reproduction, endocrine function and general development of fish (Tremblay and Van der Kraak, 1998, 1999; Honkanen, et al., 2005; Clotfelter and Rodriguez, 2006). Minor components of vegetable oils such as phytosterols need to be examined to determine whether they have positive or negative effects not only for the consumer, but also for fish health.

1.3.3 Regiospecificity of fatty acids

Lipids are important molecules in the body for storing energy and maintaining cell membrane integrity. Regiospecificity is the terminology for how individual FA are positioned on the glycerol backbone of both storage triacylglycerol (TAG) and membrane polar lipids (PL). Regiospecificity of lipids plays an important role in their function and bioavailability. Traditional profiling of lipid class and fatty acid composition provides important information, however, they do not reveal the regiospecific nature of the molecule which can play a key role in their function (Kew, et al., 2003a; Kew, et al., 2003b). With advances in analytical and computing facilities, new techniques and methods can be utilised to examine lipids with the emphasis on regiospecific distribution of FA.

The middle *sn*-2 position of the storage TAG molecule is thought to be the most bio-available position for the digestion of particular fatty acids. This was first shown in fat absorption by infants fed breast or formulated milk (Lucas, et al., 1997). How Atlantic salmon

regiospecifically store ω3 LC-PUFA has the potential to affect the bioavailability of FA the human consumer. Structural lipids, such as PL, are important components of cell membrane structure. The composition of the molecular species in the cell membrane can be influenced by many factors including temperature and diet (Bell, et al., 1986). The composition of cell membranes has a major effect on the health of the cell and therefore the health of the fish. To date, limited research has been conducted to identify changes in the regiospecific composition of membrane lipids and TAG in Atlantic salmon as a result of changes in the FA profile of their diet. The regiospecificity of both PL and TAG is further covered in chapters 6 and 7.

1.4 *Environmental influences on lipids*

1.4.1 Temperature

This thesis explores the current issue of increasing water temperatures effect on membrane and storage lipids. Temperature has a major influence on the membrane and storage lipids of exothermic animals, such as Atlantic salmon, which need to adapt to seasonal and occasional abrupt changes in environmental temperature (Hazel, 1984; Henderson and Tocher, 1987; Hazel, et al., 1991; Hazel, et al., 1992). The Tasmanian Atlantic salmon industry commonly encounters temperatures (over 19°C) that approach the upper threshold for salmon survival (Carter, et al., 2003). Replacement oil blends may assist by adapting the FA profiles of salmon diet to meet the different nutritional requirements for elevated temperatures.

Fish may exploit the structural diversity of lipids within their membranes to adapt to change in ambient water temperature. Membrane lipids may adapt in several ways to a change in temperature: by altering the unsaturation and chain-length of the fatty acids (Zwingelstein, et al., 1978); by changing the distribution of fatty acids within the phospholipid molecules (Miller, et al., 1976; Hazel, 1979); and, by altering the composition of the polar head group of the phospholipids (Hazel and Landrey, 1988; Farkas, et al., 1994). In general, colder temperatures lead to an increase in unsaturation in gill lipids, thus maintaining membrane fluidity (Hazel,

1979; Tocher and Sargent, 1990). Most studies have investigated temperature influence on

Atlantic salmon lipids within the range of 2–12°C (Grisdale-Helland, et al., 2002; Bendiksen

and Jobling, 2003; Jobling and Bendiksen, 2003; Ruyter, et al., 2003), but none have examined

salmon at higher temperatures such as 19°C. With global water temperatures increasing it is

pertinent to monitor membrane structure and oil storage in salmon at elevated temperatures.

1.5 *Potential sources of ω3 LC-PUFA for aquaculture*

Vegetable oils do not contain ω3 LC-PUFA that are an essential component of salmon diets.

Other than fish oil the current possible sources of oil that contain, or that contain precursors to

EFA, are;

- vegetable oils that contain biosynthetic precursors which can be utilised by

    Atlantic salmon to biosynthesis ω3 LC-PUFA,

- single cell oil sources which are the ω3 LC-PUFA bio-factories of the oceans, and

- vegetable oils that have undergone genetic modification to contain ω3 LC-PUFA.

Each of these sources of oil will be discussed in the following sections.

1.5.1 *Biosynthetic precursors of ω3 LC-PUFA*

Atlantic salmon have an endogenous capacity to biosynthesise ω3 LC-PUFA.

Understanding and utilising biosynthetic precursors may provide renewable and sustainable

options from specialised vegetable oils for future aquafeeds. Plants such as Patterson's curse

(*Echium plantagineum* L.) and blackcurrent (*Ribes nigrum* L.) have a Δ6 desaturase gene which

produces the ω3 LC-PUFA biosynthetic precursor SDA from ALA. Echium oil from

Patterson's curse has an SDA level >10% depending on the strain (Table 1.1). It has been

suggested that the Δ^{6}desaturation of ALA to SDA is the rate limiting step in the biosynthetic

pathway of ω3 LC-PUFA (Brenner, 1981). Dietary SDA bypasses the initial rate limiting Δ^{6}

desaturase step in the ω3 LC-PUFA biosynthetic pathway and therefore EPA and DHA may be

biosynthesised. The use of SDA rich oil from Echium as a replacement oil is explored in

Chapter 2 and 3.

The cost and availability of an SDA rich oil source, such as Echium oil from Patterson's

curse, is at present not economically viable for aquaculture. The current price of SDA rich oil

from Patterson's curse is 5–10 times the price of fish oil. In Australia, Patterson's curse is an

introduced pest species and considered a noxious weed. Despite a significant proportion of

southern Australian agricultural land being covered by Patterson's curse, there are presently no

companies in Australia looking to use this resource for its oil content. Furthermore, the ability

of salmon to digest, accumulate and biosynthesise SDA into longer ω3 LC-PUFA needs to be

assessed before it can be considered as a dietary ingredient for aquafeeds.

1.5.2 Single cell oils (SCO)

Single cell oils such as thraustochytrid oil provide a novel and renewable source of EFA, in

particular EPA and DHA. Single cell organisms, including thraustochytrids, diatoms and other

microalgae, are the ω3 LC-PUFA biofactories of the ocean. Thraustochytrids possess the ability

to produce a number of ω3 LC-PUFA, especially DHA. Thraustochytrid oil (Table 1.1) from

the species *Schizochytrium L,* has a high concentration of DHA (35%) and is commercially

available from Martek (Columbia, Maryland, USA). The use of SCO from thraustochytrid as a

replacement oil is explored in chapters 4 and 7.

Presently the production cost and quantities of single cell oil needed to replace fish oil in

salmon aquafeeds is prohibitive. Continued bio-prospecting for new strains and increased

research into fermentation techniques may provide cheaper biomass and improved quality oils

for aquaculture purposes. The intensive aquaculture industry utilises feeding regimes, such as

finishing diets, which provide high levels of ω3 LC-PUFA to fish prior to harvest. As a result,

if costs are reduced single cell biomass and/or oils may be a candidate for these finishing diets due to their high ω3 LC-PUFA content.

1.5.3 Genetic modification (GM) of oils

Transgenic oilseed crops that are engineered to produce the major ω3 LC-PUFA by the insertion of various genes encoding desaturases and/or elongases have been suggested as a source of ω3 LC-PUFA (Robert, et al., 2005; Robert, 2006). However, the requirement for coordinate expression and activity of five or more new enzymes encoded by genes from possibly diverse sources has made this goal difficult to achieve and only low yields have generally been obtained (Abbadi, et al., 2001; Sayanova and Napier, 2004; Robert, et al., 2005).

A gene encoding the Δ6 desaturase isolated from borage (*Borago officinalis*) was expressed in transgenic tobacco and *Arabidopsis*, resulting in the production of GLA and SDA, the direct precursors for LC-PUFA (Sayanova, et al., 1997; Sayanova, et al., 1999). This initial research provided only the first step to ω3 LC-PUFA, but may provide a renewable source of SDA for aquaculture and other uses. Recently more genes encoding the whole pathway have produced EPA (Qi, et al., 2004) and DHA (Robert, et al., 2005), in crops or model plants, including oilseeds. In the model plant, *Arabidopsis*, the insertion of five genes resulted in the first oil with DHA (Robert, et al., 2005). That study observed a total LC-PUFA content (ARA + EPA + DHA) of 4.2%. Further research using different genes, and seed specific promoters with soybean has produced an EPA content of 19.5% and DHA of 3.3% (Damude and Kinney, 2007). These two examples demonstrate the complicated nature and difficulties present with engineering multi-genes to produce a sustainable land plant source of ω3 LC-PUFA rich oil. GM of oil seed crops continues to be refined and developed and will likely in the future present the aquaculture sector with a promising source of ω3 LC-PUFA to meet their needs.

GM plants may provide the most economically viable source of ω3 LC-PUFA rich oil for aquaculture. It is estimated that the cost and availability of the production of oils from GM plant would be simular to that of currently available commercial oilseed crops such as canola and soy. Research in this area has the potential for significant commercial, social and environmental benefits. However, consumer and industry acceptance and passing health and safety requirements set by regulatory bodies of this biotechnology is vital for oils from GM plants before they can be accepted and utilised by the aquaculture industry.

1.6 *The aims and hypothesis of thesis*

The overall aim of this study was to assess possible alternative sources of dietary oil for Atlantic salmon aquaculture with respect to their ω3 LC-PUFA content under Tasmanian conditions. To address this aim the following approaches were adopted:

1)   to analyse biosynthetic precursors SDA and ALA as sources of ω3 LC-PUFA for Atlantic salmon

2)   to analyse single cell oil as a possible source of ω3 LC-PUFA for Atlantic salmon

3)   to examine the digestibility and accumulation of phytosterols from vegetable oil in Atlantic salmon

4)   to understand the effect of dietary oil on how Atlantic salmon store fatty acids and how they utilise dietary fatty acids in their cell membranes

5)   to understand the requirement and effect of seasonally high temperatures on lipid composition and its storage in Atlantic salmon

Each of the above approaches formed the basis of specific hypotheses that were addressed in a series of fish feeding trials. Chapters 2-7 outline these fish feeding trials and are stand alone

chapters which have been published or are under internal review in preparation for submission to a variety of international oil chemistry, nutrition and biochemistry journals.

Chapter 2 examines the first hypothesis that dietary SDA increases ω3 LC-PUFA accumulation in the tissues of Atlantic salmon parr. This study was published in Comparative Biochemistry and Physiology Part B (Miller, et al., 2007a). The promising results from this trial warranted further examination of seawater fish. In Chapter 3, a second trial examined the hypothesis that dietary SDA will increase gene expression and ω3 LC-PUFA accumulation in Atlantic salmon in seawater. This research is in internal review with planned submission to the Journal of Nutrition.

The hypothesis that dietary DHA from a single cell organism (Thraustochytrid) can replace fish oil in Atlantic salmon diets without detriment to growth is examined in Chapter 4. This part of the study was published in Comparative Biochemistry and Physiology Part A (Miller, et al., 2007b). This trial was expanded in Chapter 7, which tested the hypothesis that a high level of dietary DHA effects it storage and the cell membrane structure in the white and red muscle, liver and gills of Atlantic salmon. This research has been submitted into the internal review process before submission to the journal Lipids.

Chapter 5 examined the hypothesis that Atlantic salmon can digest and accumulate phytosterols contained in dietary vegetable oils. The research used fish from the seawater SDA replacement trial (Chapter 4). This chapter will be submitted to the journal Lipids. Finally, in chapter 6 the hypothesis examined was that elevated water temperature (19^oC) increases the amount of SFA in the fatty acid profile, cell membrane structure and alters lipid storage in Atlantic salmon parr. Tissue samples were advantageously taken from a high temperature protein/lipid requirement trial designed by Julia Barnes (UTAS, Tasmania) as a part of her PhD candidature. This research has been published in Lipids (Miller, et al., 2006).

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CHAPTER 2

Replacement of dietary fish oil for Atlantic salmon parr (*Salmo salar* L.) with a stearidonic acid containing oil has no effect on omega 3 long chain polyunsaturated fatty acid concentrations.

2.1 ABSTRACT

The worldwide increase in aquaculture production and the concurrent decrease of wild fish stocks has made the replacement of fish oil in aquafeeds an industry priority. Oil from a plant source *Echium plantagineum* L., Boraginaceae, has high levels of stearidonic acid (SDA, 18:4ω3, 14%) a biosynthetic precursor of omega-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (ω3 LC-PUFA). Atlantic salmon (*Salmo salar L.)* parr were fed a control fish oil diet (FO) or one of 3 experimental diets with 100% canola oil (CO) 100% SDA oil (SO), and a 1:1 mix of CO and SDA oil (MX) for 42 days. There were no differences in the growth or feed efficiency between the four diets. However, there were significant differences in the fatty acid (FA) profiles of the red and white muscle tissues. Significantly higher amounts of SDA, eicosapentaenoic acid (20:5ω3, EPA), docosahexaenoic acid (22:6ω3, DHA) and total ω3 FA occurred in both red and white muscle tissues of fish fed SO and FO compared with those fed CO. Feeding SO diet resulted in ω3 LC-PUFA amounts in the white and red muscle being comparable to the FO diet. This study shows that absolute concentrations (μg/g) of EPA, DHA and total ω3 have been maintained over 6 weeks for Atlantic salmon fed 14% SDA oil. The balance between increased biosynthesis and retention of ω3 LC-PUFA to maintain the concentrations observed in the SO fed fish remains to be conclusively determined, and further studies are needed to ascertain this.

2.2 INTRODUCTION

Global production of farmed fish has more than doubled in the last 15 years and this expansion places an increasing demand on supplies of wild fish harvested to provide protein and oil as ingredients for aquafeeds (Naylor, et al., 2000). The supply of seafood from global capture fisheries sources is presently around 100 million tones per annum (FAO, 2001). This amount has not increased since the mid 1980's and will not increase in the future since most fisheries are at or above sustainable levels of production (Barlow, 2000; FAO, 2001). Fish oil stocks are also under increasing demand not only from aquaculture, but also from the agriculture and nutraceutical/biomedical industries (Barlow, 2000; Naylor, et al., 2000).

Replacement oils for aquaculture of Atlantic salmon have been sourced from a variety of commercial terrestrial plant sources including sunflower (Bell, et al., 1993; Bransden, et al., 2003), canola (rapeseed) (Polvi and Ackman, 1992; Bell, et al., 2003a; Torstensen, et al., 2004), olive (Torstensen, et al., 2004), palm (Bell, et al., 2002; Fonseca-Madrigal, et al., 2005) and linseed (Bell, et al., 1993; Bell, et al., 2004). In the majority of studies, the inclusion of plant oil to replace part or all of the fish oil in Atlantic salmon feeds has not effected growth rate or feed conversion (Polvi and Ackman, 1992; Bell, et al., 2002; Bransden, et al., 2003; Bell, et al., 2004; Torstensen, et al., 2004; Fonseca-Madrigal, et al., 2005). However, salmon fed diets high in plant oil may have reduced human health benefits compared to salmon fed a fish oil diet (Seierstad, et al., 2005). This is because land plant derived oils do not contain any omega-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acid ($\omega 3$ LC-PUFA), and they can have high amounts of monounsaturated fatty acids

(MUFA), high amounts of ω6 PUFA and low ω3/ω6 ratios. Therefore feeding diets high in plant oil has the potential to reduce the important cardiovascular and other health benefits associated with eating fish and thereby consuming ω3 LC-PUFA (Seierstad, et al., 2005).

The biosynthetic pathways for PUFA are well known (Sargent, et al., 2002). Vertebrates lack Δ12 and Δ15 (ω3) fatty acid desaturases and cannot produce linoleic acid (18:2ω6, LA) and α-linolenic acid (18:3ω3, ALA) from oleic acid (18:1ω9, OA). The conversion of ALA to eicosapentaenoic acid (20:5ω3, EPA) and docosahexaenoic acid (22:6ω3, DHA) is inefficient in marine fish, which have high concentrations of LC-PUFA in their natural diet. Conversion is greater in freshwater fish, which have high concentrations of LA and ALA and limited DHA in their natural diet (Sargent, et al., 2002). High concentrations of ω3 LC-PUFA, which are found in salmon, cannot be synthesised from ALA and LA at a rate sufficient to meet essential fatty acid (EFA) requirements and therefore must be provided to the fish in their diet.

Two recent publications have examined *in vivo* diets containing stearidonic acid (SDA, 18:4ω3) as a ω3 LC-PUFA precursor for Atlantic cod (*Gadus morhua L.*) and Arctic charr (*Salvelinus alpinus L.*), but to date there have been no *in vivo* studies on Atlantic salmon (Bell, et al., 2006; Tocher, et al., 2006). Research has largely focused on enriching salmon diets by increasing the dietary supply of ALA (Bell, et al., 1993) and EPA and DHA (Harel, et al., 2002; Carter, et al., 2003a). SDA is a precursor of ω3 LC-PUFA and is derived by desaturation of ALA. The Δ^6 desaturation is the rate limiting step in the desaturation and elongation pathway of

C_{18} essential fatty acids in both the ω3 and ω6 pathways (Tocher, et al., 1998). The Δ^6 desaturase is also involved in other steps in the biosynthesis of LC-PUFA in the formation of DHA from EPA (Yamazaki, et al., 1992) and 20:4ω6 (arachidonic acid, ARA) from 18:2ω6. Therefore it is likely that the Δ^6 desaturation of ALA to SDA may be out-competed by the ω6 pathway when diets contain high concentrations of 18:2ω6 which occur in plant oils such as canola and particularly sunflower.

Oil from the plant source *Echium plantagineum* L., Boraginaceae, has high concentrations (14%) of SDA in its fatty acid profile. Our feeding experiment aimed to test whether use of the 14% SDA oil would lead to an accumulation of ω3 LC-PUFA at similar concentrations to when using fish oil. Canola oil was used as a negative control because it is a source of ALA, but contains no SDA or ω3 LC-PUFA (Carter, et al., 2003b). The experiment was terminated after 6 weeks since the principle aim was to access the accumulation of ω3 LC-PUFA. The weight of fish approximately doubled and considerable fat deposition expected.

2.3 MATERIALS AND METHODS

2.3.1 Experimental diets

Four diets were formulated to compare canola oil (CO), two different levels of stearidonic acid oil (100% (SO), 1:1 SO:CO (MX)), and fish oil (FO) (Table 2.1). Fish meal was defatted three times using a 2:1 mixture of hexane and ethanol (400ml 100g^{-1} fish meal). Soybean (Hamlet Protein A/S, Horsens, Denmark), casein (MP Biomedcals Australasia Pty Ltd, Seven Hills NSW, Australia), wheat gluten (Starch Australasia, Lane Cove, NSW, Australia) and BOIIC pre-gelatinised maize

starch (Penford Australia Limited, Lane Cove, NSW, Australia) were used as ingredients. Stearidonic acid oil was provided as Crossential SA14 (Croda Chemicals, East Yorkshire, UK). Fish oil was from jack mackerel, *Trachurus symmetricus* L., (Skretting Australia, Cambridge, Tasmania, Australia) and a domestic source of pure canola oil was used (Steric Trading Pty Ltd, Villawood, NSW, Australia). Stay-C and Rovimix E50 were supplied from Roche Vitamins Australia (Frenchs Forest, NSW, Australia), and the remaining ingredients were supplied by Sigma-Aldrich (Castle Hill, NSW, Australia). The diets were manufactured into 3 mm diameter pellets using a California Pellet Mill (CL-2), dried and stored at -5°C (Carter, et al., 2003b).

2.3.2 Growth experiment

The experiment was conducted at the School of Aquaculture, University of Tasmania (Launceston, Tasmania, Australia). Atlantic salmon (*Salmo salar* L.*)* parr (about 40g) were obtained from Wayatinah Salmon Hatchery (SALTAS, Tasmania, Australia) acclimated for 14 days 300 l tanks and fed a commercial feed (Skretting). The fish were held in a freshwater partial recirculation system (Bransden, et al., 2003). The tanks were held at a constant temperature of 15.0^{o}C under a 16:8 (light:dark) photoperiod. Water was treated through physical, UV and biofilters, with a continuous replacement of approximately 15% per day. Dissolved oxygen, pH, ammonia, nitrate, nitrite, and chlorine were monitored daily to ensure water quality remained within parameters recommended for Atlantic salmon (Wedemeyer, 1996). The experiment was conducted in accordance with the University of Tasmania Animal Ethics guidelines (Investigation A0007719).

Table 2.1: Ingredient and lipid composition (g/kg dry matter) of experimental diets

	Diet			
	CO	MX	SO	FO
Ingredient composition (g kg⁻¹)				
Fish meal (defatted)	148.7	148.7	148.7	148.7
Casein	148.7	148.7	148.7	148.7
Wheat gluten	99.1	99.1	99.1	99.1
Soybean meal	224.0	224.0	224.0	224.0
Fish oil	0.0	0.0	0.0	128.8
Canola oil	128.8	64.4	0.0	0.0
SDA oil	0.0	64.4	128.8	0.0
Pre gel starch	148.7	148.7	148.7	148.7
Vitamin mix[a]	3.0	3.0	3.0	3.0
Mineral mix[b]	5.0	5.0	5.0	5.0
Stay C[c]	3.0	3.0	3.0	3.0
Chlorine chloride	2.0	2.0	2.0	2.0
Bentonite	49.6	49.6	49.6	49.6
CMC	9.9	9.9	9.9	9.9
Sodium mono phosphate	19.8	19.8	19.8	19.8
Yttrium oxide	9.9	9.9	9.9	9.9
Chemical composition (g kg⁻¹ DM)				
Dry matter	941.2	948.1	933.5	920.4
Crude protein	387.3	390.1	387.4	390.2
Crude fat	165.1	172.2	168.1	171.8
Energy (MJ kg⁻¹ DM)	20.1	20.1	19.9	19.8
FAME (g kg⁻¹ DM)				
Total SFA	8.5	16.2	14.0	59.3
Total MUFA	103.1	74.5	53.3	43.5
18:3ω3 ALA	16.6	27.6	32.9	4.1
18:4ω3 SDA	0.0	9.6	18.5	5.5
20:5ω3 EPA	0.1	0.0	0.1	23.8
22:6ω3 DHA	0.7	0.7	0.6	14.2
Total ω3	17.6	37.9	52.0	52.3
18:2ω6	35.8	35.8	33.3	10.6
18:3ω6	0.0	7.4	13.8	1.1
Total ω6	35.8	43.3	47.5	13.4
Total PUFA	53.5	81.2	99.5	65.8

SO, stearidonic oil-Crossential SA14 from Croda chemicals; CO, canola oil; MX, 1:1 mix of canola oil and stearidonic acid oil; FO, fish oil, SFA, Saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; CMC, Carboxymethyl cellulose; DHA, Docosahexaenoic Acid; EPA, Eicosapentaenoic Acid; SDA, Stearidonic acid, ALA, ω- Linolenic acid.
[a] Vitamin mix (ASV4) supplied per kilogram of feed: 2.81 mg thiamine HCL, 1.0 mg riboflavin, 9.15 mg pyridoxine HCL, 25 mg nicotinic acid, 54.35 mg calcium D-pantothenate, 750 mg myo-inositol, 0.38 mg D-biotin, 2.5 mg folic acid, 0.03 mg

cyanocobalamin, 6350 IU retinol acetate, 2800 IU cholecalciferol, 100 IU DL α-
tocopherol acetate, 5 mg menadione sodium bisulphate, 100 mg Roche rovimix E50.
[b] Mineral mix (TMV4) to supplied per kilogram of feed:117 mg CuSO4.5H2O, 7.19
mg KI, 1815 mg FeSO4.7H2O, 307 mg MnSO4.H2O, 659 mg ZnSO4.7H2O, 3.29
mg Na2SeO3, 47.7 mg CoSO4.7H2O
[c] L-Ascorbyl-2-polyphosphate (Stay-C, Roche Vitamins Australia, French Forest,
NSW, Australia).

At the start of the experiment fish were anaesthetized (50mg L^{-1}, benzocaine),
their weight and fork length measured, and four fish killed to measure initial lipid
content and composition. Twenty five fish were randomly re-allocated into each of
twelve 300 L tanks. The four diets were fed in triplicate at a ration of 2.2% body
weight per day (% BW d^{-1}), in two equal feeds at 0900 and 1700 by automatic belt
feeders. Every three weeks all fish were anaesthetized (50mg L^{-1}, benzocaine) and
batch-weighed. Fish were starved the day prior to weighing. Every 7 days the total
feed consumption (kg DM) was estimated from the amount of feed that was not
eaten by collection in sediment collectors (Helland, et al., 1996).

At the end of the experiment fish were starved for one day prior to being
anaesthetized (50mg L^{-1}, benzocaine) and their weight and fork length measured.
Three fish per tank were killed by a blow to the head after immersion in anaesthetic.
Samples of red and white muscle (approximately 1.3g) were dissected from below
the dorsal fin and frozen at $-80^{o}C$ until analysis.

Specific growth rate (SGR) was calculated as SGR (% day^{-1}) = 100 x (ln
(W_f/W_i)) x d^{-1} where W_f and W_i are the final and initial weights (g) and d is the
number of days of the experiment. Total feed consumption (FC) was calculated as
the total average amount (g DM) consumed per fish over 42 days. The feed
efficiency ratio (FER) was calculated as FER (gg^{-1}) = total weight gain (g)/ FC (g).

The hepatosomatic index (HSI) was calculated as HSI (%) = 100 x (liver weight (g WW) / total body weight (g WW)).

The mass balance estimation was used to calculate the absolute intake and accumulation of specific FA. The estimated total amount (T_f) of individual FA in a fish after 42 days was calculated as T_f (g) = W_f x FA_{wmf} where FA_{wmf} (g/kg) is proportion of FA in the white muscle after 42 days. W_f is the weight of the fish at 42 days. Total amount of individual FA consumed in diet (T_c) was calculated as T_c (g) = FC (g) x FA_{diet}, where FA_{diet} (g/kg) was the proportion of FA in the diet and FC is total feed consumption. The total initial amount (T_i) of an individual FA was calculated as T_i (g) = W_i x FA_{wfi} where FA_{wfi} (g/kg) is the proportion of FA in the white muscle at day 1. This calculation assumes that the FA composition of the white muscle reflects the carcass composition.

2.3.3 Lipid extraction and isolation

Samples were freeze dried and extracted overnight using a modified Bligh and Dyer protocol (Bligh and Dyer, 1959). This involved a single phase extraction, $CHCl_3$/MeOH/H_2O (1:1:0.9, by vol), followed by phase separation to yield a total lipid extract (TLE).

Lipid classes were analysed by an Iatroscan MK V thin-layer chromatography-flame ionization detector (TLC-FID) analyser (Iatron Laboratories, Japan). Samples were spotted onto silica gel SIII Chromarods (5 μm particle size) and developed in a glass tank lined with pre-extracted filter paper. The solvent system used for the lipid separation was hexane: diethyl ether: acetic acid (60:17:0.1, v/v/v) (Volkman and Nichols, 1991). After development for 25 minutes, the chromarods were oven-dried and analysed immediately to minimise adsorption of atmospheric contaminants.

Lipid classes were quantified by DAPA software (Kalamunda, WA, Australia). The FID was calibrated for each compound class: phosphatidylcholine, cholesterol, cholesteryl ester, oleic acid; hydrocarbon (squalene), wax ester (derived from fish oil), triacylglycerol (derived from fish oil), and diacylglycerol ethers (DAGE) (purified from shark liver oil).

An aliquot of the TLE was trans-methylated in methanol: chloroform: hydrochloric acid (10:1:1, v/v/v) for 1 hour at 100°C. After addition of water, the mixture was extracted three times with hexane: chloroform (4:1, v/v) to obtain fatty acid methyl esters (FAME). FAME were concentrated under nitrogen and treated with N,O-bis(trimethylsilyl)-trifloroacetamide (BSFTA, 50 µl, 60°C, 1h) to convert free hydroxyl groups to their corresponding trimethylsilyl ethers . Samples were made up to a known volume with an internal injection standard (19:0 FAME) and analysed by gas chromatography (GC) using an Agilent Technologies 6890N GC (Palo Alto, California, USA) equipped with an HP-5 cross-linked methyl silicone fused silica capillary column (50 m × 0.32 mm i.d.) and an FID. Helium was used as the carrier gas. Samples were injected, by a split/splitless injector and an Agilent Technologies 7683 Series auto sampler in splitless mode, at an oven temperature of 50°C. After 1 min the oven temperature was raised to 150°C at 30°C min^{-1}, then to 250°C at 2°C per min and finally to 300°C at 5°C min^{-1}. Peaks were quantified by Agilent Technologies GC ChemStation software (Palo Alto, California, USA). The HP5 column was used as it enables separation of a wide range of FA and other components simultaneously, e.g. FA with hydrocarbons, alcohols and sterols (the latter two as TMSi ethers, data not shown). FA separation on HP5 is useful for measuring unusual VLC-PUFA ($\geq$ C24). The only separation that is not obtained is

that of 18:1ω9c and 18:3ω3, which was determined in this study using a BPX-70

column (see below).

Individual components were identified by mass spectral data and by comparing

retention time data with those obtained for authentic and laboratory standards. GC

results are typically subject to an error of ±5% of individual component area. GC-

mass spectrometric (GC-MS) analyses were performed on a Finnigan Thermoquest

GCQ GC-mass spectrometer fitted with an on-column injector and using

Thermoquest Xcalibur software (Austin, Texas, USA). The GC was fitted with a

capillary column similar to that described above.

A polar GC column was used to separate 18:1ω9 and 18:3ω3 which coeluted on

the HP5 column. FAME were analysed with a Hewlett Packard 5890 gas

chromatograph (GC) equipped with a flame ionisation detector (FID) set at 250°C.

FAME samples were injected using a split/splitless injector onto a polar BPX-70

fused-silica column (50 m x 0.32 mm i.d.). The carrier gas was helium. The GC

oven temperature was initially held at 45°C for 2 min after injection and then

increased at 30°C/min to 120°C and at 3°C/min to 240°C, then held isothermal for 10

min.

2.3.4 Chemical analysis

Standard methods were used to determine dry matter (freeze dry to constant

weight); crude fat (Bligh and Dyer, 1959); nitrogen (Kjeldahl using a selenium

catalyst; crude protein was calculated as N x 6.25) and energy (bomb calorimeter,

Gallenkamp Autobomb, calibrated with benzoic acid).

2.3.5 Statistical analysis

Mean values are reported as plus or minus the standard error of the mean. Normality and homogeneity of variance were confirmed and percentage data were arcsin transformed prior to analysis. Comparison between means was by 1-way analysis of variance (ANOVA) followed by multiple comparisons using Tukey-Kramer HSD. Significance was accepted at probabilities of 0.05 or less. Statistical analysis was performed using SPSS for Windows version 11.

2.4 RESULTS

2.4.1 Growth results

Diet had no significant effect on the final weight, weight gain, length, specific growth rate (SGR), total feed consumption (FC), feed efficiency ratio (FER), hepatosomatic index (HSI) or survival (Table 2.2). Over the 42 days of the trial, the weight of fish approximately doubled and the FER was less than 1 (Table 2.2). Survival was close to 100% on all diets.

2.4.2 Lipid class composition

After 42 days there was no statistical difference in lipid composition among the dietary groups in either red and white muscle (Tables 3 and 4). The predominant lipid class in red muscle was TAG (94.0-96.7%). There was significantly ($p<0.02$) less TAG in all treatments (42.0-67.0%) compared to the initial group (82.0%) for the white muscle.

Table 2.2: Growth and efficiencies of Atlantic salmon fed experimental diets with canola oil (CO), 14% stearidonic acid oil (SO), 1:1 CO:SO (MX) and fish oil (FO) (mean ± SE).

	CO			MX			SO			FO		
Initial weight (g)	46.2	±	2.5	44.6	±	1.1	44.8	±	1.1	42.3	±	1.2
Final weight (g)	81.4	±	8.4	80.1	±	1.9	76.9	±	2.2	76.5	±	3.3
Weight gain (g)	35.1	±	5.9	35.5	±	0.8	32.1	±	2.0	34.1	±	3.1
Initial length (cm)	15.4	±	0.5	15.7	±	0.1	15.4	±	0.4	15.1	±	0.4
Final length (cm)	17.2	±	0.7	17.5	±	0.2	17.5	±	0.3	17.4	±	0.4
SGR (% day^{-1})	1.3	±	0.2	1.4	±	0.0	1.3	±	0.1	1.4	±	0.1
Total FC (g DM)	41.4	±	2.0	41.9	±	0.8	40.5	±	0.7	38	±	1.8
FER (g/g DM)	0.8	±	0.1	0.8	±	0.0	0.8	±	0.1	0.9	±	0.0
HSI (%)	1	±	0.1	1	±	0.1	0.9	±	0.2	0.9	±	0.1
Survival	98.7	±	1.4	98.7	±	1.4	100	±	0.0	100	±	0.0

SO, stearidonic oil diet; CO, canola oil diet; MX, 1:1 mix diet of canola oil and stearidonic acid oils; FO, fish oil diet; DM, Dry matter
SGR, Specific growth rate = 100 x (ln ($W_{final(g)}$/$W_{initial(g)}$)) / number of days (*d*)
FC, Total feed consumption = Total amount (g DM) consumed by an individual over 42 days.
FER, feed efficiency ratio = total weight gain (g)/ total feed consumption (g DM).
HSI, hepatosomatic index = 100 (liver weight (g WW) / Total body weight (g WW)).
Survival = survival during experiment. Growth and feed efficiencies were not significantly different as determined by Tukey-Kramer HSD; *df* =4,11.

2.5.3 Fatty acid (FA) composition

There were significantly ($p<0.01$) greater absolute amounts of SDA, in both the white and red muscle, in the fish fed SO than in fish fed CO (Table 2.3, 2.4). The CO fish had significantly higher ($p<0.01$) absolute amounts of ALA in both muscle tissues compared with FO and more in the red muscle compared to the SO fed fish. Absolute amounts of DHA and total ω3 were significantly ($p<0.01$) greater in both muscle tissues for the FO and SO treatments compared to CO. EPA was significantly ($p<0.01$) higher in the red muscle for the FO fed fish compared to all other experimental diets. The ratio of ω3/ω6 was significantly ($p<0.01$) lower for the CO and MX fed fish than for the SO and FO fed fish.

Table 2.3: Lipid content and FA and lipid class composition (g 100g^{-1}) of red muscle samples of Atlantic salmon fed canola Oil (CO), 1:1 mix of canola oil:stearidonic oil (MX), stearidonic oil (SO) and fish oil (FO) diets

FA	Initial	±	SE	CO	±	SE	MX	±	SE	SO	±	SE	FO	±	SE	f
14:0	0.71	±	0.06a	0.76	±	0.16a	0.66	±	0.18a	0.75	±	0.02a	1.47	±	0.05b	38.9
16:0	2.97	±	0.07a	2.97	±	0.27a	2.82	±	0.26a	2.75	±	0.07a	4.70	±	0.07b	49.8
18:0	0.83	±	0.05a	0.96	±	0.04a,b	1.02	±	0.12b	0.85	±	0.01a	1.21	±	0.03c	42.1
Other SFA [e]	0.35	±	0.00a	0.40	±	0.01a,b	0.31	±	0.01a	0.35	±	0.00a	0.54	±	0.01b	12.2
Total SFA	4.87	±	0.12a	5.10	±	0.28a	4.82	±	0.09a	4.70	±	0.25a	7.92	±	0.11b	50.3
16:1ω7c	1.05	±	0.07a,b	1.14	±	0.24a,b	0.96	±	0.07a	1.11	±	0.03b	2.07	±	0.09c	31.3
18:1ω9c	2.39	±	0.09a	5.67	±	1.03c	6.48	±	0.25c	3.07	±	0.10b	4.43	±	0.33b	27.1
18:1ω7c	0.58	±	0.01a	0.76	±	0.01b	0.64	±	0.02a	0.57	±	0.01a	0.99	±	0.03c	44.6
20:1ω9c	0.24	±	0.02a,b	0.46	±	0.03b	0.29	±	0.09a,b	0.33	±	0.00a,b	0.12	±	0.12a	3.5*
Other MUFA [f]	0.45	±	0.00a	0.53	±	0.01a	0.55	±	0.01a	0.47	±	0.00a	1.19	±	0.01b	18.0
Total MUFA	4.71	±	0.02a	8.57	±	0.10c	8.92	±	0.11c	5.54	±	0.14b	8.80	±	0.1	19.1
18:3ω3 ALA	0.13	±	0.01a	1.79	±	0.32d	0.60	±	0.02b	1.09	±	0.03c	0.29	±	0.02a	28.1
18:4ω3 SDA	0.41	±	0.03a	0.49	±	0.06a	0.82	±	0.02b	0.82	±	0.06b	0.74	±	0.02b	11.4
20:4ω3	0.20	±	0.01a	0.22	±	0.02a	0.27	±	0.01b	0.28	±	0.01b	0.34	±	0.01c	30.5
20:5ω3 EPA	1.54	±	0.03b	1.10	±	0.26a	0.98	±	0.07a	1.19	±	0.03a,b	2.15	±	0.09c	29.5
22:5ω3 DPA	0.57	±	0.01a,b	0.52	±	0.13a	0.48	±	0.03a	0.59	±	0.03b	1.03	±	0.04c	24.0
22:6ω3 DHA	3.43	±	0.33b,c	2.20	±	0.15a	1.99	±	0.15a	2.77	±	0.11b	4.04	±	0.20c	20.6
Other ω3 [g]	0.18	±	0.00a	0.19	±	0.03a	0.16	±	0.01a	0.20	±	0.00a	0.37	±	0.01b	21.0
Total ω3	6.45	±	0.11b	6.52	±	0.4	5.29	±	0.23a	6.94	±	0.28b	8.96	±	0.15c	12.7
18:2ω6 LA	0.51	±	0.02a	1.75	±	0.40c	2.02	±	0.15c	1.19	±	0.10b,c	1.08	±	0.19a,b	12.1
18:3ω6	0.03	±	0.00a	0.13	±	0.05b	0.21	±	0.02c	0.13	±	0.01b	0.05	±	0.02a	11.3
20:4ω6 ARA	0.23	±	0.04b	0.12	±	0.01a	0.11	±	0.00a	0.11	±	0.00a	0.18	±	0.00b	29.1
22:5ω6	0.05	±	0.00a	0.05	±	0.01a	0.04	±	0.00a	0.05	±	0.00a	0.08	±	0.00b	15.2
Other ω6 [h]	0.13	±	0.01a	0.22	±	0.02c	0.19	±	0.00b,c	0.16	±	0.00b	0.25	±	0.00c	18.2
Total ω6	0.95	±	0.04a	2.28	±	0.21c	2.57	±	0.17c	1.63	±	0.29b	1.64	±	0.22b	12.0
Other PUFA [i]	0.86	±	0.04	0.47	±	0.04	0.62	±	0.01	0.65	±	0.02	0.75		0.02	
Total PUFA	8.25	±	0.14b	9.27	±	0.22a	8.49	±	0.7a	9.22	±	0.04a	11.35	±	0.14c	133.3
Ratios																
ω3/ω6	6.8	±	0.33c	2.9	±	0.47a	2.1	±	0.21a	4.3	±	0.49b	5.5	±	0.23c	14.1
Lipid Class																
TAG	17.2	±	0.1	22.2	±	0.3	21.2	±	0.1	18.8	±	0.1	26.4	±	0.2	
FFA	0.1	±	0.0a	0.2	±	0.1a	0.4	±	0.0a,b	0.1	±	0.0a	0.7	±	0.2b	8.00
ST	0.1	±	0.0	0.3	±	0.0	0.2	±	0.0	0.2	±	0.0	0.3	±	0.1	
PL	0.3	±	0.0	0.3	±	0.2	0.4	±	0.0	0.3	±	0.1	0.7	±	0.1	
g 100g^{-1} Wet [j]	17.8	±	1.0	22.9	±	0.7	22.2	±	1.1	19.5	±	1.8	28.1	±	5.5	
g 100g^{-1} Dry [j]	44.3	±	2.8	53.6	±	0.9	57.0	±	7.7	50.1	±	2.9	56.6	±	7.1	

SO, stearidonic oil diet; CO, canola oil diet; MX, 1:1 mix diet of canola oil and stearidonic acid oils; FO, fish oil diet SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; SDA, Stearidonic acid; LA, Linoleic acid; ALA, α- Linolenic acid; ARA, Arachidonic acid; TAG; Triacylglycerol; FFA, Free fatty acid; ST, Sterol; PL, Polar lipid; WW, Wet weight; Sig, Significance; *f*, Mean sum of squares.
[a,b,c,d] Mean values across the row not sharing a common superscript were significantly different as determined by Tukey-Kramer HSD; *df* =4,15. *p*<0.01 * indicates *p*<0.05
[e] Other SFA includes 15:0, 17:0, 20:0, 22:0 and 24:0
[f] Other MUFA includes 16:1ω9, 16:1ω5, 18:1ω5, 20:1ω7, 22:1ω9, 22:1ω11 and 24:1ω9
[g] Other ω3 PUFA include 21:5ω3 and 24:6ω3
[h] Other ω6 PUFA include 20:2ω6, 20:3ω6, 22:4ω6 and 24:5ω6
i Other PUFA include 16:2ω4, 16:3ω4 and 18:2ω9
[j] Determined by TLC-FID

In the red muscle tissues there were significantly (*p*<0.01) higher amounts of 14:0, 16:0 and total saturates in the FO fed fish compared with the CO, SO and MX fed fish. In both muscle tissues, FO and SO fed salmon had significantly (p<0.01) lower amounts of 18:1ω9 and total MUFA compared to the fish fed the CO and MX diets. There was significantly (p<0.01) greater amounts of 18:2ω6 and total ω6 FA in the fish fed CO and MX diets compared with the FO fed fish.

Table 2.4: Lipid content and FA and lipid class composition (mg g^{-1}) of white muscle samples of Atlantic salmon fed canola Oil (CO), 1:1 mix of canola oil:stearidonic oil (MX), stearidonic oil (SO) and fish oil (FO) diets

FA	Initial	±	SE	CO	±	SE	MX	±	SE	SO	±	SE	FO	±	SE	f
14:0	0.33	±	0.03c	0.21	±	0.01a,b	0.17	±	0.02a	0.21	±	0.01a,b	0.26	±	0.01b,c	9.8
16:0	1.54	±	0.03	1.34	±	0.03	1.37	±	0.02	1.54	±	0.05	1.42	±	0.02	
18:0	0.47	±	0.02	0.43	±	0.00	0.50	±	0.01	0.52	±	0.01	0.36	±	0.01	
Other SFA[e]	0.15	±	0.00	0.10	±	0.00	0.07	±	0.00	0.09	±	0.00	0.10	±	0.00	
Total SFA	2.49	±	0.09	2.08	±	0.08	2.11	±	0.15	2.37	±	0.13	2.13	±	0.04	
16:1ω7c	0.47	±	0.04c	0.28	±	0.01a,b	0.24	±	0.03a	0.29	±	0.01a,b	0.37	±	0.03b,c	10.5
18:1ω9c	1.19	±	0.06a	1.90	±	0.09b	2.15	±	0.10b	1.04	±	0.07a	0.85	±	0.08a	6.8
18:1ω7c	0.27	±	0.01c	0.26	±	0.00c	0.23	±	0.01a,b	0.20	±	0.01a,b	0.22	±	0.01b,c	10.4
20:1ω9c	0.10	±	0.02	0.14	±	0.00	0.08	±	0.04	0.09	±	0.00	0.04	±	0.03	
Other MUFA[f]	0.28	±	0.00	0.19	±	0.00	0.18	±	0.00	0.15	±	0.00	0.17	±	0.00	
Total MUFA	2.38	±	0.06a	3.54	±	0.23b	3.04	±	0.48a,b	2.35	±	0.22a,b	1.71	±	0.09a	5.9
18:3ω3 ALA	0.09	±	0.00a	0.77	±	0.03c	0.17	±	0.0a,b	0.58	±	0.04b,c	0.07	±	0.01a	5.9
18:4ω3 SDA	0.17	±	0.02a,b	0.15	±	0.00a	0.25	±	0.01a,b	0.35	±	0.03b	0.14	±	0.01a,b	3.3*
20:4ω3	0.09	±	0.00a,b	0.07	±	0.00a	0.10	±	0.00a,b	0.12	±	0.0b	0.07	±	0.00a	5.6
20:5ω3 EPA	0.63	±	0.01	0.44	±	0.03	0.48	±	0.03	0.68	±	0.05	0.62	±	0.02	
22:5ω3 DPA	0.26	±	0.01b,c	0.19	±	0.01a	0.20	±	0.01a,b	0.24	±	0.01a,b,c	0.26	±	0.01c	6.3
22:6ω3 DHA	1.68	±	0.10a,b	1.48	±	0.04a	1.65	±	0.06a,b	2.05	±	0.03b	1.74	±	0.02b	5.3*
Other ω3[g]	0.07	±	0.01	0.05	±	0.00	0.03	±	0.00	0.05	±	0.00	0.07	±	0.00	
Total ω3	2.89	±	0.02a,b	2.37	±	0.24a	2.72	±	0.16a	3.49	±	0.19b	2.90	±	0.15b	7.2
18:2ω6 LA	0.25	±	0.01a	0.69	±	0.04b	0.67	±	0.06b	0.52	±	0.06a,b	0.23	±	0.05a	4.3*
18:3ω6	0.01	±	0.00a	0.09	±	0.01b	0.10	±	0.01b	0.08	±	0.01b	0.01	±	0.00a	8.4
20:4ω6 ARA	0.11	±	0.02	0.09	±	0.00	0.12	±	0.00	0.09	±	0.01	0.06	±	0.00	
22:5ω6	0.02	±	0.00	0.03	±	0.00	0.02	±	0.00	0.03	±	0.00	0.02	±	0.00	
Other ω6h	0.11	±	0.01	0.08	±	0.00	0.05	±	0.00	0.05	±	0.00	0.03	±	0.00	
Total ω6	0.50	±	0.1a	0.98	±	0.12b	0.96	±	0.16b	0.76	±	0.13a,b	0.34	±	0.05a	6.9
Other PUFA[i]	0.14	±	0.00	0.13	±	0.00	0.17	±	0.01	0.23	±	0.01	0.12	±	0.00	
Total PUFA	3.52	±	0.03	3.48	±	0.13	3.84	±	0.27	4.48	±	0.17	3.36	±	0.15	
Ratios																
ω3/ω6	5.8	±	0.28b,c	2.4	±	0.67a	2.8	±	1.40a	4.6	±	0.87b	8.5	±	1.00c	13.2
Lipid Class																
TAG	6.93	±	0.01c	4.22	±	0.01a	6.01	±	0.00b	5.51	±	0.00b	3.65	±	0.04a	3.1*
FFA	0.14	±	0.01b	0.18	±	0.00b	0.03	±	0.00a	0.16	±	0.00b	0.04	±	0.00a	23.9
ST	0.17	±	0.00	0.36	±	0.00	0.19	±	0.00	0.35	±	0.00	0.16	±	0.00	
PL	1.20	±	0.01a	4.35	±	0.01b	2.74	±	0.01a,b	3.18	±	0.01b	3.39	±	0.04b	4.1*
mg g^{-1} Wet[j]	8.4	±	0.3	9.1	±	0.1	9.0	±	0.2	9.2	±	0.1	7.2	±	0.3	
mg g^{-1} Dry[j]	10.1	±	1.0a	15.2	±	0.4b	14.2	±	0.6b	14.9	±	0.2b	15.1	±	1.0b	8.9*

Abbreviations and other footnote definitions, see Table 2.3.

Table 2.5: Mass balance of absolute amount T$_f$ (g) and total eaten T$_e$ (g) plus total initial T$_i$ (g) of ω3 LC-PUFA in Atlantic salmon fed experimental feeds with canola oil (CO), 14% stearidonic acid oil (SO), 1:1 CO:SO (MX) and fish oil (FO).

| | Mass Balance content of Atlantic salmon | | | | | | | |
| | CO | | MX | | SO | | FO | |
FAME	T$_f$ (±SE)	T$_c$+T$_i$(±SE)	T$_f$ (±SE)	T$_c$+T$_i$(±SE)	T$_f$ (±SE)	T$_c$+T$_i$(±SE)	T$_f$ (±SE)	T$_c$+T$_i$(±SE)
18:3ω3 ALA	0.63 ± 0.04	0.67 ± 0.07	0.13 ± 0.01	1.10* ± 0.03	0.44 ± 0.01	1.44* ± 0.08	0.05 ± 0.05	0.20 ± 0.05
18:4ω3 SDA	0.12 ± 0.01	0.08 ± 0.01	0.20 ± 0.01	0.45 ± 0.08	0.27 ± 0.01	0.84* ± 0.07	0.11 ± 0.01	0.29 ± 0.01
20:4ω3 ETA	0.06 ± 0.00	0.04 ± 0.03	0.08 ± 0.01	0.04 ± 0.02	0.09 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.09 ± 0.01
20:5ω3 EPA	0.36 ± 0.08	0.29 ± 0.06	0.39 ± 0.11	0.29 ± 0.05	0.52 ± 0.06	0.29* ± 0.04	0.41 ± 0.04	1.21* ± 0.04
22:5ω3 DPA	0.15 ± 0.01	0.13 ± 0.01	0.16 ± 0.03	0.12 ± 0.01	0.52 ± 0.02	0.12* ± 0.03	0.20 ± 0.02	0.22 ± 0.01
22:6ω3 DHA	1.20 ± 0.17	0.80 ± 0.07	1.32 ± 0.09	0.80* ± 0.07	1.57 ± 0.07	0.80* ± 0.05	1.33 ± 0.05	1.32 ± 0.08

SO,stearidonic oil diet; CO, canola oil diet; MX, 1:1 mix diet of canola oil and stearidonic acid oils; FO, fish oil diet; DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; ETA, Eicosatetraenoic acid SDA, Stearidonic acid; ALA, alpha-linolenic acid

T$_f$ (g) = W_f x FA$_{wmf}$ where FA$_{wmf}$ (g/kg) is proportion of FA in the white muscle after 42 days and Wf is the weight on day 42.

T$_c$ (g) = FC (g) x FA$_{diet}$ where FA$_{diet}$ (g/kg) was the proportion of FA in diet and FC is the total feed consumption.

T$_i$ (g) = Wi x FA$_{wfi}$ where FA$_{wfi}$ is the proportion of FA in the white muscle at day 1 and W$_i$ is the weight at day 1.

* Indicates significant ($p<0.01$) difference between T$_f$ and (Te + Ti) as determined by Tukey-Kramer HSD; df=4,15

Calculation of the mass balance of FA (Table 2.5) showed there was a significant ($p<0.01$) decrease between the final absolute amount compared to the amount eaten plus initial absolute amount of ALA, SDA for fish fed the SO diet and in ALA for fish fed the MX diet. There was a significant ($p<0.01$) increase in EPA, DPA and DHA in the SO fed fish, in DHA in the MX fed fish and EPA in the FO fed fish.

2.5 DISCUSSION

The results show, to my knowledge, the first evidence that absolute amounts of total $\omega3$, and in particular DHA and EPA can be maintained in the muscle of Atlantic salmon without their inclusion as dietary FA. Larger salmon fed diets that include plant oils, which are consequently rich in ALA but low in EPA and DHA, cause significant reduction in total $\omega3$ and $\omega3$ LC-PUFA, in particular DHA and EPA (Polvi and Ackman, 1992; Bell, et al., 2003a; Bransden, et al., 2003; Bell, et al., 2004). The same effect has been shown in salmon parr, although $\omega3$ LC-PUFA synthesis in hepatocytes and enterocytes was significantly increased in fish fed plant oil (Tocher, et al., 2003). Therefore prior to this study, minimal conversion to, or negligible accumulation of, $\omega3$ LC-PUFA has been reported when salmon are fed plant oil. Our inclusion of SO at 130 or 65 g/kg of diet for Atlantic salmon parr did not significantly influence growth or feed conversion, compared to the other experimental diets, during the 42 day growth trial in freshwater. However, the inclusion of SO did significantly effect the concentrations of total $\omega3$ and $\omega3$ LC-PUFA in the muscle tissues.

The lipid class profiles showed that there was significantly less TAG in the white muscle of all experimental fish compared to the initial group. The reduced TAG was probably due to the lower oil level in the experimental diets (130 g/kg) compared to the pre-experimental diet (approx. 300 g/kg). This reduction in TAG in the white muscle and corresponding increase in percent PL may indicate fish have selectively retained the ω3 LC-PUFA in PL from the initial whole body lipids. However, absolute amounts of ω3 LC-PUFA (Table 2.3 and 2.4) and the resultant mass balance data (Table 2.5) indicate that along with retention, that a possible biosynthesis of ω3 LC-PUFA has occurred in the SO fed fish, compared to the CO fed fish, to maintain muscle FA concentrations. As previously shown (Henderson and Tocher, 1987; Sargent, et al., 2002), fish muscle FA lipid profiles in the present study were closely related to the FA profile of their diet except for the SO fed fish which had an ω3 LC-PUFA profile similar to the FO fed fish. Whatever the underlying mechanisms it was clear that inclusion of SDA oil resulted in a number of difference to the ways that FA were distributed in tissues.

There have been two recent studies looking at the use of SDA oil with in vivo trials on Arctic charr and Atlantic cod (Bell, et al., 2006; Tocher, et al., 2006). Arctic charr fed SDA oil for 16 weeks showed increases in SDA and ETA (eicosatetraenoic acid, 20:4ω3) in the FA profile of the liver and flesh and increased hepatocyte LC-PUFA synthesis determined by isotope labelled ALA and EPA (Tocher, et al., 2006). However, this increased metabolism was not translated to enhance tissue ω3 LC-PUFA concentrations in particular EPA and DHA. The SDA diet used in the Arctic charr trial contained concentrations of EPA (5.1%) and DHA (7.3%) derived from the residual oil in the fish meal and only partial (80% SDA oil and 20% fish

oil) substitution of fish oil. In Atlantic cod, an SDA diet was fed for 18 weeks and the fish showed reduced concentrations of EPA and DHA in the FA profiles of flesh and liver (Bell, et al., 2006). Desaturation of ALA and EPA to DHA in hepatocytes was increased with the SDA oil diet compared to fish oil fed fish. The Atlantic cod trial also had residual EPA (2.6%) and DHA (4.8%) in the SDA diet which was derived again from the fish meal. Both these trials were performed on very small fish (4 g initial weight grown to 30-50 g) compared to this study (42.4 to 81.4 g). Along with these results, feeding SDA oil to Atlantic cod and Arctic charr had no effect on growth, feed efficiencies or other heath indices. Marine fish, such as Atlantic cod, have a inefficient ability to convert ALA to EPA and DHA, as they have high concentrations of ω3 LC-PUFA in their natural diet (Sargent, et al., 2002). However, both studies showed that ω3 LC-PUFA metabolism has increased with the SDA oil compared to fish oil diets. This metabolism may be altered by the presence of residual EPA and DHA from fish meal in the SDA diet.

In this study, Atlantic salmon parr were at an important stage of their life cycle and undergoing physiological changes necessary for migration into seawater (Craig Clark, 2000). During smoltification, salmon undergo major changes in morphology, behaviour, physiology including in their lipid metabolism (Bell, et al., 1997). At this life stage, pre-smolt salmon increase FA desaturation and elongation activities to build up reserves of ω3 LC-PUFA to the provide EFA that are required to synthesise new cell membranes and allow successful adaptation to sea water as well as to increase energy stores and condition (Bell, et al., 1997). As appetite temporarily declines post transfer, salmon depend on their lipid reserves to meet their energy requirements (Stead, et al., 1996; Arnesen, et al., 1998). It has been suggested that

feeding salmon parr diets containing fish oil (that would not have been in their natural diets) partially inhibited the natural pre-adaptive increases in FA desaturation with possible detrimental effects on the LC-PUFA composition (Bell, et al., 1997; Tocher, et al., 2000). Wild salmon smolts contain much greater proportions of arachidonic acid (ARA, 20:4ω6), in their total lipids which may be due to increased Δ^6 desaturase activity (Ackman and Takeuchi, 1986). Eicosanoids and prostaglandins are metabolites of C_{20} PUFA and are known to mediate fluid and electrolyte fluxes in fish gill and kidney and are therefore important during saltwater transfer (Mustafa and Srivastava, 1989). Therefore increasing EPA and ARA prior to smoltification will increase the ability of salmon to adapt to the marine environment. Condition and FA composition of pre-smolt fish, especially the proportions of ω3 and ω6 LC PUFA, are vital for successful saltwater transfer.

There were significant differences in the absolute amounts of DHA and total ω3 in both the white and red muscle between the SO and the CO fed fish. Due to the use of defatted fish meal, the CO and SO diets contained only very low (<1%) amounts of ω3 LC-PUFA. Therefore preferential retention and or a biosynthesis of EPA and DHA from SDA occurred for fish fed the SO diet to maintain LC-PUFA concentrations. This is, to my knowledge, the first reported evidence of salmon maintaining absolute amounts of ω3, DHA and EPA in a plant oil replacement feeding trial.

The increased retention of DHA also may be explained in part by the reduction in relative percentage of TAG and the corresponding increase in PL in the white muscle due to reduction in dietary lipid content of the trial diets from the diet fed prior to the experiment. The influence of this diet change may be enhanced by

physiological reductions in muscle lipid as the parr approach smoltification. It is also yet to be determined whether SDA can displace ω3 LC-PUFA from the *sn*-2 position on phospholipids more effectively than oils high in ALA and/or LA, such as sunflower, soya, linseed or canola (rapeseed) oils. Further longer term studies including *in vivo* use isotopically labelled FA or analysis of stable isotope ratios are needed to ascertain whether biosynthesis and or greater retention occurred in the SO fed fish.

In this present study the absolute FA concentration results combined with the mass balance data suggested that ω3 LC-PUFA were biosynthesised by salmon fed SDA. Most significantly, the absolute amount of DHA in the SO fed fish was significantly greater in both muscle tissues than for fish fed the CO diet (Tables 2.3 and 2.4). The amount of DHA in the SO fed fish was not significantly different from the amounts in fish fed a FO diet. The mass balance data (Table 2.5) indicated that the SO fed fish showed significant differences between the amounts of EPA, DPA and DHA in the body at day 42 compared to the amount they were fed and had contained initially. The data support the hypothesis that biosynthesis of EPA, DPA and DHA had occurred. However, mass balance results are only an indication of FA synthesis as they are calculated under the assumption that the whole body has a uniform FA profile similar to that of the white muscle. Biologically active tissues such as the liver, spleen and kidney as well as fat storage tissues such as the visceral adipose tissue were not included in the white muscle calculation. However, by comparing the amount of FA in the white muscle at the beginning and the end of the trial with close attention to the amount of FA fed, an indication is obtained as to how the white muscle is storing and using particular FA.

The indication of the mass balance that biosynthesis of ω3 LC-PUFA occurred is further supported by the significant reduction of ALA and SDA in the SO fed fish, and this observation further supports the use of these FA in the biosynthesis of ω3 LC-PUFA. ALA is extensively used for β-oxidation in salmon and other fish (Tocher, 2003; Torstensen and Stubhaug, 2004; Stubhaug, et al., 2005). This study showed that monounsaturate concentrations were lower in the SO diet than the CO diet, it is possible that PUFA could be selected for oxidation in fish fed SO. Recent research has confirmed that >90% of ALA is used for β-oxidation rather than desaturation and elongation (Bell and Dick, 2005). At present there has been little research on SDA metabolism and studies on β-oxidation with salmon due to the low abundance of SDA in most plant and marine oils (Ghioni, et al., 1999; Ghioni, et al., 2002). The mass balance results are indicative of how dietary FA is being used by the salmon. More ALA and SDA were being consumed than was stored in the muscle of the SO fish over 42 day period. β-oxidation may explain this reduction in ALA and possibly the SDA, however, the increased absolute amount of ω3 LC-PUFA strongly indicates that biosynthesis has occurred. Further studies on the mass balance for the whole body of fish fed a SO diet for a longer period are needed to confirm this finding.

The conversion of ALA to SDA involves desaturation at the Δ^6 position of the carbon chain with a further chain elongation step, followed by Δ^5 desaturation to form EPA. In Atlantic salmon, synthesis of DHA from EPA is via the Sprecher pathway which requires additional chain elongation and also involves the Δ^6 desaturase in the conversion of 24:5ω3 to 24:6ω3 before chain shortening to DHA

(Sprecher and Chen, 1999; Sargent, et al., 2002). With the conversion of 18:2ω6 to 20:4ω6 also using the Δ^6 desaturase, it is possible that the large amounts of 18:2ω6 in plant oils may out compete with and therefore inhibit the conversion of ALA. An excess of 18:2ω6 will out compete ALA for the Δ^6 desaturation enzyme, and therefore minimal conversion of ALA to SDA or SDA to EPA and DHA will occur along the ω3 pathway. The reverse is also possible with an excess of ALA inhibiting the metabolism of 18:2ω6 to ARA (Horrobin, 1991). This effect has been demonstrated with Atlantic salmon fed diets containing sunflower oil (1% ALA and 40% 18:2ω6) and linseed oil (32% ALA and 12% 18:2ω6) (Bell, et al., 1993). In the salmon fed sunflower oil there were significantly greater concentrations of ω6 desaturation and elongation products of 18:2ω6 than the salmon fed linseed oil which had increased ALA and SDA (Bell, et al., 1993).

The Δ^6 desaturase is the biosynthetic hurdle in the conversion of LA to ARA in the ω6 pathway (Brenner, 1981). The conversion of ALA to SDA is the rate liming step in the synthesis of ω3 LC-PUFA (Tocher, et al., 1998). ALA is quickly oxidised and is not accumulated in tissue as quickly as LA, and the LC-PUFA derivatives of ALA are incorporated faster into cell membranes than ALA itself (Leyton, et al., 1987). Therefore ALA is not readily accumulated in the tissues and its conversion to SDA is poor. It has been shown that feeding salmon diets low in ALA, may stimulate some fatty acid desaturation and elongation, but it was insufficient to maintain concentrations of EPA and DHA in the tissue to the same concentrations as observed in FO fed fish (Tocher, et al., 2002).

In salmon cell line studies it has been shown that SDA can serve as a precursor for ETA which can effect the metabolism of eicosanoids (Ghioni, et al., 2002). The study showed that SDA was readily converted to ETA and then further desaturated and elongated to EPA but not DHA. It has also been shown that small amounts of radiolabeled [U-^{14}C] SDA can be converted to DHA (Ghioni, et al., 1999). These small amounts of converted DHA in this cell line study would be insufficient to maintain body DHA concentrations and only very small amounts progress past the conversion to EPA, but it does show it is possible that salmon cell lines can convert SDA though to DHA.

 Mammalian nutrition studies with SDA have shown similar results with conversions along the ω3 Pathway to EPA (Crozier, et al., 1987; Crozier, et al., 1989; Yamazaki, et al., 1992). For rats (*Rattus norvegicus*) a two fold increase in EPA occurred when fed SDA, compared to rats fed ALA (Yamazaki, et al., 1992). It was suggested that Δ^6 desaturation was the rate limiting step in the conversion of ALA to EPA in mammals (Yamazaki, et al., 1992). It has been shown that significantly more EPA was incorporated into TAG and PL classes of guinea pig (*Cavia porcellus)* livers which were fed black current seed oil, which has a 10% proportion of SDA in its fatty acid profile, than guinea pigs which were feed 11% ALA or no ω3 precursor (Crozier, et al., 1989). A study looking at the ingestion of ALA via linseed oil by humans showed no significant increase in plasma EPA, compared to a 10 fold increase with cod liver oil; this suggests that the human ability to elongate and desaturate ALA is minimal (Dyerburg, et al., 1980). More recent studies have shown that men have limited ability to convert ALA to EPA, but transfer to DHA is very low (Mantzioris, et al., 2000; Burdge and Calder, 2005,

2006). Women have a greater ability to covert ALA to ω3 LC-PUFA (Burdge and Calder, 2006). Although there is an important difference between sexes, these studies conclude that conversion of ALA to ω3 LC-PUFA, especially DHA, in humans appears to be limited. Dietary SDA increased EPA and DPA concentrations, but not DHA in human erythrocyte and plasma phospholipids (James, et al., 2003). This indicates that human have an enhanced ability to desaturate and elongate SDA rather than ALA, but conversion to DHA in both precursors is poor.

The high absolute amounts of ω3 LC-PUFA in the muscle of SO fed salmon may be due to the salmon parr making metabolic changes as they undergo smoltification. Salmon parr have a greater capability to elongate and desaturate ALA into ω3 LC-PUFA than smolts (Bell, et al., 1997; Zheng, et al., 2005). Therefore salmon parr fed SDA may have a increased ability for this conversion to ω3 LC-PUFA. It was also shown that post-smolts have a reduced Δ^5 desaturase capacity compared to parr, which suggests that post smolt salmon have a greater dietary need for ω3 LC-PUFA (Bell, et al., 1997). Therefore it is important prior to salt water transfer that the absolute concentrations of ω3 LC-PUFA are increased. Parr fed SO may be more capable of adapting to salt water due to their greater relative concentrations and absolute concentrations of ω3 LC-PUFA compared to CO fed fish.

Our results indicate that a 14% SDA plant oil can be used as a source of dietary oil for aquafeeds and that the use of SDA oil importantly did not affect the amount of ω3 LC-PUFA in the FA profile of salmon muscle over 6 weeks compared with FO fed fish. Recent research has focused on looking into finishing diets that are rich

in ω3 LC-PUFA to counteract their reduced tissue content due to the use of diets containing vegetable oil during the grow-out phase (Bell, et al., 2003b; Bell, et al., 2004; Torstensen, et al., 2005). However, salmon fed plant oil only partially restore concentrations of EPA and DHA to 80% of the content of salmon fed FO, when fed a finishing diet for a period (16-24 weeks) prior to harvest (Bell, et al., 2003b; Bell, et al., 2004; Torstensen, et al., 2005). It has not yet been shown that salmon fed vegetable oil can recover their ω3 LC-PUFA content by using a finishing diet compared to fish fed FO diet, but it has been possible in the European sea bass (*Dicentrarchus labrax* L.) over 14 week period (Montero, et al., 2005; Mourente, et al., 2005).

Further studies using plant-derived SDA oil, such as from blackcurrant seed and *Echium plantagineum* (Patterson's curse), are needed to confirm this initial result. Trials with larger Atlantic salmon will confirm whether fish can maintain their concentrations of ω3 LC-PUFA over a longer period of time. Fish health and flesh quality should be closely monitored. It has been shown that salmon fed plant oil have no adverse sensory (taste/smell) affect (Turchini, et al., 2003; Turchini, et al., 2004; Torstensen, et al., 2005). The recent report of DHA being incorporated, although presently at only low relative concentrations, in a model seed crop, *Arabidopsis*, via genetic modification (GM), indicates the possibility of future sources of DHA-containing oil in other GM oil seed crops (Robert, et al., 2005). Our study demonstrates that enhanced concentrations of SDA in vegetable oil such as canola also could provide a source for ω3 LC-PUFA in salmon.

2.6 CONCLUSION

The Δ^6 desaturation of ALA to SDA is a major biosynthetic hurdle and the rate limiting step in the production of ω3 LC-PUFA in Atlantic salmon. Our results suggest that Atlantic salmon can be grown on a diet in which fish oil is replaced with a plant oil containing 14% SDA, without apparent detriment to fish growth or health or a reduction in concentration of specific key FA, in particular EPA and DHA. Unlike previous oil replacement trials, the muscle FA profile of the SO fed fish was not significantly different to the FO fed fish and therefore retained the nutritional value derived from the presence of elevated concentrations of ω3 LC-PUFA. This study was only conducted for a short period of time and on salmon parr and further longer studies on larger fish are needed to confirm whether the maintenance of ω3 LC-PUFA in particular EPA and DHA occurred due to biosynthesis rather than lipid retention.

2.7 ACKNOWLEDGEMENTS

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CHAPTER 3

Effect of stearidonic acid enriched diet on growth, fatty acid composition and elongase and desaturase gene expression in seawater Atlantic salmon (*Salmo salar* L.)

3.1 ABSTRACT

Alternative oils that provide omega 3 long chain ($\geq C_{20}$) polyunsaturated fatty acids (ω3 LC-PUFA) are a priority for the aquaculture industry to reduce reliance on fish oil. Understanding and utilising the endogenous capacity of Atlantic salmon (*Salmo salar L.*) to produce ω3 LC-PUFA when fed specific vegetable oils containing biosynthetic precursors has potential for developing ingredients for sustainable aquafeeds. The hypothesis tested was that dietary biosynthetic precursors, α-linolenic acid (18:3ω3, ALA) in canola oil (CO) and stearidonic acid (SDA, 18:4ω3) in Echium oil (SO) would stimulate biosynthesis as demonstrated by up regulation of the expression of genes involved in FA synthesis resulting in a net accumulation of ω3 LC-PUFA in seawater Atlantic salmon. Comparison was made to a diet containing fish oil (FO) that provided ω3 LC-PUFA, a reflection of their natural diet. Gene expression of liver elongase and Δ^5 desaturase was up-regulated on SO compared to the FO fish, while the CO fish up-regulated/ expression of elongase and Δ^6 and of Δ^5 desaturases in the liver and Δ^6 desaturase in the white muscle compared to FO fish. In all sampled tissues dietary SDA affected the fatty acid profile with increased concentration of SDA as well as eicosatetraenoic acid (20:4ω3, ETA), the direct biosynthetic product of the elongation of SDA. Increased concentrations of eicosapentaenoic acid (20:5ω3, EPA) occurred in the liver of the SO fish compared to the CO fish. However, the SO fish did not accumulate ω3 LC-PUFA at concentrations as high as FO fish. This experiment indicated that Atlantic salmon up regulate expression of genes involved in FA synthesis in response to dietary stimuli. However, the high concentrations of ω3 LC-PUFA found in seawater Atlantic salmon fed diets rich in fish oil cannot be provided via biosynthesis from ALA or SDA present in diets containing specific vegetable oils.

3.2 INTRODUCTION

Due to concerns with worldwide fish oil supply, its replacement in aquafeeds has become a priority for the aquaculture industry. Fish oil supply from wild fisheries is under pressure because it will not meet increasing demand from use in nutraceutical and agriculture industries, as well as the continued growth of aquaculture (Barlow, 2000; FAO, 2004; Carter, 2007). Seawater Atlantic salmon (*Salmo salar*) require dietary omega-3 long chain ($\geq C_{20}$)-polyunsaturated fatty acids ($\omega 3$ LC-PUFA) which are largely supplied from marine fishery products. Alternative oil sources are needed to ensure the sustainability of intensive aquaculture of carnivorous fish, particularly Atlantic salmon which dominates intensive marine finfish production. It is recognised that Atlantic salmon can grow on diets with up to 75% fish oil replaced with vegetable oils without compromising growth and performance or significantly affecting fish health or welfare (Rosenlund, 2001; Tocher, et al., 2003b; Torstensen, et al., 2005). Vegetable oils generally contain higher concentrations of saturated, monounsaturated and $\omega 6$ PUFA, but do not contain any $\omega 3$ LC-PUFA (Beardsell, et al., 2002). Atlantic salmon fed vegetable oils have a reduced $\omega 3$ LC-PUFA content which is a reflection of their diet (Torstensen, et al., 2000; Bell, et al., 2001; Rosenlund, 2001; Bell, et al., 2003; Bendiksen, et al., 2003; Bransden, et al., 2003; Tocher, et al., 2003b; Bell, et al., 2004; Torstensen, et al., 2004). However, beneficial effects of $\omega 3$ LC-PUFA are being increasingly recognised in human nutrition (Shahidi and Miraliakbari, 2004; Brouwer, et al., 2006; MacLean, et al., 2006). Atlantic salmon offer a good source of $\omega 3$ LC-PUFA for human consumption but reduced concentrations of $\omega 3$ LC-PUFA may

compromise their nutritional benefit to consumers (Seierstad, et al., 2005b).

Alternative oils that potentially utilise the endogenous biosynthetic capacity of

salmon may provide renewable and sustainable ω3 LC-PUFA sources for future

aquafeeds (Bell, et al., 2006; Tocher, et al., 2006; Miller, et al., 2007a).

The biosynthesis of eicosapentaenoic acid (20:5ω3, EPA) and docosahexaenoic

acid (22:6ω3, DHA) from α-linolenic acid (18:3ω3, ALA) is inefficient in marine

fish, an evolutionary consequence of a natural diet rich in ω3 LC-PUFA (Sargent, et

al., 2002; Tocher, 2003). Conversion is greater in freshwater fish, due to higher

concentrations of ALA and limited DHA in their natural diet (Sargent, et al., 2002).

Consequently changes in the fatty acid metabolism of Atlantic salmon that migrate

from fresh water to sea water are of interest (Tocher, et al., 1997; Bell, et al., 2002;

Tocher, et al., 2003a). The biosynthetic pathways for PUFA are well known, but the

mechanism underpinning ω3 LC-PUFA biosynthesis from precursors is not entirely

clear (Sargent, et al., 2002; Tocher, 2003). The Δ^6 desaturation step, which has a

multi-functional role in the conversion of ALA to DHA, has been shown to be the

rate limiting step (Brenner, 1981). Diets rich in the ω3 LC-PUFA biosynthetic

precursor stearidonic acid (18:4ω3, SDA) bypass the initial Δ^6 desaturase in the ω3

LC-PUFA biosynthetic pathway and theoretically can be converted more readily

through to EPA and DHA (Brenner, 1981). It is of particular interest to determine

whether dietary SDA can affect elongase and desaturase gene expression in the ω3

LC-PUFA biosynthetic pathway. Real-time quantitative (RT-PCR) allows a

quantitative measurement of the expression of the genes involved in ω3 LC-PUFA

biosynthesis. To the best of my knowledge, this study is the first experiment

examining the *in vivo* gene expression of Atlantic salmon fed a SDA rich diet.

Oil from the plant source *Echium plantagineum* L., Boraginaceae, has high concentrations (14%) of SDA and may provide an oil source for fish oil replacement diets. Previous dietary replacement studies with SDA have examined different species of fish, Atlantic cod (*Gadus morhua L.*) and Arctic charr (*Salvelinus alpinus L.*), and have shown fatty acid conversion through to eicosatetraenoic acid (20:4ω3, ETA), but not to EPA and DHA (Bell, et al., 2006; Tocher, et al., 2006). We have recently shown that in freshwater Atlantic salmon parr can maintain ω3 LC-PUFA, in particular EPA and DHA, concentrations in muscle tissue over a six week period when fed a diet rich in SDA but with only trace levels of ω3 LC-PUFA (Miller, et al., 2007a). This result indicated that SDA rich aquafeeds may have potential as an alternative to replace direct ω3 LC-PUFA sources such as fish oil in freshwater aquaculture and warranted further investigation in seawater salmon. The majority of the Atlantic salmon production cycle occurs in seawater where fish are grown from around 100 g to over 3 kg. The effect of dietary SDA, in regard to ω3 LC-PUFA biosynthesis, in seawater Atlantic salmon is yet to be determined. Dietary SDA has the potential to be a sustainable oil source for Atlantic salmon culture which could provide ω3 LC-PUFA endogenously and overcome shortfalls in fish oil production.

The present study aimed to determine whether dietary SDA and ALA affected elongase and desaturase gene expression in seawater Atlantic salmon and the resultant affect on the accumulation or biosynthesis of ω3 LC-PUFA. In this trial all fish were labelled so that individual growth could be assessed to allow the sampling of fish with similar performance (doubling of initial weight). This selection strategy was applied to measure changes in gene expression and relate these changes to individual performance and ω3 LC-PUFA concentrations, in particular EPA and

DHA, in tissue and organs. An SDA rich oil diet from Echium (SO) was compared

to a canola oil (CO) diet as it has no ω3 LC-PUFA, but has high concentrations of

ALA, the precursor to SDA. A fish oil diet (FO) was used which represents a

traditional aquafeed and is rich in EPA and DHA.

3.3 MATERIALS AND METHODS

3.3.1 Experimental diets

Three diets were formulated to compare canola oil (CO), SDA rich oil from

Echium (SO) and fish oil (FO) (Table 3.1). Fish meal was defatted three times using

a 2:1 mixture of hexane and ethanol (400 mL 100g^{-1} fish meal). Echium oil

containing SDA was supplied as Crossential SA14 (Croda Chemicals, East

Yorkshire, UK). Fish oil was from jack mackerel, *Trachurus symmetricus* L.,

(Skretting Australia, Cambridge, Tasmania, Australia), and a domestic source of

pure canola oil was used (Steric Trading Pty Ltd, Villawood, NSW, Australia). The

diets were manufactured into 3 mm diameter pellets using a California Pellet Mill

(CL-2), dried and stored at 5$^{\text{o}}$C (Carter, et al., 2003b).

Table 3.1: Ingredient and lipid composition (g/kg dry matter) of experimental diets

	Diet		
	CO	SO	FO
Ingredient composition (g kg⁻¹)			
Fish meal (defatted)[1]	150	150	150
Casein[2]	150	150	150
Wheat gluten[3]	100	100	100
Soybean meal[4]	180	180	180
Fish oil[1]	200	0	0
Canola oil[5]	0	200	0
SDA oil[6]	0	0	200
Pre gel starch[7]	127	127	127
Vitamin mix[8]	3	3	3
Mineral mix[9]	5	5	5
Stay C[10]	3	3	3
Choline chloride[11]	2	2	2
Sipernat[12]	40	40	40
CMC[11]	10	10	10
Sodium monophosphate[11]	20	20	20
Yttrium oxide[11]	10	10	10
Chemical composition (g kg⁻¹ DM)			
Dry matter	940.1	943.1	938.1
Crude protein	351.7	340.0	344.1
Crude fat	247.9	255.2	250.8
Energy (MJ kg⁻¹ DM)	19.3	19.0	19.1
FAME (g kg⁻¹ DM)			
Total SFA	22.1	25.7	68.2
Total MUFA	114.6	41.2	53.7
18:3ω3 ALA	13.1	52.3	1.2
18:4ω3 SDA	0.3	20.0	1.8
20:5ω3 EPA	0.0	0.0	24.2
22:5ω3 DPA	0.1	0.0	2.5
22:6ω3 DHA	0.0	0.2	18.2
Total ω3	13.5	72.5	49.9
18:2ω6 LA	45.2	42.1	11.6
18:3ω6 GLA	0.3	17.1	5.3
Total ω6	49.0	60.3	20.3
Total PUFA	63.2	132.8	77.1

[1] Skretting Australia, Cambridge, Tasmania, Australia
[2] MP Biomedicals Australasia Pty Ltd, Seven Hills, NSW, Australia
[3] Starch Australasia, Lane Cove, NSW, Australia
[4] Hamlet Protein A/S, Horsens, Denmark
[5] Croda Chemicals, East Yorkshire, UK
[6] Steric Trading Pty Ltd, Villawood, NSW, Australia
[7] Penford Australia Limited, Lane Cove, NSW, Australia
[8] Vitamin mix (ASV4) supplied per kilogram of feed: 2.81 mg thiamine HCL, 1.0 mg riboflavin, 9.15 mg pyridoxine HCL, 25 mg nicotinic acid, 54.35 mg calcium D-pantothenate,

750 mg myo-inositol, 0.38 mg D-biotin, 2.5 mg folic acid, 0.03 mg cyanocobalamin, 6350 IU retinol acetate, 2800 IU cholecalciferol, 100 IU DL α-tocopherol acetate, 5 mg menadione sodium bisulphate by Sigma-Aldrich, Castle Hill, NSW, Australia and 100 mg Roche rovimix E50.

[9] Mineral mix (TMV4) to supplied per kilogram of feed:117 mg $CuSO_4.5H_2O$, 7.19 mg KI, 1815 mg $FeSO_4.7H_2O$, 307 mg $MnSO_4.H_2O$, 659 mg $ZnSO_4.7H_2O$, 3.29 mg Na_2SeO_3, 47.7 mg $CoSO_4.7H_2O$ by Sigma-Aldrich, Castle Hill, NSW, Australia

[10] L-Ascorbyl-2-polyphosphate Roche Vitamins Australia, Frenchs Forest, NSW, Australia.

[11] Sigma-Aldrich, Castle Hill, NSW, Australia

[12] Degussa, Frankfurt, Germany

SO, stearidonic acid oil diet; CO, canola oil diet; FO, fish oil diet, SFA, Saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; CMC, Carboxymethyl cellulose; DHA, Docosahexaenoic Acid; EPA, Eicosapentaenoic Acid; SDA, Stearidonic acid, ALA, α-Linolenic acid; LA, Linolenic acid: GLA, γ-Linolenic acid.

3.3.2 Growth experiment

The experiment was conducted at the School of Aquaculture, University of Tasmania (Launceston, Tasmania, Australia). Atlantic salmon (_Salmo salar_ L.) parr (≈87.9 g) were obtained from Springfield Fisheries hatchery (Scottsdale, Tasmania, Australia) acclimated for 14 d in 300 L tanks and fed a commercial feed (Skretting). Prior to the experiment the fish were slowly adapted to seawater over a 21 day period. The tanks were held at a constant temperature of 12.0°C under a natural photoperiod. Water was treated through physical, UV and biofilters. Dissolved oxygen, pH, ammonia, nitrate, nitrite, and salinity were monitored daily to ensure water quality remained within parameters recommended for Atlantic salmon (Wedemeyer, 1996). The fish were held in an average of 27.4 ± 0.2 ppm saltwater. The experiment was conducted in accordance with the University of Tasmania Animal Ethics guidelines (Investigation A0008392).

At the start of the experiment fish were anaesthetized (50 mg L^{-1}, benzocaine), their weight and length measured, and four fish killed to measure initial lipid content and composition. Twenty five fish were randomly re-allocated into each of twelve 300 L tanks. Fish were marked on the ventral surface by a Panjet (Hart and Pitcher, 1969; McCarthy, et al., 1992) so individual performance could be assessed. The

three diets were fed in quadruplicate by hand at a ration of 1.1% body weight per day (% BW d^{-1}). Every three weeks all fish in each tank were anaesthetized (50 mg L^{-1}, benzocaine) and batch-weighed. Fish were starved on the day prior to weighing. Total feed consumption (kg DM) was estimated daily from the amount of feed supplied that was not eaten.

At the end of the experiment fish were starved on the day prior to being anaesthetized (50 mg L^{-1}, benzocaine) and their weight and length measured. Due to high variability in growth, fish for final sampling were selected based on similar performance. The first three fish which had at least doubled their known initial weight were sampled from each tank and killed by a blow to the head after immersion in anaesthetic. Samples of red (mean 0.5 ± 0.0g) and white muscle (0.8 ± 0.0g), dissected from below the dorsal fin, and liver (2.6 ± 0.1g) were frozen at -80°C until analysis (Miller, et al., 2006). A further two fish, which had doubled their initial weight, per tank were taken, killed (as described above) and frozen for total carcass analysis.

Specific growth rate (SGR) was calculated as SGR (% d^{-1}) = 100 x (ln (W_f/W_i)) x d^{-1} where W_f and W_i are the final and initial weights (g) and d is the number of days of the experiment. Total feed consumption (FC) was calculated as the total amount (g DM) consumed per tank over 84 days. The feed efficiency ratio (FER) was calculated as FER (g g^{-1}) = total weight gain (g)/ FC (g). The hepatosomatic index (HSI) was calculated as HSI (%) = 100 x (liver weight (g WW) / total body weight (g WW)) for sampled fish.

3.3.3 Digestibility

The diets included yttrium oxide (10 g kg^{-1}) as a digestibility marker. On days 88-90, faecal samples from all tanks were collected from the sediment collectors attached to the tanks between 1100-1700 and 1900-0900 h, freeze-dried and used in the analysis of digestibility (Cho, et al., 1982; Carter, et al., 2003a; Ward, et al., 2005). Apparent digestibility coefficient (ADC) was calculated using the standard formula ADC (%) = 100 − [100((Y_{diet}/Y_{faeces})x ((FA_{faeces}/FA_{diet}))] where Y is percentage of yttrium oxide and FA is the % of particular fatty acids (Maynard and Loosli, 1969).

3.3.4 Lipid extraction and isolation

Samples were freeze dried and extracted overnight using a modified Bligh and Dyer protocol (Bligh and Dyer, 1959). This involved a single phase extraction, $CHCl_3/MeOH/H_2O$ (1:1:0.9, by vol), followed by phase separation to yield a total lipid extract (TLE).

Lipid classes were analysed by an Iatroscan MK V thin-layer chromatography-flame ionization detector (TLC-FID) analyser (Iatron Laboratories, Japan). Samples were spotted onto silica gel SIII Chromarods (5 μm particle size) and developed in a glass tank lined with pre-extracted filter paper. The solvent system used for the lipid separation was hexane: diethyl ether: acetic acid (60:17:0.1, v/v/v) (Volkman and Nichols, 1991). After development for 25 min, the chromarods were oven-dried and analysed immediately to minimise adsorption of atmospheric contaminants. Lipid classes were quantified by DAPA software (Kalamunda, WA, Australia). The FID was calibrated for each compound class: phosphatidylcholine, cholesterol, oleic acid; hydrocarbon (squalene), wax ester (derived from fish oil), triacylglycerol

(derived from fish oil), and diacylglycerol ethers (DAGE) (purified from shark liver oil).

An aliquot of the TLE was trans-methylated in methanol: chloroform: hydrochloric acid (10:1:1, v/v/v) for 1 h at 100°C. After addition of water, the mixture was extracted three times with hexane: chloroform (4:1, v/v) to obtain fatty acid methyl esters (FAME). Samples were made up to a known volume with an internal injection standard (19:0 FAME) and analysed by gas chromatography (GC) using an Agilent Technologies 6890N GC (Palo Alto, California, USA) equipped with an Equity™-1 fused silica capillary column (15 m × 0.1 mm i.d., 0.1μm film thickness), an FID, a split/splitless injector and an Agilent Technologies 7683 Series auto sampler and injector. Helium was used as the carrier gas. Samples were injected in splitless mode at an oven temperature of 120°C. After injection, the oven temperature was raised to 250°C at 10°C per min and finally to 270°C at 3°C min^{-1}. Peaks were quantified with Agilent Technologies ChemStation software (Palo Alto, California, USA).

Individual components were identified by mass spectral data and by comparing retention time data with those obtained for authentic and laboratory standards. GC results are typically subject to an error of up to ±5% of individual component area. GC-mass spectrometric (GC-MS) analyses were performed on a Finnigan Thermoquest GCQ GC-mass spectrometer fitted with an on-column injector and using Thermoquest Xcalibur software (Austin, Texas, USA). The GC was equipped with an HP-5 cross-linked methyl silicone fused silica capillary column (50 m × 0.32 mm i.d.). Helium was used as the carrier gas, with operating conditions previously described (Miller, et al., 2006).

3.3.5 Chemical analysis

Standard methods were used to determine dry matter (freeze dry to constant weight); crude fat (Bligh and Dyer, 1959); nitrogen (Kjeldahl using a selenium catalyst; crude protein was calculated as N x 6.25) and energy (bomb calorimeter, Gallenkamp Autobomb, calibrated with benzoic acid).

3.3.6 RNA isolation and preparation

Total RNA was extracted from white muscle and liver tissue stored in an RNA preservation reagent (25 mM sodium citrate, 10 mM EDTA, 10M ammonium sulphate, pH 5.2) and purified using TRI Reagent® (Molecular Research Center, Cincinnati, OH, USA) including DNAse treatment (DNA-*free*™, Ambion, Austin, TX, USA). RNA yield (A_{260}) and purity ($A_{260/230}$ and $A_{260/280}$) were determined spectrophotometrically and the integrity of the RNA was estimated from gel electrophoresis on a 1% agarose gel.

3.3.7 Reverse transcription

First strand cDNA was synthesised from total RNA (1 μg) using a SensiMix kit (Quantace, NSW, Australia) with Oligo $(dT)_{18}$ priming according to the manufacturer's instructions. The reactions were incubated at 65°C for 10 min then 42°C for 50 min before the reverse transcriptase enzyme was inactivated at 70°C for 15 min. First strand cDNA reactions (20 μL) were diluted to 80 μL using nuclease-free water (Sigma-Aldrich, NSW, Australia) and stored at -20°C until quantitative PCR was performed.

Table 3.2: Characteristics of the real-time PCR primers

Gene		Sequence	Accession no.	Amplicon
EF1A	F	TGATTGTGCTGTGCTTATCG	AF321836	173
	R	AACGCTTCTGGCTGTAGG		
β-actin	F	TTGCGGTATCCACGAGAC	AF012125	158
	R	TAGAGGGAGCCAGAGAGG		
RPL2	F	TAACGCCTGCCTCTTCACGTTGA	CA049789	112
	R	ATGAGGGACCTTGTAGCCAGCAA		
PolyUb	F	TCTTCATCTGGTCCTGCGTCTC	SGP.Contig7470[*]	111
	R	AATGGGTGGGATTGGAGGTAA		
FAD5	F	GACCTATATTTCCAGCATTATCC	AF478472	192
	R	TCACTCATCTACAAATAGTATTCC		
FAD6	F	CATCTGATTCTGATTCCATTCC	AY458652	127
	R	CTCTGCTCCACTCACACC		
FAE	F	GACACCCACGGAAACCATTAC	AY170327	111
	R	CTCTCCTAGCGACATTACATACAG		

GenBank accession numbers used to design real-time primers with respective amplicon sizes (bp).
* denotes primer pair designed from an EST contig found in the Salmon Genome Project (SGP) database (http://www.salmongenome.no).

3.3.8 Quantitative PCR

Real-time PCR primers (Table 3.2) were designed using gene sequences

available on GenBank and a 1147 bp EST contig (SGP.Contig7470) identified as

PolyUb (94% nucleotide identity to *O. mykiss* polyubiquitin: accession no.

AF361365.1) by searching the salmon genome project (SGP) database

(http://www.salmongenome.no). The RPL2 primers were designed previously

(Jorgensen, et al., 2006) from a 556 bp salmon sequence (accession no. CA049789).

Quantitative PCR was performed using SYBR® Green chemistry on a MyiQ™ Real-

Time PCR Detection System (Bio-Rad, NSW, Australia). Each reaction (25 µL)

contained primers (200 nM each), 1× SensiMix*Plus* SYBR & Fluorescein PCR

master mix (Quantace) and 2 µL cDNA. All samples were assayed for each gene in

duplicate with no-template controls and a 5-step, 2-fold cDNA dilution series for

PCR efficiency calculation on the same plate. The reaction was incubated at 95°C

for 10 min to activate the heat-activated Taq DNA polymerase followed by 40

cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 25 s. At the end of the 40 cycles

a melt curve analysis was performed to test the specificity of reaction.

3.3.9 Relative expression

mRNA expression levels were normalized using the geometric mean of four

stably expressed reference genes (EF1A, β-actin, RPL2 and PolyUb) as determined

by the geNorm software (Vandesompele, et al., 2002). Automated analysis of real-

time quantitative PCR data was performed using qBase software (Hellemans, et al.,

2007) which employs a modified delta delta-Ct relative quantification model with

PCR efficiency correction and multiple reference gene normalisation.

3.3.10 Statistical analysis

Mean values are reported as plus or minus the standard error of the mean (SEM).

Normality and homogeneity of variance were confirmed and percentage data were

arcsin transformed prior to analysis. Comparison between treatments of fatty acid

concentration means was by 1-way analysis of variance (ANOVA) followed by

multiple comparison using Tukey-Kramer HSD. Significance was accepted at

probabilities of 0.05 or less. Correlations were determined by a 1-tailed Spearman's

bi variate correlation (n=18). Statistical analysis was performed using SPSS for

Windows version 11. The normalised relative quantities (NRQ) generated from the

qBase software were exported to SigmaStat 3.5 (SPSS) and the non-parametric

Mann-Whitney U-test (n=6) was used to evaluate the significant difference in mean NRQ of the CO and SO treatments compared to the FO treatment. Differences in the expression level of a gene were considered statistically significant at $P < 0.05$.

3.4 RESULTS

3.4.1 Growth results

Diet had no significant effect on final weight, final length, specific growth rate (SGR), feed consumption (FC), feed efficiency ratio (FER), hepatosomatic index (HSI) or survival (Table 3.3). A random sample of fish that had at least doubled their initial weight were taken for fish oil composition and gene expression analyse. There was no statistical difference in the final weight or growth of the sampled fish between diet treatments (Table 3.3). Further analysis and results concerns this group of randomly sampled fish.

3.4.2 Lipid class composition

There was no significant differences in lipid class composition of whole carcass and white muscle among fish fed the three diets (Table 3.4, 3.5). However, in the liver there were significantly ($P<0.01$) greater concentrations of free fatty acids (FFA) and sterols (ST) and lower concentrations of polar lipid (PL) in the CO fed fish (here after termed CO fish and similarly for the other treatments) compared to SO and FO fish (Table 3.6). The dominant lipid class was triacylglycerol (TAG) in both whole carcass (103.0-107.8 mg g^{-1}) and white muscle (17.7-20.3 mg g^{-1}) compared to PL in the liver (31.1-32.2 mg g^{-1}).

Table 3.3: Growth and efficiencies of Atlantic salmon fed experimental diets with canola oil (CO), 14% stearidonic acid oil (SO) and fish oil (FO) (mean ± SEM).

All Fish	CO (±SEM)		SO (±SEM)		FO (±SEM)	
Initial weight (g)	107.7 ±	1.6	107.9 ±	1.6	104.7 ±	1.5
Final weight (g)	156.2 ±	7.1	160.6 ±	9.0	158.4 ±	4.3
Weight gain (g)	48.5 ±	8.1	52.8 ±	5.3	53.8 ±	4.9
Initial length (cm)	21.8 ±	0.5	21.8 ±	0.4	21.7 ±	0.3
Final length (cm)	24.8 ±	0.3	24.6 ±	0.3	25.1 ±	0.3
Total feed consumption (g DM)	2469 ±	69.4	2697.6 ±	41.5	2520.9 ±	33.4
SGR (% day-1) [1]	0.4 ±	0.1	0.4 ±	0.1	0.5 ±	0.0
FC (mg DM g^{-1} d^{-1}) [2]	8.8 ±	0.6	11.4 ±	1.5	9.8 ±	0.6
FER (g/g DM) [3]	0.5 ±	0.1	0.5 ±	0.1	0.5 ±	0.0
Survival (%) [4]	89.0 ±	3.8	76.0 ±	14.0	87.0 ±	5.7
Sampled fish	**CO (±SEM)**		**SO (±SEM)**		**FO (±SEM)**	
Initial weight (g)	113.0 ±	3.6	108.8 ±	4.2	108.0 ±	3.5
Weight (g)	239.6 ±	5.1	247.2 ±	14.0	223.0 ±	8.4
Growth (g)	126.6 ±	5.3	138.4 ±	11.4	115.1 ±	7.3
HSI (%) [5]	1.4 ±	0.1	1.2 ±	0.6	1.6 ±	0.8

DM, Dry matter [1]SGR, Specific growth rate = 100 x (ln ($W_{final(g)}/W_{initial(g)}$)) x number of days $(d)^{-1}$

[2]FC, feed consumption = Total feed consumption (g DM)/ Σ individual mid weight (g)/ 84 days.

[3]FER, feed efficiency ratio = total weight gain (g)/ total feed consumption (g DM).

[4]Survival during growth experiment. [5]HSI, hepatosomatic index = 100 (liver weight (g WW) / Total body weight (g WW)). Mean values across the rows were not significantly different as determined by Tukey-Kramer HSD ($df = 2,241$, $n = 242$ for all fish; $df = 2,57$, n = 58 for sampled fish).

3.4.3 Fatty acid (FA) composition

There were significant ($P<0.01$) differences between the FA profiles of the whole carcass, white muscle and liver for the three diet treatments (Table 3.4, 3.5, 3.6). Generally, the FO fish had significantly ($P<0.01$) increased concentrations of saturated fatty acids (SFA), in particular 16:0, 14:0 and 18:0, compared to the SO and CO fed fish. The CO fish had significantly ($P<0.01$) higher concentrations of monounsaturated fatty acids (MUFA), in particular 18:1ω9 (oleic acid, OA), compared to the SO and FO fish. The FO and initial fish had a significantly ($P<0.01$) higher ω3/ω6 ratio than the SO and CO fish in all samples.

Table 3.4: Fatty acid content and lipid class composition of the whole carcass of Atlantic salmon smolt fed canola oil (CO), stearidonic acid oil (SO) diets and fish oil (FO) (mg g^{-1} total fatty acids)

FA (mg g^{-1})	Initial (±SEM)		CO (±SEM)		SO (±SEM)		FO (±SEM)		f
14:0	3.7 ±	0.2b	1.7 ±	0.2a	1.7 ±	0.3a	6.0 ±	0.3c	61.5
16:0	15.2 ±	0.9a	11.9 ±	0.6a	13.2 ±	19a	22.4 ±	1.4b	12.9
18:0	4.3 ±	0.2	4.1 ±	0.2	5.6 ±	0.8	5.7 ±	0.4	
Other SFA[1]	1.2 ±	0.1a,b	1.5 ±	0.2b	0.7 ±	0.1a	1.9 ±	0.2b	7.0
Total SFA	24.4 ±	1.4a	19.3 ±	1.1a	21.2 ±	3.0a	35.9 ±	2.1b	13.3
16:1ω7c	6.7 ±	0.4b	3.2 ±	0.4a	3.0 ±	0.5a	11.0 ±	0.6c	68.5
18:1ω9c	16.4 ±	1.6a	53.8 ±	1.7b	22.3 ±	3.2a	24.8 ±	1.7a	55.1
18:1ω7c	3.7 ±	0.a,b	4.7 ±	0.2b,c	2.4 ±	0.4a	5.8 ±	0.4c	19.8
20:1ω9c	1.4 ±	0.1a	2.3 ±	0.1b	1.4 ±	0.2a	1.7 ±	0.2a,b	4.9
Other MFA[2]	1.5 ±	0.2	1.9 ±	0.1	1.2 ±	0.2	1.4 ±	0.2	
Total MFA	29.7 ±	2.4a	65.8 ±	2.2c	30.3 ±	4.4a	44.7 ±	2.5b	29.3
18:3ω3 ALA	0.7 ±	0.2a	2.8 ±	0.2a	13.4 ±	1.9b	1.3 ±	0.2a	36.7
18:4ω3 SDA	1.7 ±	0.2a	2.0 ±	0.1a	9.8 ±	1.5b	2.8 ±	0.2a	22.9
20:4ω3 ETA	0.9 ±	0.1a,b	0.5 ±	0.1a	1.6 ±	0.2c	1.2 ±	0.2b,c	8.7
20:5ω3 EPA	5.4 ±	0.3b	2.3 ±	0.2a	2.7 ±	0.5a	9.0 ±	0.5c	57.8
22:5ω3 DPA	2.2 ±	0.1b	1.0 ±	0.1a	1.1 ±	0.2a	2.9 ±	0.4b	13.1
22:6ω3 DHA	9.3 ±	0.4b	1.6 ±	0.8a	1.4 ±	0.9a	12.3 ±	1.0b	42.9
Other ω3[3]	0.4 ±	0.0b	0.1 ±	0.0a	0.1 ±	0.0a	0.4 ±	0.1b	11.3
Total ω3	20.6 ±	1.1b	10.3 ±	1.0a	30.2 ±	4.8b	30.0 ±	2.2b	11.1
18:2ω6 LA	4.3 ±	0.5a	16.3 ±	0.6b	15.8 ±	2.2b	7.6 ±	0.5a	21.0
18:3ω6 GLA	0.2 ±	0.1a	1.3 ±	0.1a	5.5 ±	0.8b	0.3 ±	0.1a	33.6
20:3ω6	0.2 ±	0.1a	1.1 ±	0.0b	1.6 ±	0.2c	0.3 ±	0.0a	32.6
20:4ω6 ARA	0.7 ±	0.0	0.9 ±	0.1	0.6 ±	0.1	0.9 ±	0.1	
22:5ω6	0.2 ±	0.0a	2.8 ±	0.9b	2.8 ±	0.8b	0.1 ±	0.0a	6.6
Other ω6[4]	0.1 ±	0.0a,b	0.0 ±	0.0a	0.0 ±	0.0a	0.2 ±	0.0b	5.2*
Total ω6	5.8 ±	0.6a	22.4 ±	1.2b	26.4 ±	3.4b	9.4 ±	0.6a	24.9
Other PUFA[5]	1.3 ±	0.1b	0.6 ±	0.1a	0.5 ±	0.1a	2.0 ±	0.3b	17.2
Total PUFA	27.7 ±	1.8a	33.3 ±	1.6a	57.2 ±	8.3b	41.4 ±	2.9a,b	6.9
ω3/ω6	3.8 ±	0.4c	0.5 ±	0.1a	1.1 ±	0.1b	3.2 ±	0.2c	38.9
Lipid Class (mg g^{-1})									
TAG	77.5 ±	7.3a	107.8 ±	8.9b	103.0 ±	9.8a,b	100.5 ±	9.9b	3.1*
FFA	0.9 ±	0.2	0.2 ±	0.1	0.9 ±	0.6	0.2 ±	0.0	
ST	1.2 ±	0.1b	0.6 ±	0.1a	0.8 ±	0.1a,b	0.6 ±	0.1a	6.5*
PL	7.8 ±	0.7	7.9 ±	0.9	7.7 ±	0.9	9.2 ±	0.9	
Lipid content (mg g^{-1})[6]									
Wet	81.8 ±	5.1a	118.4 ±	4.8a,b	108.7 ±	15.8a,b	122.0 ±	6.4b	3.2*
Dry	264.9 ±	15.5a	317.5 ±	12.8a,b	318.1 ±	12.4a,b	347.8 ±	19.1b	4.4

SO, stearidonic acid rich oil diet; CO, canola oil diet; FO, fish oil diet; SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; ETA, Eicosatetraenoic acid; SDA, Stearidonic acid; LA, Linoleic acid; ALA, α-Linolenic acid; GLA, γ-Linolenic acid, ARA, Arachidonic acid; TAG; Triacylglycerol; FFA, Free fatty acid; ST, Sterol; PL, Polar lipid; WW, Wet weight; f, Mean sum of squares.
[a,b,c,d] Mean values across the row not sharing a common superscript were significantly different as determined by Tukey-Kramer HSD; df =3,30. $P<0.01$ * indicates $P<0.05$
[1] Includes 15:0, 17:0, 20:0, 22:0 and 24:0
[2] Includes 16:1ω9, 16:1ω5, 18:1ω5, 20:1ω7, 22:1ω9, 22:1ω11 and 24:1ω9
[3] Includes 21:5ω3 and 24:6ω3
[4] Includes 20:2ω6, 22:4ω6 and 24:5ω6
[5] Includes 16:2ω4, 16:3ω4 and 18:2ω9
[6] Determined gravimetrically and confirmed by TIC-FID

The SO fish had a significantly ($P<0.01$) higher ω3/ω6 ratio than the CO fish in whole carcass and liver. In white muscle and whole carcass there were significantly higher ($P<0.01$) concentrations of total PUFA in SO and FO fish compared to the CO fish.

3.4.4 Omega 3 biosynthetic pathway

The concentration of SDA was significantly ($P<0.01$) increased in the SO fish compared to other diets (Table 3.4, 3.5, 3.6). The concentrations of ETA were significantly ($P<0.01$) increased in all tissues of the SO fish compared to both the FO and CO fish. The most important difference was that the concentrations of EPA were significantly ($P<0.01$) increased in the liver of SO (2.3 mg g^{-1}) fish compared to CO fish (1.1 mg g^{-1}), although both values are significantly ($P<0.01$) reduced compared to the FO fish (3.2 mg g^{-1}). The concentrations of DHA were significantly ($P<0.01$) increased in all tissues in the FO fish compared to the other diets. There were significantly higher ($P<0.01$) concentrations of total ω3 in the initial, FO and SO fish compared to the CO fish.

Table 3.5: Fatty acid content and lipid class composition of the white muscle samples of Atlantic salmon smolt fed canola oil (CO), stearidonic acid oil (SO) diets and fish oil (FO) (mg g⁻¹ total fatty acids)

FA (mg g⁻¹)	Initial (±SEM)		CO (±SEM)		SO (±SEM)		FO (±SEM)		f
14:0	0.7 ±	0.1a,b	0.3 ±	0.1a	0.3 ±	0.1a	1.3 ±	0.3b	48.2
16:0	3.5 ±	0.4	3.0 ±	0.4	3.7 ±	0.6	4.8 ±	0.8	
18:0	0.9 ±	0.1	1.0 ±	0.2	1.5 ±	0.3	1.1 ±	0.2	
Other SFA[1]	0.3 ±	0.0	0.3 ±	0.1	0.1 ±	0.0	0.4 ±	0.1	
Total SFA	5.4 ±	0.6	4.7 ±	0.7	5.6 ±	1.0	7.7 ±	1.4	
16:1ω7c	1.2 ±	0.2a,b	0.5 ±	0.1a	0.4 ±	0.1a	2.1 ±	0.5b	8.0
18:1ω9c	3.4 ±	0.9a	12.8 ±	2.3b	5.3 ±	1.3a	4.5 ±	0.9a	8.0
18:1ω7c	0.8 ±	0.1a,b	1.0 ±	0.1a,b	0.5 ±	0.1a	1.2 ±	0.2b	3.9*
20:1ω9c	0.3 ±	0.0	0.5 ±	0.1	0.3 ±	0.1	0.4 ±	0.1	
Other MFA[2]	0.2 ±	0.1	0.4 ±	0.1	0.4 ±	0.1	0.4 ±	0.1	
Total MFA	5.9 ±	1.0a	15.2 ±	2.6b	6.9 ±	1.6a	8.5 ±	1.7a,b	4.6
18:3ω3 ALA	0.0 ±	0.0a	0.8 ±	0.1a	4.4 ±	1.0b	0.3 ±	0.1a	14.5
18:4ω3 SDA	0.3 ±	0.0a	0.5 ±	0.1a	3.1 ±	0.7b	0.6 ±	0.1a	12.1
20:4ω3 ETA	0.2 ±	0.0a	0.1 ±	0.0a	0.5 ±	0.1b	0.3 ±	0.1a,b	5.7
20:5ω3 EPA	1.3 ±	0.2a,b	0.6 ±	0.1a	0.7 ±	0.1a	2.2 ±	0.4b	9.4
22:5ω3 DPA	0.4 ±	0.1a,b	0.2 ±	0.0a	0.2 ±	0.0a	0.7 ±	0.1b	8.5
22:6ω3 DHA	2.9 ±	0.3b	1.4 ±	0.1a	1.4 ±	0.2a	3.9 ±	0.5b	15.5
Other ω3[3]	0.1 ±	0.0a,b	0.0 ±	0.0a	0.0 ±	0.0a	0.1 ±	0.0b	7.0
Total ω3	5.2 ±	0.6a,b	3.6 ±	0.3a	10.4 ±	2.1b	8.1 ±	1.3b	4.9
18:2ω6	0.9 ±	0.2a	3.3 ±	0.5a,b	4.6 ±	1.0b	1.5 ±	0.3a	6.1
18:3ω6	0.0 ±	0.0a	0.4 ±	0.1a	1.7 ±	0.4b	0.1 ±	0.0a	13.5
20:3ω6	0.0 ±	0.0a	0.4 ±	0.1b,c	0.6 ±	0.1c	0.1 ±	0.0a,b	11.7
20:4ω6 ARA	0.2 ±	0.0	0.2 ±	0.0	0.2 ±	0.0	0.2 ±	0.0	
22:5ω6	0.0 ±	0.0a,b	0.0 ±	0.0a	0.0 ±	0.0a,b	0.1 ±	0.0b	4.9
Other ω6[4]	0.0 ±	0.0a	0.0 ±	0.0a,b	0.0 ±	0.0a,b	0.1 ±	0.0b	3.5*
Total ω6	1.2 ±	0.2a	4.3 ±	0.5a,b	7.2 ±	1.6b	2.0 ±	0.5a	7.6
Other PUFA[5]	0.3 ±	0.0a,b	0.1 ±	0.0a	0.1 ±	0.0a	0.5 ±	0.1b	8.1
Total PUFA	6.6 ±	0.8a	8.0 ±	0.5a	17.7 ±	3.6b	10.6 ±	1.9a,b	4.3
ω3/ω6	4.8 ±	0.4b	1.1 ±	0.3a	1.5 ±	0.0a	4.7 ±	0.4b	53.1
Lipid Class (mg g⁻¹)									
TAG	11.7 ±	4.6	20.0 ±	5.9	17.7 ±	6.0	20.3 ±	5.0	
FFA	0.7 ±	0.2b	0.1 ±	0.0a	0.1 ±	0.0a	0.1 ±	0.0a	15.9
ST	0.2 ±	0.0	0.2 ±	0.1	0.1 ±	0.1	0.1 ±	0.0	
PL	5.3 ±	1.3	5.4 ±	0.6	7.2 ±	1.0	6.3 ±	0.5	
Lipid content (mg g⁻¹)[6]									
Wet	17.9 ±	2.9	28.0 ±	3.4	30.1 ±	6.2	26.8 ±	4.9	
Dry	80.4 ±	12.9	109.8 ±	9.7	108.3 ±	20.4	108.0 ±	17.4	

df =3,40. *P*<0.01 * indicates *P*<0.05

Abbreviations and other footnote definitions, see Table 3.4.

Table 3.6: Fatty acid content and lipid class composition of the liver of Atlantic salmon smolt fed canola oil (CO), stearidonic acid oil (SO) diets and fish oil (FO) (mg g^{-1} total fatty acids)

FA (mg g^{-1})	Initial (±SEM)			CO (±SEM)			SO (±SEM)			FO (±SEM)			f
14:0	0.5	±	0.0a	0.4	±	0.0a	0.4	±	0.1a	0.8	±	0.1b	12.9
16:0	6.6	±	0.5a,b	5.4	±	0.4a	6.7	±	0.5a,b	8.0	±	0.8b	3.6*
18:0	1.9	±	0.1a	2.8	±	0.1b,c	3.5	±	0.3c	2.6	±	0.2a,b	8.0
Other SFA[1]	0.3	±	0.0	0.4	±	0.1	0.2	±	0.0	0.4	±	0.1	
Total SFA	9.3	±	0.7	9.0	±	0.4	10.7	±	0.8	11.7	±	1.1	
16:1ω7c	0.9	±	0.1b,c	0.5	±	0.1a,b	0.5	±	0.1a	1.2	±	0.1c	12.3
18:1ω9c	3.6	±	0.4b	19.0	±	2.7b	7.0	±	0.7b	4.2	±	0.4b	24.1
18:1ω7c	1.0	±	0.1a,b	1.6	±	0.2c	0.7	±	0.1a	1.5	±	0.1b,c	14.5
20:1ω9c	0.2	±	0.1a	1.0	±	0.2b	0.4	±	0.1a	0.5	±	0.2a,b	6.2
Other MFA[2]	0.3	±	0.1a	1.0	±	0.1b	0.5	±	0.0a	0.5	±	0.1a	11.2
Total MFA	6.0	±	0.6a	23.2	±	3.1b	9.0	±	0.9a	7.8	±	0.7a	20.8
18:3ω3 ALA	0.1	±	0.0a	0.5	±	0.1a	2.0	±	0.2b	0.1	±	0.0a	40.8
18:4ω3 SDA	0.2	±	0.0a	0.4	±	0.1a	1.6	±	0.2b	0.2	±	0.0a	24.5
20:4ω3 ETA	0.2	±	0.0a	0.3	±	0.0a	1.0	±	0.1b	0.2	±	0.0a	48.8
20:5ω3 EPA	2.2	±	0.1a,b	1.1	±	0.1a	2.3	±	0.2b	3.2	±	0.3c	13.3
22:5ω3 DPA	0.8	±	0.0b	0.5	±	0.1a	0.4	±	0.1a	1.0	±	0.1b	12.7
22:6ω3 DHA	7.7	±	0.4b	4.1	±	0.4a	3.5	±	0.3a	8.8	±	0.7b	27.0
Other ω3[3]	0.1	±	0.0b	0.0	±	0.0a	0.0	±	0.0a	0.1	±	0.0b	10.1
Total ω3	11.3	±	0.5b	7.1	±	0.6a	10.7	±	0.7b	13.5	±	1.1b	11.6
18:2ω6	1.0	±	0.2a	4.6	±	0.4b	3.7	±	0.3b	1.1	±	0.1a	37.6
18:3ω6	0.0	±	0.0a	0.7	±	0.3b	0.8	±	0.1b	0.0	±	0.0a	7.4
20:3ω6	0.1	±	0.0a	1.7	±	0.1b	1.9	±	0.2b	0.1	±	0.0a	88.4
20:4ω6 ARA	1.0	±	0.1a	2.5	±	0.2b	1.3	±	0.1a	1.4	±	0.1a	14.8
22:5ω6	0.0	±	0.0a	0.2	±	0.0b	0.0	±	0.0a	0.0	±	0.0a	8.2
Other ω6[4]	0.1	±	0.0a,b	0.1	±	0.0b	0.0	±	0.0a	0.0	±	0.0a,b	4.6
Total ω6	2.2	±	0.3a	9.7	±	0.5c	7.8	±	0.5b	2.7	±	0.2a	75.9
Other PUFA[5]	0.1	±	0.0	0.1	±	0.0	0.0	±	0.0	0.1	±	0.0	
Total PUFA	13.6	±	0.7a	16.9	±	0.9a,b	18.5	±	1.1b	16.2	±	1.4a,b	3.0
ω3/ω6	5.3	±	0.4c	0.8	±	0.0a	1.4	±	0.1b	5.1	±	0.1c	255.7
Lipid Class (mg g^{-1})													
SQ	0.3	±	0.1	0.3	±	0.1	1.8	±	1.1	0.2	±	0.0	
TAG	0.4	±	0.2a	6.8	±	2.5b	3.9	±	1.6a,b	1.6	±	0.8a,b	3.1*
FFA	7.0	±	0.7c	3.2	±	0.6b	1.4	±	0.2a	0.7	±	0.1a	30.4
ST	1.4	±	0.2a	2.6	±	0.3b	1.4	±	0.2a	1.4	±	0.1a	7.9
PL	19.9	±	1.1a	32.2	±	1.9b	31.1	±	1.8b	31.9	±	2.5b	7.2
Lipid content (mg g^{-1})[6]													
Wet	29.0	±	2.2a	49.1	±	3.5b	38.3	±	2.4a,b	35.8	±	2.7a	7.3
Dry	150.8	±	10.1	196.3	±	11.7	155.8	±	9.5	155.0	±	15.2	

df =3,40. *P*<0.01 * indicates *P*<0.02 SQ; Squalene. Abbreviations and other footnote definitions, see Table 3.4.

Table 3.7: Apparent digestibility coefficient (ADC) for the crude protein (N), energy (kJ), and fatty acids for diets containing different oils

ADC (%)	CO ± (SEM)		SO ± (SEM)		FO ± (SEM)		$p<$	f
N	93.5	± 1.7	90.7	± 1.1	93.7	± 0.4		
kJ	90.4	± 1.1	86.6	± 3.2	89.4	± 1.1		
Fatty acids								
14:0	100.0	± 0.0	91.6	± 4.2	92.7	± 1.3		
16:0	96.3	± 0.3b	95.4	± 0.4b	88.2	± 2.5a	0.02	8.8
18:0	95.4	± 0.3b	95.1	± 0.3b	85.6	± 3.3a	0.02	8.2
Total SFA	95.3	± 0.2b	95.1	± 0.2b	88.8	± 2.4a	0.02	8.5
16:1ω7c	98.6	± 0.1	96.1	± 0.7	96.8	± 0.8		
18:1ω9c	96.1	± 0.8	96.2	± 0.5	95.1	± 0.9		
18:1ω7c	95.5	± 0.7	95.1	± 0.4	94.6	± 1.0		
Total MUFA	96.1	± 0.8	95.8	± 0.3	95.6	± 0.7		
18:3ω3 ALA	97.0	± 0.5	96.7	± 0.6	81.5	± 11.0		
18:4ω3 SDA	100.0	± 0.0b	96.8	± 0.7b	99.3	± 1.8a		
20:4ω3 ETA	100.0	± 0.0	99.1	± 9.2	98.8	± 1.1		
20:5ω3 EPA	100.0	± 0.0	100.0	± 0.0	97.6	± 0.7		
22:5ω3 DPA	100.0	± 0.0	100.0	± 0.0	98.8	± 0.7		
22:6ω3 DHA	99.9	± 0.1	99.9	± 0.0	99.3	± 0.7		
Total ω3	98.9	± 0.6	98.7	± 0.7	97.7	± 0.3		
18:2ω6 LA	96.6	± 0.7	96.3	± 0.6	95.1	± 1.0		
18:3ω6 GLA	100.0	± 0.0b	96.5	± 0.6a	98.5	± 0.9a,b	0.02	7.7
20:4ω6 ARA	99.7	± 0.2b	100.0	± 0.0b	97.2	± 0.7a	0.01	12.1
Total ω6	98.9	± 0.6	99.2	± 0.5	96.4	± 0.8		
Total PUFA	98.9	± 0.61	98.8	± 0.57	97.2	± 0.31		

SO, stearidonic acid oil diet; CO, canola oil diet; FO, fish oil diet; SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; ETA, Eicosatetraenoic acid; Stearidonic acid; LA, Linoleic acid; ALA, α-Linolenic acid; GLA, γ-Linolenic acid; ARA, Arachidonic acid; Each value is the mean (±SEM, n=3). Means with the same symbol are not significantly different (Tukey-Kramer HSD multiple comparison)

3.4.5 Omega 6 biosynthetic pathway

The concentrations of 18:2ω6 were significantly ($P<0.01$) increased in CO and SO fish tissues compared to the FO fish (Table 3.4, 3.5, 3.6). The SO fish had significantly higher ($P<0.01$) concentrations of 18:3ω6 in the white muscle and

whole carcass compared to FO and CO fish. CO and SO fish had significantly

higher ($P<0.01$) concentrations of 18:3ω6 in the liver than FO fish. The SO fish had

significantly higher ($P<0.01$) concentrations of 20:3ω6 in the whole carcass than FO

and CO fish. The liver of CO and SO fish had significantly higher ($P<0.01$)

concentrations of 20:3ω6 than the FO fish. However, there was a significantly lower

($P<0.01$) concentration of arachidonic acid (20:4ω6, ARA) in the liver in SO fish

compared to the CO and FO fish. There was a significantly higher ($P<0.01$)

concentration of total ω6 in the liver in CO fish compared to SO fish, which was

significantly higher ($P<0.01$) than the FO fish. There was a significantly higher

($P<0.01$) concentration of total ω6 in the whole carcass in SO and CO fish compared

to FO fish.

3.4.6 Digestibility

There was no significant difference in apparent digestibility of energy or crude

protein between diets (Table 3.7). The were significant ($P<0.05$) differences in

digestibility of some SFA between the FO and the CO and SO diets, in particular

16:0, 18:0 and total SFA. The digestibility of 18:3ω6 in SO diet was significantly

lower compared to CO diet.

3.4.7 Gene expression

Measurements of mRNA abundance by real-time quantitative RT-PCR

showed gene expression in both the liver and white muscle was affected by dietary

oil. In the liver of the SO fish, there were significantly higher elongase and Δ^5

desaturase gene activities compared to the FO fish (Figure 3.1). There were

significantly higher elongase and both Δ^6 and Δ^5 desaturase gene expression in the

liver of CO fish compared to the FO fish. In the white muscle there was a

significantly higher Δ^6 desaturase gene expression in the CO fish compared to FO

fish (Figure 3.2).
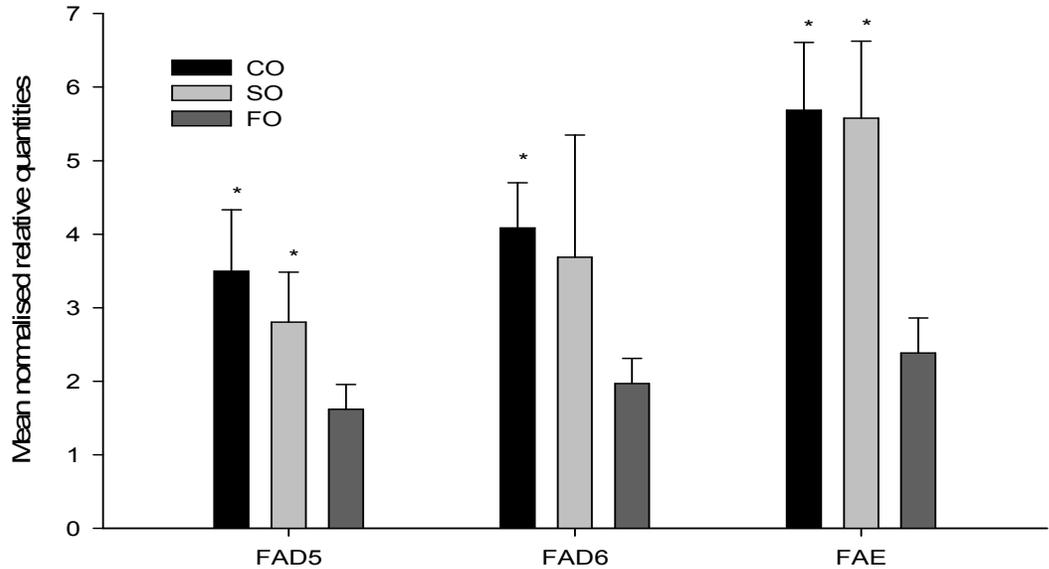
Figure 3.1: Differential gene expression in livers of Atlantic salmon fed diets rich in stearidonic rich oil (SO); canola oil (CO) and fish oil (FO).

Changes in the mean normalised expression (MNE, n=6) values of fatty acid Δ^5 desaturase (FAD5), Δ^6 desaturase (FAD6), and FA elongase (FAE) mRNA were determined by real-time PCR analysis. Results were calculated using qBase software and were normalised using the geometric mean of four stably expressed reference genes. * Indicates significant differences compared to the control fish oil diet (FO) as determined by the non-parametric Mann-Whitney test ($P<0.05$). SO, stearidonic oil rich diet; CO, canola oil diet; FO, fish oil diet

In the liver there was a positive correlation between dietary linoleic acid (18:2ω6

LA) concentration and the expressions of elongase (Spearman's coefficient (ρ)

=0.66, $P<0.01$), Δ^6 (ρ=0.62, $P<0.01$) and Δ^5 (ρ=0.55, $P<0.01$) desaturase genes.

There was a positive correlation between dietary ALA concentrations and the

expression both elongase (ρ=0.53, $P<0.01$) and Δ^5 (ρ=0.47, $P<0.02$) desaturase

genes. There was a negative correlation between the dietary SDA with Δ^6 (ρ=-0.45,

$P<0.03$) desaturase gene expression.
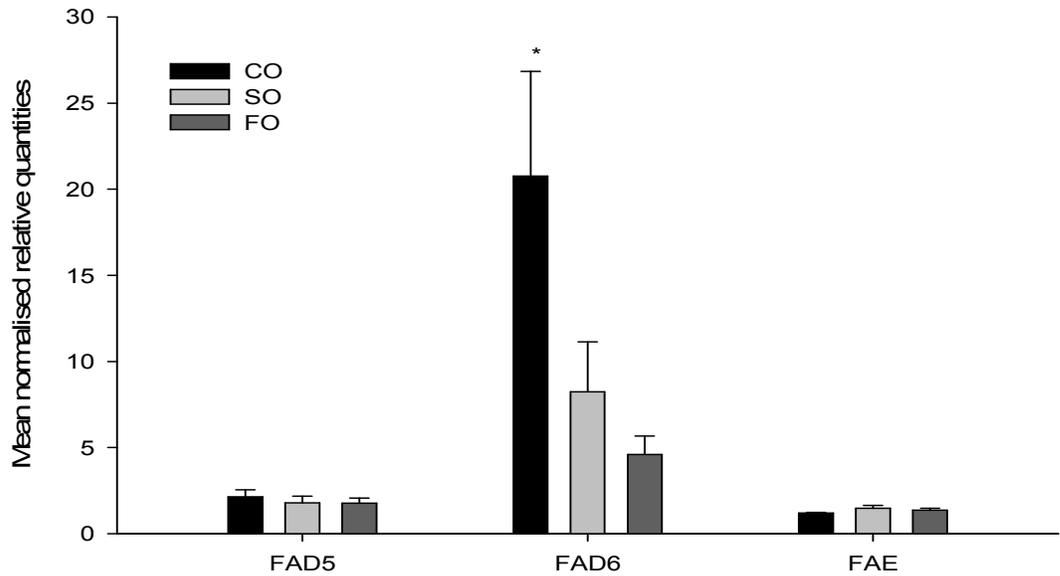
Figure 3.2: Differential gene expression in white muscle of Atlantic salmon fed diets rich in stearidonic rich oil (SO); canola oil (CO) and fish oil (FO).

Changes in the mean normalised expression (MNE, n=6) values of fatty acid Δ^5 desaturase (FAD5), Δ^6 desaturase (FAD6), and FA elongase (FAE) mRNA were determined by real-time PCR analysis. Results were calculated using qBase software and were normalised using the geometric mean of four stably expressed reference genes. * Indicates significant differences compared to the control fish oil diet (FO) as determined by the non-parametric Mann-Whitney test ($P<0.05$). SO, stearidonic oil rich diet; CO, canola oil diet; FO, fish oil diet

There was a modest negative correlation in the liver between dietary ω3 LC-PUFA concentrations with the expression of elongase (EPA, ρ=-0.68, $P<0.01$, DHA ρ=-0.66, $P<0.01$), Δ^5 (EPA, ρ=-0.59, $P<0.01$, DHA ρ=-0.55, $P<0.01$) and Δ^6 (EPA, ρ=-0.45, $P<0.03$, DHA ρ=-0.62, $P<0.01$) desaturase genes.

3.5 DISCUSSION

The present study described the effect of dietary oil on digestibility, tissue fatty acid profiles and gene expression of principle desaturation and elongation enzymes involved in the FA biosynthetic pathway in Atlantic salmon. Feeding SDA and ALA

to seawater Atlantic salmon increased gene expression in the liver for genes involved in the ω3 LC-PUFA biosynthetic pathway. However, increased expression did not appear to be reflected in the deposition of LC-PUFA, since ω3 LC-PUFA concentrations in SO fish were lower compared to the FO fish. Previously we demonstrated that Atlantic salmon in fresh water were able to maintain high concentrations of ω3 LC-PUFA when fed SDA (Miller, et al., 2007a). The current experiment was in saltwater and demonstrated increased conversion of SDA through to ETA in the SO fish. Also, the liver fatty acid profiles indicated increased production of EPA in the SO fish compared to the CO fish. Most significantly the current experiment demonstrated that in Atlantic salmon in sea water, the enhanced gene expression due to dietary SDA will not produce ω3 LC-PUFA concentrations comparable to diets rich in fish oil.

3.5.1 Fatty acid profiles

The concentrations of individual FA in salmon indicate how fish store dietary FA in tissues, and they may also indicate an endogenous biosynthetic capacity. Defatted fish meal removes virtually all LC-PUFA from the diet which allows the assumption that differences in C_{20} LC-PUFA concentrations in tissues between the CO and SO fish are due to biosynthesis rather than accumulation. There was a significant increase of EPA concentration in the liver of the SO fish compared to the CO fish which indicates that if the Δ^6 desaturase was bypassed with dietary SDA, increased biosynthesis occurs through to EPA. In all sampled tissues, fish fed the SO diet increased ETA and 20:3ω6, the immediate biosynthetic elongation products of SDA and γ-linolenic acid (18:3ω6, GLA) which are provided by the SO diet. The

FA profiles of the muscle and carcass of the SO fish suggest C_{18} PUFA were readily biosynthesised to their direct elongase product in both the ω3 and ω6 LC-PUFA biosynthetic pathways. Overall, the FA profiles of the sampled tissues reflected the FA profile of their diet. There was a reduction of ω3 LC-PUFA in the tissues of fish fed SO and CO diets, increased oleic acid (18:1ω9, OA) in fish fed the CO diet and increased SDA and GLA, in fish fed the SO diet. In many oil replacement trials the FA profile of Atlantic salmon reflects their diet (Torstensen, et al., 2000; Rosenlund, 2001; Bransden, et al., 2003; Carter, et al., 2003b; Bell, et al., 2004; Torstensen, et al., 2004; Miller, et al., 2007b). In the present study, compared to the FO fish, the SO fish did not attain whole carcass, muscle or liver concentrations of ω3 or ω6 LC-PUFA, in particular EPA, DHA and ARA that was shown with salmon parr (Miller, et al., 2007a).

The conversion of SDA through to EPA has been shown previously in the plasma and liver of other animal models (Yamazaki, et al., 1992), as well as in the plasma of humans (James, et al., 2003). In a salmon cell line study, SDA was converted in vitro to ETA and then further desaturated and elongated to EPA (Ghioni, et al., 2002). Furthermore, radiolabelled [U-^{14}C] SDA was transferred in vitro to DHA in cell culture (Ghioni, et al., 1999) and indicated SDA can be converted all the way along the ω3 pathway to DHA. The liver FA profiles suggest that the salmon have an increased ability to convert SDA through to EPA, compared to ALA. However, tissue FA profiles indicate that further biosynthesis in the ω3 pathways is inefficient.

The FA results indicate that elongase activity is greater for the ω6 pathway than the ω3 pathway. As CO and SO diets provide virtually no LC PUFA, conversion can

be estimated from calculations of biosynthesis from the C_{18} precursors. The conversion from GLA to $20:3\omega6$ was greater than for SDA to ETA in the both the CO and SO fish in all tissues. This increased conversion resulted in significantly higher concentrations of ARA in the white muscle and the liver of the CO fish compared to the SO fish even though SO fish had significantly higher concentrations of the precursor product $20:3\omega6$. The FA results are consistent with an increased biosynthesis in the $\omega6$ pathway in the CO fish for the production of ARA. ARA is the primary eicosanoid precursor in fish and is important in the production of leukotrienes (Bell and Sargent, 2003). The reason for the increase in $\omega6$ LC-PUFA in the CO fish and not in the SO fish is presently unclear.

3.5.2 Gene expression

All vertebrates, including fish, lack the Δ^{12} and Δ^{15} desaturases and therefore LA and ALA are the shortest chain length FA that can be included in diets and still allow biosynthesis of LC PUFA. As mentioned above marine fish have a reduced ability to biosynthesis LC PUFA from these precursors and are required as essential dietary FA (Sargent, et al., 2002; Tocher, 2003). In Atlantic salmon expression of desaturase and elongase genes in the $\omega3$ LC-PUFA biosynthetic pathway are increased by dietary vegetable oils (Zheng, et al., 2004a; Zheng, et al., 2005b). There are many potential mechanisms by which FA, in particular $\omega3$ LC-PUFA, can affect gene expression including changes in membrane composition and signalling, eicosanoid production, oxidant stress, nuclear receptor activation and /or covalent modification of specific transcription factors (Jump, et al., 1999). Dietary FA can regulate gene expression by controlling the activity or abundances of key

transcription factors (Jump, et al., 2005). Some of the transcription factors that are targets for FA regulation include peroxisome proliferators-activated receptors (PPARα, β, γ1 and γ2), sterol regulatory element protein-1c (SREP-1c), hepatic nuclear factors (HNF-4α and γ), retinoid X receptor (RXRα), liver X receptor (LXRα) and possibly others, and in this fashion act like hydrophobic hormones to control gene expression (Jump, 2004; Jump, et al., 2005). Fatty acids can also can affect gene expression indirectly through specific enzyme-mediated pathways, such as cyclooxygenase, lipoxygenase, protein kinase C, and/or sphingomyelinase signal transduction pathways, or through pathways that involve changes to membrane lipid/lipid raft composition that affect the G-proteins receptor or tyrosine kinase-linked receptor signalling (Jump, 2004). All this demonstrates that dietary FA affects structural, metabolic and regulatory components of cells through a number of direct and indirect processes.

It has yet to be established what mechanisms are involved in the increased elongase and desaturase activity shown in the liver of both the SO and CO fish. It is known that dietary FA, environmental factors and life stage (hormonal) can affect elongase and desaturase activity (Jump, et al., 1996; Jump, et al., 1999; Zheng, et al., 2004b; Zheng, et al., 2005b). Each step along the ω3 LC-PUFA biosynthesis pathway is dependant on the amount of substrate, and therefore the activity of the preceding enzymatic step if it is not supplied by the diet, but also the removal of the subsequent products (Zheng, et al., 2004b). Therefore increased elongase and desaturase activity could be influenced by either increased dietary concentrations of the substrate (ALA in CO; ALA and SDA in SO) or the removal or absence of the product (SO and CO diets having no ω3 LC-PUFA).

The presence of SDA in the diet did influence the molecular mechanisms involved in the biosynthesis of ω3 LC-PUFA. The presence of SDA did affect expression in the liver of elongase and Δ^5 desaturase genes compared to FO fish (Figure 3.1). Furthermore, ALA in the CO diet also influenced the expression of elongase and Δ^5 and Δ^6 desaturase genes. However, these results could also be interpreted as the absence of dietary ω3 LC-PUFA in the CO and SO diets resulted in increased expression of elongase and Δ^5 and Δ^6 desaturase genes. The altered expression in the genes involved with ω3 LC-PUFA biosynthetic pathway resulted in compositional changes of FA concentrations in tissues as previously described. However the elongase and Δ^5 and Δ^6 desaturase genes also act upon the ω6 LC-PUFA biosynthetic pathway. Increased expression of elongase and Δ^5 and Δ^6 desaturase genes measured could be the result of the production of LA and GLA through to AA. The FA profiles and conversion ratios indicate that a there is a preference for the ω6 pathway in particular for the CO fish. This study demonstrated that both dietary concentrations of substrate had a positive correlation of expression in both the ω3 and ω6 classes and a negative correlation to the presence of ω3 LC-PUFA in particular EPA, DPA and DHA. However, there are more factors than just substrate/product presence/absence in the diet to alter enzymatic activity.

There was virtually no ω3 LC-PUFA present in the SO and CO diets which will lead, over a short period of time, to deficiencies in these important fatty acids. Deficiencies in ω3 LC-PUFA can lead to a multitude of nutritional "diseases" or pathologies in Atlantic salmon (Bell, et al., 1991; Tacon, 1996; Seierstad, et al., 2005a). These deficiencies may trigger an endogenous response to increase ω3 LC-

PUFA production as both EPA and DHA play important roles in cell membranes. Increased concentrations of DHA favourably alter numerous properties of membranes including fluidity, phase behaviour, elastic compressibility, permeability, fusion, flip-flop and protein activity (Stillwell and Wassall, 2003). To preserve cell health and function, an increased biosynthetic activity to maintain $\omega 3$ LC-PUFA concentrations is necessary. However, the high concentrations of $\omega 3$ LC-PUFA which are associated with salmon fed fish oil will not be able to be provided by increased biosynthetic activity from dietary ALA or SDA.

There have been conflicting results reported recently involving the expression of FA elongase expression in salmon liver for replacement of dietary fish oil with vegetable oil (Zheng, et al., 2004b, 2005a; Zheng, et al., 2005b). It has been shown that FA elongase gene expression increased with a graded replacement of linseed oil, in the liver (Zheng, et al., 2004b). However, in a more recent trial, liver FA elongase gene expression was not increased by a vegetable oil blend (Zheng, et al., 2005a). Our study confirmed the earlier result (Zheng, et al., 2004b) with liver FA elongase gene expression increasing for both alternative oil diets (SO and CO) compared to FO.

In the white muscle a different pattern of gene expression was observed to that of the liver (Figure 3.2). In the white muscle there was no difference in the expression of the elongase and Δ^5 desaturase genes between both the CO and SO and the FO fish. However, the CO fish had enhanced expression of the Δ^6 desaturase gene compared to the FO fish. This result provides evidence that the presence of the substrate ALA without SDA in the CO diet may have enhanced expression of the Δ^6

desaturase gene. The presence of SDA in the SO diet eliminates the Δ^6 desaturase rate limiting step and therefore decreased expression was observed.

In a recent experiment we demonstrated that Atlantic salmon parr fed a SDA rich oil had comparable levels of ω3 LC-PUFA in all tissues to that of FO fed fish (Miller, et al., 2007a). The six week trial indicated that parr fed the SDA rich diet which bypasses the Δ^6 desaturase enabled the fish to attain levels of ω3 LC-PUFA comparable to a FO diet (Miller, et al., 2007a). However, freshwater fish and salmon parr approaching smoltification have an enhanced ability to biosynthesise ω3 LC-PUFA (Sargent, et al., 2002; Zheng, et al., 2005b). As the majority of salmon production occurs in a marine environment it is fundamental to ascertain how SDA is further biosynthesised and whether ω3 LC-PUFA concentrations can be maintained in seawater fish. This current study demonstrates that, although SDA fed smolt have increased endogenous metabolism due to elevated Δ^5 desaturase and FA elongase gene expression, they are unable to maintain high concentrations of ω3 LC-PUFA in body tissues. Salmon in this trial were previously fed a commercial fish oil based diet throughout their life including transfer to sea water and smoltification. The history of the presence of ω3 LC-PUFA in their diet may have influenced the expression of FA elongase and desaturase genes. It would be interesting to determine the FA profile and elongase and desaturase gene expression over a life time of use of a SO diet. It may be possible that under such dietary history the increased elongase and desaturase activity prior to smoltification may carry through to the seawater growout period.

3.6 CONCLUSION

This study showed that dietary FA can up-regulate expression of the liver elongase and Δ^6 and Δ^5 desaturase and the white muscle Δ^6 desaturation genes for Atlantic salmon smolt fed vegetable oils. Dietary SDA enhanced elongase and Δ^5 desaturation gene expression compared to the FO treatment. Dietary SDA demonstrated increased concentrations of EPA, ETA and GLA in the liver of Atlantic salmon smolt and increase concentrations of ETA and GLA in the white muscle and whole carcass of the SO fish compared to the CO fish. However, the SO fish did not attain ω3 LC-PUFA concentrations simular to that of FO fish as demonstrated in Atlantic salmon parr. The high concentrations of ω3 LC-PUFA found in FO fish will not be provided by increased metabolism of diets rich in ALA or SDA.

3.7 ACKNOWLEDGEMENTS

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CHAPTER 4

Replacement of fish oil with thraustochytrid *Schizochytrium sp. L* oil in Atlantic salmon parr (*Salmo salar* L) diets

4.1 ABSTRACT

Replacing fish oil with that from a docosahexaenoic acid (22:6ω3, DHA) rich single cell micro-organism, thraustochytrid *Schizochytrium sp. L,* in diets for Atlantic salmon was investigated. Four experimental diets containing 100% thraustochytrid oil (TO), 100% palm oil (PO) and a 4:1 palm and thraustochytrid oil mixture (MX) were compared to a fish oil (FO) diet over 9 weeks. A saltwater transfer challenge occurred at the end of the trial for 14 days to test the diet treatments on the ability of salmon to smolt. There were no significant differences in the feed consumption of the diets or the digestibility of the ω3 or ω6 PUFA, indicating that there were no differences in the digestibility of fatty acids between diets. There were no significant differences between the growth of fish on the four diet treatments. There was a significant difference in the fatty acid profiles of the fish muscle tissues between all diets. Fish on the TO diet had a significantly greater percentage of DHA in muscle tissue compared with fish on all other diets. Blood osmolarity, which is inversely related to the ability of salmon to smolt, from the TO and FO fed fish was significantly lower than that of fish on the PO diet. This study showed that thraustochytrid oil can be used to replace fish oil in Atlantic salmon diets without detriment to the growth of parr. Including thraustochytrid oil in fish diets significantly increases the amount of DHA in the Atlantic salmon muscle and therefore is a candidate for use in oil blends for salmon diets. Thraustochytrid oil provides a renewable source of essential fatty acids, in particular DHA, for aquafeeds.

4.2 INTRODUCTION

Raw feed materials of marine origin, particularly fish oil, are under pressure from both an increase in world population and the continued growth of aquaculture worldwide (FAO, 2004). Fish oil is a finite resource and by 2010 it is estimated that ≥90% of global fish oil supplies will be used for aquafeeds (Barlow, 2000). However a continued increase in its use by the nutraceutical/biomedical industries for fortified/functional foods and supplements will limit this resource. A major challenge for aquaculture production is to identify and validate stable, predictable and high quality sources of alternative oils for the manufacture of aquafeeds. Farmed Atlantic salmon have traditionally been fed diets that contain up to 40% marine fish oil, but more recently oil blends (marine, terrestrial plant and animal) have been used. Marine fish oil diets have high concentrations of omega-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acid (ω3 LC-PUFA) especially eicosapentaenoic acid (20:5ω3, EPA) and docosahexaenoic acid (22:6ω3, DHA), which closely resembles the nutritional composition of their natural diet during the marine phase of their life cycle. Diets containing replacement oils such as terrestrial plant and animal oil have reduced concentrations of EPA and DHA.

Atlantic salmon flesh has naturally high concentrations of ω3 LC-PUFA, especially EPA and DHA, when it is provided in their diet. The importance of ω3 LC-PUFA in human health as an inhibitor of cardiovascular disease, renal disease, rheumatoid arthritis, cancer and skin diseases is being increasingly recognised (Shahidi and Miraliakbari, 2004; Brouwer, et al., 2006; MacLean, et al., 2006). Oily fish such as Atlantic salmon are generally regarded as good sources of dietary ω3

LC-PUFA. The replacement of fish oil with vegetable oils such as canola, sunflower and linseed has been extensively researched, but these oils are limited by the absence of ω3 LC-PUFA (Bell, et al., 1993; Bransden, et al., 2003; Bell, et al., 2004; Miller, et al., 2007). Atlantic salmon tend to accumulate the lipid profile of their diet and a corresponding reduction in the level of ω3 LC-PUFA in the salmon flesh has been shown with various vegetable oil trials (Polvi and Ackman, 1992; Bell, et al., 1993; Bransden, et al., 2003; Tocher, et al., 2003; Bell, et al., 2004). This reduction of ω3 LC-PUFA in salmon flesh as a result of the use of vegetable oil diets, has been shown to reduce the protection, to human consumers, against cardiovascular disease associated with eating salmon (Seierstad, et al., 2005). There was however still a cardiovascular benefit from eating salmon fed vegetable oil diet rather than not eating fish (Seierstad, et al., 2005).

Thraustochytrids are heterotrophic protists, commonly found in marine and other saline environments; they can be detritivores, bacterivores and/or parasites (Lewis, et al., 1999). Originally thought to be closely related to primitive fungi, thraustochytrids have more recently been assigned to the subclass Thraustochytridea (Chromista, Heterokonta), aligning them with heterokont algae such as diatoms and brown algae (Cavalier-Smith, et al., 1994). Thraustochytrids possess the ability to produce a number of ω3 LC-PUFA, especially DHA. Thraustochytrids show potential as a source of oil for aquaculture (Barclay and Zeller, 1996; Nichols, et al., 1996; Lewis, et al., 1998; Lewis, et al., 1999; Carter, et al., 2003b). Large scale culture of thraustochytrids may be suitable for commercial aquafeeds as they produce a relatively high biomass and have high percentage of ω3 LC-PUFA rich lipid (Lewis, et al., 1999). Optimising strain selection and growth conditions can

provide single cell oils with specific qualities such as high DHA (concentrations up to 60%), low ω6 (in particular docosapentaenoic acid DPA-6, 22:5ω6), high ω3 LC-PUFA, and high ω3/ω6 ratios (Nichols, et al., 2004). Thraustochytrid biomass is already being used commercially as feed for rotifers (*Brachionus spp)* and brine shrimp (*Artemia)* prior to feeding them to finfish larvae (Barclay and Zeller, 1996; Nichols, et al., 1996; Lewis, et al., 1998) and has been used in a trial for Atlantic salmon as a possible partial replacement for fish oil (Carter, et al., 2003b). In changing the dietary oil source it is important to examine the ability for salmon to absorb and digest large concentrations of DHA.

Research on thraustochytrids as an oil source for use in salmonid aquafeeds is presently limited to a single study to partially replace fish meal and fish oil with Atlantic salmon (Carter, et al., 2003b; Carter, et al., 2003a). This earlier study concluded that there was no detrimental effect of using thraustochytrid biomass as an ingredient in salmon feed, but it was only tested at a low inclusion (10% of feed) (Carter, et al., 2003b). Higher incorporation of thraustochytrids in feeds is required to ascertain if they can be of wider use to the aquaculture industry. The aim of this experiment was to assess the ability of Atlantic salmon to digest and incorporate high concentrations of thraustochytrid derived DHA into cellular phospholipids and/or storage. A commercially available source of thraustochytrid oil from the species *Schizochytrium L,* which has a high (35%) concentration of DHA, was assessed against palm oil used as a negative control as it contains no ω3 LC-PUFA and no DHA. A 4:1 blend of palm oil and thraustochytrid oil (MX) provided an additional diet with similar concentrations of DHA to that of fish oil.

4.3 MATERIALS AND METHODS

4.3.1 Experimental diets

Four diets were formulated to compare palm oil (PO), two different concentrations of thraustochytrid oil (100% (TO), 1:4 TO:PO (MX)), and fish oil (FO) (Table 4.1). Fish meal was defatted three times using a 2:1 mixture of hexane and ethanol (400ml 100g^{-1} fish meal). Soybean (Hamlet Protein A/S, Horsens, Denmark), casein (MP Biomedicals Australasia Pty Ltd, Seven Hills, NSW, Australia), wheat gluten (Starch Australasia, Land Cove, NSW, Australia) and BOIIC pre-gelatinised maize starch (Penford Australia Limited, Land Cove, NSW, Australia) were used as ingredients. Thraustochytrid oil was provided as DHASCO$^{®}$-S (Martek, Columbia, Maryland, USA). Fish oil was from jack mackerel (Skretting Australia, Cambridge, Tasmania, Australia) and a domestic source of pure palm oil was used (Aoroma, Hallam, Victoria, Australia). Stay-C and Rovimix E50 were supplied from Roche Vitamins Australia (Frenchs Forest, NSW, Australia), and the remaining ingredients were supplied by Sigma-Aldrich (Castle Hill, NSW, Australia). Yttrium oxide was used as a digestibility marker (Carter, et al., 2003a). The diets were manufactured into 3 mm diameter pellets using a California Pellet Mill (CL-2), dried and stored at -5°C (Carter, et al., 2003b).

Table 4.1: Ingredient and lipid composition (g/kg dry matter) of experimental diets

	Diet			
	PO	MX	TO	FO
Ingredient composition (g kg⁻¹)				
Fish meal (defatted)	150	150	150	150
Casein	150	150	150	150
Wheat gluten	100	100	100	100
Soybean meal	226	226	226	226
Fish oil	0	0	0	130
Palm oil	130	104	0	0
Thraustochytrid oil	0	26	130	0
Pre gel starch	150	150	150	150
Vitamin mix[a]	3	3	3	3
Mineral mix[b]	5	5	5	5
Stay C[c]	3	3	3	3
Chlorine chloride	2	2	2	2
Bentonite	50	50	50	50
CMC	10	10	10	10
Sodium mono phosphate	20	20	20	20
Yttrium oxide	10	10	10	10
Chemical composition (g kg⁻¹ DM)				
Dry Matter	956.4	961.7	960.5	957.9
Crude protein	389.9	391.6	390.2	387.8
Crude fat	147.6	146.0	150.7	149.3
Energy *(MJ kg⁻¹ DM)*	20.0	20.0	19.8	20.0
FAME (g kg⁻¹ DM)				
14:0	2.1	4.4	13.0	10.2
16:0	61.4	43.5	38.5	31.4
18:0	7.0	7.1	1.6	5.7
Total SFA	71.3	56.1	54.7	54.4
16:1ω7c	0.5	0.6	1.0	10.7
18:1ω9c OA	48.9	48.5	3.4	19.2
Total MUFA	51.2	51.1	6.1	43.5
20:5ω3 EPA	0.3	0.8	3.4	18.5
22:6ω3 DHA	1.1	9.8	53.5	11.1
Total ω3	1.7	11.2	60.0	37.2
18:2ω6 LA	22.8	23.4	7.1	8.8
20:4ω6 ARA	0.0	0.1	0.0	1.1
22:5ω6 DPA−6	0.6	3.9	21.2	0.4
Total ω6	23.4	27.6	29.4	11.4
Total PUFA	25.1	38.8	89.4	48.6

TO, Thraustochytrid oil DHASCO®-S from Martek; PO, palm oil; MX, 4:1 mix of palm oil and thraustochytrid oil; FO, fish oil, SFA, Saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; CMC, Carboxymethyl cellulose; DHA, Docosahexaenoic Acid; EPA, Eicosapentaenoic Acid; DPA, Docosapentaenoic acid; OA, oleic acid; LA, Linoleic acid; ARA, Arachidonic acid.

[a] Vitamin mix (ASV4) supplied per kilogram of feed: 2.81 mg thiamine HCL, 1.0 mg riboflavin, 9.15 mg pyridoxine HCL, 25 mg nicotinic acid, 54.35 mg calcium D-pantothenate, 750 mg myo-inositol, 0.38 mg D-biotin, 2.5 mg folic acid, 0.03 mg cyanocobalamin, 6350 IU retinol acetate, 2800 IU cholecalciferol, 100 IU DL α-tocopherol acetate, 5 mg menadione sodium bisulphate, 100 mg Roche rovimix E50.
[b] Mineral mix (TMV4) to supplied per kilogram of feed:117mg $CuSO_4.5H_2O$, 7.19 mg KI, 1815 mg $FeSO_4.7H_2O$, 307 mg $MnSO_4.H_2O$, 659 mg $ZnSO_4.7H_2O$, 3.29 mg Na_2SeO_3, 47.7 mg $CoSO_4.7H_2O$
[c] L-Ascorbyl-2-polyphosphate (Stay-C, Roche Vitamins Australia, French Forest, NSW, Australia).

4.3.2 Growth experiment

The experiment was conducted at the School of Aquaculture, University of Tasmania (Launceston, Tasmania, Australia.). Atlantic salmon (*Salmo salar* L.*)* parr were obtained from Wayatinah Salmon hatchery (SALTAS, Tasmania, Australia) and randomly stocked into twelve 300 L tanks at 24 fish per tank where they were acclimated for 14 days. The tanks were held at a average temperature of 14.8^{o}C and natural spring photoperiod. The fish were held in a freshwater partial recirculation system (Bransden, et al., 2003).

Water was treated through physical, UV and biofilters, with a continuous replacement of approximately 15% per day. DO, pH, ammonia, nitrate, nitrite, and chlorine were monitored daily to ensure water quality remained within parameters recommended for Atlantic salmon (Wedemeyer, 1996). The experiment was conducted in accordance with the University of Tasmania Animal Ethics guidelines (Investigation A0007719).

At the start of the experiment fish were anaesthetized (50 mg L^{-1}, benzocaine), weight and length measured, and four fish were killed to measure initial lipid content and composition. Samples of red (~ 0.7 g) and white (~ 1.3 g) muscle, dissected from below the dorsal fin, liver and gill arch (~ 0.6 g) were frozen at -80°C until analysis. The four diets were fed in triplicate at a ration of 1.8% body weight

per day (% BW d^{-1}) in two equal feeds at 0900 and 1700 by automatic belt feeders. Every three weeks all fish were anaesthetized (50 mg L^{-1}, benzocaine) and batch-weighed. Fish were starved the day prior to weighing. Every 7 days the total feed consumption (kg DM) was estimated from the amount of uneaten feed in Guelph-type sediment collectors (Helland, et al., 1996; Carter, et al., 2003a).

At the end of the experiment (day 63) fish were starved for one day prior to being anaesthetized (50 mg L^{-1}, benzocaine) and their weight and length measured. Three fish per tank were killed by a blow to the head after immersion in anaesthetic and red and white muscle, liver and gill samples were taken as described above. Blood for osmolarity testing was taken from three fish per tank with a syringe from the caudal vein behind the anal fin.

On day 63 a 14 day post trial saltwater challenge occurred on the remaining 21 fish per tank. Over 72 hours freshwater was replaced with seawater to 32 ppt. On day 77, three fish per tank were anaesthetized, killed and blood taken below the anal fin. Blood was centrifuged at 600 rpm for 5 min and plasma osmolarity was tested on a Vapro 5250 vapour pressure osmometer.

Specific growth rate (SGR) was calculated as SGR (% day^{-1}) = 100 x (ln (W$_f$/W$_i$)) x d^{-1} where W$_f$ and W$_i$ are the final and initial weights (g) and d is the number of days (63 days) of the experiment. Total feed consumption was calculated as the total amount (g DM) consumed over 63 days. The feed consumption (FC) was calculated as the total feed consumption (g DM)/ Σ individual mid weight (g)/ 63 days. The feed efficiency ratio (FER) was calculated as FER (gg^{-1}) = total weight gain (g)/ FC (g). The hepatosomatic index (HSI) was calculated as HSI (%BW) = 100 x (liver weight (g WW) / total body weight (g WW)). The diets included

yttrium oxide (10 g kg^{-1}) as a digestibility marker. On day 58 and 59, faecal samples from all tanks were collected from the sediment collectors between 1100-1700 and 1900-0900 h, freeze-dried and used in the analysis of digestibility (Carter, et al., 2003a). The apparent digestibility coefficients (ADC) were calculated using the standard formula ADC (%) = 100 − [100((Y_{diet}/Y_{faeces})x ((FA_{faeces}/FA_{diet}))] where Y is percentage of yttrium oxide and FA is the % of particular fatty acids (Maynard and Loosli, 1969).

4.3.3 Lipid extraction and isolation

Samples were freeze dried and extracted using a modified Bligh and Dyer protocol (Bligh and Dyer, 1959). This involved a single phase overnight extraction, $CHCl_3/MeOH/H_2O$ (1:1:0.9, by vol), followed by phase separation to yield a total lipid extract (TLE).

Lipid classes were analysed by an Iatroscan MK V thin-layer chromatography-flame ionization detector (TLC-FID) analyser (Iatron Laboratories, Japan). Samples were spotted onto silica gel SIII Chromarods (5 μm particle size) and developed in a glass tank lined with pre-extracted filter paper. The solvent system used for the lipid separation was hexane: diethyl ether: acetic acid (60:17:0.1, v/v/v) (Volkman and Nichols, 1991). After development for 25 min, the chromarods were oven-dried and analysed immediately to minimise adsorption of atmospheric contaminants. Lipid classes were quantified by DAPA software (Kalamunda, WA, Australia). The FID was calibrated for each compound class: phosphatidylcholine, cholesterol, cholesteryl ester, oleic acid; hydrocarbon (squalene), wax ester (derived from orange roughy oil), triacylglycerol (derived from fish oil), and diacylglycerol ethers (DAGE) (purified from shark liver oil).

An aliquot of the TLE was trans-methylated in methanol/chloroform/ hydrochloric acid (10:1:1, by vol) for 1 hour at 100°C. After addition of water, the mixture was extracted three times with hexane: chloroform (4:1, v/v) to obtain fatty acid methyl esters (FAME) which are concentrated under nitrogen. Samples were made up to a known volume with an internal injection standard (19:0 FAME) and analysed by gas chromatography (GC) using an Agilent Technologies 6890N GC (Palo Alto, California, USA) equipped with an HP-5 cross-linked methyl silicone fused silica capillary column (50 m × 0.32 mm i.d.) and an FID. Helium was used as the carrier gas. Samples were injected, by a split/splitless injector and an Agilent Technologies 7683 Series auto sampler in splitless mode, at an oven temperature of 50°C. After 1 min the oven temperature was raised to 150°C at 30°C min^{-1}, then to 250°C at 2°C per min and finally to 300°C at 5°C min^{-1}. Peaks were quantified by Agilent Technologies GC ChemStation software (Palo Alto, California, USA). FA separation on the HP5 column is useful for measuring unusual VLC-PUFA ($\geq$ C24) and sterols, both of which are not eluted on polar columns.

Individual component identification was confirmed by mass spectral data and by comparing retention time data with those obtained for authentic and laboratory standards. GC results are typically subject to an error of ±5% of individual component area. GC-mass spectrometric (GC-MS) analyses were performed on a Finnigan Thermoquest GCQ GC-mass spectrometer fitted with an on-column injector and using Thermoquest Xcalibur software (Austin, Texas, USA). The GC was fitted with a capillary column similar to that described above.

4.3.4 Chemical analysis

Standard methods were used to determine dry matter (freeze dry to constant weight); crude fat (Bligh and Dyer, 1959); nitrogen (Kjeldahl using a selenium catalyst; crude protein was calculated as N x 6.25) and energy (bomb calorimeter, Gallenkamp Autobomb, calibrated with benzoic acid).

4.3.5 Statistical analysis

Mean values are reported as plus or minus the standard error of the mean. Normality and homogeneity of variance were confirmed and percentage data were arcsin transformed prior to analysis. Comparison between means was by 1-way analysis of variance (ANOVA) followed by multiple comparisons using Tukey-Kramer HSD. Significance was accepted at probabilities of 0.05 or less. Statistical analysis was performed using SPSS for Windows version 11.

4.4 RESULTS

4.4.1 Growth results

There was a significant (p<0.01) difference in the final weight of the FO fed fish

(here after termed FO fish and similarly for the other treatments) compared with the

MX fish (Table 4.1). Diet had no significant effect on the weight gain, specific

growth rate (SGR), feed consumption (FC), feed efficiency ratio (FER),

hepatosomatic index (HSI) or survival (Table 4.2). Over the 63 days, weight of fish

approximately doubled and the FER was less than 1 (Table 4.2).

**Table 4.2: Growth and efficiencies of Atlantic salmon fed experimental
diets with palm oil (PO), thraustochytrid oil (TO), 4:1 PO:TO
(MX) and fish oil (FO) (mean ± SE).**

	Feed											
	PO			MX			TO			FO		
Initial weight (g)	40.0	±	1.1	38.5	±	1.7	38.8	±	0.8	38.6	±	1.1
Final weight (g)	78.4	±	1.8[a,b]	74.5	±	2.6[a]	80.0	±	1.0[a,b]	83.1	±	3.2[b]
Weight gain (g)	38.4	±	2.0	36.0	±	2.0	41.2	±	1.1	44.5	±	2.4
Total feed consumption (g DM)	1603.7	±	44.6	1576.5	±	45.3	1639.6	±	30.9	1549.5	±	34.4
SGR (% day^{-1})	1.14	±	0.63	1.04	±	0.04	1.17	±	0.02	1.16	±	0.03
FC (mg DM g^{-1} d^{-1})	19.5	±	0.3	20.3	±	0.4	19.9	±	0.4	18.7	±	0.3
FER (g/g DM)	0.55	±	0.02	0.53	±	0.04	0.58	±	0.03	0.66	±	0.04
HSI (%)	0.76	±	0.03	0.68	±	0.04	0.72	±	0.04	0.73	±	0.04
Survival	97.2	±	2.8	98.6	±	1.4	98.6	±	1.4	97.2	±	0.0

DM, Dry matter
SGR, Specific growth rate = 100 x (ln (Wfinal(g)/Winitial(g))) x number of days (d)-1
FC, feed consumption = Total feed consumption (g DM)/ Σ individual mid weight (g)/ 63 days.
FER, feed efficiency ratio = total weight gain (g)/ total feed consumption (g DM).
HSI, hepatosomatic index = 100 (liver weight (g WW) / Total body weight (g WW)).
Survival during growth experiment. [a,b] Mean values across the row not sharing a common superscript were significantly different as determined by Tukey-Kramer HSD; df =4,260, p<0.01.

4.4.2 Lipid class composition

There was no significant difference in the lipid class composition between the two muscle types for fish fed the four diet treatments (Table 4.3, 4.4). The dominant lipid class was TAG in both red muscle (95.8 - 97.2%) and white muscle (50.8 - 57.8%).

4.4.3 Fatty acid (FA) composition

There were significant ($p<0.01$) differences between the FA profiles of the red and white muscle for the four diet treatments (Table 4.3, 4.4). The percentage concentrations of DHA were significantly increased in both the red and white muscle in the TO fish compared to the other diets (Table 4.3, 4.4).

The TO fish also had increased arachidonic acid (ARA, $20:4\omega6$) and DPA-6. This led to significantly increased concentrations of total $\omega3$, total $\omega6$ and total PUFA in the TO fish as well as a significantly lower percentage of total MUFA in particular oleic acid (OA, $18:1\omega9c$).

The FO and the initial fish had a very similar FA profile. Compared to the other diets, the FO and initial fish had significantly increased concentrations of EPA and docosapentaenoic acid (DPA-3, $22:5\omega3$) in both red and white muscle. The MX fish had fish had identical concentrations of DHA to the FO fish in white muscle tissues but significantly decreased concentrations in the red muscle.

Table 4.3: Lipid content and FA and lipid class composition (as percent of total) of red muscle samples of Atlantic salmon fed palm oil (PO), 4:1 mix of palm oil: thraustochytrid oil (MX), thraustochytrid oil (TO) and fish oil (FO) diets

FA	Initial	±	SE	PO	±	SE	MX	±	SE	TO	±	SE	FO	±	SE	f
14:0	6.3	±	0.6	3.6	±	0.1[a]	4.1	±	0.0[b]	5.8	±	0.13[c]	5.6	±	0.1[c]	130.9
16:0	12.5	±	4.2	20.0	±	0.2[c]	20.6	±	0.2[c]	18.2	±	0.3[b]	16.7	±	0.1[a]	667.7
18:0	4.4	±	0.1	4.5	±	0.1[b]	4.3	±	0.0[b]	3.4	±	0.1[a]	4.2	±	0.1[b]	34.7
Other SFA[e]	2.7	±	0.0	1.3	±	0.0[a]	1.4	±	0.0[a]	1.5	±	0.0[a]	2.6	±	0.0[b]	214.1
Total SFA	25.8	±	1.0	29.4	±	0.2	30.4	±	0.2	28.9	±	0.3	29.1	±	0.3	
16:1ω7c	8.0	±	0.6	4.7	±	0.1[a]	4.6	±	0.1[a]	4.3	±	0.1[a]	7.4	±	0.1[b]	180.7
18:1ω9c[f]	17.8	±	0.4	26.5	±	0.7[d]	24.4	±	0.3[c]	12.2	±	0.4[a]	16.3	±	0.3[b]	223.6
18:1ω7c	3.9	±	0.2	2.6	±	0.1[b]	2.5	±	0.0[b]	2.3	±	0.1[a]	3.3	±	0.0[c]	89.6
20:1ω9c	3.3	±	0.0	2.4	±	0.1	2.2	±	0.0	2.0	±	0.1	2.1	±	0.3	
Other MUFA[g]	6.0	±	0.0	3.3	±	0.0[a]	3.4	±	0.0[a,b]	3.3	±	0.0[a]	5.0	±	0.0[b]	129.1
Total MUFA	38.9	±	0.7	39.4	±	0.5[d]	37.1	±	0.3[c]	24.0	±	0.6[a]	34.0	±	0.1[b]	230.9
18:4ω3	2.5	±	0.2	1.6	±	0.1[a]	1.5	±	0.0[a]	1.5	±	0.1[a]	2.7	±	0.0[b]	163.7
20:4ω3	1.3	±	0.0	0.9	±	0.0[a]	0.8	±	0.0[a]	1.1	±	0.0[b]	1.2	±	0.0[c]	68.5
20:5ω3	7.4	±	0.7	4.3	±	0.2[a]	4.1	±	0.1[a]	4.6	±	0.1[a]	7.6	±	0.1[b]	58.1
22:5ω3	3.1	±	0.2	2.0	±	0.1[a,b]	1.9	±	0.1[a]	2.2	±	0.1[b]	3.3	±	0.0[c]	82.1
22:6ω3	13.2	±	0.5	9.8	±	0.4[a]	11.3	±	0.2[b]	23.4	±	0.4[d]	12.8	±	0.2[c]	405.6
Other ω3[h]	0.6	±	0.0	0.4	±	0.0[a]	0.3	±	0.0[a]	0.3	±	0.0[a]	0.7	±	0.0[b]	115.7
Total ω3	28.2	±	1.1	18.9	±	0.7[a]	19.9	±	0.4[a]	33.1	±	0.2[c]	28.2	±	0.4[b]	228.8
18:2ω6	3.1	±	0.2	8.1	±	0.2[c]	8.0	±	0.2[c]	3.9	±	0.1[a]	4.5	±	0.1[b]	292.5
18:3ω6	0.2	±	0.0	0.4	±	0.0[b]	0.2	±	0.0[a]	0.2	±	0.0[a]	0.2	±	0.0[a]	191.1
20:2ω6	0.3	±	0.0	0.7	±	0.0[c]	0.7	±	0.0[c]	0.3	±	0.0[a]	0.4	±	0.0[b]	153.9
20:3ω6	0.2	±	0.0	0.6	±	0.0[c]	0.3	±	0.0[b]	0.3	±	0.0[b]	0.2	±	0.0[a]	147.7
20:4ω6	0.8	±	0.1	0.7	±	0.0[a]	0.7	±	0.0[a]	1.6	±	0.0[b]	0.7	±	0.0[a]	334.7
22:5ω6	0.3	±	0.0	0.5	±	0.0[a]	1.3	±	0.0[b]	6.0	±	0.2[c]	0.3	±	0.0[a]	450.9
Other ω6[i]	0.2	±	0.0	0.2	±	0.0[a]	0.2	±	0.0[a,b]	0.4	±	0.0[b]	0.2	±	0.0[a,b]	115.6
Total ω6	5.0	±	0.2	11.1	±	0.2[b]	11.4	±	0.2[b]	12.6	±	0.4[c]	6.5	±	0.1[a]	118.7
Other PUFA[j]	2.1	±	0.0	1.2	±	0.0[a]	1.2	±	0.0[a]	1.5	±	0.0[a]	2.3	±	0.0[b]	42.1
Total PUFA	35.3	±	1.2	31.2	±	0.5[a]	32.5	±	0.4[a]	47.1	±	0.6[c]	36.9	±	0.4[b]	217.2
Ratio																
ω3/ω6	5.6	±	0.1	1.7	±	0.1[a]	1.8	±	0.1[a]	2.6	±	0.1[b]	4.4	±	0.1[c]	324.7
Lipid Class																
TAG	84.6	±	3.7	95.9	±	0.4	96.4	±	0.3	95.8	±	0.7	97.2	±	0.2	
FFA	4.2	±	1.2	1.1	±	0.1	1.0	±	0.1	1.1	±	0.1	0.8	±	0.1	
ST	2.6	±	0.6	0.8	±	0.1	0.8	±	0.1	0.8	±	0.1	0.7	±	0.1	
PL	8.7	±	3.3	2.2	±	0.2	1.8	±	0.1	2.3	±	0.1	1.3	±	0.1	
mg/g Wet[k]	213.0	±	1.5	238.9	±	1.5	220.5	±	1.4	236.8	±	2.5	252.7	±	2.2	
mg/g Dry[k]	523.5	±	6.3	566.2	±	2.1	548.7	±	2.2	539.4	±	4.7	559.6	±	2.8	

SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids;TAG; Triacylglycerol; FFA, Free fatty acid; ST, Sterol; PL, Polar lipid; f, Mean sum of squares.

[a,b,c,d] Mean values across the row not sharing a common superscript were significantly different as determined by Tukey-Kramer HSD; df =4,40, $p<0.001$

[e] Other SFA include 15:0, 17:0, 20:0, 22:0 and 24:0

[f] Includes 18:3ω3

[g] Other MUFA include 16:1ω9, 16:1ω5, 18:1ω5, 20:1ω7, 22:1ω9, 22:1ω11 and 24:1ω9

[h] Other ω3 PUFA include 21:5ω3 and 24:6ω3

[i] Other ω6 PUFA include 20:2ω6, 20:3ω6, 22:4ω6 and 24:5ω6

[j] Other PUFA include 16:2ω4, 16:3ω4 and 18:2ω9

[k] Determined by gravimetrically.

In both muscle tissues, the PO and the MX fish had significantly higher percentage of linoleic acid (LA 18:2 ω6) compared to the other two diets and the initial fish. The PO and MX fish also had a significantly increased percentage of total MUFA, particularly OA.

The ω3/ω6 ratio in both muscle tissues was significantly higher in the initial and the FO fish than for all other experimental diets. The red muscle of the TO fish had a significantly higher ω3/ω6 ratio than that of the PO and MX fish.

Table 4.4: Lipid content and FA and lipid class composition (as percent of total) of white muscle samples of Atlantic salmon fed palm oil (PO), 4:1 mix of palm oil: thraustochytrid oil (MX), thraustochytrid oil (TO) and fish oil (FO) diets

FA	Initial	±	SE	PO	±	SE	MX	±	SE	TO	±	SE	FO	±	SE	f
14:0	4.6	±	0.3	1.3	±	0.3[a]	1.0	±	0.2[a]	3.2	±	0.4[b]	3.3	±	0.3[b]	15.5
16:0	18.7	±	0.5	20.4	±	0.7[b,c]	21.3	±	0.6[c]	19.9	±	0.3[a,b]	18.4	±	0.4[a]	5.9
18:0	4.8	±	0.1	5.5	±	0.1[c]	5.5	±	0.1[c]	4.1	±	0.1[a]	5.0	±	0.1[b]	37.6
Other SFA[e]	2.1	±	0.0	0.6	±	0.0[a]	0.4	±	0.0[a]	0.8	±	0.0[a]	1.8	±	0.0[b]	38.1
Total SFA	30.2	±	0.4	27.8	±	0.8	28.3	±	0.6	27.9	±	0.6	28.4	±	0.5	
16:1ω7c	5.7	±	0.4	2.7	±	0.2[a]	2.4	±	0.3[a]	2.6	±	0.1[a]	5.4	±	0.2[b]	46.1
18:1ω9c[f]	14.1	±	0.9	24.0	±	1.4[c]	21.7	±	1.2[c]	9.6	±	0.5[a]	14.2	±	0.6[b]	45.9
18:1ω7c	3.4	±	0.1	2.3	±	0.1[a]	1.8	±	0.0[a]	1.7	±	0.2[a]	3.3	±	0.1[b]	16.5
20:1ω9c	2.4	±	0.2	1.9	±	0.1[c]	1.1	±	0.3[a,b]	1.2	±	0.2[b]	0.8	±	0.3[a]	4.6
Other MUFA[g]	4.6	±	0.0	2.0	±	0.0[b]	1.3	±	0.0[a]	2.0	±	0.0[b]	4.2	±	0.0[c]	33.4
Total MUFA	30.2	±	1.1	32.9	±	1.6[c]	28.2	±	1.3[b]	17.0	±	0.4[a]	27.7	±	0.9[b]	35.4
18:4ω3	1.8	±	0.1	1.0	±	0.0[a]	0.9	±	0.1[a]	1.0	±	0.0[a]	2.2	±	0.1[b]	108.6
20:4ω3	1.1	±	0.1	0.7	±	0.0[a,b]	0.5	±	0.1[a]	0.9	±	0.0[b]	1.1	±	0.0[c]	17.3
20:5ω3	7.5	±	0.3	4.9	±	0.3[b]	4.7	±	0.2[a,b]	4.1	±	0.1[a]	8.3	±	0.2[c]	95.7
22:5ω3	3.1	±	0.1	2.1	±	0.1[a]	1.9	±	0.1[a]	2.0	±	0.1[a]	3.6	±	0.1[b]	130.7
22:6ω3	20.1	±	2.1	18.2	±	1.9[a]	23.0	±	1.2[a]	33.1	±	0.9[b]	20.4	±	1.2[a]	19.0
Other ω3[h]	0.5	±	0.0	0.2	±	0.0[a]	0.1	±	0.0[a]	0.2	±	0.0[a]	0.5	±	0.0[b]	30.2
Total ω3	34.0	±	1.5	27.0	±	2.1[a]	31.0	±	1.9[a,b]	41.2	±	0.9[c]	36.1	±	1.2[b]	14.1
18:2ω6	2.3	±	0.1	7.6	±	0.4[b]	7.2	±	0.3[b]	3.2	±	0.1[a]	3.9	±	0.1[a]	90.6
18:3ω6	0.1	±	0.0	0.4	±	0.0[c]	0.1	±	0.0[b,c]	0.1	±	0.0[a]	0.2	±	0.0[a,b]	35.9
20:2ω6	0.4	±	0.1	0.7	±	0.0[c]	0.6	±	0.2[b,c]	0.2	±	0.0[a]	0.4	±	0.1[a,b]	11.3
20:3ω6	0.0	±	0.0	0.9	±	0.1[c]	0.4	±	0.1[b]	0.3	±	0.0[a,b]	0.2	±	0.0[a]	53.9
20:4ω6	0.9	±	0.1	1.2	±	0.1[b]	1.2	±	0.1[b]	1.9	±	0.0[c]	0.9	±	0.0[a]	49.1
22:5ω6	0.4	±	0.0	1.1	±	0.1[b]	2.6	±	0.2[c]	7.0	±	0.2[d]	0.4	±	0.0[a]	407.6
Other ω6[i]	0.2	±	0.1	0.2	±	0.0[b]	0.1	±	0.0[b]	0.4	±	0.1[b]	0.2	±	0.0[a,b]	7.3
Total ω6	4.3	±	0.0	11.9	±	0.4[b]	12.1	±	0.3[b]	13.1	±	0.3[b]	6.1	±	0.2[a]	87.5
Other PUFA[j]	1.4	±	0.0	0.4	±	0.0[a]	0.3	±	0.0[a]	0.8	±	0.0[a]	1.7	±	0.0[b]	22.0
Total PUFA	39.7	±	1.5	39.3	±	2.0[a]	43.5	±	1.8[a]	55.0	±	0.9[b]	43.8	±	1.1[a]	189
Ratio																
ω3/ω6	8.0	±	0.4	2.3	±	0.2[a]	2.6	±	0.2[a]	3.2	±	0.1[a]	6.0	±	0.4[b]	38.0
Lipid Class																
TAG	51.4	±	11.9	55.7	±	5.4	50.8	±	6.8	58.1	±	3.7	57.5	±	8.1	
FFA	12.9	±	4.0	2.4	±	0.3	3.0	±	0.7	2.7	±	0.4	2.0	±	0.3	
ST	2.1	±	0.4	1.9	±	0.2	1.7	±	0.2	1.6	±	0.3	10.5	±	8.6	
PL	33.6	±	9.9	40.0	±	5.1	44.5	±	6.8	37.7	±	3.8	29.9	±	6.3	
mg/g Wet[k]	10.6	±	0.6	16.0	±	0.3	12.9	±	0.3	16.1	±	0.1	16.5	±	0.3	
mg/g Dry[k]	66.6	±	1.3	64.4	±	1.2	51.5	±	1.0	65.9	±	0.6	69.2	±	1.1	

Abbreviations and other footnote definitions, see Table 4.3.

4.4.4 Digestibility

The lipid digestibility of the four diets was in the order ω3 PUFA ≥ ω6 PUFA > monounsaturated FA (MUFA) > saturated FA (SFA) (Table 4.5). The ω3 PUFA had apparent digestibilities of 90.9 to 96.5 and there was no difference between diets in the digestibility of EPA, DHA or total ω3 PUFA. There was a significant difference between the apparent digestibility of the total ω6 PUFA between the FO and both the PO and MX diets. MUFA digestibility showed a significant (p<0.01) difference between the TO (74.6) and the other diets (87.4-91.0). SFA had the lowest apparent digestibility across all diets (71.2 – 77.6). There was a significant difference in the SFA digestibility between the PO (77.6) and both the MX (71.4) and the TO (71.2) diets.

Table 4.5: Apparent digestibility coefficients (ADC) for different fatty acid fractions for diets containing different oil sources

Sample	PO		SE	MX		SE	TO		SE	FO		SE	Sig	f
Total SFA	77.6	±	2.8b	71.4	±	2.4a	71.2	±	0.2a	76.3	±	4.1a,b	0.02	12.9
Total MUFA	91.8	±	1.8b	91.4	±	1.4b	74.6	±	1.2a	87.4	±	2.8b	0.00	51.9
20:5ω3 EPA	n.d.	±	n.d.	93.4	±	1.0	90.9	±	1.0	96.3	±	7.9		
22:6ω3 DHA	96.7	±	1.2	96.4	±	3.3	97.1	±	5.8	95.6	±	4.3		
Total ω3 PUFA	96.5	±	0.2	96.0	±	0.2	96.5	±	1.2	96.0	±	0.7		
18:2ω6 LA	99.0	±	1.5	99.0	±	1.1	99.9	±	0.7	98.9	±	1.3		
22:5ω6 DPA-6	n.d.	±	n.d.	96.5	±	2.4	96.9	±	2.4	n.d.	±	n.d.		
Total ω6 PUFA	95.2	±	0.1b	95.2	±	0.6b	93.4	±	0.4a,b	92.1	±	0.3a	0.05	9.5

TO, Thraustochytrid oil DHASCO®-S from Martek; PO, palm oil; MX, 4:1 mix of palm oil and thraustochytrid oil; FO, fish oil, SFA, Saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; LA, Linoleic acid.
[a,b,c] Mean values across the row not sharing a common superscript were significantly different as determined by Tukey-Kramer HSD; df =4,16,

4.4.5 Saltwater challenge

There was a significant (p<0.01) difference in the osmolarity of the FO and TO fish compared with the PO fish prior to the saltwater challenge on day 63 of the trial (Figure 4.1). There was no difference in the blood osmolarity between the four diets after 2 weeks (day 77) in 32 ppm seawater. There was no difference in survival, feed consumption or growth during the seawater challenge between dietary treatments.

4.5 DISCUSSION

The present study demonstrated the potential of oils from a single cell source for inclusion into Atlantic salmon feeds. Single cell oils represent a source of renewable lipid that is high in essential fatty acids (EFA). Currently commercial Atlantic salmon feeds rely on fish oil from wild fish stocks, vegetable oil and other oil sources to provide EFA with vegetable oils not containing the longer chain (C≥20) EFA. Single cell oils such as thraustochytrid oil, although currently considered uneconomical to use in aquaculture grow-out, show great potential to provide a renewable source of EFA. Single cell organisms such as microalgae, including thraustochytrids, diatoms and other groups can produce high concentrations of ω3 LC-PUFA. Thraustochytrids can synthesise ω3 LC-PUFA via the polyketide synthases (PKS) pathway which can produce high concentrations of DHA (Metz, et al., 2001). DHA is then bio-accumulated up the food web to larger vertebrates such as salmon. Cultures of these single cell organisms could provide a reliable renewable source of ω3 LC-PUFA in particular DHA.

Thraustochytrids are already used as live feeds for rotifers (*Brachionus spp)* and brine shrimp (*Artemia)* as a source or DHA during a critical phase of the production cycle and show potential for feeds for aquaculture (Barclay and Zeller, 1996; Nichols, et al., 1996; Lewis, et al., 1998). Being heterotrophic they can be fermented to produce large amounts of biomass. Fermentation on a large scale can produce biomass with a high percentage of lipid, and of that lipid, a high proportion is ω3 LC-PUFA (Lewis, et al., 1999). With the ability to strain select and optimise growth conditions, the use of different thraustochytrid species could be adapted to suit different aquaculture requirements. Some strains of single cell organisms have characteristics such as high DHA (up to 60% of FA), low ω6 levels, or high ω3 LC-PUFA, and high ω3/ω6 ratios (Nichols, et al., 2004). These features could be advantageous to different production/life cycle stages of salmonoids and other aquaculture species that include broodstock, larvae and production fish. The extraction of oil from single cell biomass increases production costs and influences whether it is used in commercial aquafeeds. However, compared to the isolated single cell oil, the biomass may be a more affordable and readily available source of oil and EFA in addition to marine protein.

There has been only one previous trial looking at feeding thraustochytrids to salmon (Carter, et al., 2003b). This previous trial looked at inclusion of thraustochytrid biomass at low concentration (10% thraustochytrid biomass). This inclusion equated to 4% of the protein component and 18% of the oil component of the diet, with the remainder of the oil supplied as canola oil. It was shown that feeding thraustochytrid biomass had no effect on growth and growth efficiency, or on measures of performance such as survival, immune response or nutrient

digestibility. Replacement of fish oil with 100% thraustochytrid oil in this study showed that there was no effect compared to fish oil on growth or feed efficiencies, or on any measurement of fish performance such as survival, nutrient digestibility or osmolality. The major difference between treatments in this trial was in the FA profile of the Atlantic salmon, in particular the EFA including both the ω3 and ω6 LC PUFA.

This is the first experiment on Atlantic salmon to use a single cell oil as the sole oil source. It is also, to my knowledge, the highest percentage of DHA ever fed to salmon in a nutrition experiment albeit in a parr diet. Salmon diets are required to provide EFA through a number of fatty acids belonging to the ω3 and ω6 families. Failing to do this causes the fish to suffer reduced growth and feed utilisation potentially leading, in extreme cases, to the appearance of deficiency symptoms (Tacon, 1996). Marine fish and anadromous fish in their marine phase, such as Atlantic salmon, require ω3 LC-PUFA in particular EPA and DHA as well as ARA as their elongation and desaturation capacity is insufficient to meet their needs from α-linolenic (ALA 18:3ω3) or LA alone. Commercial aquafeeds have used blends of vegetable oils such as canola, soybean, linseed and palm oil, with fish and other oils. Due to commercial and availability considerations, these oil blends are used by feed companies to ease the pressure on fish stocks, to maintain nutritional requirements, to optimise digestibility and importantly to reduce the price of the feed.

Palm oil (PO) was used as the negative control as it is deficient in ω3 PUFA and is a rich source of 16:0 and 18:1ω9. It has low levels of ω6 PUFA such as 18:2ω6 and does not contain any ω3 LC-PUFA in particular DHA. Due to increasing production of PO it is predicted that within 10 years it being the world's most

abundant vegetable oil and therefore considered as a potentially cheap source of oil for aquaculture (Gunstone, 2001). It has been shown that PO can substitute for FO in Atlantic salmon feed with no apparent negative effect on growth, feed conversion, or fish health (Bell, et al., 2002). However, a high inclusion of PO significantly reduces muscle EPA, DHA and the $\omega 3/\omega 6$ ratio (Bell, et al., 2002). Our study also followed these trends with reduced EPA, DHA and $\omega 3/\omega 6$ ratio observed in the PO fish. In contrast the FO fish had a similar FA profile to the initial fish that had been fed a fish oil based feed prior to the experiment.

The MX diet was formulated with a 1:4 inclusion of thraustochytrid oil and palm oil in order to achieve a comparable percentage of DHA (6.7% in MX) to the FO (7.5%) diet. The DHA concentration of the MX diet was 9.8 g kg^{-1} DM which is similar to the 11.1 g kg^{-1} DM of the FO diet. The TO diet had elevated concentrations (53.5 g kg^{-1} DM), while the PO had very little DHA (1.1 g kg^{-1} DM). This resulted in large differences in the concentrations of DHA in the red and white muscle in the PO fish (Tables 3,4). The TO fish had double the percentage of DHA than for the PO and MX fish and almost double that of the FO fish in the red muscle. The MX fish had statistically comparable concentrations of DHA in both muscle tissues to the FO fish. Therefore the partial inclusion of thraustochytrid oil can give comparable concentrations of DHA in muscle tissues. This experiment shows that salmon can incorporate high concentrations and relative proportions of dietary DHA and store it as TAG without detriment to their growth.

It has been reported that, under experimental conditions, increased concentrations of $\omega 3$ LC-PUFA have shown a deleterious effect on fish survival after infection (Erdal, et al., 1991). In that study the increased dietary $\omega 3$ LC-PUFA

exerted immunosuppressive action on the experimental salmon, which resulted in
fish exhibiting a significantly lower survival and lower antibody levels after
vaccinations (Erdal, et al., 1991). It was thought that changes in the membrane
structure and fluidity due to increased ω3 LC-PUFA incorporation increased
erythrocyte cell wall strength and influenced the immune function (Erdal, et al.,
1991). However, fish lacking dietary ω3 LC-PUFA, in particular the ω3/ω6 ratio,
may be more prone to illnesses and disease under conditions of stress (Tacon, 1996).
It is important to note that ω3 LC-PUFA are precursors in the synthesis of
eicosanoids, which in turn are mediators in inflammatory reactions and are also
involved in the regulation of the immune response (Dias, 1995). Our results, for
Atlanitc salmon parr, show that there was no difference in survival or growth
performance between high or very low concentrations of dietary ω3 LC-PUFA.

4.5.1 Digestion

Atlantic salmon can absorb fatty acids (FA) selectively during digestion
(Johnsen, et al., 2000). Digestibility of FA increases as their unsaturation increases
so that SFA have the lowest digestibility, then MUFA, with PUFA being absorbed
best by fish (Johnsen, et al., 2000). Our results showed the same pattern with the
apparent digestibility coefficient being greatest for the most unsaturated FA in the
order of ω3 PUFA ≥ ω6 PUFA > MUFA > SFA (Table 4.5). Feeding DHA at high
(53.5 g kg^{-1} DM) amounts had no effect on the ability of the fish to digest this FA.
This indicates that high concentrations of DHA can be fed to salmon and
digestibility is not reduced. TO fish had significantly lower apparent digestibility of
total MUFA which may have been due to ω3 LC-PUFA, in particular DHA, being
preferably absorbed by the digestive tract and used/transferred to cell membranes,

stored in TAG or used for energy. Dietary oils containing saturated fatty acids primary in the *sn*-1 and *sn*-3 positions such as PO, can have different biological consequences such as their ability to be absorbed and used through digestion (Decker, 1996). The ability of salmon to digest a particular FA is influenced not only by nutritional, but also environmental factors such as temperature (Ng, et al., 2003; Zheng, et al., 2005). The apparent digestibility coefficient of total saturated fat in the PO fish was not statistically different to FO fish and significantly greater than for the MX and TO fish. Therefore no differences was observed due to regiospecificity on the adsorption of PO saturated fats; this would be due to TAG being hydrolysed to free fatty acids in the gastrointestinal tract prior to their absorption in salmon.

4.5.2 Smoltification

Smoltification is an important stage in the lifecycle of Atlantic salmon, with freshwater parr going through complex biological and physical transformations to become saltwater smolt (Craig Clark, 2000). Plasma osmolality is a measure of the concentration of ions in blood and a useful measurement of whether a Atlantic salmon parr is ready to smolt adapt to salt water conditions (Craig Clark, 2000). The ability of anadromous fish to smolt is very important at this parr stage of their life cycle.

Parr that are not adapted to marine conditions can, during saltwater transfer, suffer high mortalities with survivors exhibiting stunted growth (Duston, 1994). Smolt have the ability to osmoregulate adequately to maintain body salt levels at or slightly above baseline freshwater levels. Parr that have not quite completed the transformation have greater difficulty in regulating body salt levels and their

corresponding plasma osmolarity or sodium levels will be much higher. Fish

nutrition, in particular lipid nutrition, can influence the ability to recover from

smolting as fat stores can deplete post transfer.
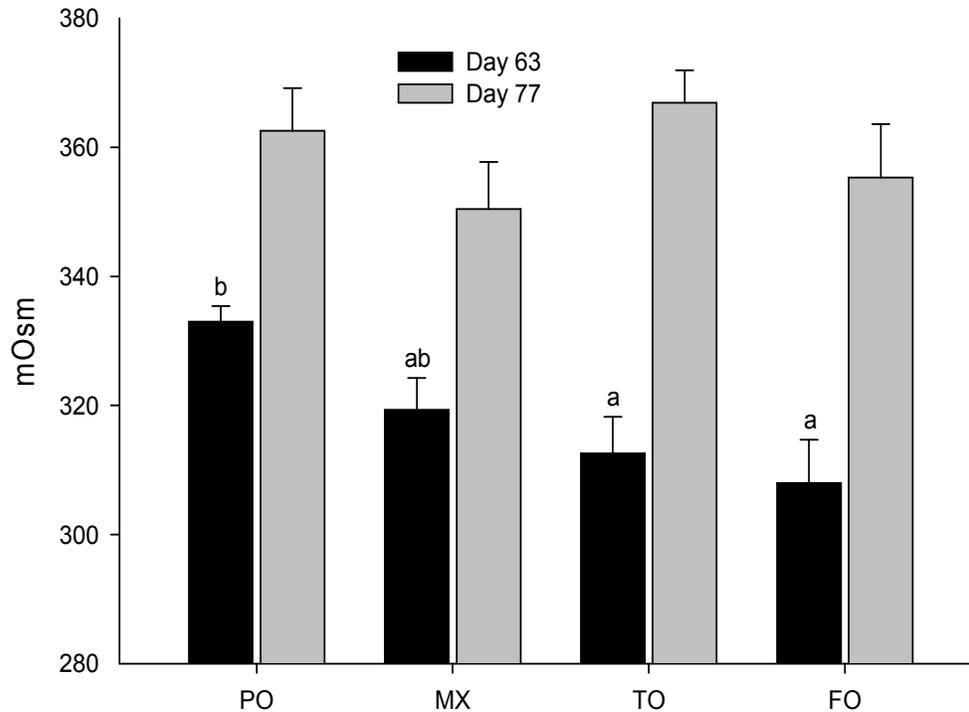
Figure 4.1: Blood osmolarity (mOsm) of Atlantic salmon on last day of trial (day 63) and 14 days later (day 77) after the salinity was raised from 0 to 32 ppm over four diets.
Each value is the mean (n=9) (±SE).
[a,b] Mean values across the row not sharing a common superscript were significantly different as determined by Tukey-Kramer. TO, Thraustochytrid oil DHASCO®-S from Martek; PO, palm oil; MX, 4:1 mix of palm oil and thraustochytrid oil; FO, fish oil.

Atlantic salmon smolt generally display an osmolarity below 320 mOsm and

levels of about 380-400 mOsm may indicate that the fish have not completed

smoltification or are still parr (Conte, 1969; Bonds, 1996). Lower blood osmolarity

in parr is an indication of a successful saltwater transfer. Our results indicate that at

the 63rd day the osmolarities of FO and TO fish were below 320 mOsm, and are significantly lower than that of the PO fish (333 mOsm). This finding indicates that TO and FO fish were more biologically advanced in their adaptation to the saltwater transfer. This result indicates that in this pre-smoltification stage of the salmon's life cycle, feeding a diet high in ω3 LC-PUFA, including DHA, increases their ability for saltwater transfer. DHA is not the only factor in driving osmolarity/smoltification and therefore this observation could be due to factors such as a higher ω3/ω6 ratio in the TO and FO fish compared to the PO fish.

4.5.3 ω3/ω6 ratio

Differences in dietary concentrations of ARA, EPA and the ω3/ω6 ratio may influence smolt physiology and health status via the complex effects of synthesis of various eicosanoids from ARA and EPA (Bell, et al., 1996). Tissue concentrations of ARA in the TO fish were most likely elevated due to retro-conversion of the high proportion of DPA−6 in the TO diet. The percentage of EPA was greatest in the muscle of FO fish, with the FO diet containing the highest dietary source of EPA. Coincidently, the TO fish had the lowest percentage of EPA in the muscle tissues. TO fish may be more likely to use ARA as a source for eicosanoid production compared to EPA in FO fish due to the high concentration of ARA in muscle derived from possible retro-conversion of DPA−6. There was no difference in the ARA/EPA ratio in any tissue, including liver and gill (data not shown) in the PO, MX or TO fish; therefore it is suggested that eicosanoid production via EPA or ARA did not affect the ability salmon have to smolt and the ω3/ω6 ratio may have a more significant role.

It has been increasingly recognised that that the ω3/ω6 ratio plays a vital role in human health (Gibson, et al., 1994; Goodstine, et al., 2003). It was estimated that the diet of paleolithic hunter-gatherers contained an approximated ω3/ω6 ratio of 1.29 (Eaton, et al., 1998). It has been estimated that the modern Australian diet has a very low ω3/ω6 ratio (0.13) and this is constant over age group or gender (Meyer, et al., 2003). It has been suggested (Meyer, et al., 2003) that the current situation needs to be addressed by increasing ω3 intake, reducing ω6 intake or a combination of both, resulting in a increase in this modern Australian ω3/ω6 ratio. Eating oily fish such as salmon is thought to be a good way to address this problem. However, if replacement oils contain low ω3 and high ω6 such as sunflower, palm and soybean oil, the fatty acid profile of salmon will reflect this and have a low ω3/ω6 ratio, making these dietary changes redundant. It is important to note that replacement oil fed salmon still remain a very valuable source of ω3 LC-PUFA. However further improvement, in particular to the ω3/ω6 ratio, can be achieved with ω3 rich diets. In this study, the initial and FO fish had the highest ω3/ω6 ratio as their diet contained a ω3/ω6 ratio of 3.26. The TO diet had a ω3/ω6 ratio of 2.04, due to high percentage of DHA together with a high percentage of DPA−6. Feeding thraustochytrid oil to salmon resulted in a ω3/ω6 ratio of 3.15 and 2.63 in white and red muscle, respectively. The ratio for white muscle (3.15) was significantly ($p<0.01$) different compared to the MX (2.56) and PO (2.26) fish. Strains of thraustochytrids with lower amounts of DPA−6 would be able to increase this ratio further making it similar to that of FO salmon. Although this experiment used parr and not production

size fish, these ω3/ω6 ratio findings are good indicators of the possible nutritional benefit of thraustochytrid enriched diets.

Recently there has been increased research on finishing diets (Bell, et al., 2003; Bell, et al., 2004; Torstensen, et al., 2005). Finishing diets increase the amount of EFA in particular EPA and DHA in the final weeks of fish life prior to harvest. The rationale behind this is that increased amounts of cheap and renewable vegetable and animal oil can be used in a blend during the growout period and the FA profile can be corrected by provision of high doses of EFA during the finishing period. However, salmon fed plant oil only partially restore concentrations of EPA and DHA, to 80% of the content of salmon fed a FO, when fed FO rich finishing diet for a period (16-20 weeks) prior to harvest (Bell, et al., 2003; Bell, et al., 2004; Torstensen, et al., 2005). It has not yet been shown that salmon can recover their ω3 LC-PUFA content to similar concentrations as those of fish fed a FO diet alone, although recovery is possible in other less oily species such as European sea bass (*Dicentrarchus labrax* L.) (Montero, et al., 2005; Mourente, et al., 2005). Although this study was on salmon parr, the results indicate that thraustochytrid oil or whole cell biomass may provide a good source of oil for these finishing diets as it provides elevated percentages of these EFA, in particular DHA in salmon.

At present the economics of using thraustochytrids as an oil is beyond the scope of a commercially viable ingredient for the aquaculture industry. Fish oil prices are increasing due to increased demand, decreasing wild fish stocks, uncertainty in the wild fish catch due to such climatic events as El Niño and a greater demand from nutraceutrical industries (Barlow, 2000; FAO, 2004). In comparison thraustochytrid oil/biomass prices may reduce in the future due to increased competition, the

discovery of new and better strains, increased knowledge of growth conditions and better fermentation techniques (Lewis, 2001). A major advantage of obtaining dietary ω3 LC-PUFA from single cell oils compared to fish oil is that there are no fat soluble xenobiotics such as dioxins and dioxin like PCB (poly-chlorinated bi-phenyls) which is problem with some fish oil sources (Jacobs, et al., 2002). With aquaculture feed companies using blends of oils in their diet formulations, thraustochytrid oil or thraustochytrid biomass may be used in small amounts to provide EFA when blended with vegetable oils. Currently there is no demand from industry to increase ω3 LC-PUFA content of commercial aquafeeds. However, with increased knowledge of the benefits of these important FA, a more health conscience market may occur and enhance the need for value-added (with the emphasis on the ω3 LC-PUFA) aquaculture products.

4.6 CONCLUSION

Our study shows that thraustochytrid oil can be used at high inclusion levels to in part or totally replace fish oil in the diets of Atlantic salmon parr without detriment to growth of fish. Thraustochytrid oil provides a renewable source of EFA for salmon feeds. Further longer duration experiments looking at thraustochytrid oil replacement with larger fish are needed with an emphasis on the effect of diet on the health of the fish, in particular innate immune responses and histopathology. Feeding thraustochytrid oil to salmon increased the amount of DHA and ω3 LC-PUFA in red and white muscle and, if maintained over the saltwater on-growing phase, would make them nutritionally beneficial to the human consumer.

4.7 ACKNOWLEDGEMENTS

4.8 REFERENCES

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CHAPTER 5

The digestibility and accumulation of dietary phytosterols in Atlantic salmon (*Salmo salar* L) smolt fed diets with replacement plant oils.

5.1 ABSTRACT

Phytosterols can occur in relatively high concentration in the oil of canola (*Brassica napus* L.) and in other vegetable oils such as oil from the borage plant Echium (*Echium plantagineum* L.). The study investigated how different naturally occurring abundances of phytosterols effect their digestion and accumulation in salmon muscle and liver. Phytosterols are known to have a cholesterol-lowering function and reduce the risk of coronary heart disease in humans. Phytosterols have also been shown to affect fish behaviour and sexual maturation, with reduced expression of secondary sexual characteristics of fish that are exposed to them via the environment. Three experimental diets containing canola oil (CO), Echium oil (SO) and fish oil (FO) were fed to Atlantic salmon smolt over 9 weeks. We demonstrated that the digestibility of natural abundances of phytosterols by Atlantic salmon was poor compared to cholesterol. However, phytosterols accumulated in both the liver and the white muscle of Atlantic salmon. There were significantly increased concentrations of 24-methylenecholesterol, 24-methylcholesterol, 24-ethylcholesterol and total phytosterol in the liver of SO fed fish compared to the FO fed fish. 24-Methylcholesterol concentrations were increased in the CO fed fish compared to those fed the FO diet. This study demonstrated that natural abundances of dietary phytosterols are digested by and accumulated in the liver and white muscle of Atlantic salmon smolt.

5.2 INTRODUCTION

Phytosterols, also known as plant sterols, is a general term given to a large number of plant-derived sterols found in legumes, seeds, fruits and vegetables (Moreau, et al., 2002; Ostlund, 2002). Phytosterols are natural, organic compounds with a molecular nucleus of 17 carbon atoms and a characteristic three-dimensional arrangement of 4 rings. Phytosterols can act as a structural component in the plant cell membrane, a role played in mammalian cells by cholesterol (Ostlund, 2002). Unlike cholesterol, which has a side chain of 8 carbon atoms, the side chain of phytosterols generally contains 9 or 10 carbon atoms with alkyl substitution at C_{24}. Phytosterols have wide bioactivity in humans, and in particular are considered an efficacious cholesterol-lowering agent and consequently may have a preventive role against vascular disease (Ostlund, 2002, 2004). Phytosterols may also have a role in cancer prevention (Ostlund, 2002; Kritchevsky and Chen, 2005). Consequently, many margarines, butters, spreads and breakfast cereals are enriched with phytosterols which are promoted as "functional foods" (Law, 2000; Kuhlmann, et al., 2005) .

Due to increasing demand and prices, reduced availability, the possibility of organic contaminants (such as dioxins and polychlorinated biphenyls (PCB)), and increased knowledge about fishing impacts, fish oil is being replaced in part with plant oils such as canola in formulated/commercial feeds for aquaculture species (Miller, et al., 2007a). Replacement oil diets have become an industry priority and so over the last 20 years there has been increased scientific activity focused on the effects of substituting fish oil with plant oils, including effects on fish growth and

health and on flesh quality. Limited research has been performed examining phytosterols in farmed Atlantic salmon. The replacement of fish oil with plant oils in aquafeeds will increase dietary amounts of phytosterols. However, it has yet to be evaluated how Atlantic salmon digest and accumulate phytosterols from dietary plant oils.

Pulp and paper mill effluent has been shown to affect reproductive and endocrine function in fish and this is thought to be due to the large amount of phytosterols, in particular 24-ethylcholesterol (referred to as β-sitosterol or simply sitosterol when C_{24} stereochemistry is defined). *In vivo* and *in vitro* studies suggest that large amounts of 24-ethylcholesterol can affect fish endocrine activity and reproduction via many mechanisms/actions on numerous pathways (Tremblay and Van der Kraak, 1998).

The digestibility of phytosterols is poor in humans, and to my knowledge has yet to be evaluated in Atlantic salmon (*Salmo salar* L.) or in any other fish species. With the increased use of plant oils and meal in aquafeeds, minor components such as phytosterols will be increasingly introduced into aquaculture diets and it is necessary to examine how they are digested by salmon. Phytosterols have been shown to have many potential benefits to the human consumer in particular as a cholesterol-lowering agent. Therefore farmed Atlantic salmon fed a replacement plant oil diet may be a novel and further delivery source of phytosterols to humans. Assessment of how they are accumulated and concentrated in tissues such as liver and muscle of Atlantic salmon fed on replacement oil diets is needed to gauge any possible advantageous affect to the consumer. This study is the first to assess digestibility and accumulation of phytosterols from two experimental replacement

oil diets (canola and Echium) compared to a traditional fish oil diet in white muscle and liver from Atlantic salmon smolt.

5.3 MATERIALS AND METHODS

5.3.1 Experimental diets

Three diets were formulated to compare canola oil (CO), Echium oil (SO) and fish oil (FO) (Table 5.1). Fish meal was defatted three times using a 2:1 mixture of hexane and ethanol (400 ml 100 g^{-1} fish meal). Soybean meal (Hamlet Protein A/S, Horsens, Denmark), casein (MP Biomedicals Australasia Pty Ltd, Seven Hills NSW, Australia), wheat gluten (Starch Australasia, Lane Cove, NSW, Australia) and BOIIC pre-gelatinised maize starch (Penford Australia Limited, Lane Cove, NSW, Australia) were used as ingredients. Echium oil was supplied as Crossential SA14 (Croda Chemicals, East Yorkshire, UK).

Fish oil was from jack mackerel, *Trachurus symmetricus* L., (Skretting Australia, Cambridge, Tasmania, Australia) and a domestic source of pure canola oil was used (Steric Trading Pty Ltd, Villawood, NSW, Australia). Stay-C and Rovimix E50 were supplied from Roche Vitamins Australia (Frenchs Forest, NSW, Australia), and the remaining ingredients were supplied by Sigma-Aldrich (Castle Hill, NSW, Australia). Yttrium oxide was used as a digestibility marker. The diets were manufactured into 3 mm diameter pellets using a California Pellet Mill (CL-2), dried and stored at $5^{o}C$ (Carter, et al., 2003b).

5.3.2 Growth experiment

The experiment was conducted at the School of Aquaculture, University of Tasmania (Launceston, Tasmania, Australia). Atlantic salmon (*Salmo salar* L.) parr

($\approx$87.9 g) were obtained from Springfield Fisheries hatchery (Scottsdale, Tasmania, Australia) acclimated for 14 days in 300 L tanks and fed a commercial feed (Skretting). Prior to the experiment the fish were slowly adapted to seawater over a 21 day period. The tanks were held at a constant temperature of 12.0^{o}C under a natural photoperiod. Water was treated through physical, UV and biofilters. Dissolved oxygen, pH, ammonia, nitrate, nitrite, and salinity were monitored daily to ensure water quality remained within parameters recommended for Atlantic salmon (Wedemeyer, 1996). The fish were held in an average of 27.4 $\pm$ 0.2 ppm saltwater. The experiment was conducted in accordance with the University of Tasmania Animal Ethics guidelines (Investigation A0008392).

At the start of the experiment fish (average weight 106.9g) were anaesthetized (50 mg L^{-1}, benzocaine), their weight and length measured, and three fish were killed to measure initial lipid content and composition. Twenty five fish were randomly re-allocated into each of twelve 300 L tanks, and four tanks were randomly allocated to each dietary treatment. The four diets were fed in triplicate at a ration of 1.8% body weight per day (% BW d^{-1}) in two equal feeds at 0900 and 1700 by automatic belt feeders. Every 7 days the total feed consumption (kg DM) was estimated from the amount of uneaten feed in Guelph-type sediment collectors (Helland, et al., 1996; Carter, et al., 2003a). Every three weeks all fish were anaesthetized (50 mg L^{-1}, benzocaine) and batch-weighed. Fish were starved the day prior to weighing.

Table 5.1: Ingredient, lipid and sterol composition (g/kg dry matter) of experimental diets.

	Diet		
	CO	SO	FO
Ingredient composition (g kg^{-1})			
Fish meal (defatted)	150	150	150
Casein	150	150	150
Wheat gluten	100	100	100
Soybean meal	180	180	180
Fish oil	200	0	0
Canola oil	0	200	0
Echium oil	0	0	200
Pre gel starch	127	127	127
Vitamin mix[a]	3	3	3
Mineral mix[b]	5	5	5
Stay C[c]	3	3	3
Chlorine chloride	2	2	2
Supernat	40	40	40
CMC	10	10	10
Sodium mono phosphate	20	20	20
Yttrium oxide	10	10	10
Chemical composition (g kg^{-1} DM)			
Dry Matter	940.1	943.1	938.1
Crude protein	351.7	340.0	344.1
Crude fat	247.9	255.2	250.8
Energy (MJ kg^{-1} DM)	19.3	19.0	19.1
FAME (g kg^{-1} DM)			
Total SFA	22.1	25.7	68.2
Total MUFA	114.6	41.2	53.7
Total ω3	13.5	72.5	49.9
Total ω6	49.0	60.3	20.3
Total PUFA	63.2	132.8	77.1
Sterols and stanols (g kg^{-1} DM)			
Cholesterol[d]	1.6	2.6	6.9
Cholestanol[e]	nd	nd	0.1
24-Methylenecholesterol[f]	0.3	0.6	0.3
Phytosterols (g kg^{-1} DM)			
Brassicasterol[g]	0.6	nd	0.0
24-Methylcholesterol[h]	2.1	1.8	0.4
Stigmasterol[i]	0.1	nd	nd
24-Ethylcholesterol[j]	3.5	2.4	1.2
Isofucosterol[k]	0.4	1.3	0.2
Other minor sterols[l]	0.3	0.2	0.2
Total phytosterols	7.0	5.7	2.0

SO, Echium oil Crossential SA14 from Croda chemicals; CO, canola oil; FO, fish oil, SFA, Saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; CMC, Carboxymethyl cellulose, nd, Not determined.

[a] Vitamin mix (ASV4) supplied per kilogram of feed: 2.81 mg thiamine HCL, 1.0 mg riboflavin, 9.15 mg pyridoxine HCL, 25 mg nicotinic acid, 54.35 mg calcium D-pantothenate, 750 mg myo-inositol, 0.38 mg D-biotin, 2.5 mg folic acid, 0.03 mg cyanocobalamin, 6350 IU retinol acetate, 2800 IU cholecalciferol, 100 IU DL α-tocopherol acetate, 5 mg menadione sodium bisulphate, 100 mg Roche rovimix E50.
[b] Mineral mix (TMV4) supplied per kilogram of feed:117 mg $CuSO_4.5H_2O$, 7.19 mg KI, 1815 mg $FeSO_4.7H_2O$, 307 mg $MnSO_4.H_2O$, 659 mg $ZnSO_4.7H_2O$, 3.29 mg Na_2SeO_3, 47.7 mg $CoSO_4.7H_2O$
[c] L-Ascorbyl-2-polyphosphate (Stay-C, Roche Vitamins Australia, Frenchs Forest, NSW, Australia).
[d] Cholesta-5-en-3β-ol
[e] Cholestan-3β-ol
[f] 24-Methylcholest-5-en-24(28)- 3β-ol
[g] 24-Methylcholesta-5,22E-dien-3β-ol
[h] 24-Methylcholest-5-en-3β-ol
[i] 24-Ethylcholesta-5,22E-dien-3β-ol
[j] 24-Ethylcholest-5-en-3β-ol
[k] 24-Ethylcholesta-5,24(28)Z-dien-3β-ol
[l] Other minor sterols included 24-ethyl-5α-cholest-7-en-3β-ol, 4,4,14-trimethyl-5α-cholesta-8,24-dien-3β-ol (lanosterol), and other undetermined sterols

At the end of the experiment, fish were starved for one day prior to being anaesthetized (50 mg L^{-1}, benzocaine) and their weight and length measured. Three fish, which had doubled their initial weight, per tank were killed by a blow to the head after immersion in anaesthetic. Samples of white muscle (approx 0.7 g) , dissected from below the dorsal fin, and liver (avg 2.7 g) were taken and frozen at -80°C until analysis (Miller, et al., 2007a).

On days 86 to 90, faecal samples were collected from sediment collectors between 1100-1700 and 1900-0900 h, freeze-dried and used in the analysis of digestibility (Carter, et al., 2003a). The apparent digestibility coefficients (ADC) were calculated using the standard formula ADC (%) = 100 − [100((Y_{diet}/Y_{faeces})x ((ST_{faeces}/ST_{diet}))] where Y is percentage of yttrium oxide and ST is the % of

particular sterols (Maynard and Loosli, 1969). This calculation does not take into account possible dealkylation of phytosterols to cholesterol.

5.3.3 Sterol extraction and isolation

All samples (tissue, faeces and diets) were freeze dried and extracted overnight using a modified Bligh and Dyer protocol (Bligh and Dyer, 1959). This involved a single phase extraction, $CHCl_3$/MeOH/H_2O (1:1:0.9, by vol), followed by phase separation to yield a total lipid extract (TLE).

Lipid classes were analysed using an Iatroscan MK V thin-layer chromatography-flame ionization detector (TLC-FID) analyser (Iatron Laboratories, Japan). Samples of the TLE were spotted onto silica gel SIII Chromarods (5 μm particle size) and developed in a glass tank lined with pre-extracted filter paper. The solvent system used for the lipid separation was hexane:diethyl ether:acetic acid (60:17:0.1, v/v/v) (Volkman and Nichols, 1991). After development for 25 minutes, the chromarods were oven-dried and analysed immediately to minimise adsorption of atmospheric contaminants. Lipid classes were quantified by DAPA software (Kalamunda, WA, Australia). The FID was calibrated from a standard of cholesterol.

An aliquot of the TLE was treated with 2 ml of 5% w/v KOH in 80:20 MeOH:H_2O ($60^{o}C$, 3h, milli-Q water). Following the addition of water, sterols were extracted into hexane/chloroform (4:1 v/v, 3 x 1.5 ml), transferred to vials, reduced under a stream of nitrogen and stored in chloroform. Samples were treated with N,O-bis(trimethylsilyl)-trifluroacetamide (BSTFA) to form trimethylsilyl (TMS) ether derivatives (of free sterols) prior to instrument analysis.

Samples were made up to a known volume and analysed by gas chromatography (GC) using an Agilent Technologies 6890N GC (Palo Alto, California, USA) equipped with an Equity ™-1 fused silica capillary column (15 m × 0.1 mm i.d., 0.1 μm film thickness), an FID, a split/splitless injector and an Agilent Technologies 7683 Series auto sampler. Helium was the carrier gas. Samples were injected in splitless mode at an oven temperature of 120ºC. After 3 min, the oven temperature was raised to 270ºC at 10ºC per min and finally to 290ºC at 5ºC min^{-1} holding at 290ºC for 5 minutes. Peaks were quantified with Agilent Technologies GC ChemStation software (Palo Alto, California, USA).

Individual components were identified from mass spectral data and by comparing retention time data with those obtained for authentic and laboratory standards. GC-mass spectrometric (GC-MS) analyses were performed on a Finnigan Thermoquest GCQ GC-mass spectrometer fitted with an on-column injector and using Thermoquest Xcalibur software (Austin, Texas, USA). The GC was equipped with an HP-5 cross-linked methyl silicone fused silica capillary column (50 m × 0.32 mm i.d.). Helium was used as the carrier gas, with operating conditions previously described (Miller, et al., 2006). Sterols were identified from comparison to known spectra and major ion fragments (Jones, et al., 1994). The stereochemistry was not determined for the C_{24} position.

5.3.4 Statistical analysis

Mean values are reported as plus or minus the standard error of the mean. Normality and homogeneity of variance were confirmed and percentage data were arcsin transformed prior to analysis. Comparison between means was by 1-way analysis of variance (ANOVA) followed by multiple comparisons using Tukey-

Kramer HSD. Significance was accepted at probabilities of 0.05 or less. Statistical

analysis was performed using SPSS for Windows version 11.

5.4 RESULTS

5.4.1 Growth results

There was no significant difference in the final weight, final length or growth

between fish fed the three experimental diets. Fish of an average weight (106.8 g)

were grown on the three experimental diets for 12 weeks to an average sampled

weight of 236.5g.

Table 5.2: Sterol content of white muscle and livers of Atlantic salmon smolt fed canola oil (CO), Echium oil (SO) and fish oil (FO).

Sterol content (mg/100g)	Initial (±SE)		CO (±SE)		SO (±SE)		FO (±SE)		f
White muscle	21.6 ±	4.8	15.0 ±	6.0	12.4 ±	6.5	13.9 ±	3.1	
Liver	139.9 ±	18.4a	263.8 ±	34.3b	144.7 ±	18.0a	139.7 ±	11.8a	7.9

[a,b] Mean values across the row not sharing a common superscript were significantly different as determined by Tukey-Kramer HSD; df =3,40, $P<0.01$

5.4.2 Sterol (ST) composition

There was no significant difference in the sterol content in the white muscle

between dietary treatments as determined by TLC-FID analysis (Table 5.2). There

was significantly higher total sterol content in the liver (263.8 mg/100g) of the fish

that were fed CO diet. Cholesterol was the major sterol in all tissues, irrespective of

diet, but the amounts of minor sterols varied. There were significantly ($P<0.01$)

higher concentrations of total phytosterols in the liver and white muscle in both the

CO and SO fed fish compared to the FO or initial fish (Tables 3, 4). This equated to

a 4 fold increase in the white muscle and a 2 fold increase in the liver in total

phytosterol concentrations in SO and CO fish compared to the FO fish. There were

significant differences in the sterol composition of the liver (Table 5.4). There was a

7 fold significant (*P*<0.01) increase in concentrations of 24-methylcholesterol (full

chemical names for all sterols are contained in the footnote for Table 5.1) in the liver

of both the CO and SO fish compared to the FO or initial fish. There were

significant (*P*<0.01) increases in concentrations of 24-ethylcholesterol and 24-

methylenecholesterol in the liver of SO fish compared to that of the FO or initial

fish. This equated to a 7 fold increase in 24-ethylcholesterol in the SO fish compared

with the FO fish. There were significantly (*P*<0.01) higher concentration of

cholestanol in the initial fish compared to the CO and SO fish. There was an

significant (*P*<0.01) 5-9 fold increased concentrations of other minor sterols

including 24-ethyl-5α-cholest-7-en-3β-ol, lanosterol, and other undetermined sterols

in the liver of CO and SO fish.

Table 5.3: Sterol composition (mg/100g) of white muscle of Atlantic salmon smolt fed canola oil (CO), Echium oil (SO) and fish oil (FO) diets

Sterol	Initial (±SE)			CO (±SE)			SO (±SE)			FO (±SE)			*f*
Cholesterol	25.7	±	2.2	15.4	±	2.0	15.2	±	2.0	13.6	±	2.8	
Cholestanol	0.2	±	0.2	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	
Brassicasterol	0.0	±	0.0	0.2	±	0.1	0.1	±	0.1	0.1	±	0.0	
24-Methylenecholesterol	0.2	±	0.1	0.1	±	0.1	0.4	±	0.1	0.1	±	0.0	
24-Methylcholesterol	0.1	±	0.1	0.2	±	0.0	0.2	±	0.1	0.0	±	0.0	
Stigmasterol	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	
24-Ethylcholesterol	0.0	±	0.0	0.3	±	0.2	0.5	±	0.3	0.0	±	0.0	
Isofucosterol	0.0	±	0.0	0.1	±	0.0	0.0	±	0.0	0.0	±	0.0	
Other minor sterols[d]	0.1	±	0.0	0.1	±	0.0	0.0	±	0.0	0.0	±	0.0	
Total Phytosterols[e]	0.2	±	0.1a	0.9	±	0.2b	0.8	±	0.3b	0.2	±	0.1a	8.5

[a,b,] Mean values across the row not sharing a common superscript were significantly different as determined by Tukey-Kramer HSD; *df*=3,36, *P*<0.01. Chemical names for sterols contained in the footnote for Table 5.1.

[d] Other minor sterols including 24-ethyl-5α-cholest-7-en-3β-ol, lanosterol, and other undetermined sterols.

[e] Includes all C_{28} and C_{29} sterols excluding 24-methylenecholesterol

There was no significant difference in the sterol composition (%) between the

white muscle for fish fed the three diet treatments (Table 5.3). However, although

not significant, the same trends in increased concentration of individual phytosterols that occurred in the liver were observed in the white muscle. There was a 3-5 fold increase in the amount of 24-ethylcholesterol and a 2 fold increase in 24-methylcholesterol in the white muscle of fish fed SO and CO diets compared with the FO fish.

Table 5.4: Sterol composition (mg/100g) of liver of Atlantic salmon smolt fed canola oil (CO), Echium oil (SO) and fish oil (FO) diets

Sterol	Initial (±SE)		CO (±SE)		SO (±SE)		FO (±SE)		f
Cholesterol	99.2 ±	7.0	222.5 ±	21.8	180.1 ±	16.8	151.4 ±	12.5	
Cholestanol	0.7 ±	0.2c	0.1 ±	0.0a	0.2 ±	0.1a,b	0.4 ±	0.1b,c	9.2
Brassicasterol	0.0 ±	0.0	0.5 ±	0.1	0.3 ±	0.1	0.4 ±	0.0	
24-Methylenecholesterol	0.3 ±	0.1a	1.7 ±	0.1a,b	3.2 ±	0.8b	0.9 ±	0.1a	5.8
24-Methylcholesterol	0.2 ±	0.1a	4.6 ±	0.7b	4.9 ±	0.9b	0.7 ±	0.1a	6.5
Stigmasterol	0.0 ±	0.0	0.1 ±	0.0	0.8 ±	0.3	0.3 ±	0.1	
24-Ethylcholesterol	0.0 ±	0.0a	0.1 ±	0.1a,b	0.7 ±	0.2b	0.0 ±	0.0a	6.5
Isofucosterol	0.0 ±	0.0	0.2 ±	0.2	0.0 ±	0.0	0.0 ±	0.0	
Other Sterols	0.3 ±	0.0a,b	0.5 ±	0.0b	0.9 ±	0.0c	0.1 ±	0.0a	6.4
Total Phytosterols[e]	0.5 ±	0.2a	6.1 ±	1.1b,c	7.5 ±	1.0c	3.3 ±	0.4a,b	6.8

[a,b,c], Mean values across the row not sharing a common superscript were significantly different as determined by Tukey-Kramer HSD; df =3,36, *P*<0.01. Chemical names for sterols contained in the footnote for Table 5.1.

[d] Other minor sterols including 24-ethyl-5α-cholest-7-en-3β-ol, lanosterol, and other undetermined sterols

[e] Includes all C_{28} and C_{29} sterols excluding 24-methylenecholesterol

5.4.3 Digestibility of phytosterols

There was no significant difference between the apparent digestibility coefficients (ADC) of cholesterol between the three diet treatments (Table 5.5). There were no significant differences between the ADC of any phytosterol between the three diet treatments. Cholesterol has an ADC of 46.7-65.8 % across the three diets. In general phytosterols had a lower ADC than cholesterol. Specifically, C_{28} phytosterols such as brassicasterol and 24-methylcholesterol had a relative absorption of 9.2% to 18.1%, 3-6 fold lower than that of cholesterol. 24-Methylenecholesterol was excluded from the calculation as it is a metabolite of

cholesterol and is usually not associated with plant phytosterols. C_{29} phytosterols including 24-ethylcholesterol, stigmasterol, and isofucosterol have a relative absorption of 1.1% to 7.1%, 7-60 fold lower than that of cholesterol

Table 5.5: Apparent digestibility coefficients (ADC) (%) for the different sterols in canola oil (CO), Echium oil (SO) and fish oil (FO) diets fed to Atlantic salmon

ADC (%)	CO (±SE)		SO (±SE)		FO (±SE)	
Cholesterol	57.5 ±	4.4	46.7 ±	5.4	65.8 ±	7.2
Cholestanol	nd ±		nd ±		52.7 ±	4.1
Brassicasterol	9.2 ±	8.0b	nd ±		nd ±	
24-Methylenecholesterol	12.4 ±	3.5	27.3 ±	5.2	17.0 ±	4.1
24-Methylcholesterol	14.4 ±	2.2	15.2 ±	2.1	18.1 ±	1.6
Stigmasterol	4.7 ±	3.2	nd ±		nd ±	
24-Ethylcholesterol	7.1 ±	2.1	4.1 ±	4.3	1.1 ±	2.0
Isofucosterol	7.6 ±	1.1	2.4 ±	2.7	8.5 ±	2.6
Other Sterols [a]	3.4 ±	3.1	7.5 ±	3.3	5.2 ±	3.1

Chemical names for sterols contained in the footnote for Table 5.1.
[a] Other minor sterols including 24-ethyl-5α-cholest-7-en-3β-ol, lanosterol, and other undetermined sterols. nd, Not determined.

5.5 DISCUSSION

Due to the decreasing availability and increasing price of fish oil, renewable land plant based oils have been increasingly incorporated into aquafeeds, and the replacement of fish oil has become an industry priority. Plant based oils contain phytosterols which are not a part of the natural diet of Atlantic salmon. Therefore it is important to investigate how natural abundances of phytosterols in replacement oils are digested and incorporated in different tissues of salmon. We demonstrated that both the liver and the white muscle accumulated significantly increased amounts of total phytosterols with both plant oil diets (CO and SO) compared to FO fed fish. Differences in individual phytosterols were demonstrated in the liver and white muscle of fish fed plant oil diets compared to the FO and initial fish. This study indicates that small amounts of phytosterols can be digested and accumulated

in both the white muscle and liver of fish when fed replacement plant oil over a 12 week period.

5.5.1 Digestibility of phytosterols in Atlantic salmon

Although cholesterol and phytosterols have similar structures, phytosterols have significantly reduced absorption compared to cholesterol and it has been shown that negligible amounts of phytosterol are absorbed by humans (Trautwein, et al., 2003; Rozner and Garti, 2006). There have been numerous studies looking at cholesterol and phytosterol adsorption in humans (Salen, et al., 1970; Heinemann, et al., 1993; Ostlund, et al., 2002a; Ostlund, et al., 2002b). From these studies cholesterol has an absorption range of 33-60%, with phytosterols having reduced absorption, 2-10% for 24-methylcholesterol and 0.5-5% for 24-ethylcholesterol often referred to as campesterol and β-sitosterol when C_{24} stereochemistry is defined (Salen, et al., 1970; Heinemann, et al., 1993; Ostlund, et al., 2002a; Ostlund, et al., 2002b). The digestibility of sterols and phytosterols in Atlantic salmon show similar patterns to those in humans. Our study demonstrates that cholesterol has an apparent digestibility coefficient (ADC) of 46.7-65.8 %. A reduced digestibility was demonstrated with phytosterols. An observed ADC of 14-18.1% for 24-methylcholesterol and 1.1-7.1% for 24-ethylcholesterol was shown across the diets. C_{29} phytosterols, such as 24-ethylcholesterol and isofucosterol, had a reduced digestibility to that of C_{28} phytosterols such as 24-methylcholesterol. Phytosterols may be dealkylated to cholesterol which will reduce their ADC. It is yet to be assessed how Atlantic salmon convert phytosterols to cholesterol. This study showed no difference in the digestibility of individual phytosterols across diets,

which suggests that dietary concentrations does not affect their digestibility by Atlantic salmon.

5.5.2 Accumulation of phytosterols in Atlantic salmon

We determined that 0.8-0.9 mg of total phytosterols per 100 g of tissue were accumulated in the white muscle and 6.1-7.5 mg/100g in the liver of fish fed plant oil replacement diets over a 12 week period. Phytosterol accumulation is dependant on dietary concentration as they are unable to be in vivo biosynthesized. The major phytosterols accumulated in the liver and white muscles were 24-methylcholesterol and 24-ethylcholesterol. Generally the relative concentrations of individual phytosterols in the liver and white muscle reflected that of their diet. However, C_{29} phytosterols had a reduced digestibility compared with C_{28} phytosterols and therefore lower amounts accumulated in the liver of white muscle in both the CO and SO fish.

Phytosterols are very expensive ingredients and therefore economically not possible to be added to aquafeeds. However replacement plant oils, such a canola, contain phytosterols as minor components. Canola is a prime candidate for fish oil replacement as it contains high levels of $\omega 3$ PUFA, largely as α-linolenic acid (ALA, 18:3ω3) and is grown in sufficient quantities to meet future aquaculture demands. A recent study has shown that Echium oil also may be a candidate as it contains an unusual $\omega 3$ PUFA profile containing stearidonic acid (SDA, 18:4ω3), which was shown to maintain concentrations of important omega 3 long chain PUFA ($\omega 3$ LC-PUFA) in Atlantic salmon parr (Miller, et al., 2007a). Both these plant oils have phytosterol compositions which are dependent on genotype, planting

location and temperature (Vlahakis and Hazebroek, 2000; Hamama, et al., 2003; Gul and Seker, 2006).

In this study all the experimental diets had minor concentrations of phytosterols from the meal and oil sources. In the CO and SO diets the major source of phytosterols came from the plant oil. All the diets contained plant based proteins which consisted of soybean meal, casein and wheat gluten. As phytosterols act in cell walls and membranes of plants, the plant meals will have residual concentrations of phytosterols. The FO diet contained 2.0 g/kg DM phytosterols, while the CO (7.0 g/kg DM) and the SO (5.7 g/kg DM) had 3.5 and 2.9 times the amount of total phytosterols (Table 5.1).

In the present study sterols represented only about 1% of the total lipid of salmon, and 92-99% of that 1% is present as cholesterol, therefore the amount of phytosterols in Atlantic salmon smolt is relatively small. Phytosterols cannot be synthesized by humans or fish, therefore they are supplied in the diet. Phytosterols, due to their lipophilic nature, have been added to margarines and spreads which are known as "functional foods". Oily fish, such as Atlantic salmon, may provide another possible delivery source. Phytosterols in combination with the high levels of omega 3 long chain ($\geq C_{20}$) polyunsaturated fatty acids (ω3-LC PUFA) found in salmon may act in unison to deliver an enhanced benefit against CHD. Additionally and unlike ω3-LC PUFA, phytosterols are very stable compounds and are not readily oxidised even under severe conditions, such as deep frying (Dutta, 1997).

Most human nutrition studies with phytosterols assessed an intake of 0.8-4.0 g of phytosterol as a daily dose which reduced the lower density lipoprotein (LDL) cholesterol between 6-15% which reduces the risk of coronary heart disease (CHD)

(Ostlund, 2002; Patel and Thompson, 2006). Dietary intake of phytosterols ranges from 150-400 mg/day, with typically 65% derived from 24-ethylcholesterol, 30% 24-methylcholesterol and 5% stigmasterol (Ostlund, 2002; Trautwein, et al., 2003). The content of phytosterol from this experiment in white muscle of Atlantic salmon smolt is 0.8-0.9 mg/100g which is well below the concentration range of dietary phytosterol generally being supplied in functional foods for CHD prevention. This result will be elevated in larger commercial cultured fish, where oil content (7-20%) of white muscle is markedly higher than observed in the smolt (2-3%) examined here. Due to use of extrusion technologies commercial salmon aquafeeds have an oil content of 30-40%. Our study used an inclusion of 20% oil which will underestimate the phytosterol content compared to commercial diet. It was not possible for this experiment to obtain the high lipid content of commercial aquafeeds. However, even at this low inclusion, it was possible to demonstrate that phytosterols significantly accumulated in the tissues of Atlantic salmon. Our experiment was for a 12 week period. It is plausible to suggest that over a life time of replacement oil diets that Atlantic salmon will accumulate considerably more phytosterols. However, at this point it is yet to be substantiated how Atlantic salmon accumulated phytosterols over a lifetime. The phytosterol concentrations in the white muscle (0.8-0.9 mg/100g) of plant oil fed fish in this experiment is several orders of magnitude less than the recommended daily serving size of functional foods such as spreads and margarines (phytosterols total ≈ 200 mg/day) (Noakes, et al., 2005). However, in a balanced diet, this additional amount of phytosterols (estimated to be ≈ 20-50 mg/serve in a large commercial size fish) is a 10-25% increase in the average adult human dietary intake of phytosterols.

5.5.3 Environmental effects of phytosterols

Several studies have shown that phytosterols in effluent, in particular 24-ethylcholesterol, produced estrogenic effects in maturing fish as well as a reduced cholesterol level (Tremblay and Van der Kraak, 1998, 1999). Cholesterol is a precursor of steroid hormones and therefore the presence of structurally similar molecules or the reduction of cholesterol may effect maturation in fish. Phytosterols, in particular 24-ethylcholesterol, are present in pulp and paper mill effluents and have been associated with different reproductive responses in fish including reduction in gonad size, delayed sexual maturation, and reduced expression of secondary sexual characteristics (Munkittrick, et al., 1994; McMaster, et al., 1996; Tremblay and Van der Kraak, 1999). Recent studies have shown for Atlantic cod (*Gadus morhua*) that phytosterols might have been the active compounds in changes to gonad development (Pickova and Mørkøre, 2007). It has also been suggested that increased sterol-like compounds may have contributed in the postponed spawning of Baltic cod in the Baltic sea (Pickova and Mørkøre, 2007). However, most farmed fish are harvested prior to maturation and a postponing of maturation may be advantageous to the aquaculture industry. Research has focused on the effect of phytosterols as a waterborne contaminant on fish health, reproduction and endocrine function (Munkittrick, et al., 1994; McMaster, et al., 1996; Tremblay and Van der Kraak, 1999). The effects of dietary sources of phytosterols on fish have yet to be assessed, however, dietary concentrations are significantly lower than that of the effluent experiments.

There have been many well designed and executed experimental aquaculture trials with replacement oil, which have all shown no difference in growth,

performance or health of fish (Bell, et al., 1993; Bransden, et al., 2003; Tocher, et al., 2003; Bell, et al., 2004; Torstensen, et al., 2004; Miller, et al., 2007a; Miller, et al., 2007b). Further research looking at dietary phytosterol accumulation over a longer period and in particular reproductive and endocrine function such as the production of vitellogenin will provide further insight on the accumulation and function of these ingredients. The use of vegetable oils in aquafeeds is now commonplace throughout the aquaculture industry and the accumulation of phytosterols in Atlantic salmon demonstrated will be advantageous to their continued use.

5.6 CONCLUSION

While it has been shown that phytosterols in pulp mill effluent can be detrimental to fish health, low concentrations that occur in plant oils most likely will not affect health and performance of fish, and ultimately may provide health benefits to the consumer. Although the digestibility of phytosterols is low and their accumulation in fish of commercial size is likely to be at a level lower than used in functional food such as spreads and margarines, phytosterols along with the high content of ω3 LC-PUFA may give increased CHD protection to consumers.

5.7 ACKNOWLEDGEMENTS

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CHAPTER 6

Regiospecificity profiles of storage and membrane lipids from the gill and muscle tissue of Atlantic salmon (*Salmo salar L*) grown at elevated temperature

6.1 ABSTRACT

Regiospecific and traditional analysis, of both the storage and membrane lipids, was performed on gill, white muscle and red muscle samples taken from Atlantic salmon (*Salmo salar*) to gauge the effect of elevated water temperature. The fish, fed a commercial diet, were held at an elevated water temperature of 19°C. Total ω3 polyunsaturated fatty acids (PUFA), total PUFA, ω3/ω6 and unsaturated/saturated fatty acid (UFA/SFA) ratios in the fatty acid profile of the total lipid extract in the white muscle were comparatively low compared to fish grown at 15°C. Adaptation of structural and storage lipids at elevated temperatures was shown by a significant ($p>0.01$) reduction in PUFA especially in the percentage of eicosapentaenoic acid (EPA) (6-8%). Further adaptation was indicated by the percentage of SFA which were significantly ($p>0.05$) higher in gill (56%) and white muscle (58%) polar lipid fraction and coincided with lower percentage of ω3, ω6 and total PUFA. The regiospecific profiles indicated a high affinity of docosahexaenoic acid (DHA) to the *sn*-2 position in both the triacylglycerol (61-68%) and polar lipid (35-60%) fractions. The combination of detailed regiospecific and lipid analyses demonstrated adaptation of cell membrane structure in Atlantic salmon grown at elevated water temperature.

6.2 INTRODUCTION

Examination of the regiospecificity profile of the lipid classes of Atlantic salmon (*Salmo salar* L.) at and elevated temperature of 19°C has yet to be fully investigated. Regiospecificity is the position of individual fatty acids (FA) on the glycerol backbone of both storage and membrane lipids. Both membrane and storage lipids are present and act in the body as complex molecules and therefore, should be studied as whole molecules. Traditional lipid class and fatty acid profiling provide important compositional information, however, they do not reveal the regiospecific details of the molecule which can play a key role in their function including bioactivity in humans and other animals (Lucas, et al., 1997; Kew, et al., 2003a; Kew, et al., 2003b).

It is being increasingly recognized that not only is the FA composition of lipids important, so too are the particular combinations of fatty acids within individual phospholipid classes (Roy, et al., 1999). Up to 70 different polar lipid molecular species have been identified for a range of different fish species (Bell, 1989; Bell and Tocher, 1989; Bell and Dick, 1990, 1991), and their diversity and abundances can depend on species, tissue sampled, dietary and environmental factors (Tocher, 1995). The composition of these molecular species and their role in the cell membrane can be influenced by many factors, but little is known about how they function in fish.

With advances made in analytical and computing facilities, new techniques and methods can be used to examine intact lipids and focus on the regiospecific distribution of specific fatty acids. Electrospray ionization reversed-phase liquid

chromatography-mass spectrometry (ESI RP-LC-MS) provides a rapid and accurate method to measure molecular species. This method provides regiospecific information on how membranes adapt to different factors such as temperature, hormonal states and photoperiod. This method has only had limited application to Atlantic salmon (Hvattum, et al., 2000) and it has yet to be used to explore a range of tissues.

The regiospecific distribution of the TAG fraction of salmon has been examined by ^{13}C nuclear magnetic resonance (^{13}C NMR) (Aursand and Grasdalen, 1992). The regiospecific distribution of the TAG fraction may affect its physical characteristics and potentially have a profound influence on function and resultant bioactivity to consumers (Kew, et al., 2003a; Kew, et al., 2003b). It was first shown in infant nutrition that the regiospecific distribution of fatty acids in the diet affects their absorption (Lucas, et al., 1997). Therefore absorption of the long chain polyunsaturated fatty acids (LC-PUFA) by human consumers of salmon may be affected by position on the TAG molecule.

Previous regiospecific analysis has been performed for other fish species *via* destructive and/or time consuming methods, such as using lipases (Hazel and Zerba, 1986; Bell, et al., 1993) and thin layer chromatography (TLC) combined with high pressure liquid chromatography (HPLC) (Bell and Tocher, 1989; Bell and Dick, 1990; Bell and Henderson, 1990; Bell and Dick, 1991). These two new techniques allow a rapid and detailed examination of intact membrane and storage lipids. In studying intact molecules it is possible to determine the regiospecificity of the fatty acids and whether the molecular structure plays a role in the molecules function, storage and absorption, in particular in regard to ω3 LC-PUFA.

The physical properties of cell membranes and their lipid content can be affected by temperature (Hazel, 1984; Hazel and Zerba, 1986; Hazel and Landrey, 1988; Hazel, et al., 1991; Hazel, et al., 1992). Fish may exploit the structural diversity of lipids within their membranes to adapt to physical properties, such as change in ambient temperature. The tailoring of membrane lipid composition is probably the most ubiquitous and continuously graded cellular response to temperature change (Hazel and Zerba, 1986). Most studies have investigated Atlantic salmon lipids within a temperature range of 2 – 12°C (Grisdale-Helland, et al., 2002; Bendiksen and Jobling, 2003; Jobling and Bendiksen, 2003; Ruyter, et al., 2003), but none have measured salmon at higher temperatures such as 19°C, a temperature now commonly encountered by farmed salmon in Tasmanian waters and which is approaching their upper threshold for survival (Carter, et al., 2003).

This is the first detailed examination, including regiospecific profiling, of the membrane and storage lipids of salmon from a feeding experiment performed at an elevated temperature of 19°C. This study aims to combine and apply these two regiospecific analyses for both storage and structural lipids from a range of different tissues. With global waters increasing in temperature, this study will indicate possible adaptive changes in salmon lipid makeup at elevated temperature.

6.3 MATERIAL AND METHODS

6.3.1 Experimental system

The experiment was conducted at the School of Aquaculture, University of Tasmania. Atlantic salmon parr were obtained from Wayatinah Salmon hatchery

(SALTAS, Tasmania, Australia) and 14 fish per tank were randomly stocked into three 300 litre tanks in a partial recirculation system (Carter and Hauler, 2000). The tanks were held at a constant temperature of 19°C in 30 ppt seawater (Carter and Hauler, 2000) with a photoperiod of 16:8, light:dark. The water was treated through physical, biological and UV filters. Dissolved oxygen, pH, ammonia, nitrate and nitrite were monitored daily to ensure water quality remained within parameters recommended for Atlantic salmon (Wedemeyer, 1996). A commercially extruded feed (Skretting) was fed to excess twice a day (0900-1000 and 1600-1700) by automatic belt feeders. The salmon had an initial weight of 139.6 ± 1.5 g (mean + S.E; n = 6), were grown for 105 days, and reached an average final weight of 510.2 ± 13.2 g. Salmon of 420.2 ± 6.3 g n=3, maintained at 15°C and with a similar history and fed on the same diet provided a reference group that represented baseline temperature conditions.

6.3.2 Sampling

On the last day (day 104) of the trial, fish were killed by a blow to the head after immersion in anaesthetic (benzocaine, 100 mg/l) and 2 fish were sampled from each tank. Samples of gill tissue were dissected from the first gill arch. Red (average sample weight 1.3 ± 0.2 g, n = 6) and white muscle (average sample weight 2.4 ± 0.1 g, n = 6) samples were taken from below the dorsal fin. Samples were immediately frozen and stored at -80°C until analysis. Salmon at 15°C were similarly sampled. The experiment was conducted in accordance with the University of Tasmania Animal Ethics guidelines (Investigation A0007719).

6.3.3 Lipid extraction, fractionation and fatty acid analysis

Samples were freeze dried and extracted by a modified Bligh and Dyer protocol(Bligh and Dyer, 1959). A single phase extraction, $CHCl_3$/MeOH/H_2O (1:1:0.9, by vol), was used to yield a total lipid extract (TLE).

Lipid classes were analysed by an Iatroscan MK V thin-layer chromatography-flame ionization detector (TLC-FID) analyser (Iatron Laboratories, Japan). Samples were spotted onto silica gel SIII Chromarods (5 μm particles size) and developed in a glass tank lined with pre-extracted filter paper. The solvent system used for the lipid separation was hexane: diethyl ether: acetic acid (60:17:0.1, v/v/v) (Volkman and Nichols, 1991). After development for 25 minutes, the chromarods were oven-dried and analysed immediately to minimise adsorption of atmospheric contaminants. Lipid classes were quantified by DAPA software (Kalamunda, W.A., Australia). The FID was calibrated for each compound class: phosphatidylcholine; cholesterol; cholesteryl ester; oleic acid; hydrocarbon (squalene); wax ester (derived from fish oil); triacylglycerol (derived from fish oil); and DAGE (diacylglycerol, purified from shark liver oil). To analyse the polar lipid (PL) fraction, a second solvent system, chloroform:methanol:water (80:35:3 v,v,v), was used (Innis and Clandinin, 1981). The Iatroscan was calibrated with phosphatidylcholine with equivalent response factors assumed across the PL classes. Retention factors were measured for each phospholipid and compared to standards (Sigma-Aldrich).

An aliquot of TLE was trans-methylated in methanol: chloroform: hydrochloric acid (10:1:1, v/v/v) for 1 hour at 100°C. After addition of water the mixture was extracted three times with hexane: chloroform (4:1, v/v) to obtain fatty acid methyl esters (FAME). FAME were concentrated under nitrogen and treated with N,O-

bis(trimethylsilyl)-trifloroacetamide (BSFTA, 50μl, 60°C, 1h) to convert hydroxyl groups to their corresponding trimethyl silyl ethers. Samples were made up to a known volume with an internal injection standard (23:0 or 19:0 FAME) and analysed by gas chromatography (GC) using an Agilent Technologies 6890N GC (Palo Alto, California, USA) equipped with an HP-5 cross-linked methyl silicone fused silica capillary column (50m×0.32mm i.d.), and an FID. Helium was used as the carrier gas. Samples were injected, by a split/splitless injector and an Agilent Technologies 7683 Series auto sampler in splitless mode, at an oven temperature of 50°C. After 1 min the oven temperature was raised to 150°C at 30°C min^{-1}, then to 250°C at 2°C per min and finally to 300°C at 5°C min^{-1}. Peaks were quantified by Agilent Technologies GC ChemStation software (Palo Alto, California, USA). Individual components were identified by mass spectral data and by comparing retention time data with those obtained for authentic and laboratory standards. GC results are typically subject to an error of ±5% of individual component area. GC-mass spectrometric (GC-MS) analyses were performed on a Finnigan Thermoquest GCQ GC-mass spectrometer fitted with an on-column injector with Thermoquest Xcalibur software (Austin, Texas, USA). The GC was fitted with a capillary column similar to that described above.

Fractionation of lipid classes was accomplished by silicic acid column chromatography. The TLE was applied to a 1 g column of silicic acid (preheated to 100°C, 1 h) and separated into neutral lipids, glycolipids and polar lipids in a stepwise elution of chloroform (10 ml), acetone (20 ml) and methanol (10 ml) respectively (Kates, 1986).

6.3.4 Membrane lipid analysis – ESI-RP-LCMS

Polar lipid (PL) species were analysed by LC-MS by negative ion electrospray ionization. A Waters Alliance 2690 HPLC fitted with a Nova-Pak reverse-phase C18 column (3.9mm x 150 mm) was coupled to a Finnigan LCQ ion trap mass spectrometer fitted with an electrospray source. The mobile phase gradient was methanol: 0.25M ammonium acetate (pH 5), (96:4 v,v) to methanol: 0.25M ammonium acetate (pH 5): hexane (86:4:10 v,v,v) at 20 minutes, then to methanol: hexane (80:20 v,v) at 25 minutes, which was held for 10 min. The flow rate was 1 ml min^{-1}. Alternating normal scans in the range m/z 600 to 950 and data-dependent MS/MS product ion scans were acquired. The MS/MS scans isolated the strongest ion observed, and a collision energy of 40% was applied with all product ions monitored. The capillary temperature was 275°C, sheath gas pressure was 600 kPa, auxiliary gas pressure 275 kPa and the needle voltage was 4 kV. Minor molecular species (>0.1 across all tissues) are not included to simplify the analysis. PL species and regiospecificity was identified by MS and MS/MS breakdown products according to published data (Hvattum, et al., 2000).

6.3.5 Storage lipid analysis - ^{13}C NMR spectroscopy

Acyl group positional distributions on TAG were determined quantitatively by ^{13}C NMR on a 400 MHz Varian (Palo Alto, USA) Inova Wide Bore spectrometer, with either a Broadband 10 mm probe or a 3 mm MAS Nanoprobe at 25.0 ± 0.1°C. A π pulse at 100.516 MHz, 128K acquisition with 2.5 s relaxation delay and full nuclear overhauser effect (NOE) enhancement and decoupling sequence was employed. Samples were dissolved in 99.96% deuterated chloroform (CDCl$_3$) with *ca* 0.025M Cr(acac)$_3$ as a relaxation agent and brought to a volume of 0.6 ml. TAG

fractions which were <10 mg were dissolved in 20-35 μl of CDCl$_3$ / Cr(acac)$_3$ in a

glass rotor and filled to a total volume of 40 μl and spun at 3.000 ± 0.002 KHz. 512

to 8192 transients were collected. A 0.12 Hz Gaussian and a 0.15 Hz Lorentzian

window function was applied to the free induction decay (FID) prior to

transformation and polynomial baseline correction and drift correction was applied

to each spectrum. Peak chemical shifts were referenced to CDCl$_3$ at 77.16 ppm

(Gottlieb, et al., 1997).

6.3.6 Statistical analysis

Mean values were reported plus or minus standard error of the mean.

Percentage data were arcsin transformed prior to analysis. Normality and

homogeneity of variance were confirmed and a comparison between means was

achieved by 1-way analysis of variance (ANOVA). Multiple comparisons were

achieved by Turkey-Kramer HSD (honestly significant difference). Significance was

accepted as probabilities of 0.05 or less. Statistical analysis was performed using

SPSS (statistical package for social science) for windows version 11.

6.4 RESULTS

6.4.1 Lipid class

TAG accounted for the largest proportion of the lipid in the diet (88.2%) and in

the tissues (81.9-94.1%) (Table 6.1). The red muscle had significantly ($p<0.05$)

higher percentage of TAG compared to the gill and white muscle, but diet was not

significantly different to any sampled tissue. The PL varied across the tissues and

was significantly ($p<0.05$) higher in the gills (15.8%) and the white muscle (15.5%),

than in red muscle (4.8%). The diet contained 8.4% PL and was not significantly different from any tissues. FFA were significantly ($p<0.01$) higher in the diet (1.7%).

6.4.2 Fatty acid profiles of total lipid

The major (>10%) dietary fatty acids were 16:0, 18:1ω9, EPA and DHA, with 14:0 and 16:1ω7 present at moderate percentage abundances (5% to 10%) (Table 6.1). Of all the FA, 16:0, 18:1ω9 and DHA were at high proportions across all tissues whereas 14:0, 16:1ω7 and EPA were present at moderate percentage. In addition, 18:0 was present at moderate abundance in white muscle and gills. The TLE fatty acid profile of the three tissues reflected closely the profiles of the diet. This was judged by the small statistical difference between the diet and the tissues. There were no significant differences in the relative percentage of MUFA in any tissue or the diet. The major exception was EPA which showed significantly ($p<0.01$) lower percentage in all the salmon tissues compared with the diet.

A significantly ($p<0.05$) lower unsaturated fatty acid/saturated fatty acid (UFA/SFA) ratio in the white muscle and significantly ($p<0.05$) lower ω3/ω6 ratios were observed in all tissues compared to the diet. There was a significant percentage reduction in total ω3 PUFA ($p<0.01$), total ω6 PUFA ($p<0.05$) and total PUFA ($p<0.01$) in white muscle compared to the diet and red muscle.

Table 6.1: FA and lipid class composition and content of the total lipid fraction of a commercial formulated diet and white and red muscle and gill tissue of Atlantic salmon *Salmo salar* fed that diet when held at 19oC

% FA	Diet			Gill			White Muscle			Red Muscle		
SFA												
14:0	6.4	±	0.1[b]	5.2	±	0.1[a]	6.3	±	0.3[b]	6.5	±	0.2[b]
16:0	19.4	±	0.3[a]	19.0	±	0.1[a]	20.3	±	0.4[b]	19.3	±	0.4[a,b]
18:0	4.2	±	0.1[a]	5.4	±	0.1[b]	5.0	±	0.0[a,b]	4.6	±	0.1[a,b]
Other SFA[d]	1.9	±	0.0	1.9	±	0.0	2.4	±	0.0	2.3	±	0.0
Total SFA	32.0	±	0.4	31.5	±	0.1	34.0	±	0.7	32.6	±	0.6
MUFA												
16:1ω7c	7.0	±	0.1	6.7	±	0.1	7.3	±	0.4	8.0	±	0.3
18:1ω9c[e]	14.1	±	0.3	15.7	±	0.2	16.7	±	0.5	16.7	±	0.3
18:1ω7c	3.0	±	0.0	3.5	±	0.0	3.6	±	0.2	3.6	±	0.1
20:1ω9c	1.6	±	0.1	1.6	±	0.0	1.8	±	0.1	1.8	±	0.0
Other MUFA[f]	3.6	±	0.0	3.9	±	0.0	3.2	±	0.0	2.9	±	0.0
Total MUFA	29.3	±	0.1	31.3	±	0.0	32.6	±	0.2	33.1	±	0.1
ω3 PUFA												
18:4ω3	2.8	±	0.1[b]	2.2	±	0.0[b]	0.4	±	0.4[a]	2.4	±	0.0[b]
20:4ω3	0.9	±	0.0	1.2	±	0.0	1.0	±	0.0	1.2	±	0.0
20:5ω3 EPA	12.7	±	0.3[b]	8.0	±	0.2[a]	6.7	±	0.6[a]	7.3	±	0.3[a]
22:6ω3 DHA	11.0	±	0.9[a,b]	13.1	±	0.3[b]	10.4	±	1.2[a,b]	11.0	±	0.6[a]
22:5ω3 DPA	2.0	±	0.0	3.2	±	0.1	2.7	±	0.2	3.0	±	0.1
Other ω3 PUFA[g]	0.6	±	0.0	0.8	±	0.0	0.7	±	0.0	0.8	±	0.0
Total ω3 PUFA	30.0	±	0.2[b]	28.6	±	0.1[b]	21.9	±	0.1[a]	25.6	±	0.2[b]
ω6 PUFA												
18:2ω6	4.2	±	0.1	4.0	±	0.0	4.1	±	0.2	4.4	±	0.1
20:4ω6	0.8	±	0.0	1.2	±	0.0	0.7	±	0.1	0.7	±	0.0
22:5ω6	0.4	±	0.0[b]	0.4	±	0.0[b]	0.3	±	0.0[a]	0.3	±	0.0[a]
Other ω6 PUFA[h]	0.6	±	0.0	1.2	±	0.0	0.9	±	0.0	0.9	±	0.0
Total ω6 PUFA	6.0	±	0.0[a]	6.9	±	0.0[b]	6.0	±	0.1[a]	6.3	±	0.0[a,b]
Other PUFA[i]	1.8	±	0.0	1.8	±	0.0	3.8	±	0.5	2.4	±	0.0
Total PUFA	37.8	±	0.2[b]	37.2	±	0.3[b]	31.7	±	0.2[a]	34.3	±	0.1[b]
ω3/ω6	5.0	±	0.1[c]	4.2	±	0.1[b]	3.7	±	0.1[a]	4.1	±	0.2[a,b]
UFA/SFA	2.1	±	0.0[b]	2.2	±	0.0[b]	1.9	±	0.0[a]	2.1	±	0.0[b]
Lipid Class												
TAG	88.2	±	0.4[a,b]	81.8	±	1.0[a]	83.8	±	4.9[a]	94.1	±	1.0[b]
FFA	1.7	±	0.1[c]	0.6	±	0.0[b]	0.2	±	0.1[a]	0.6	±	0.1[b]
ST	1.3	±	0.1[b]	1.4	±	0.1[b]	0.5	±	0.1[a]	0.5	±	0.1[a]
PL	8.4	±	0.5[a,b]	15.8	±	1.0[b]	15.5	±	4.9[b]	4.8	±	0.9[a]
Total Lipid (g/kg WW)[j]	262.1	±	3.8	69.4	±	3.2	58.2	±	5.8	220.0	±	12.2

SFA, Saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; DHA, Docosahexaenoic Acid; DPA, Docosapentaenoic Acid; EPA, Eicosapentaenoic Acid; TAG; Triacylglycerol; FFA, free fatty acid; ST, sterol; PL, polar lipid; WW, wet weight; UFA, Unsaturated fatty acid;
[a,b,c] Mean values across the row not sharing a common superscript were significantly different (P<0.05) as determined by Turkey-Kramer HSD; n =6.
[d] Other SFA includes 15:0, 17:0, 20:0, 22:0 and 24:0
[e] Includes 18:3ω3 (minor component)
[f] Other MUFA includes 16:1ω9, 16:1ω5, 18:1ω5, 20:1ω7, 22:1ω9, 22:1ω11 and 24:1ω9
[g] Other ω3 PUFA include 21:5ω3 and 24:6ω3
[h] Other ω6 PUFA include 20:2ω6, 20:3ω6, 22:4ω6 and 24:5ω6
[i] Other PUFA include 16:2ω4, 16:3ω4 and 18:2ω9
[j] Determined by TLC-FID

6.4.3 15°C Fish

Lipid class and fatty acid profiles for fish grown at a water temperature of 15°C are shown in Table 6.2. These fish had a very similar history and results are included to provide comparison to the fish grown at the elevated temperature of 19°C. The lipid class profiles for the 15°C fish are very simular to 19°C fish (Table 6.1). The fatty acid profiles showed that in the red and white muscle there was decreased SFA and increased ω3 PUFA and total PUFA in the 15°C fish (Figure 6.1).

6.4.4 Lipid class composition of polar lipid

The major (>10%) phospholipid classes in all samples were phosphatidylethanolamine, PE, and phosphatidylcholine, PC (Table 6.3). Both PE and phosphatidylserine (PS) were significantly ($p<0.05$) lower in all tissues compared with the diet. Phophatidylinositol (PI) was significantly ($p<0.05$) higher in red muscle. Unidentified polar lipid (UPL) consisted of several very polar peaks which may have included sphingomyelin and lysophosphatidyl derivatives. UPL was significantly higher in all salmon tissues ($p<0.05$) compared to the diet.

Table 6.2: FA and lipid class composition and content of the total lipid fraction of white and red muscle and gill tissue of Atlantic salmon *Salmo salar* fed commercial formulated diet when held at 15°C

	Percentage composition								
SFA	Gill 15°C			White 15°C			Red 15°C		
14:0	4.8	±	0.5	5.9	±	0.1	5.8	±	0.2
16:0	21.3	±	0.4	17.3	±	0.1	18.7	±	0.5
18:0	5.9	±	0.4	4.1	±	0.1	2.8	±	1.4
Other SFA[d]	2.1	±	0.2	2.1	±	0.2	2.0	±	0.1
Total SFA	34.1	±	1.0	29.4	±	0.3	29.3	±	0.6
MUFA									
16:1ω7c	7.4	±	0.5	8.5	±	0.1	8.1	±	0.1
18:1ω9c[e]	15.9	±	0.4	15.6	±	0.4	14.7	±	0.4
18:1ω7c	4.2	±	0.1	3.7	±	0.0	3.7	±	0.0
20:1ω9c	1.6	±	0.1	1.7	±	0.1	1.4	±	0.1
Other MUFA[f]	3.2	±	0.1	3.3	±	0.1	3.2	±	0.1
Total MUFA	32.2	±	1.1	32.9	±	0.6	31.1	±	0.5
ω3 PUFA									
18:4ω3	1.9	±	0.2	2.5	±	0.0	2.4	±	0.1
20:4ω3	1.1	±	0.1	1.3	±	0.0	0.9	±	0.4
20:5ω3 EPA	7.1	±	0.1	7.1	±	0.3	7.9	±	0.5
22:6ω3 DHA	14.1	±	1.5	13.9	±	0.3	16.2	±	0.6
22:5ω3 DPA	2.2	±	0.2	3.4	±	0.1	3.2	±	0.1
Other ω3 PUFA[g]	0.8	±	0.1	1.3	±	0.1	1.1	±	0.1
Total ω3 PUFA	27.2	±	1.2	29.4	±	0.7	31.8	±	0.8
ω6 PUFA									
18:2ω6	4.3	±	0.3	5.2	±	0.1	4.9	±	0.0
20:4ω6	2.2	±	0.5	0.8	±	0.0	0.9	±	0.1
22:5ω6	0.3	±	0.0	0.4	±	0.0	0.4	±	0.0
Other ω6 PUFA[h]	0.9	±	0.1	1.5	±	0.1	1.1	±	0.1
Total ω6 PUFA	7.7	±	0.2	7.8	±	0.1	7.3	±	0.0
Other PUFA[i]	0.5	±	0.1	1.0	±	0.1	0.9	±	0.2
Total PUFA	35.4	±	1.2	38.2	±	0.7	40.1	±	0.7
ω3/ω6	3.5	±	0.2	3.8	±	0.1	4.3	±	0.1
UFA/SFA	2.0	±	0.1	2.4	±	0.0	2.4	±	0.1
Lipid Class									
TAG	79.2	±	0.3	84.2	±	2.1	96.1	±	2.1
FFA	0.7	±	0.0	0.2	±	0.1	0.4	±	0.1
ST	1.6	±	0.1	0.6	±	0.2	0.3	±	0.1
PL	18.5	±	0.2	15.0	±	0.8	3.2	±	0.7
Total Lipid (g/kg WW)[j]	31.5	±	8.4	57.9	±	3.2	222.7	±	6.7

Abbreviations and other footnote definitions, see Table 6.1

6.4.5 Fatty acid profiles of polar lipid

The major (>10%) dietary polar lipid fatty acids (PLFA) were 16:0, 18:1ω9, 20:5ω3 and 22:6ω3, with 14:0, 16:1ω7 and 18:2ω6 present at moderate percentage (from 5% to 10%) (Table 6.3). The major PLFA for the gills were 16:0, 18:0 and 18:1ω9, with 14:0, 16:1ω7 and 22:6ω3 also present at moderate abundance. The major PLFA for white muscle were 16:0, 18:0, 18:1ω9 and 22:6ω3 with 14:0, and 20:5ω3 also present at moderate abundance. The major FA for red muscle PL were 16:0, 18:1ω9 and 22:6ω3, with 14:0, 18:0 and 20:5ω3 also present at moderate abundance.

Accumulation of PUFA was not observed in the PL fraction. This was shown by the decrease in total PUFA percentage across all tissues compared to the FA profile of the 19°C fish. Over 50% of PLFA in the gill and white muscle were saturates (especially 16:0, 34.4% and 37.1% respectively) which were significantly ($p>0.01$) higher than in the red muscle.

Gill PLFA showed statistically higher percent of MUFA, especially 18:1ω9 (17.7%) and 18:1ω7 (3.9%) and total MUFA (30.2%). This coincided with significantly ($p>0.01$) lower relative abundances of ω3, ω6 and total PUFA. Lower percentages of ω3, ω6 and total PUFA occurred in the white muscle compared with red muscle.

Table 6.3: FA and lipid class percentage composition comparison and content of the polar lipid fraction of white and red muscle and gill tissue of Atlantic salmon *Salmo salar* fed commercial formulated diet when held at 19°C

Sample	Gill			White Muscle			Red Muscle		
SFA									
14:0	5.6	±	0.3	5.2	±	0.3	5.0	±	0.4
16:0	34.4	±	0.5[b]	37.1	±	5.8[b]	23.2	±	0.5[a]
18:0	12.8	±	1.8	14.1	±	5.6	7.4	±	1.1
Other SFA[d]	3.0	±	0.1	2.3	±	0.1	1.9	±	0.0
Total SFA	55.8	±	0.7[b]	58.6	±	3.0[b]	37.6	±	0.5[a]
MUFA									
16:1ω7c	5.3	±	0.2[b]	3.8	±	0.1[a]	4.7	±	0.5[a,b]
18:1ω9c[e]	17.7	±	0.3[b]	12.1	±	1.2[a]	12.3	±	0.8[a]
18:1ω7c	3.9	±	0.1[b]	3.0	±	0.5[a,b]	2.7	±	0.3[a]
20:1ω9c	0.9	±	0.1[b]	0.2	±	0.3[a]	0.9	±	0.1[b]
Other MUFA[f]	2.6	±	0.0	1.0	±	0.1	3.0	±	0.0
Total MUFA	30.2	±	0.1[c]	20.2	±	0.4[a]	23.7	±	0.4[b]
ω3 PUFA									
18:4ω3	0.3	±	0.2[a]	0.5	±	0.4[a,b]	1.2	±	0.0[b]
20:4ω3	0.0	±	0.0[a]	0.3	±	0.3[b]	0.8	±	0.0[c]
20:5ω3 EPA	3.5	±	0.8	5.9	±	3.3	7.8	±	1.3
22:6ω3 DHA	5.2	±	1.7[a]	10.5	±	6.8[a,b]	20.1	±	3.2[b]
22:5ω3 DPA	0.4	±	0.3[a]	1.0	±	0.8[a]	2.75	±	0.1[c]
Other ω3 PUFA[g]	0.0	±	0.0	0.0	±	0.0	0.3	±	0.0
Total ω3 PUFA	9.4	±	0.5[a]	18.1	±	1.9[b]	33.0	±	0.8[c]
ω6 PUFA									
18:2ω6	1.9	±	0.1	1.6	±	0.2	2.3	±	0.3
20:4ω6	2.1	±	0.5[b]	0.8	±	0.6[a]	1.4	±	0.1[a,b]
22:5ω6	0.0	±	0.0	0.2	±	0.2	0.6	±	0.0
Other ω6 PUFA[h]	0.0	±	0.0	0.0	±	0.0	0.2	±	0.1
Total ω6 PUFA	3.9	±	0.1	2.6	±	0.2	4.5	±	0.1
Other PUFA[i]	0.7	±	0.0	0.5	±	0.1	1.2	±	0.0
Total PUFA	14.0	±	0.2[a]	21.2	±	0.8[b]	38.8	±	0.3[c]
w3/w6	2.4	±	0.3[a]	7.1	±	1.1[b]	7.3	±	0.4[b]
UFA/SFA	0.8	±	0.2[a]	0.7	±	0.3[a]	1.7	±	0.4[b]
Lipid Class									
PE	58.4	±	9.4	31.0	±	9.1	49.3	±	9.1
PI	2.4	±	0.4[a]	2.7	±	0.0[a,b]	3.4	±	0.1[b]
PS	1.4	±	0.4[a]	0.7	±	0.1[a]	2.5	±	0.3[b]
PC	32.3	±	11.6	27.2	±	10.9	34.4	±	10.1
UPL	5.6	±	1.4[a]	38.4	±	2.7[b]	10.4	±	1.2[a]
Total Lipid (g/kg WW)[j]	10.96	±	1.3	9.02	±	1.8	10.9	±	0.9

PE, phosphatidylethanolamine; PI, phophatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; UPL undetermined polar lipid; Abbreviations and other footnote definitions, see Table 6.1

Significantly ($p>0.01$) lower percent of DHA was present in the gills (5.2%), than white muscle (10.5%), but red muscle was significantly ($p<0.05$) higher (20.1%). The UFA/SFA ratio was significantly ($p<0.05$) lower in the gill and white muscle fractions. The ω3/ω6 ratio was significantly ($p<0.05$) lower in the gills compared to the red and white muscle ($p.>0.05$).

6.4.6 Regiospecific analysis of the membrane lipids.

The major PL (>10%) species for the gills were PC 16:0/18:1 (denotes 16:0 at *sn-1* and 18:1 at *sn-2* on the PC molecule), PE 18:0/18:1 and PE 18:0/22:6, with moderate percentages (5% to 10%) of PC 16:0/22:6 and PE 16:0/22:6 (Table 6.4a,b). The gills had the highest proportion of SFA/MUFA, MUFA/MUFA and MUFA/PUFA profiles. The major PL species for white muscle was PE 18:0/22:6 with moderate percentages of PC 16:0/18:1, PC 16:0/22:6 and PE 18:0/20:5. The major PL species for red muscle were PC 16:0/22:6, PE 16:0/22:6 and PE 18:0/22:6, with moderate percentages of PE 18:0/20:5 and PE 18:1/22:6.

Faint signals of phosphatidylglycerol (PG) species was detected by LC-MS, in particular PG 16:0/18:2, PG 16:0/18:1, PG 18:0/18:2 and PG 18:0/18:1, in some samples. Small amounts of PG 16:0/18:1 were found in all samples, as was sphingomyelin.

Table 6.4a: Composition of the polar lipid fraction of white and red muscle and gill tissue of Atlantic salmon *Salmo salar* fed commercial formulated diet when held at 19°C

	Percentage Composition								
PC	Gills			White Muscle			Red Muscle		
16:0/18:1	10.2	±	2.4	6.8	±	2.1	3.9	±	0.9
14:0/22:6	0.7	±	0.2	1.0	±	0.6	1.6	±	0.4
16:0/20:5	4.4	±	1.2	4.7	±	0.6	4.9	±	1.1
16:1/22:6	0.9	±	0.2	0.5	±	0.4	0.8	±	0.1
16:0/22:6	7.0	±	1.7	8.2	±	1.5	11.6	±	3.1
16:0/22:5	2.8	±	0.8	1.9	±	0.5	3.1	±	0.8
18:1/22:6	1.3	±	0.3	0.7	±	0.2	1.7	±	0.5
18:0/22:6	0.8	±	0.2	0.8	±	0.5	0.5	±	0.1
20:5/22:6	0.3	±	0.1	0.5	±	0.0	1.7	±	0.4
22:6/22:6	0.5	±	0.1	tr	±	0.0	1.0	±	0.2
18:0/18:1	3.1	±	0.8	1.2	±	1.0	1.4	±	0.2
15:0/22:6	0.4	±	0.1	0.4	±	0.3	2.1	±	0.1
PC Total	32.3			27.2			34.4		
SFA /MUFA[a]	13.3			8.0			5.3		
SFA / PUFA[b]	16.0			17.0			23.8		
MUFA / PUFA[c]	2.2			1.2			2.6		
PUFA / PUFA[d]	0.8			0.5			2.7		
PE	Gills			White Muscle			Red Muscle		
14:0/22:6	tr	±	0.0	0.3	±	0.1	0.6	±	0.1
16:0/20:5	1.1	±	0.2	0.9	±	0.2	1.7	±	0.3
18:1/18:1	1.2	±	0.1	0.2	±	0.0	0.4	±	0.1
18:0/18:1	14.7	±	2.9	3.3	±	0.5	2.8	±	0.6
18:2/20:5	0.2	±	0.0	0.3	±	0.1	0.6	±	0.1
16:0/22:6	6.0	±	1.5	4.9	±	1.3	12.9	±	3.3
16:0/22:5	1.1	±	0.3	tr	±	0.0	tr	±	0.0
18:0/20:5	7.8	±	2.2	5.0	±	1.2	6.4	±	1.1
18:2/22:6	1.4	±	0.2	0.6	±	0.2	1.0	±	0.3
18:1/22:6	6.2	±	1.7	2.6	±	0.4	5.3	±	1.5
18:0/22:6	13.5	±	1.1	11.5	±	3.0	13.5	±	2.4
20:5/22:6	0.7	±	0.2	0.4	±	0.1	2.1	±	0.5
22:6/22:6	4.6	±	0.6	1.1	±	0.2	2.0	±	0.5
PE Total	58.4			31.0			49.3		
SFA / MUFA	14.7			3.3			2.8		
SFA / PUFA	29.5			22.5			35.0		
MUFA/MUFA[e]	1.2			0.2			0.4		
MUFA / PUFA	6.2			2.6			5.3		
PUFA / PUFA	5.4			1.8			4.7		

Continued next page. For Abbrivations see Table 6.4b

Table 6.4b: Composition of the polar lipid fraction of white and red muscle and gill tissue of Atlantic salmon *Salmo salar* fed commercial formulated diet when held at 19°C

Percentage Composition								
PI	Gills			White Muscle			Red Muscle		
16:0/18:2	0.0	±	0.0	0.0	±	0.0	0.1	±	0.0
16:0/20:5	0.0	±	0.0	0.0	±	0.0	0.1	±	0.0
16:0/20:4	0.2	±	0.1	0.1	±	0.0	0.1	±	0.0
16:0/22:6	0.2	±	0.1	0.2	±	0.1	0.2	±	0.1
18:0/20:5	0.1	±	0.0	0.6	±	0.1	0.5	±	0.1
18:0/20:4	1.5	±	0.4	0.7	±	0.2	0.8	±	0.1
18:1/22:6	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0
18:0/22:6	0.3	±	0.0	0.9	±	0.2	1.6	±	0.3
PI Total	2.4			2.7			3.4		
SFA / PUFA	2.3			2.6			3.3		
MUFA / PUFA	0.1			0.1			0.1		
PS	Gills			White Muscle			Red Muscle		
16:0/22:6	0.4	±	0.1	0.2	±	0.0	0.7	±	0.0
18:1/22:6	0.1	±	0.0	0.0	±	0.0	0.2	±	0.0
18:0/22:5	tr	±	0.0	0.1	±	0.0	0.9	±	0.2
20:1/22:6	0.1	±	0.0	0.1	±	0.0	0.3	±	0.0
18:0/22:6	0.8	±	0.2	0.2	±	0.0	0.5	±	0.1
PS Total	1.4			0.7			2.5		
SFA / PUFA	1.2			0.6			2.1		
MUFA / PUFA	0.2			0.1			0.4		
Total	Gills			White Muscle			Red Muscle		
SFA/ MUFA	28.0			11.3			8.0		
SFA / PUFA	49.0			42.7			64.2		
MUFA / MUFA	1.2			0.2			0.4		
MUFA /PUFA	8.6			4.0			8.4		
PUFA / PUFA	6.2			2.4			7.4		

SFA, Saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; PE, phosphatidylethanolamine; PI, phophatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; tr, trace amounts; n = 4.

[a] Percentage of lipid with the SFA in the *sn*-1 and MUFA in the *sn*-2 position
[b] Percentage of lipid with the SFA in the *sn*-1 and PUFA in the *sn*-2 position
[c] Percentage of lipid with the MUFA in the *sn*-1 and PUFA in the *sn*-2 position
[d] Percentage of lipid with the PUFA in the *sn*-1 and PUFA in the *sn*-2 position
[e] Percentage of lipid with the MUFA in the *sn*-1 and MUFA in the *sn*-2 position

6.4.7 Regiospecific analysis of storage lipid - ^{13}C NMR

Where random distribution of a FA occurs across the TAG molecule, 33% of

any FA would be expected at the *sn*-2 position. Since the TAG molecule has a

symmetric plane the relative proportions at the *sn*-1 and *sn*-3 positions cannot be identified by ^{13}C NMR. DHA had a high affinity with the *sn*-2 position (62.1-68.3% across the tissues). 18:4ω3 (42.1-46.3%) and DPA (37.3-39.6%) also had an affinity to the *sn*-2 position. EPA (34.2-36.0% *sn*-2) and 20:4 (32.6-35.3% *sn*-2) had even distribution across the molecule and did not show a preferred regiospecific position (Table 6.5).

Table 6.5: Percentage of fatty acid in the *sn*-2 position in the triacylglycerol fraction from Atlantic salmon *Salmo salar* tissues when held at 19°C and fed a commercial formulated diet determined by ^{13}C NMR spectroscopy

	Diet			Gills			White Muscle			Red Muscle		
DHA	66.6	±	3.4	68.3	±	0.1	62.1	±	1.3	64.2	±	0.2
DPA	50.8	±	1.6[b]	37.3	±	0.7[a]	37.8	±	1.1[a]	39.6	±	0.2[a]
EPA	23.4	±	1.2[a]	34.3	±	1.3[a,b]	36.0	±	0.4[b]	34.7	±	1.3[b]
20:4ω6	26.2	±	1.8	nd			32.6	±	2.3	35.3	±	0.4
18:4ω3	35.0	±	1.7[a]	42.1	±	0.6[a,b]	45.3	±	0.8[b]	46.3	±	0.1[b]

DHA, Docosahexaenoic Acid; DPA, Docosapentaenoic Acid; EPA, Eicosapentaenoic Acid; nd, not determined.
[a,b,c] Mean values across the row not sharing a common superscript were significantly different (P<0.05) as determined by Turkey-Kramer HSD; n = 4.

EPA and 18:4ω3 abundance at the *sn*-2 position were significantly ($p<0.01$) higher in the tissues relative to the diet, whereas DPA was significantly ($p<0.01$) lower. The percentage distribution of all the FA did not show significant differences across the three tissues. However, there were significant increases ($p<0.01$) in EPA and 18:4ω3 and a significant decrease in DPA ($p<0.01$) in the *sn*-2 position relative to the diet.

6.5 DISCUSSION

This is the first detailed examination of the lipid profile of Atlantic salmon at an elevated temperature (19°C). We describe the effect elevated temperature has on the composition of storage and structural lipids of three key tissues in seawater Atlantic salmon. The water temperature of 19°C was considerably above the optimum although representative of environmental conditions now experienced by an established industry in Tasmania, Australia (Carter, et al., 2003). As often observed the fatty acid profile of fish, particularly the storage (TAG) fraction, is strongly influenced by diet (Henderson and Tocher, 1987; Sargent, et al., 2002). Many studies performed at lower temperatures have shown that salmon accumulate ω3 (with a corresponding reduction in SFA) (Bendiksen and Jobling, 2003; Jobling and Bendiksen, 2003; Bell, et al., 2004). We observed, in the red and white muscle, lower levels of total ω3 PUFA and total PUFA and higher SFA in the FA profile in the TLE of the 19°C fish compared to fish grown at 15°C (Figure 6.1).

Environmental factors, particularly temperature, have a more significant influence on the structural lipid (PL) fraction (Hazel, 1979, 1984, 1990; Hazel, et al., 1991). Elevated temperature raised fish had higher SFA in the PLFA and lower levels of ω3, ω6 and total PUFA in the gill and white muscle. These changes suggest adaptation of the cell membrane structure has occurred, which could be explained as a response to the elevated water temperature. In other species it has been shown that there is more PUFA present and less SFA in fish that live in colder temperatures (Dunstan, et al., 1999). Consideration of both membrane and storage lipids, including regiospecifity, allowed further examination of these adaptive changes.
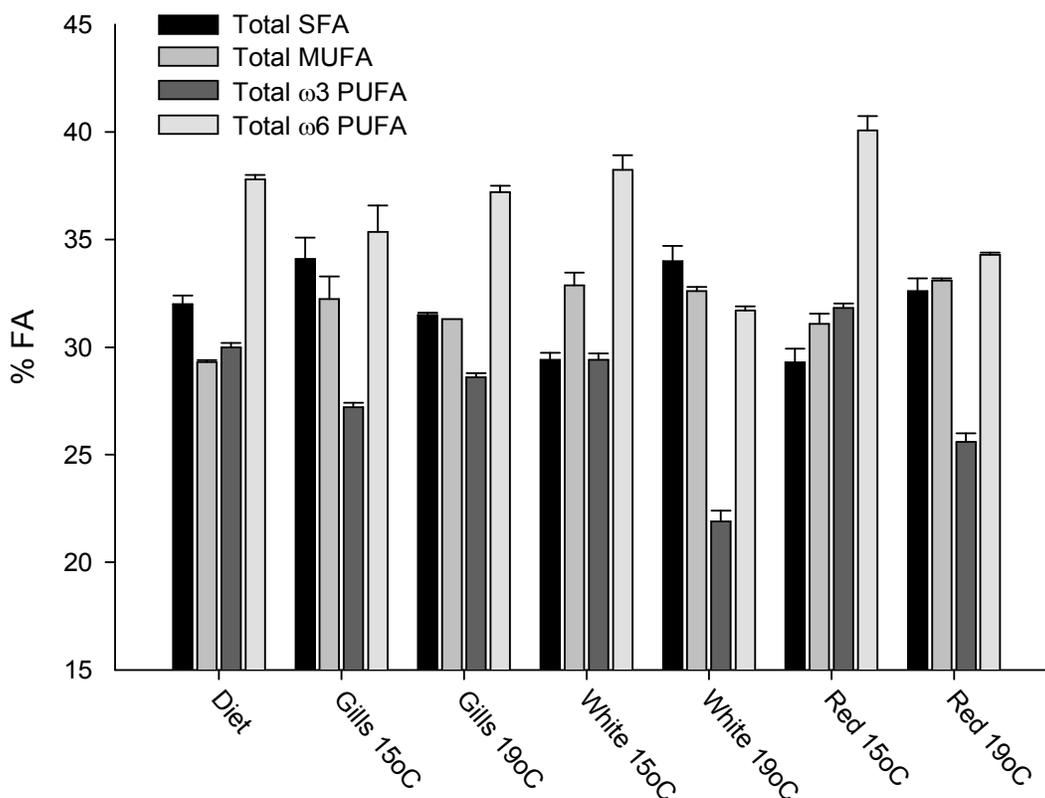
Figure 6.1: Abundances of SFA, MUFA, total ω3 PUFA and total PUFA from the total lipid extract (TLE) for the diet, red muscle, white muscle and gills from Atlantic salmon *Salmo* salar grown at 15 and 19°C.
Values are means with ± SE.

6.5.1 Membrane Lipids

Phospholipids, the membrane lipids that form the cell bi-layers, consist of two

fatty acid moieties on a glycerol backbone generally with one of 5 different

functional phosphatidyl head groups attached. Specific fatty acids chains can be

attached at either the *sn*-1 or *sn*-2 positions. Fish phospholipids generally contain

16:0 and 18:1ω9 at the *sn*-1 position and 20:5ω3 and 22:6ω3 at the *sn*-2 position

(Sargent, et al., 2002). This generalization is dependent on the phospholipid class

and also on the particular tissue (Sargent, et al., 2002). In the present study 16:0 (33.4%, 17.0%, 39.3% gill, white muscle, red muscle (G,W,R)) and 18:0 (41.1%, 23.6%, 28.1 G,W,R) were the major PLFA at the *sn*-1 position with 18:1 (8.9%, 3.6, 7.7% G,W,R) present at low percentage as determined by LCMS. The major PLFA at the *sn*-2 position were 22:6ω3 (46.1%, 35.1%, 60.7% G,W,R), 20:5ω3 (13.6%, 11.5%, 14.2% G,W,R) and 18:1 (29.2 %, 11.5%, 8.5% G,W,R).

In the present study the PLFA profile showed a reduced number of statistical differences between tissue FA profiles in the gills and white muscle when compared to the TLE FA profile. This observation is consistent with the PL profile being more functional (membrane structure role) and therefore more adaptive to environmental change and also less influenced by diet. The PLFA profile is also very dependent on the particular tissue analysed.

Phospholipids generally contain high levels of 16:0, 18:1ω9, 20:5ω3 and 22:6ω3 as principle fatty acids in the membrane bi-layer (Sargent, et al., 2002). The PLFA fractions from the present study on Atlantic salmon contained these principle FA, but also contained an enhanced percentage of 18:0 with a significantly lower percentage ($p>0.01$) of 18:2ω6 compared with the diet. Significantly lower percentages ($p>0.05$) of ω3 PUFA, ω6 PUFA and total PUFA occurred in the gills and white muscle in the PLFA compared with red muscle and the diet (Table 6.3). The relative percentage of DHA was lower in the gill (5.2%) and in white muscle (10.5%), but was significantly higher ($p>0.05$) in red muscle (20.1%). In the gill, the percentages of ω3 PUFA, ω6 PUFA and total PUFA were lower leading to higher percentages of 16:0, 18:1 and 18:0 FA which are important pulmonary surfactants (Holm, et al., 1996). Higher percent levels of DHA in the gill TAG may be

attributed to the presence of residue blood in the gill tissue. The reduction of PUFA in white muscle is most likely due to the effect of elevated temperature.

The regiospecific distribution of membrane polar lipids cannot be determined by traditional FA analysis. ESI-RP-LCMS provides a rapid method to compare relative percentage of the individual intact lipids across tissues. There is only limited work published on phospholipid molecular species distribution in salmonoid tissues determined by tandem mass spectroscopy (Chen and Claeys, 1996; Hvattum, et al., 2000). These studies, on the distribution of individual molecular species across phospholipid species, focused on the liver (Chen and Claeys, 1996) and head kidney (Hvattum, et al., 2000) tissues. We examined the molecular species distribution of the gill, white and red muscle.

The principle molecular species in muscle PC in most fish is 16:0/22:6 (Tocher, 1995). Increased PC 16:0/22:6 tends to fluidize the membrane and reduce the temperature sensitivity of electrolyte permeation (Hazel, et al., 1991). PC 16:0/22:6 is the predominate lipid in the PC fraction of the muscle tissues of Atlantic salmon, but not in the gills. The PI fraction was relatively high in 18:0 and low in PUFA, which was predominantly 20:4ω6; this has previously been observed in fish and was suggested as due to a role in eicosanoid metabolism (Tocher, 1995).

Studies on the molecular diversity of wild Atlantic cod muscle showed that SFA/PUFA and MUFA/PUFA dominated, especially PC 16:0/20:5, PC 16:0/22:6, PE 16:0/22:6 and PE 18:1/22:6 (Bell and Dick, 1991). The PUFA/PUFA totalled between 21% to 38% in the PC and PE fractions for cod (Bell and Dick, 1991). Our results showed very little PUFA/PUFA, 0.5 % PC, 1.8% PE and 4% total for white muscle and 2.7% PC, 4.7% PE and 8.4% total in red muscle. This finding is

consistent with the elevated water temperature and lower PUFA percentages noted earlier for the FAME profiles. The SFA/PUFA species dominated (42.7 – 64.2%) throughout the tissues, with the gill showing low (28%) levels of SFA/MUFA.

6.5.2 Storage lipids

Along with the structural membrane bi-layer function, lipids play an important role in energy storage in fish (Corraze, 1999) in the form of TAG. Fish oil, which is predominately TAG, is nutritionally important for the supply of omega-3 LC-PUFA in the human diet. However, in salmon it is recognised that dietary TAG are hydrolysed to FFA in the gastrointestinal tract prior to absorption. The regiospecific position of the dietary lipids is less important in fish than in mammals, including humans. It has been well documented that consuming quantities of LC-PUFA in the form of TAG is nutritionally beneficial (Sargent, 1997), with recent reports suggesting the position of the LC-PUFA on the glycerol backbone is also important in human nutrition (Kew, et al., 2003b). In general, fish oils have SFA and MUFA preferentially located at the *sn*-1 and *sn*-3 positions on the glycerol backbone, whereas LC-PUFA are generally preferentially located in the *sn*-2 position (Sargent, et al., 2002).

The original speculation that TAG structure might influence lipid absorption was based on observed differences in fat absorption by infants fed breast or formulated milk (Lucas, et al., 1997). It was shown that palmitate (16:0) in the *sn*-2 position is generally not hydrolysed by pancreatic lipase and the remaining monoglyceride was well absorbed. However, when palmitate was in the *sn*-1 and *sn*-3 positions it was hydrolysed forming free fatty acids, which were poorly absorbed (Lucas, et al., 1997). The same argument could be used for the delivery of LC-PUFA in human

diet. LC-PUFA of TAG from Atlantic salmon are largely at the *sn*-2 position and are not hydrolysed by pancreatic lipases and therefore can be absorbed into the intestinal mucosal cells of the small intestine.

It has also been shown that in whale oil EPA and DHA in the *sn*-1 and *sn*-3 positions showed a steric hindrance to pancreatic lipase hydrolysis, which may decrease their intestinal absorption (Bottino, et al., 1967). Therefore fish oil with large quantities of LC-PUFA in the *sn*-2 will be nutritionally more desirable since they are at the preferred position for absorption into the body.

The effect of regiospecific distribution of the TAG molecule on absorption and metabolism has not been thoroughly investigated in fish oils due to the wide diversity of molecular species. There have been few studies showing the relative percentages of EPA and DHA across the glycerol backbone in marine oils. Available results include fish oil (DHA 79% and EPA 53%, *sn*-2 position) (Ando, et al., 1996); salmon oil (DHA 72.6 and EPA 37.8%, *sn*-2) (Aursand, et al., 1995b); this study (62.1-68.3% DHA and EPA 34.2-36.3%, *sn*-2), and cod liver oil (DHA 74.4% and EPA 39.7%, *sn*-2) (Aursand, et al., 1995b). Seal oil shows contrasting regiospecificity (DHA 4.4% and EPA 4.4%, *sn*-2) (Ando, et al., 1996).

There was no significant difference in the regiospecific composition of TAG across the three tissues in Atlantic salmon in the present study. The majority of DHA for all tissues is at the *sn-2* position (62.1–68.3%) which is slightly lower compared to the 15°C fish (68.4-69.7%) and other literature for salmon oil (72.6%) (Aursand, et al., 1995b). Our results suggest a reduced percentage of DHA at the *sn*-2 position at elevated temperature but further work is needed to ascertain this effect. EPA showed a significant ($p > 0.01$) increase in the percentage at the *sn*-2 position

(34.7-36.0%) compared to the diet (23.4%). The percentage of EPA at the *sn*-2

position are comparable to results from previous work on Atlantic salmon (Aursand,

et al., 1995a). Interestingly the proportions of 18:4ω3 at the *sn*-2 position (42.1 -

46.3 %) significantly (*p*>0.05) increased compared to the diet (35.0%). The

significant (*p*>0.05) changes observed in the regiopecific distribution of DPA,

18:4ω3 and EPA, in salmon tissues compared with diet, showed that this distribution

is not just a reflection of diet. Further biosynthesis or some adaptive changes have

taken place in the storage of lipids as TAG in Atlantic salmon.

6.5.3 Temperature effects

Temperature plays a major influence on the membrane and storage lipids of

ectothermic animals such as Atlantic salmon which need to adapt to seasonal and

occasional abrupt changes in environmental temperature (Hazel, 1984; Henderson

and Tocher, 1987; Hazel, et al., 1991; Hazel, et al., 1992). To counteract these

changes, membrane lipids may adapt in several ways: by altering the unsaturation

and chain-length of the fatty acids (Zwingelstein, et al., 1978); by changing the

distribution of fatty acids within the phospholipid molecules (Miller, et al., 1976;

Hazel, 1979); and by altering the composition of the polar head group of the

phospholipids (Hazel and Landrey, 1988; Farkas, et al., 1994). Gill fatty acid

profiles have been shown to vary with environmental temperature (Cossins, 1977).

In general, colder temperatures lead to an increase in unsaturation in gill lipids thus

maintaining its membrane fluidity (Hazel, 1979; Tocher and Sargent, 1990).

Introducing a double bond to 18:0 changes the melting point from about 70°C to

20°C (Corraze, 1999), with additional double bonds further reducing melting point

(22:6ω3, -45°C; 20:5ω3, -52°C) (Corraze, 1999). By exploiting the diversity of the

lipid structure, Atlantic salmon changes its membranes to provide physical

properties to suit the environmental temperature. Since different mixtures of

phospholipid molecular species, which share the same overall fatty acid

composition, can display a marked difference in physical properties, remodelling of

molecular species may also help fish adapt to differences in temperature.

It is well documented that tissue FA composition of salmon, especially muscle

tissue, reflects closely the FA composition of their diet (Henderson and Tocher,

1987; Bell, et al., 2001; Bell, et al., 2002; Sargent, et al., 2002; Bell, et al., 2003;

Bell, et al., 2004). In the present study, of salmon grown at 19°C, significant

differences between diet and tissue profiles were observed. Significantly higher

($p>0.05$) percentage of 16:0 occurred in white muscle, with significantly higher

($p>0.05$) percentage of 18:0 also observed in white muscle as well as the gills. The

major differences were significantly ($p>0.05$) lower percentage of PUFA, in

particular EPA, across all tissues compared to the diet. It has been shown that at

lower temperatures (5-15°C) that LC-PUFA, especially DHA, showed preferential

deposition and retention regardless of the concentration present in diet (Bell, et al.,

2001; Bell, et al., 2003; Bell, et al., 2004). Comparing these results with those for

fish grown at 15°C (Table 6.2) shows the higher temperature increased the

percentage of SFA while reducing total PUFA and ω3 PUFA in particular DHA

(Figure 6.1). These findings indicate that there was major adaptation of the total

body lipid due to extreme temperature. In this study, at the elevated water

temperature, there were also significantly ($p>0.05$) lower percentage of ω3 PUFA

and total PUFA in white muscle tissue. It is presently unclear and remains to be

validated whether the PUFA has been directed preferentially towards metabolism,

presumably for energy production, rather than to storage in muscle tissue in the form

of TAG.

Cold climate has been associated with an increase in the proportions of PE and

decreased proportions of PC (Miller, et al., 1976; Tocher and Sargent, 1990). Our

results show PE was the major phospholipid in Atlantic salmon grown at 19°C. The

ratio of UFA/SFA provides an assessment of the proportion of both MUFA and

PUFA to SFA, and the ratio will be unchanged if MUFA replace PUFA (Jobling and

Bendiksen, 2003). At lower temperatures it has been shown that there has been an

accumulation of UFA with a reduction of SFA which led to an increase in the

UFA/SFA ratio (Jobling and Bendiksen, 2003). As mean water temperature

increases, a reduction in the UFA/SFA ratio for Atlantic salmon occurs at 10°C (4.0

UFA/SFA ratio) (Bell, et al., 2004), 11°C (3.4) (Bell, et al., 2003) and at 12°C (2.5)

(Bransden, et al., 2003). A trial looking at dietary lipids at two temperatures (2 and

8°C), showed high UFA/SFA ratio for the muscle fraction where the diet was most

like a commercial diet (high fat, fish oil). The trial separated both the polar and

neutral lipids with UFA/SFA being 7.1 at 2°C and 7.0 at 8°C for polar lipids and 5.9

at 2°C and 5.3 at 8°C for the neutral lipids (Jobling and Bendiksen, 2003). The 15°C

fish had a higher UFA/SFA ratio (2.4) in the red and white muscle which is due to

an accumulation of UFA (especially PUFA) in the muscle at the lower temperatures.

Our results for fish grown at a elevated temperature are consistent with these

patterns with UFA/SFA ratios of 0.7-1.6 observed for the polar lipids and 1.9-2.2 for

the TLE, the latter also largely neutral (TAG) lipid (81-94% of TLE). At 19°C a

significant ($p > 0.05$) decrease in UFA/SFA occurred in the white muscle for the TLE

fraction, with a highly significant ($p>0.05$) decrease in the white muscle and gill tissues also occurring in the polar lipid.

A study looking at Atlantic salmon cell culture and the effect on phospholipid class and its metabolism showed that temperature significantly affected the incorporation of PUFA in a phospholipid class (Tocher and Sargent, 1990). There was higher PUFA incorporation into PC at 10°C than at 22°C, clearly showing temperature to have an important consequence for membrane structure and possible function (Tocher and Sargent, 1990). In cell culture there was an increase in levels of PUFA, altered distribution of PUFA within the phospholipid classes, and decreased metabolism of C_{18} PUFA for cells grown at 10°C than 22°C (Tocher and Sargent, 1990). It has also been shown, in the marine dinoflagellate *Crypthecodinium cohnii* grown at different temperatures (16-27°C), that at the lower temperature there was a decrease in SAT/SAT and an increase in SAT/PUFA species (Bell and Henderson, 1990).

There is more PUFA present and less SFA in species of fish which live in colder regions (Tocher and Sargent, 1990; Dunstan, et al., 1999). High levels of SFA in diets have been associated with poor human health and recommendations have been made to reduce SFA levels in diets (German and Dillard, 2004). It is also well known that eating foods containing MUFA and specific ω3 and ω6 LC-PUFA have associated health benefits (Belluzzi, et al., 2000; Ruxton, et al., 2004; Shahidi and Miraliakbari, 2004; Nettleton and Katz, 2005). As a source of these MUFA and PUFA, fish and especially fish oil have been traditionally the major resource. Increased SFA in white muscle has implications for the salmon aquaculture industry

in regions where water temperature can reach temperatures of 19°C and beyond in the warmer months.

6.6 CONCLUSION

The results from this study suggest a reduction in the need for dietary PUFA over warmer months. Economically this would be advantageous to the aquaculture industry due to the high cost involved with supplying LC-PUFA rich fish oil in the diet. Conversely, if harvest occurs over these warmer months, the percentage of LC-PUFA in these fish will be lower and will reduce the nutritional quality of the product with respect to ω3 LC-PUFA content. The precise physiological significance of the phospholipid distribution and how incorporation of these results into feed formulation may occur remains to be developed. With new methods such as LC-MS and ^{13}C NMR becoming more accessible and automated, regiospecific analysis can be routinely applied in investigations of marine oils. Such information, as it becomes available, will ultimately provide improved understanding on the distribution, function and bioactivity of LC-PUFA containing oils.

6.7 ACKNOWLEDGEMENTS

6.8 REFERENCES

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CHAPTER 7

The effect on the cell membrane structure and lipid storage of Atlantic salmon (*Salmo salar* L.) fed high levels of docosahexaenoic acid.

7.1 ABSTRACT

This study determined how elevated amounts of dietary docosahexaenoic acid ($22:6\omega3$, DHA) affected the storage of lipids and how it is incorporated in cell membranes of Atlantic salmon (*Salmo salar*). A single cell microorganism, the thraustochytrid *Schizochytrium sp.* L offers a source of DHA rich oil. Four experimental diets containing 100% thraustochytrid oil (TO, 54.5 g kg^{-1} DHA), 100% palm oil (PO, 1.1 g kg^{-1} DHA), a 4:1 palm and thraustochytrid oil mixture (MX, 9.8 g kg^{-1} DHA) and fish oil (FO, 11.1 g kg^{-1} DHA) were fed to Atlantic salmon parr over 9 weeks. Elevated dietary DHA increased the relative proportion of DHA in the fatty acid profile and the cell membrane molecular species. Principal components analysis (PCA) demonstrated that dietary fatty acid composition is the major influence on total lipid profile in salmon. Diet was also the major factor affecting the polar lipid fatty acid profile of muscle tissues. However, exogenous alteration of membranes between salmon at the start and end of the trial contributed to the majority of the differences in the liver and gills for both the polar lipid fatty acids and intact cell membrane molecular species. It is theorised that as salmon approach smoltification at the end of the trial, endogenous alterations in the molecular species of polar lipids in cell membranes occurred in both the liver and gills. Increased dietary DHA increased the absolute amount of DHA stored, which in turn increased the amount in the *sn*-2 "middle" position on the triacylglycerol molecule. This result has biochemical implications for the consumer because the *sn*-2 position is the most readily digested by humans.

7.2 INTRODUCTION

Single cell oils, derived from bacteria, thraustochytrids, diatoms and other micro-algae are the major source of omega 3 long chain polyunsaturated fatty acids (ω3 LC-PUFA) in the marine food web and could provide an alternative and renewable sources of oil for aquaculture (Barclay and Zeller, 1996; Nichols, et al., 1996; Lewis, et al., 1999; Carter, et al., 2003b). Thraustochytrids are heterotrophic and therefore can be fermented to produce a high biomass with large amounts of ω3 LC-PUFA rich lipid in particular docosahexaenoic acid (22:6ω3, DHA) (Lewis, et al., 1999). The ω3 LC-PUFA, particularly DHA, play important roles in cell membranes and it has been demonstrated that increased incorporation into membranes has many positive functional benefits for cells including permeability and fluidity (Stillwell and Wassall, 2003). The endogenous biosynthesis of ω3 LC-PUFA in marine fish is inefficient and therefore must be supplied by their diet (Sargent, et al., 2002; Tocher, 2003). Large scale culture of thraustochytrids has potential for commercial aquafeeds, in particular for use in finishing diets fed prior to harvest to optimise the amount of ω3 LC-PUFA in the product. We have recently shown that replacing fish oil with 100% thraustochytrid oil in the diet did not affect the growth of Atlantic salmon parr and increased their ability to adapt to smoltification (Miller, et al., 2007b). Thraustochytrid oils contain high concentrations of dietary ω3 LC-PUFA, particularly DHA, although it is yet to be assessed how salmon store and incorporate these key FA into membranes.

Recent advances allow examination of the regiospecific distribution of FA in the intact molecular species of cell membranes. Electrospray ionization reversed-phase

liquid chromatography-mass spectrometry (ESI RP-LC-MS) provides a rapid and accurate method to measure PL molecular species. The diversity and abundance of polar lipid molecular species in fish are dependent on species, tissue sampled, dietary and environmental factors such as temperature (Bell and Tocher, 1989; Bell and Dick, 1990; Hvattum, et al., 2000; Miller, et al., 2006). The LC-MS method provides regiospecific and abundance information on molecular species in membranes and has only had limited application to Atlantic salmon (Hvattum, et al., 2000; Miller, et al., 2006). It is known that DHA is readily incorporated into polar lipid (PL) and can directly affect cell membrane properties (Stillwell and Wassall, 2003). Therefore it is important to understand how high concentrations of dietary DHA are incorporated into membrane composition of different tissues compared to traditional fish oil based diets.

The regiospecific distribution of FA, in particular DHA, on the storage lipid triacylglycerol (TAG), can be examined by ^{13}C nuclear magnetic resonance (^{13}C NMR) and only limited research has been performed on Atlantic salmon (Aursand and Grasdalen, 1992; Aursand, et al., 1997; Miller, et al., 2006). The beneficial effects of ω3 LC-PUFA, particularly DHA, are being increasingly recognised in human nutrition and farmed Atlantic salmon are considered a good source for human consumption (Shahidi and Miraliakbari, 2004; Brouwer, et al., 2006; MacLean, et al., 2006). It has been demonstrated that the regiospecific nature of dietary TAG affects the ability of individual FA to be digested by humans as well as Atlantic salmon (Lucas, et al., 1997; Oxley, et al., 2007). Biochemically it is of scientific interest to examine how large amounts of dietary DHA are

regiospecifically incorporated into storage lipid by salmon, in particular in regards to the bioavailability to the consumer.

This study examined how Atlantic salmon incorporated dietary DHA into cellular phospholipids and storage TAG. This experiment used Atlantic salmon from a previous experiment (Miller, et al., 2007b). However, all data presented are original to this study which further explores lipid composition with advanced analytical techniques. The fatty acid and intact molecular distribution of fish lipid were examined by principal components analysis (PCA) to indicate the major influences in membrane structure and lipid profiles. A commercially available source of thraustochytrid oil from the species *Schizochytrium L,* which has a high (35%) concentration of DHA, was assessed against three diets. Palm oil was used as a negative control as it contains no ω3 LC-PUFA and is a cheap, available resource for aquafeeds (Ng, et al., 2007). A 4:1 blend of palm oil and thraustochytrid oil (MX) provided an additional diet with similar concentrations of DHA to that of fish oil diets, and the fish oil diet (FO) represented a traditional ω3 LC-PUFA rich aquafeed. Comparison between initial fish sampled on day one of the experiment with all diet treatments sampled 63 days later allowed insight on the adaptation of membranes in different tissues as salmon approach salt water transfer.

7.3 MATERIALS AND METHODS

7.3.1 Experimental diets

Four diets were formulated to compare thraustochytrid oil (TO), with palm oil (PO), a 4:1 mixture of PO: TO (MX) oil and fish oil (FO) (Table 7.1). Fish meal was defatted three times using a 2:1 mixture of hexane and ethanol (400 ml 100g^{-1} fish meal). Soybean (Hamlet Protein A/S, Horsens, Denmark), casein (MP Biomedicals Australasia Pty Ltd, Seven Hills, NSW, Australia), wheat gluten (Starch Australasia, Land Cove, NSW, Australia) and BOIIC pre-gelatinised maize starch (Penford Australia Limited, Land Cove, NSW, Australia) were used as ingredients. Thraustochytrid oil was provided as DHASCO®-S (Martek, Columbia, Maryland, USA). Fish oil was from jack mackerel *Trachurus symmetricus* L. (Skretting Australia, Cambridge, Tasmania, Australia) and a domestic source of pure palm oil was used (Aoroma, Hallam, Victoria, Australia). Stay-C and Rovimix E50 were supplied from Roche Vitamins Australia (Frenchs Forest, NSW, Australia), and the remaining ingredients were supplied by Sigma-Aldrich (Castle Hill, NSW, Australia). The diets were manufactured into 3 mm diameter pellets using a California Pellet Mill (CL-2), air dried and stored at 5°C (Carter, et al., 2003a).

Table 7.1: Ingredient and lipid composition (g/kg dry matter) of experimental diets.

	Diet			
	PO	MX	TO	FO
Ingredient composition (g kg^{-1})				
Fish meal (defatted)	150	150	150	150
Casein	150	150	150	150
Wheat gluten	100	100	100	100
Soybean meal	226	226	226	226
Fish oil	0	0	0	130
Palm oil	130	104	0	0
Thraustochytrid oil	0	26	130	0
Pre gel starch	150	150	150	150
Vitamin mix[a]	3	3	3	3
Mineral mix[b]	5	5	5	5
Stay C[c]	3	3	3	3
Chlorine chloride	2	2	2	2
Bentonite	50	50	50	50
CMC	10	10	10	10
Sodium mono phosphate	20	20	20	20
Yttrium oxide	10	10	10	10
Chemical composition (g kg^{-1} DM)				
Dry Matter	956.4	961.7	960.5	957.9
Crude protein	389.9	391.6	390.2	387.8
Crude fat	147.6	146.0	150.7	149.3
Energy	20.0	20.0	19.8	20.0
FAME (g kg^{-1} DM)				
16:0	61.4	43.5	38.5	31.4
Total SFA	71.3	56.1	54.7	54.4
16:1ω7c	0.5	0.6	1.0	10.7
18:1ω9c OA	48.9	48.5	3.4	19.2
Total MUFA	51.2	51.1	6.1	43.5
20:5ω3 EPA	0.3	0.8	3.4	18.5
22:6ω3 DHA	1.1	9.8	53.5	11.1
Total ω3	1.7	11.2	60.0	37.2
18:2ω6 LA	22.8	23.4	7.1	8.8
20:4ω6 ARA	0.0	0.1	0.0	1.1
22:5ω6 DPA−6	0.6	3.9	21.2	0.4
Total ω6	23.4	27.6	29.4	11.4
Total PUFA	25.1	38.8	89.4	48.6

TO, Thraustochytrid oil DHASCO®-S from Martek; PO, palm oil; MX, 4:1 mix of palm oil and thraustochytrid oil; FO, fish oil, SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; CMC, carboxymethyl cellulose; DHA, docosahexaenoic acid; EPA, eicosapentaenoic Acid; DPA, docosapentaenoic acid; OA, oleic acid; ARA, Arachidonic acid; LA, linoleic acid..[a] Vitamin mix (ASV4) supplied per kilogram of feed: 2.81 mg thiamine HCL, 1.0 mg riboflavin, 9.15 mg pyridoxine HCL, 25 mg nicotinic acid, 54.35 mg calcium D-pantothenate, 750 mg myo-inositol, 0.38 mg D-biotin, 2.5 mg folic

acid, 0.03 mg cyanocobalamin, 6350 IU retinol acetate, 2800 IU cholecalciferol, 100 IU DL α-tocopherol acetate, 5 mg menadione sodium bisulphate, 100 mg Roche rovimix E50. [b] Mineral mix (TMV4) to supplied per kilogram of feed:117mg $CuSO_4.5H_2O$, 7.19 mg KI, 1815 mg $FeSO_4.7H_2O$, 307 mg $MnSO_4.H_2O$, 659 mg $ZnSO_4.7H_2O$, 3.29 mg Na_2SeO_3, 47.7 mg $CoSO_4.7H_2O$. [c] L-Ascorbyl-2-polyphosphate (Stay-C, Roche Vitamins Australia, Frenchs Forest, NSW, Australia

7.3.2 Growth experiment

The experiment was conducted at the School of Aquaculture, University of Tasmania (Launceston, Tasmania, Australia.). Atlantic salmon (*Salmo salar* L.) parr were obtained from Wayatinah Salmon hatchery (SALTAS, Tasmania, Australia) and randomly stocked into twelve 300 L tanks at 24 fish per tank where they were acclimated for 14 days. The tanks were held at an average temperature of 14.8 ± 0.2^oC and natural spring photoperiod. The fish were held in a freshwater partial recirculation system (Miller, et al., 2007a). Water was treated through physical, UV and biofilters, with a continuous replacement of approximately 15% per day. DO, pH, ammonia, nitrate, nitrite, and chlorine were monitored daily to ensure water quality remained within parameters recommended for Atlantic salmon (Wedemeyer, 1996). The experiment was conducted in accordance with the University of Tasmania Animal Ethics guidelines (Investigation A0007719).

At the start of the experiment fish were anaesthetized (50 mg L^{-1}, benzocaine), weight and length measured, and four fish were killed to measure initial lipid content and composition. Samples of red (~ 0.7 g) and white (~ 1.3 g) muscle, dissected from below the dorsal fin, liver (~ 0.8 g) and gill arch (~ 0.6 g) were frozen at -80^oC until analysis (Miller, et al., 2007a). The four diets were fed in triplicate at a ration of 1.8% body weight per day (% BW d^{-1}) in two equal feeds at 0900 and 1700 by automatic belt feeders. At the end of the experiment (day 63), fish were starved for one day prior to being anaesthetized (50mg L^{-1}, benzocaine) and

their weight and length measured. Three fish per tank were killed by a blow to the head after immersion in anaesthetic and red and white muscle, liver and gill samples were taken as described above.

7.3.3 Lipid extraction and isolation

Samples were freeze dried and extracted using a modified Bligh and Dyer protocol (Bligh and Dyer, 1959). This involved a single phase overnight extraction, $CHCl_3/MeOH/H_2O$ (1:1:0.9, by vol), followed by phase separation to yield a total lipid extract (TLE). Fractionation of lipid classes was by silicic acid column chromatography. The TLE was applied to a 1 g column of silicic acid (preheated to 100^oC, 1 h) and separated into neutral lipids, glycolipids and PL in a stepwise elution of chloroform (10 ml), acetone (20 ml) and methanol (10 ml) respectively (Miller, et al., 2006).

An aliquot of the TLE and PL was trans-methylated in methanol/chloroform/ hydrochloric acid (10:1:1, by vol) for 1 hour at 100^oC. After addition of water, the mixture was extracted three times with hexane: chloroform (4:1, v/v) to obtain fatty acid methyl esters (FAME) which where concentrated under nitrogen. Samples were analysed by gas chromatography (GC) and confirmed by mass spectroscopy (MS) as described previously (Miller, et al., 2007b).

7.3.4 Membrane lipid analysis – ESI-RP-LCMS

Polar lipid (PL) species were analysed by LC-MS by negative ion electrospray ionization. A Waters Alliance 2690 HPLC fitted with a Nova-Pak reverse-phase C18 column (3.9mm x 150 mm) was coupled to a Finnigan LCQ ion trap mass spectrometer fitted with an electrospray source. The mobile phase gradient was methanol: 0.25M ammonium acetate (pH 5) (96:4 v,v) to methanol: 0.25M

ammonium acetate (pH 5): hexane (86:4:10 v,v,v) at 20 min, then to methanol:

hexane (80:20 v,v) at 25 min, which was held for 10 min. The flow rate was 1 ml

min^{-1}. Alternating normal scans in the range m/z 600 to 950 and data-dependent

MS/MS product ion scans were acquired. The MS/MS scans isolated the strongest

ion observed, and a collision energy of 40% was applied with all product ions

monitored. The capillary temperature was 275°C, sheath gas pressure was 600 kPa,

auxiliary gas pressure 275 kPa and the needle voltage was 4 kV. Minor molecular

species (<1% across all tissues) are not included to simplify the analysis.

7.3.5 Storage lipid analysis - ^{13}C NMR spectroscopy

Acyl group positional distributions on TAG were determined quantitatively by

^{13}C NMR on a 400 MHz Varian (Palo Alto, USA) Inova Wide Bore spectrometer,

with either a Broadband 10 mm probe or a 3 mm MAS Nanoprobe at 25.0 ± 0.1°C.

A π pulse at 100.516 MHz, 128K acquisition with 2.5 s relaxation delay and full

nuclear overhauser effect (NOE) enhancement and decoupling sequence was

employed. Samples were dissolved in 99.96% deuterated chloroform (CDCl$_3$) with

ca 0.025M Cr(acac)$_3$ as a relaxation agent and brought to a volume of 0.6 ml. TAG

fractions which were <10 mg were dissolved in 20-35 μl of CDCl$_3$ / Cr(acac)$_3$ in a

glass rotor and filled to a total volume of 40 μl and spun at 3.000 ± 0.002 KHz. 512

to 8192 transients were collected. A 0.12 Hz Gaussian and a 0.15 Hz Lorentzian

window function was applied to the free induction decay (FID) prior to

transformation and polynomial baseline correction and drift correction was applied

to each spectrum. Peak chemical shifts were referenced to CDCl$_3$ at 77.16 ppm

(Gottlieb, et al., 1997).

7.3.6 Principal components analysis (PCA)

The overall ESI RP-LC-MS, PL FA, and the TLE FA profiles were compared using PCA tested in five dimensions by the PRIMER6 package. No transformations were used because this would inappropriately give artificial weight to FA that made only minor contributions to the FA profiles.

7.3.7 Statistical analysis

Mean values were reported plus or minus standard error of the mean. Percentage data were arcsin transformed prior to analysis. Normality and homogeneity of variance were confirmed and a comparison between means was achieved by 1-way analysis of variance (ANOVA). Multiple comparisons were achieved by Turkey-Kramer HSD. Significance was accepted as probabilities of 0.05 or less. Statistical analysis was performed using SPSS for windows version 11.

7.4 RESULTS

7.4.1 Growth experiment and fatty acid profiles of total lipid extracts

This trial used fish from a preceding experiment and growth, performance and muscle total lipid extract (TLE) fatty acid composition data has been previously described (Miller, et al., 2007b).

7.4.2 Fatty acid profiles of polar lipid extracts

There were significant ($P<0.01$) differences between the FA profiles of the PL extracts in all sampled tissues for the four diet treatments (Table 7.2, 7.4) (data not shown for liver and red muscle). The relative levels (%) of DHA were significantly ($P<0.01$) higher in white muscle in the TO fish compared to the MX and PO fish (Table 7.2). The TO fish also had ($P<0.01$) increased levels of DPA-6. These differences led to TO fish having significantly ($P<0.01$) enhanced proportions of total PUFA compared to the PO and MX fish. PO fish had significantly ($P<0.01$) higher proportion of oleic acid (OA, 18:1ω9c), arachidonic acid (20:4ω6 ARA) and linoleic acid (18:2ω6 LA). The FO fish had significantly ($P<0.01$) higher concentrations of eicosapentaenoic acid (20:5ω3 EPA).

In the gills there were significantly ($P<0.01$) higher concentrations of EPA in the FO fish and the initial fish compared to other diet treatments (Table 7.5). There was, however, no difference in the gills in the total SFA, MFA, total ω3 PUFA, total ω6 PUFA and total PUFA between the four diet treatments.

Table 7.2: FA content and lipid class composition of the polar lipid extract of white muscle samples of Atlantic salmon fed: palm oil (PO), 1:4 mix of thraustochytrid oil: palm oil (MX), thraustochytrid oil (TO) and fish oil (FO) diets. Initial fish (Int) were fed a FO diet.

FA (%)	Initial		SE	PO		SE	MX		SE	TO		SE	FO		SE	f
14:0	0.7	±	0.0	0.4	±	0.1	0.3	±	0.0	0.9	±	0.2	0.7	±	0.1	
16:0	18.6	±	1.1	20.9	±	1.1	20.5	±	0.1	20.3	±	0.2	18.2	±	1.2	
18:0	14.3	±	1.0b	10.0	±	1.5a,b	10.0	±	0.2a,b	8.3	±	0.9a	8.2	±	0.0a	4.7*
Other SFA[d]	0.7	±	0.0	0.4	±	0.0	0.1	±	0.0	0.5	±	0.0	0.8	±	0.0	
Total SFA	34.2	±	2.5	31.7	±	0.6	30.9	±	0.3	30.0	±	0.8	28.0	±	1.5	
16:1ω7c	1.1	±	0.1	0.8	±	0.2	0.3	±	0.0	0.4	±	0.1	0.8	±	0.2	
18:1ω9c OA[e]	7.5	±	0.1b,c	11.0	±	0.9d	8.8	±	0.2c,d	3.8	±	0.3a	5.3	±	0.2a,b	26.8
18:1ω7c	2.9	±	0.0c	1.4	±	0.1a	1.4	±	0.1a	1.1	±	0.1a	2.1	±	0.1b	80.5
Other MUFA[f]	0.1	±	0.0	0.6	±	0.0	0.7	±	0.0	0.0	±	0.0	0.5	±	0.0	
Total MUFA	11.6	±	0.3c,d	13.8	±	1.1d	11.1	±	0.4b,c	5.4	±	0.2a	8.8	±	1.9b	27.3
18:4ω3 ALA	0.1	±	0.1a,b	0.4	±	0.0b,c	0.0	±	0.0a	0.0	±	0.0a	0.7	±	0.0c	19.1
20:4ω3 SDA	0.2	±	0.2	0.0	±	0.0	0.0	±	0.0	0.2	±	0.1	0.5	±	0.2	
20:5ω3 EPA	6.3	±	0.1a,b	2.3	±	1.9a	5.2	±	0.2a,b	2.3	±	1.0a	9.0	±	0.2b	6.1
22:5ω3 DPA	3.8	±	0.1c	2.8	±	0.2b	2.2	±	0.1a,b	1.6	±	0.1	4.0	±	0.2c	38.4
22:6ω3 DHA	40.6	±	1.0a,b	37.9	±	1.2a	39.7	±	0.8a,b	50.3	±	1.5c	45.1	±	0.9b,c	13.4
Other ω3[g]	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.1	±	0.1	
Total ω3	51.1	±	1.8a,b,c	43.3	±	3.2a	47.1	±	0.6a,b	54.4	±	0.5b,c	59.4	±	1.3c	11.7
18:2ω6 LA	0.8	±	0.0a	4.4	±	0.6b	3.2	±	0.1b	0.9	±	0.1a	1.4	±	0.1a	24.8
18:3ω6	0.0	±	0.0	0.1	±	0.1	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	
20:2ω6	0.0	±	0.0a	0.6	±	0.1b	0.4	±	0.2a,b	0.0	±	0.0a	0.0	±	0.0a	6.9
20:3ω6	0.0	±	0.0	0.3	±	0.2	0.4	±	0.2	0.0	±	0.0	0.0	±	0.0	
20:4ω6 ARA	2.2	±	0.0a,b	3.0	±	0.4b	2.3	±	0.1a,b	2.5	±	0.1a,b	1.5	±	0.1a	5.0*
22:5ω6 DPA-6	0.2	±	0.2a	2.5	±	0.5b	4.5	±	0.1c	6.6	±	0.3d	0.6	±	0.1a	58.2
Other ω6[h]	0.0	±	0.0	0.2	±	0.1	0.0	±	0.0	0.2	±	0.1	0.2	±	0.1	
Total ω6	3.2	±	0.2a	11.1	±	1.7b	10.8	±	0.8b	10.2	±	0.5b	3.7	±	0.3a	19.0
Other PUFA[i]	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	
Total PUFA	54.3	±	2.0a	54.4	±	3.6a	57.9	±	1.2b	64.6	±	1.0c	63.2	±	2.1c	11.7

SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; SDA, Stearidonic acid; OA, oleic acid; LA, Linoleic acid; ALA, alpha linolenic acid; ARA, Arachidonic acid; Sig, Significance; *f*, Mean sum of squares.
[a,b,c] Mean values across the row not sharing a common superscript were significantly different as determined by Tukey-Kramer HSD; $df = 4,14$. $p < 0.01$ * denotes $p < 0.05$
[d] Other SFA include 15:0, 17:0, 20:0, 22:0 and 24:0
[e] Includes 18:3ω3
[f] Other MUFA include 16:1ω9, 16:1ω5, 18:1ω5, 20:1ω7, 22:1ω9, 22:1ω11 and 24:1ω9
[g] Other ω3 PUFA include 21:5ω3 and 24:6ω3
[h] Other ω6 PUFA include 22:4ω6 and 24:5ω6
[i] Other PUFA include 16:2ω4, 16:3ω4 and 18:2ω9

7.4.3 Regiospecific analysis of the polar lipids extracts

Only the molecular species at >1% from phosphatidylcholine (PC),

phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phophatidylinositol

(PI) were identified and quantified by ESI RP-LCMS for all sampled tissues. The

percentage composition of each individual PL is shown in Table 7.3 and 7.4 (data

for red muscle and liver are not shown). In the white muscle of the TO fish there

were significantly (P <0.01) higher levels of PC 22:6/22:6, PE 22:6/22:6 and PE

22:6/22:5 compared to all other diet treatments (Table 7.3). In the white muscle of

the PO fish there were significantly (P <0.02) higher relative levels (%) of PC

16:0/18:1, PC 18:0/18:1, PC 18:1/22:6 and PE 18:1/22:6, compared to the TO diet.

In the white muscle of the PO fish there were significantly (P <0.01) higher levels of

PC 18:2/22:6, PE 18:1/18:1 and PG 18:1/22:6 compared to both TO and FO fish. In

the white muscle of the FO fish there were significantly (P<0.01) higher levels of

PC 20:5/22:6 and PE 20:5/22:6 compared to all other diet treatments and

significantly (P<0.02) higher levels of PC 14:0/22:6 and PG 22:6/22:6 compared to

the PO fish. In the FO fish there was significantly (P<0.02) higher proportions of PI

16:0/20:5 compared to the TO fish

Table 7.3: Percentage composition of molecular species of phospholipids as determined by ESI RP LC-MS of the white muscle of Atlantic salmon

PC	Int	±	SE	PO	±	SE	MX	±	SE	TO	±	SE	FO	±	SE	f
16:0/18:1	10.4	±	0.9a,b	14.9	±	1.8b	11.1	±	1.3a,b	7.0	±	0.3a	10.6	±	0.8a,b	6.2
14:0/22:6	1.9	±	0.1a	2.0	±	0.5a	2.1	±	0.2a	4.5	±	0.5b	4.0	±	0.45b	9.6
16:0/20:5	7.2	±	0.5b	9.3	±	0.8b	7.2	±	0.6b	4.0	±	0.3a	9.6	±	0.5b	15.8
18:0/18:1	1.2	±	0.2a,b	1.8	±	0.2b	1.3	±	0.2a,b	0.8	±	0.0a	1.2	±	0.1a,b	5.3*
15:0/22:6	18.8	±	0.6	12.2	±	0.6	11.5	±	1.8	11.5	±	1.0	8.3	±	3.4	
16:0/22:6	35.4	±	0.7	38.5	±	1.4	38.7	±	2.4	42.0	±	0.7	41.0	±	1.5	
16:0/22:5	2.6	±	0.1a	4.2	±	0.9a,b	5.8	±	0.3b	6.4	±	1.0b	2.2	±	0.1a	9.3
18:4/22:6	0.5	±	0.2a	0.7	±	0.1a	0.5	±	0.0a	0.5	±	0.1a	2.0	±	0.2b	19.9
18:3/22:6	0.4	±	0.0a	0.7	±	0.0b	0.5	±	0.0a,b	0.4	±	0.1a	1.0	±	0.0c	32.6
18:2/22:6	0.6	±	0.1a	1.9	±	0.2b	1.8	±	0.1b	1.0	±	0.1a	1.1	±	0.1a	22.8
18:1/22:6	3.4	±	0.2a,b	4.7	±	0.4b	4.3	±	0.3b	2.7	±	0.4a	3.8	±	0.2a,b	6.0
18:0/22:6	1.3	±	0.1	1.9	±	0.1	7.6	±	5.6	1.6	±	0.2	1.7	±	0.1	
20:5/22:6	2.5	±	0.5a	2.9	±	1.3a,b	1.9	±	0.0a	3.1	±	0.4a,b	5.8	±	0.5b	4.9*
22:6/22:6	4.9	±	1.0b,c	1.4	±	0.2a	2.0	±	0.1a,b	5.8	±	1.7c	3.0	±	0.2b	5.2
22:6/22:5	9.0	±	2.4b	2.7	±	0.6a	3.5	±	0.2a,b	8.7	±	1.4a,b	4.8	±	0.3a,b	5.0*
PE	Int		SE	PO		SE	MX		SE	TO		SE	FO		SE	
16:0/18:1	0.9	±	0.1	0.9	±	0.1	0.9	±	0.0	0.6	±	0.0	0.7	±	0.0	
16:0/20:5	3.4	±	0.2b	3.5	±	0.2b	2.7	±	0.3b	1.2	±	0.2a	2.8	±	0.1b	10.7
18:1/18:1	0.6	±	0.1a	1.4	±	0.2b	1.3	±	0.0b	0.4	±	0.0a	0.6	±	0.1a	14.1
16:1/22:6	1.5	±	0.1b	1.8	±	0.2b	1.4	±	0.1a,b	0.7	±	0.1a	1.7	±	0.0b	9.2
16:0/22:6	35.1	±	1.5	32.0	±	1.5	31.0	±	3.7	39.7	±	1.5	33.7	±	0.7	
18:0/20:5	3.7	±	0.2a,b	4.9	±	0.6a,b,c	6.7	±	0.2c	5.4	±	0.3b,c	2.7	±	0.1a	10.2
18:2/22:6	7.2	±	3.7	7.1	±	0.3	6.8	±	0.2	3.5	±	0.2	3.7	±	0.2	
18:1/22:6	14.9	±	0.3b	16.6	±	0.6b	16.9	±	0.4b	6.4	±	2.2a	12.0	±	0.1a,b	8.4
18:0/22:6	16.8	±	1.2b	10.9	±	0.4a	12.1	±	0.4a	11.2	±	0.1a	12.3	±	0.2a	8.1
20:5/22:6	3.5	±	0.4a	6.1	±	0.5a	5.2	±	0.3a	3.0	±	1.0a	11.4	±	0.3b	19.1
22:6/22:6	8.2	±	0.9a	9.4	±	0.7a,b	7.7	±	2.4a	18.0	±	1.9c	11.8	±	0.8b	17.4
22:6/22:5	3.0	±	0.4a	4.6	±	0.4a,b	6.3	±	0.4b,c	8.7	±	0.6c	5.4	±	0.4a,b	12.0
PG	Int		SE	PO		SE	MX		SE	TO		SE	FO		SE	
14:0/22:6	5.1	±	0.1	3.8	±	0.9	3.7	±	0.3	6.6	±	1.1	3.8	±	1.1	
16:0/20:5	10.5	±	0.5b	11.7	±	2.3b	8.5	±	1.2a,b	4.6	±	0.4a	8.0	±	0.3a,b	5.1*
18:0/18:2	4.5	±	0.2	7.4	±	2.3	3.8	±	1.0	3.2	±	1.3	0.8	±	0.5	
18:0/18:1	1.3	±	0.4	2.5	±	1.8	1.0	±	0.3	3.3	±	2.1	1.0	±	0.5	
16:0/22:6	54.3	±	2.0b	21.4	±	8.6a	36.4	±	2.1a,b	31.5	±	3.4a	28.8	±	4.1a	6.8
16:0/22:5	2.0	±	0.8	4.6	±	0.5	6.9	±	0.5	6.7	±	1.3	7.0	±	3.2	
18:1/22:6	7.9	±	0.7a	17.7	±	3.7b	12.9	±	1.0a,b	8.1	±	0.8a	9.2	±	0.4a	5.4
22:6/18:1	3.2	±	1.6	5.1	±	0.6	4.2	±	0.1	6.5	±	0.8	2.6	±	0.6	
20:5/22:6	5.8	±	1.0	8.2	±	1.6	5.6	±	0.0	3.7	±	0.7	7.6	±	1.2	
22:6/22:6	5.3	±	2.7a	17.6	±	1.5b	17.1	±	3.0b	26.0	±	2.7b,c	31.1	±	1.0c	17.7
PI	Int		SE	PO		SE	MX		SE	TO		SE	FO		SE	
16:0/20:5	3.0	±	0.3b	2.8	±	0.3a,b	2.3	±	0.1a,b	1.5	±	0.2a	3.1	±	0.4b	5.0*
16:0/20:4	1.9	±	0.4	2.4	±	0.4	1.9	±	0.3	1.9	±	0.1	1.7	±	0.1	
16:0/22:6	12.8	±	0.6a	14.9	±	1.4a,b	15.2	±	1.2a,b	18.4	±	0.1b	12.3	±	0.8a,b	6.6
18:0/20:5	25.9	±	0.7	14.6	±	6.3	18.1	±	0.9	16.0	±	0.9	27.6	±	0.4	
18:0/20:4	17.6	±	0.5b	17.7	±	1.2b	15.9	±	0.5a,b	14.0	±	0.1a,b	9.6	±	2.9a	5.4
18:1/22:6	2.8	±	0.3	9.1	±	4.4	5.0	±	0.2	3.2	±	0.4	2.9	±	0.2	
18:0/22:6	36.0	±	0.1	38.6	±	1.9	41.7	±	2.8	45.1	±	0.9	42.8	±	2.8	

The four experiment diets included palm oil (PO), 4:1 mix of palm oil: thraustochytrid oil (MX), thraustochytrid oil (TO), fish oil (FO). Initial fish (Int) were fed a FO diet. PE, phosphatidylethanolamine; PI, phosphatidylinositol; PC, phosphatidylcholine; PG, phosphatidylglycerol. $p<0.01$, * demotes $p<0.05$. For all other abbreviations see Table 7.2.

In gill PI molecular species there were significantly ($P<0.01$) higher levels of PI 16:0/20:5 in the initial fish compared to the four diet treatments. The TO fish had significantly ($P<0.01$) higher proportion of PI 18:0/20:4 compared to all other diet treatments. All dietary treatments had significantly ($P<0.01$) higher proportions of PI 18:0/20:4 than the initial fish. The TO fish contained significantly ($P<0.04$) lower proportions of PI 18:0/20:5 and PI 18:0/22:6 compared to the initial fish. LC-MS also detected faint signals of sphingomyelin in all samples, but these have not been reported.

7.4.4 Principal components analysis (PCA)

PCA was performed for the FA profile of the TLE of the red muscle, white muscle, liver and gills (Figure 7.1). PCA of FA profiles of the PL extracts (Figure 7.2) and the ESI RP-LCMS profiles of the PL extract (Figure 7.3) are also shown. Directional component loadings are presented in these plots in the two dimensions shown and expressed as a percentage of influence. The major eigenvectors, the coefficients in the liner combinations of variables making up principal components, are shown and listed in descending order of importance. A 1-way analysis of variance (ANOVA) was performed on the first and second principal component scores to assess overall difference between diets (Table 7.4).

In the PCA of the TLE (Figure 7.1), in all tissues the major eigenvectors (>0.3) in the x dimension that explained 63.0-76.2% of the variance were described by the

difference between the levels of DHA and OA. In the y dimension the major

eigenvectors (>0.3) that explained 20.0-22.8% of the variance were the differences

between EPA and/or 16:1ω7 compared to DPA-6, 16:0 and/or DHA. The major

eigenvectors in the y dimension (22.1% of variation) in the white muscle were EPA

and 16:1ω7 compared to OA, LA and DHA.
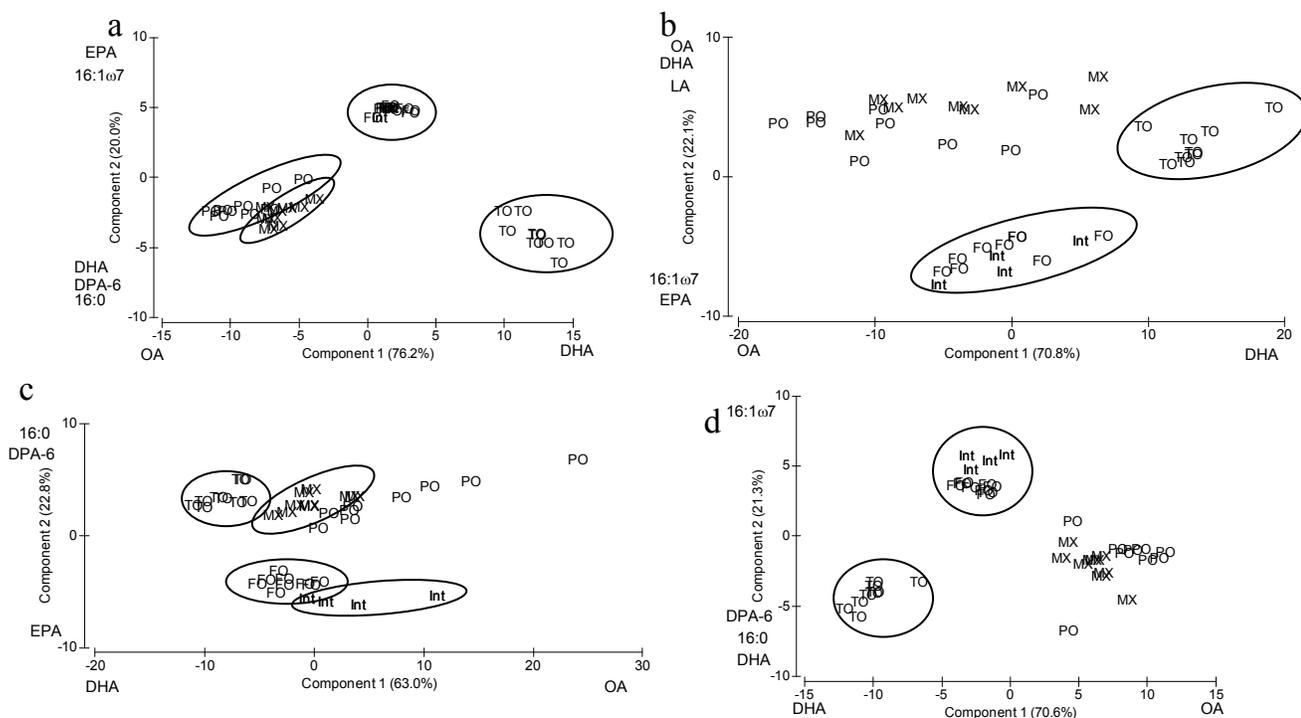
Figure 7.1: Principal component analysis of the fatty acid composition of the total solvent extract of Atlantic salmon: a) red muscle b) white muscle c) liver and d) gill - fed 4 experimental diets; palm oil (PO), 4:1 mix of palm oil: thraustochytrid oil (MX), thraustochytrid oil (TO) and fish oil (FO).
Circles indicate statistical significance (*P*<0.05) DHA, Docosahexaenoic acid; DPA-6, Docosapentaenoic acid (ω6); EPA, Eicosapentaenoic acid; OA, Oleic acid; LA, Linoleic acid

In the PCA of the FA analysis of the PL extract (Figure 7.2) of the gill and both muscle tissues, the major eigenvectors (>0.3) in the x dimension which explained 55.3-68.3% of the variance were described by the difference between the concentrations of DHA and OA, with DPA-6 pairing with DHA in the red muscle and OA in the gills. In the liver, the major eigenvectors (>0.3) that explained 56.8% of the variance are described by the difference between 16:0 and 18:0.
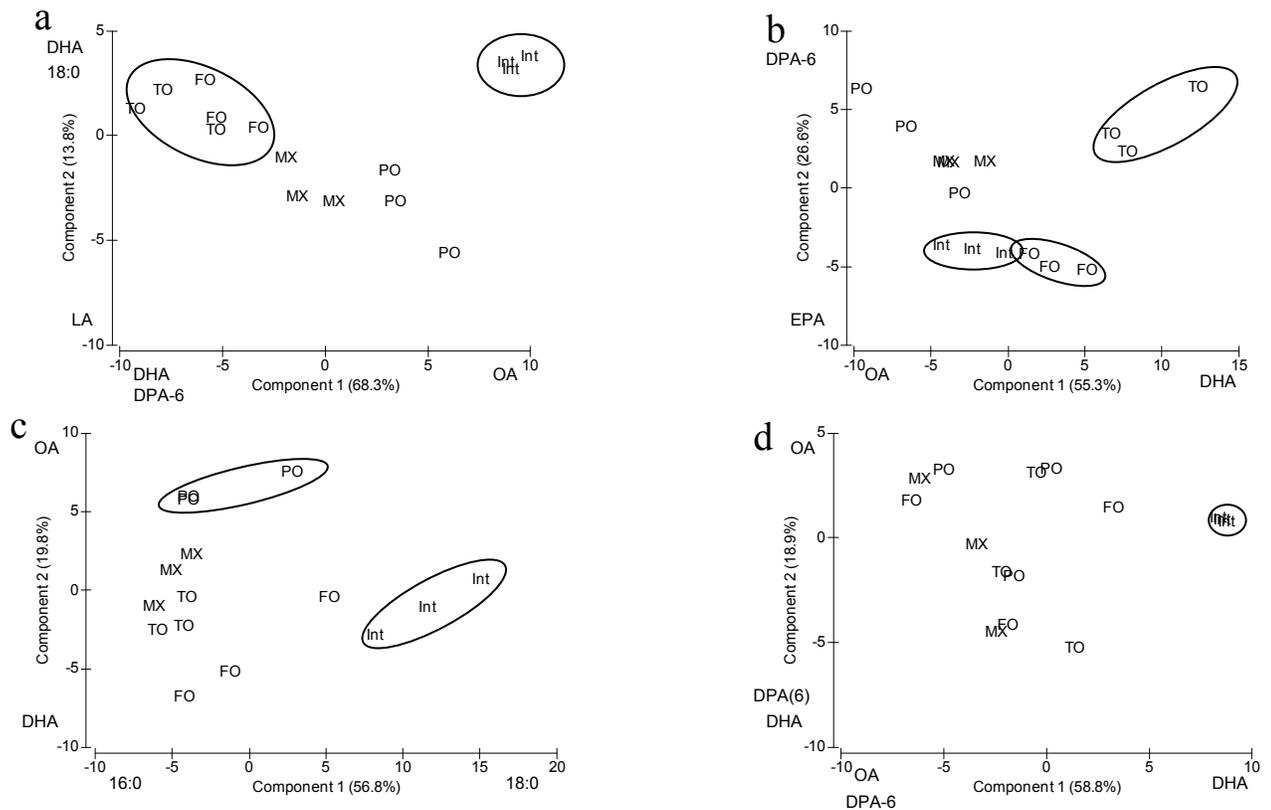

Figure 7.2: Principal component analysis of the fatty acid composition of the polar lipid extract of Atlantic salmon: a) red muscle b) white muscle c) liver and d) gill - fed 4 experimental diets; palm oil (PO), 4:1 mix of palm oil: thraustochytrid oil (MX), thraustochytrid oil (TO) and fish oil (FO).
Abbreviations and other footnote definitions, see Figure 7.1

In the PCA of the ESI RP-LCMS analysis of the PL extract (Figure 7.3) of the red muscle, the major eigenvectors (>0.25) in the x dimension which explained 42.6% of the variance were described by the differences between the relative levels of PI 18:0/22:6 compared with PI 18:0/20:5 and PG 16:0/22:6. In the white muscle the major eigenvectors (>0.25) that explained 44.1% of the variance were PG 22:6/22:6 compared to PG 16:0/22:6. In the liver the major eigenvectors (>0.25) that explained 44.1% of the variance were PG 16:0/22:6 compared to PG 22:6/22:6 and PE 22:6/22:6. In the gills the major eigenvectors (>0.25) that explained 61.7% of the variance were PI 18:0/20:4 compared to PI 18:0/20:5, PI 18:0/22:6 and PE 22:6/22:6.

There was significant difference in the diet treatments when an ANOVA was performed on their principal component scores (Table 7.4). Significant differences are shown in the principal component 1 and the principal component 2 scores (visually illustrated in Figure 7.1, 7.2 and 7.3 as the x and y dimensions). Significant differences between diet treatments are shown in Figures 7.1-7.3 as circled groups in both the x and y dimensions.
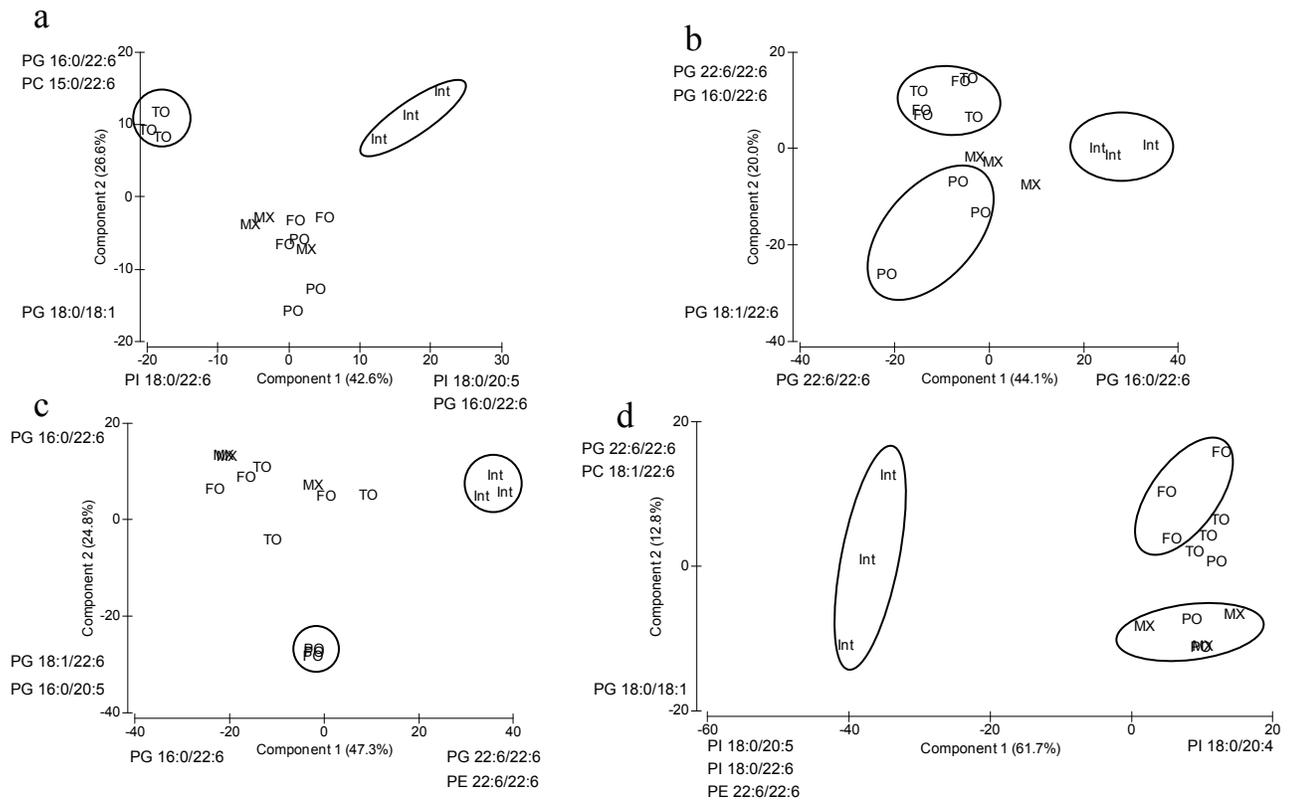
Figure 7.3: Principal component analysis of the molecular species from liquid chromatography-mass spectrometry of the polar lipid extract of Atlantic salmon: a) red muscle b) white muscle c) liver and d) gill - fed 4 experimental diets; palm oil (PO), 4:1 mix of palm oil: thraustochytrid oil (MX), thraustochytrid oil (TO) and fish oil (FO).
Circles indicate statistical significance (*P*<0.05)
Eigenvectors of >0.25 are shown with vector score in descending order.
PE, phosphatidylethanolamine; PI, phophatidylinositol; PC, phosphatidylcholine; PG, phosphatidylglycerol

Table 7.4: Frequencies and statistical significance for one way ANOVA for principal component scores

Fatty acid analysis of the total lipid extract (TLE)

	Red muscle		White muscle		Liver		Gills	
Source of variation	F	p	F	p	F	p	F	p
Main effects								
PC1 Diet[*]	307.2	<0.01	597.8	<0.01	17.3	<0.01	185.3	<0.01
PC2 Diet[*]	279.3	<0.01	151.4	<0.01	156.3	<0.01	102.7	<0.01
Tukeys HSD								
PC1 Diet	$PO^a, MX^b, FO^c, Int^c, TO^d$		$PO^a, MX^a, FO^{a,b}, Int^b, TO^c$		$TO^a, FO^{a,b}, MX^{b,c}, Int^c, PO^d$		$TO^a, FO^b, Int^b, MX^c, PO^d$	
PC2 Diet	$TO^a, PO^b, MX^b, Int^c, FO^c$		$Int^a, FO^a, TO^a, PO^{b,c}, MX^c,$		$Int^a, FO^b, TO^c, PO^c, MX^c,$		$TO^a, MX^b, PO^b, FO^b, Int^b$	

Fatty acid analysis of the polar lipid extract (PL)

	Red muscle		White muscle		Liver		Gills	
Source of variation	F	p	F	p	F	p	F	p
Main effects								
PC1 Diet[#]	4.9	0.02	18.7	<0.01	13.1	0.01	9.4	0.02
PC2 Diet[#]	2.5	0.11	15.9	<0.01	12.6	0.01	0.4	0.81
Tukeys HSD								
PC1 Diet	$TO^a, FO^a, MX^{a,b}, PO^{a,b} Int^b$		$PO^a, MX^{a,b}, Int^{a,b}, FO^{b,c}, TO^c$		$MX^a, TO^a, PO^a, FO^a, Int^b$		$MX^a, PO^a, FO^a, TO^a, Int^b$	
PC2 Diet	-		$Int^a, FO^a, MX^b, PO^b, TO^b$		$FO^a, TO^a, Int^a, MX^a, PO^b,$		-	

ESI RP LCMS analysis of the polar lipid extract (PL)

	Red muscle		White muscle		Liver		Gills	
Source of variation	F	p	F	p	F	p	F	p
Main effects								
PC1 Diet[#]	49.7	<0.01	16.6	<0.01	15.1	<0.01	85.7	<0.01
PC2 Diet[#]	30.4	<0.01	12.7	0.01	45.7	<0.01	3.9	0.04
Tukeys HSD								
PC1 Diet	$TO^a, MX^b, PO^b, FO^b, Int^c$		$FO^a, PO^a, TO^a, MX^a, Int^b$		$MX^a, FO^a, TO^a, PO^a, Int^b$		$Int^a, FO^b, MX^b, PO^b, TO^b$	
PC2 Diet	$PO^a, MX^a, FO^a, TO^b, Int^b,$		$PO^a, MX^{a,b}, Int^{b,c}, FO^{b,c}, TO^c$		$PO^a, TO^b, Int^b, FO^b, MX^b,$		MX^a, FO^b	

PO, palm oil; MX, 1:4 mix of thraustochytrid oil: palm oil; TO, thraustochytrid oil; FO, fish oil.
[a,b,c,d] Mean values across the row not sharing a common superscript were significantly different as determined by Tukey-Kramer HSD [*] denotes *df* of 4,43 [#] denotes *df* of 4,14

7.5.5 Regiospecificity of storage lipids

DHA had a high affinity with the *sn*-2 position (56.1-70.4% across the diets) (Figure 7.4). There was a significantly (*P*<0.01) decreased percentage of DHA in the *sn*-2 position in the TO fish compared to the other diets. SDA (41.5-79.0%) and DPA (34.7-43.6%) also had an increased affinity to the *sn*-2 position for the TAG of

the red muscle of fish fed experimental diets. There was a significantly (*P*<0.01)

increased percentage of SDA in the *sn*-2 position of the TO fish. EPA (31.7-35.0%

sn-2) and ARA (30.3-34.3% *sn*-2) had even distribution across the TAG molecule

and did not show a preferred regiospecific position.
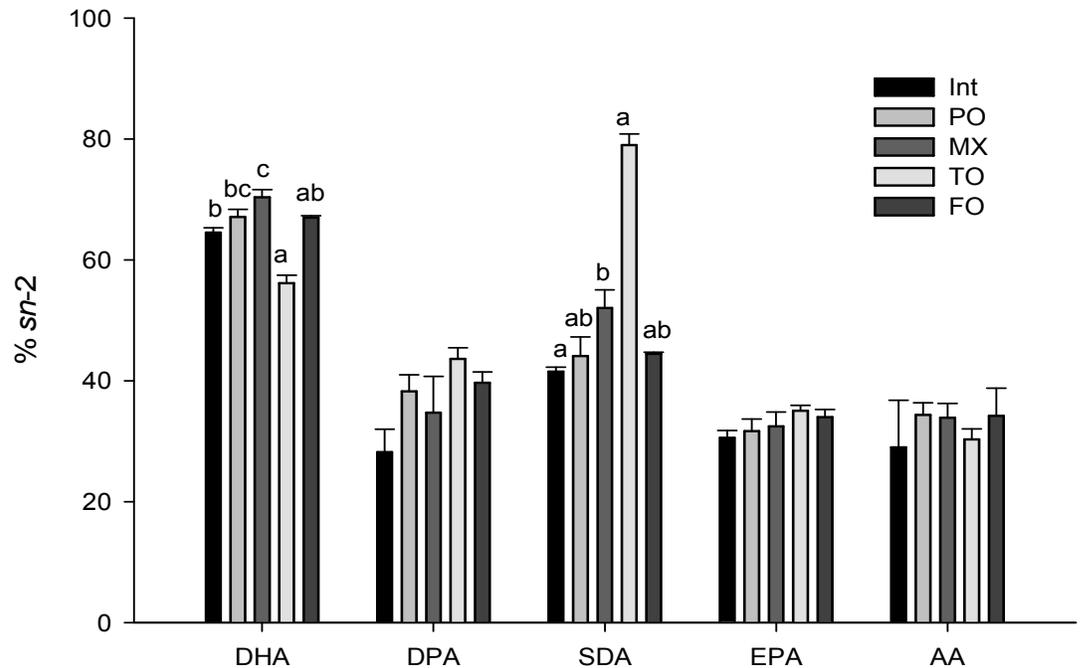
Figure 7.4: Percentage of fatty acids in the *sn*-2 position in the triacylglycerol fraction from Atlantic salmon *Salmo salar* red muscle fed: palm oil (PO) 1:4 mix of thraustochytrid oil: palm oil (MX), thraustochytrid oil (TO) diets and fish oil (FO).
Determined by ^{13}C NMR spectroscopy
DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; SDA, Stearidonic acid; EPA, Eicosapentaenoic acid; ARA, Arachidonic acid
[a,b,c] Mean values not sharing a common superscript were significantly different as determined by Tukey-Kramer HSD; *df*=4,14.

7.5 DISCUSSION

Dietary fatty acids are the major factor that influences the lipid profiles and molecular species that make up cell membranes of muscle tissues. However, along with diet, life stage can influence membrane molecular species in all tissues. DHA rich dietary oil sources, such as thraustochytrid oils, are incorporated into the membrane structure of Atlantic salmon by adaptation of their molecular species. Increased incorporation of molecular species containing DHA was demonstrated in all tissues fed the TO diet. High concentrations of dietary DHA significantly increased the amount of this key FA in the middle (*sn*-2) position in storage TAG. The growth details of this trial have been previously reported (Miller, et al., 2007b) and this study has focused on intact lipids (storage and structural) using advanced chromatography and statistical methods to clarify compositional changes.

7.5.1 Membrane Lipids

Salmon phospholipids generally contain 16:0, OA, EPA and DHA as major fatty acids in the membrane bilayer which is a reflection of their diet (Sargent, et al., 2002). The PL FA (Table 7.2) fractions from the present study on Atlantic salmon contained these major FA, but fish fed PO had enhanced levels of LA and 18:0, while the TO fish had enhanced DHA and DPA-6 concentrations which mirrors the FA profile of their diet. The initial fish were fed a fish oil based diet and the PL FA reflects that of the FO fish. Due to the large data set generated by the detailed lipid analysis undertaken, only the total PL FA and ESI-RP-LC-MS results for the white muscle (Table 7.2 and 7.3) and for the PI for the gills (Table 7.5) are shown.

Table 7.5: FA percentage composition and phosphatidylinositol molecular species of the polar lipid extract of gill samples of Atlantic salmon fed: palm oil (PO) 1:4 mix of thraustochytrid oil: palm oil (MX), thraustochytrid oil (TO) and fish oil (FO) diets. Initial fish (Int) were fed a FO diet.

FA (%)	Int	SE	PO	SE	MX	SE	TO	SE	FO	SE	f
Total SFA[d]	42.7	± 4.6	38.6	± 0.7	39.9	± 1.3	40.4	± 1.1	39.0	± 1.1	
Total MFA[e]	16.2	± 1.4	22.5	± 1.6	23.6	± 0.9	19.9	± 2.0	22.6	± 1.2	
20:5ω3 EPA	4.6	± 1.0b,c	3.5	± 0.9a,b	2.3	± 0.4a	2.3	± 0.4a	4.9	± 0.2c	4.8*
22:6ω3 DHA	28.7	± 1.1	22.3	± 3.1	21.8	± 2.5	22.6	± 4.6	22.8	± 3.2	
Total ω3[f]	35.8	± 0.9b	27.2	± 2.6a,b	25.0	± 1.7a	26.0	± 2.5a	29.8	± 3.2a,b	5.0
20:4ω6 ARA	3.2	± 1.9	5.1	± 1.4	4.7	± 1.5	6.3	± 1.3	4.4	± 1.0	
Total ω6[g]	4.1	± 2.0	11.7	± 3.4	11.5	± 1.5	13.7	± 0.4	8.5	± 2.8	
Total PUFA[h]	39.8	± 2.7	38.9	± 4.2	36.6	± 2.8	39.7	± 2.7	38.4	± 4.2	
PI Molecular species											
16:0/20:5	4.5	± 1.4b	0.9	± 0.3a	1.1	± 0.2a	0.5	± 0.1a	2.1	± 0.4a	5.6
16:0/20:4	6.1	± 2.1	12.0	± 0.8	12.5	± 0.9	9.8	± 3.6	12.7	± 1.0	
16:0/22:6	5.7	± 0.5	10.9	± 1.1	10.9	± 0.7	8.9	± 3.6	11.3	± 0.3	
18:0/20:5	29.2	± 1.3b	8.3	± 0.94a	10.4	± 2.2a	3.9	± 1.5a	9.0	± 1.4a	39.3
18:0/20:4	28.0	± 2.4a	55.0	± 0.8b	52.5	± 2.0b	67.2	± 1.8c	52.6	± 1.4b	5.7
18:1/22:6	2.1	± 1.0	0.9	± 0.3	1.5	± 0.2	0.6	± 0.3	1.3	± 0.1	
18:0/22:6	24.4	± 5.6b	12.0	± 1.0a,b	11.2	± 1.1a,b	9.0	± 3.7a	11.0	± 0.5a,b	4.0*

Abbreviations and other footnote definitions, see Table 7.2

Diet influences molecular species in the tissues of salmon. In white muscle, high dietary concentrations of DHA statistically elevated PC 22:6/22:6, PE 22:6/22:6 and PE 22:6/22:5 in the TO fish compared to all other diet treatments (Table 7.3). It is known that dietary DHA is incorporated into a variety of cells in different organs and tissues but primarily into PL (Stillwell and Wassall, 2003). Dietary FA influence can be further demonstrated in the molecular species of the PO fish which had elevated dietary OA. PO fish had statistically increased proportions of PE 18:1/18:1 and PG 18:1/22:6 compared to both TO and FO fish, and PC 16:0/18:1, PC 18:0/18:1, PC 18:1/22:6 and PE 18:1/22:6 than the TO fish. This trend is further highlighted by the enhanced levels of EPA in FO fish in PC 20:5/22:6 and PE 20:5/22:6 percentages. Therefore dietary FA are readily incorporated into the molecular species by adaptations in both the *sn*-1 and *sn*-2 position and is not

dependant on PL class. High dietary DHA increases its relative concentrations in PL and therefore can affect membrane function. In cell membranes, DHA favourably alters many basic properties including fluidity, phase behaviour, elastic compressibility, permeability, fusion, flip-flop and protein activity (Stillwell and Wassall, 2003). Theoretically, the high incorporation of DHA into the membranes of the TO fish may enhance specific cell function and affect immune response which therefore could improve fish health. However, this study did not assess cell or fish health between dietary treatments.

The ESI RP-LCMS results also show that life stage can influence the relative abundance of molecular species in Atlantic salmon. An example of this is in the PI in gills where increases in PI 18:0/20:4 occurred in all dietary treatments in day 63 fish compared to the initial fish (Table 7.5). It has been shown previously in Atlantic salmon fed vegetable oils that ARA concentrations in liver PL increased consistently leading up to smoltification, while EPA concentrations decreased significantly before rising at seawater transfer (Bell, et al., 1997; Tocher, et al., 2000). The enzymes involved with eicosanoid production occur widely in fish tissues, with the major production in the leucocytes and gills (Bell, et al., 1992; Holland, et al., 1999). ARA is the primary eicosanoid precursor and is acknowledged for providing benefits to fish physiology and biochemistry (Bell and Sargent, 2003). Fish with higher ARA levels in gill PL tend to adapt better to the challenge of smoltification by reducing plasma chloride levels (Bell, et al., 1997; Tocher, et al., 2000). In this study PI 18:0/20:4 increased in all pre-smolt fish and this finding supports the concept of adaptive changes in the gills leading up to saltwater transfer. The TO

treatment had elevated PI 18:0/20:4 compared to all other diets and is possibly a

factor in the decreased blood osmolarity described previously (Miller, et al., 2007b).

7.5.2 PCA

Due to the large amount of information generated by fatty acid profiling and the

analysis of individual molecular species by advanced chromatography, PCA

techniques were employed to interpret these data sets to identify the most important

influences on the lipids in different tissues of Atlantic salmon between dietary

treatments. The PCA of the FA profiles of the TLE extracts demonstrated that diet

influenced the majority of the variance (Figure 7.1). The initial fish and the FO

treatment grouped together in the PCA as they have similar dietary FA. This

indicates that life stage of the fish is not a factor in the variance between the TLE

FA profiles in all tissues. The major (over 63%) differences between dietary

treatments were described by the two FA, DHA and OA, in all tissues. The second

principal component accounted for 20.0-22.8% of the remaining variance and

generally separated the FO and the initial fish from the other treatments. This was

driven by differences between concentrations of 16:1ω7 and EPA verses DHA,

DPA-6, 16:0 and OA. The key dietary FA were the eigenvectors that influenced the

difference in the TLE in all tissues which indicates diet as the major influence. TLE

contains predominately storage TAG, and in some tissues such as red muscle it is

the major (≈90%) lipid class, therefore it would be predicted that the TLE would

reflect the FA profile of the diet.

In the PL, the major FA in the red and white muscle influencing the variance are

again DHA and OA (Figure 7.2). The differences between DHA and OA cause the

majority (over 68.3% and 55.3% in red and white muscle respectively) of the

variance in the PL fraction of the muscle tissues. The dietary groups were tightly

clustered, in both muscle tissues, in the PCA analysis which indicated diet being the

major factor in their profile. However, the PCA analysis of the PL in the liver and

gills did not show differences between dietary treatments in the primary principal

component. The PCA of the PL FA highlighted the differences between life stages;

between fish parr at day one and smolt at day 63 in the gill and liver. It has been

shown that salmon undergoing smoltification will endogenously increase FA

biosynthesis in the liver (Zheng, et al., 2005), as well as adaptation to gill lipid FA

profile/membrane structure to prepare for saltwater transfer (Bell, et al., 1996; Bell,

et al., 1997). Therefore, due to smoltification being the over-riding influence on

adaptation and changes in liver and gill tissues, diet played a relatively reduced role

in the PL FA profile of these tissues.

Analysis of individual molecular species by PCA demonstrated that the major

polar lipid class causing variance between dietary treatments was

phosphatidylglycerol (PG) (Figure 7.3). More specifically the molecules responsible

for the variance were PG 16:0/22:6, PG 16:0/20:5, PG 22:6/22:6 and PG 18:1/22:6.

The PL, PG, is a minor class in salmon membranes, especially compared to the

abundant PC and PE. However, the changes in molecular species in PG are

nevertheless important. In the white muscle, the PCA results of the membrane

molecular species demonstrated that diet treatments clustered together. This

indicates dietary FA is the major factor of variance which confirms the earlier PL

FA result. However, the major variance in all tissues in the molecular species of

membrane was the differences between initial fish and fish at day 63. This indicates

that the sensitive ESI-RP LC-MS technique was able to detect small molecular

changes in membranes that occurred in all tissues as they approach saltwater transfer. However, dietary FA was a minor contributor to the variance between molecular species in all tissues which is demonstrated in the secondary principal component (y axis).

7.5.3 Storage lipids

Health benefits of ω3 LC-PUFA, in particular DHA, are related to not only the amount consumed in the diet, but also the positional distribution within the TAG molecule (Hunter, 2001; Mu and Hoy, 2004). FA in the *sn*-2, "middle" position of the glycerol backbone are the most bio-available for human digestion (Hunter, 2001; Mu and Hoy, 2004; Wijesundera, 2005). This was first shown in human infant feeding trials where 16:0 at the *sn*-2 position in breast milk had significantly enhanced absorption than from the *sn*-1,3 position in infant formula (Lucas, et al., 1997). Dietary FA in the *sn*-2 position are also preferentially utilised for accumulation into TAG in Atlantic salmon (Oxley, et al., 2007). Biochemically it is of interest to know how salmon regiospecifically store ω3 LC-PUFA for the consumer to access it.

Fish oils have SFA and MUFA usually located at the *sn*-1,3 positions of glycerol, whereas LC-PUFA in contrast are located in the *sn*-2 position (Aursand, et al., 1995; Miller, et al., 2006). If the distribution of a FA across glycerol was random, a 33.3% abundance of any FA would occur at the *sn*-2 position. In salmon, DHA is predominantly located in the *sn*-2 position (62.1-72.6%) where as EPA has a more even distribution across the TAG molecule with 34.7-37.8% in the *sn*-2 position (Miller, et al., 2006). High dietary input of DHA statistically reduced its percentage (56.1%) in the *sn*-2 position (Figure 7.4). In TO fish the concentration of

DHA (55.4 g/100g) in the red muscle was significantly greater than the concentration for fish fed other diets (23.0-32.2 g/100g) (Miller, et al., 2007b). A decrease in percentage of DHA in the *sn*-2 position of the TO fish is not due to a reduction in DHA in the *sn*-2 position, but to an increase of DHA in the *sn*-1,3 positions. Calculations demonstrate that 31.1 mg/g of DHA is in the *sn*-2 position in TO fish which is significantly greater than FO (21.5 mg g^{-1}), MX (17.4 mg g^{-1}) and PO (15.6 mg g^{-1}) fish. High amounts of dietary DHA can be stored by salmon as TAG. However, possibly due to the physical properties of TAG, the increase in stored DHA is accommodated in the *sn*-1,3 position. Furthermore, increased dietary DHA increases the absolute amount of DHA in the most biological available *sn*-2 position.

Fish fed a TO diet, high in dietary DHA, also had a significant increase in the proportion of SDA in the *sn*-2 position. SDA content in the red muscle of salmon in the TO fish (2.5 mg g^{-1}) was not significantly different to the PO (2.1 mg g^{-1}), MX fish (1.8 mg g^{-1}) and FO (3.0 mg g^{-1}) fish. It is unclear why SDA in the TO fish showed significantly increased affinity with the *sn*-2 position. This is the first time, to my knowledge, that dietary FA in particular DHA, have been shown to affect the relative distribution of FA across the TAG molecule in salmon. Individual TAG can be further examined by RP-LCMS and this may provide insight onto the position of SDA on TAG in the TO fish (Fauconnot, et al., 2002). However, the TAG profile of Atlantic salmon is complicated due to large number of FA species and therefore interpretation can be difficult.

7.6 CONCLUSIONS

Enhanced concentrations of dietary DHA affected the fatty acid profile of both the TLE and PL extracts and increased concentration of DHA in salmon tissues. It is well known that FA profile of diet influences the FA profile of the fish. PCA analysis has shown graphically and statistically that dietary FA is the major influence on total lipid profile. Diet was also the major factor affecting the PL FA profile of the two muscle tissues. However, in the liver and gill for both the PL FA and intact molecular species, life stage contributed to most of the differences. This result may indicate exogenous alteration of membranes in the liver and gills due to adaptation by salmon for smoltification. Therefore, both diet and life stage play important roles in membrane structure of different tissues in Atlantic salmon. Elevated dietary DHA increased the amount of DHA in storage TAG, which in turn increased the amount in the sn-2 position considered more bioavailable to the consumer. The incorporation of high concentrations of dietary DHA into the membrane structure and storage molecules was achieved by adaptation of the molecular species of the PL and TAG lipids.

7.7 ACKNOWLEDGEMENTS

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CHAPTER 8

GENERAL DISCUSSION

8.1 GENERAL DISCUSSION

This thesis investigated alternative sources of oil for Atlantic salmon aquafeeds with a focus on the delivery of omega 3 long chain polyunsaturated fatty acids (ω3 LC-PUFA) and understanding important components of the underlying nutritional physiology and biochemistry. More specifically, this thesis examined the main challenges associated with changing the dietary oil source of aquafeeds to one that is more sustainable and economically viable in the long term. The following general discussion will discuss ω3 LC-PUFA replacement oils in regards to their commercial viability, further use of advance analytical/molecular techniques and the broader issues surrounding the sustainability of intensive aquaculture.

8.1.1 Capacity for the endogenous production of ω3 LC-PUFA

Atlantic salmon parr (Chapter 2) were demonstrated to have an endogenous capacity to biosynthesise ω3 LC-PUFA from the dietary precursor stearidonic acid (SDA, 18:4ω3). Increased concentrations of ω3 LC-PUFA during the freshwater parr stage is likely to be due to increased biosynthesis leading up to smoltification. Smolting involves a series of morphological, physiological and behavioural changes which include both increased lipid deposition and increased accumulation of LC-PUFA, in particular ARA, prior to saltwater transfer (Rowe, et al., 1991; Bell and Sargent, 2003). The biosynthesis of LC-PUFA in Atlantic salmon shows greatest activity prior to saltwater transfer (Zheng, et al., 2005). Therefore it is during this freshwater parr stage that the use of dietary precursor (SDA) oils, such as Echium oil may prove most beneficial in aquafeeds. Further data presented in this thesis (Chapter 3) suggested that increased gene expression due to SDA rich diets is not enough to maintain concentrations of ω3 LC-PUFA in seawater Atlantic salmon fed SDA at equivalent levels to

that of fish oil fed fish. As such it would be interesting to further investigate the effect on both the gene expression and fatty acid profile of Atlantic salmon fed an SDA rich diet over their whole life cycle. It is possible that the increased biosynthesis prior to smoltification may carry through to the saltwater life stage if SDA diet was fed over this entire period. However, currently there is no evidence that the increase in ω3 LC-PUFA biosynthesis that has been shown for SDA fed fish leading up to smoltification can be maintained in saltwater.

Results presented with Atlantic salmon smolt are analogous to findings for other marine fish species fed SDA rich oil (Bell, et al., 2006; Tocher, et al., 2006). Atlantic salmon selective breeding programs have focused on characteristics such as health and growth requirements but now contain product quality factors such as flesh colour, fat content and fat distribution (Gjedrem, 2000). In the future selection traits may be widened to include biosynthesis of ω3 LC-PUFA and/or ability to store large amounts the ω3 LC-PUFA oil in the fillet. Molecular techniques examining the expression of the key desaturase and elongase genes could provide a tool for this criteria. However, there are many levels on which variation can occur in these traits, including on a genetic level, through expression of genes and other biological aspects and therefore selection of salmon may be difficult. Family lines of salmon with an elevated ability for ω3 LC-PUFA biosynthesis and/or storage may lead to a reduced need for the provision of dietary ω3 LC-PUFA.

8.1.2 Advanced biochemistry and molecular biology

This thesis has contributed to the development and application of three techniques that may prove advantageous to the aquaculture industry and future lipid nutrition research. To the best of my knowledge these techniques have not been used in unison in fish nutrition experiments and presently only have limited use on Atlantic salmon. These techniques were: 1) advanced chromatography and mass spectroscopy to examine intact membrane lipids, 2) nuclear magnetic

resonance (^{13}C NMR) to assess the regiospecific distribution of ω3 LC-PUFA in oil, and 3) molecular RT-PCR techniques to investigate endogenous ω3 LC-PUFA production. The major advantage with these new techniques is that the composition and regiospecificity of the intact molecules can be investigated. Individual molecular species of cell membranes can be examined without the use of traditional destructive and laborious techniques. Information gained by these new techniques may highlight changes in membrane structure and possibly indicate cell health. There are advantages and disadvantages compared to traditional techniques and as such the overall accuracy and speed of their analysis warrant further investigation.

The bioavailability of ω3 LC-PUFA is of vital importance if Atlantic salmon is to be marketed as a good source of omega 3. How dietary and possibly endogenously biosynthesised ω3 LC-PUFA is regiospecifically stored as TAG by Atlantic salmon is yet to be understood fully by fish nutritionists. Non destructive ^{13}C NMR techniques ascertain where on the TAG molecule ω3 LC-PUFA is stored and therefore evaluate the accessibility for the consumer. Understanding the complex interaction between gene expression, production of enzymes, and FA composition will give a better understanding of biological responses at the cellular level, including for example, how Atlantic salmon endogenously produce, use and store ω3 LC-PUFA. Such knowledge will be vital in the future expansion of aquaculture of large carnivorous.

8.1.3 Replacement options for ω3 LC-PUFA oils and their commercial viability

Single cell oils, such as oil from thraustochytrids, provide a major renewable source of ω3 LC-PUFA for aquaculture (Chapter 4). These oil 'bio-factories' of the ocean could potentially provide a source of the ω3 LC-PUFA without any foreseeable negative impact on wild fish stocks. Currently, manufactures of single cell oils do not have the production capacity and these oils are too expensive to be considered for use as a replacement oil in diets for Atlantic salmon.

However, there is still considerable scope for the discovery of novel strains with other advantageous properties including high ω3 LC-PUFA concentrations. With the continual discovery of new strains and improvements with fermentation techniques, it may be possible to produce oil with sufficient amounts of ω3 LC-PUFA and at a price suitable to meet the growing demand in aquaculture. It is likely that increased use in biomedical fields will cover the high initial cost of this new biotechnology allowing aquaculture to access it when production capacity is increased and the price is reduced. Single cell biomass rather than extracted oil is a logical candidate for aquafeeds as it provides ω3 LC-PUFA rich oil with accompanying marine proteins at a significantly lower price due to a reduction of the processing costs.

The aquaculture industry exists in a very competitive commercial environment. Economic considerations are most important when selecting new ingredients for aquafeeds. Aquafeed companies require oil sources that can provide large volumes of a consistent quality, that are readily available, renewable and economical and that also can adapt to the growing industry demands. Global fish oil production is not increasing, however, recent improvements in fisheries management may be able to provide or contribute to a continued source of fish oil to maintain the ω3 LC-PUFA requirement of aquafeeds. As demand has grown from the aquaculture, agricultural and biomedical industries, the price of fish oil has risen; this has over the last few years resulted in some feed sectors ceasing or reducing their use of fish oil. This increased price and demand allows alternative sources of ω3 LC-PUFA rich oil to competitively enter the market place. Due to the economic constraints coinciding with limited consumer knowledge, Atlantic salmon diets with very high levels of ω3 LC-PUFA are presently not an industry priority. With increased scientific and community knowledge of the benefits of ω3 LC-PUFA on human health, a consumer led push for "omega 3" enriched Atlantic salmon may be plausible. In addition, market forces can alter due to an increased

environmental awareness in consumer choices. This has been shown in other commodities such as coffee where fair trade organisations have changed some consumer demands to more environmentally friendly products that promote fair labour and social policy in the areas of production. Environmental considerations coinciding with an increased consumer demand for elevated ω3 LC-PUFA concentrations may open the market to the use of single cell biomass in aquaculture.

The genetic modification (GM) of plants to contain ω3 LC-PUFA provides an oil replacement option that will be relatively cheap, renewable, competitive and secure. The complex multi gene modification of oil seed crops to contain ω3 LC-PUFA has already been demonstrated and there are a number of corporations and scientific groups actively conducting research and development. However, the consumer acceptance of GM ω3 LC-PUFA rich products is yet to be evaluated in a real market and the anti GM movement is well known, and is particularly strong in Europe, the traditional home of salmon aquaculture. Recent assessment of perceived consumer acceptance of GM land plant ω3 LC-PUFA technologies reported that farmed fish was a preferred delivery mechanism compared to capsules or functional foods (Cox, et al., 2007a, b). There are several factors that need addressing before the inclusion can occur of ω3 LC-PUFA from GM plants as an ingredient in aquafeed in Australia. These include removal of the state and federal government moratoriums on GM crops being grown in Australia, independent statutory agencies such as Food Standards Australia New Zealand (FSANZ) regulating the use of GM products in aquaculture, and the perception of aquafeed and aquaculture companies believing that consumers will purchase salmon fed oil from a GM plant source. Ultimately, as demand for fish oil intensifies, knowledge about fishing impacts and human health benefits of ω3 LC-PUFA increases, and salmon prices being able to decrease due

to reduced ingredient cost, consumers may eventually accept oil from a GM crop as an ingredient of aquafeeds.

8.1.4 Broader issues of sustainable intensive aquaculture

Two broader issues that sustainable intensive aquaculture faces were assessed by this thesis. They are the consequences of minor components of replacement oils (Chapter 5) and the effect elevated temperature and diet have on membrane and storage lipids (Chapter 6 and 7). Plant based meal and oil products will continue to be a constituent of aquafeeds. The high inclusion of oils in aquafeeds for the long marine grow out period of Atlantic salmon will continue as they promote retention of protein for growth and reduce nitrogen excretion into the environment. A feeding regime of vegetable oil based diet throughout the grow out period, with an ω3 LC-PUFA rich finishing diet, has been suggested to reduce reliance of aquaculture on fish oil, reduce costs of aquafeeds and provide nutritionally beneficial products (Bell, et al., 2003; Bell, et al., 2004). With the increased use of vegetable oils, increased natural abundances of phytosterols will occur in Atlantic salmon diets. Research in this thesis is the first to demonstrate that Atlantic salmon can digest and accumulate low concentrations of phytosterols. Although these concentrations are small, it is yet to be established how phytosterols accumulate in salmon fed replacement oil throughout their life, or how this increased dietary concentration affects fish performance and health. The low amount of phytosterols available in the diet, coupled with their low digestibility, suggests at this stage that Atlantic salmon are unlikely to be a major delivery source of this cholesterol lowering agent. However, in combination with the health benefits of ω3 LC-PUFA, phytosterols may act in unison to increase the benefits of eating farmed salmon for the consumer.

The scientific community and now governments are slowly starting to accept that an increase in ocean temperature is the result of climate change. The salmon aquaculture industry

in Tasmania is moving into an era where water temperatures are approaching the upper

threshold for Atlantic salmon survival in the warmer summer and autumn months. Nutrition,

especially the amount and type of oil, can play a vital role in the health and performance of fish

during these periods. Data presented in this study (Chapter 6) shows that in Atlantic salmon, the

requirements for FA change with elevated temperature ($>19^{o}$C as in now commonly

encountered in Tasmanian waters). This thesis established that cell membrane structures adapt

to high temperatures and also to the FA dietary source. As water temperature rises the need for

high levels of ω3 LC-PUFA in polar lipids to maintain optimal cell membrane function is

reduced. The converse relationship is true for decreasing water temperatures. These results

suggest that it may be beneficial to change diets, in particular the amount of ω3 LC-PUFA, in

those periods of high water temperature to maintain health and performance of fish

8.1.5 Future security of ω3 LC-PUFA oils and sustainable aquaculture

Having access to secure sources of ω3 LC-PUFA rich oils is vital for the continued

sustainability and growth of the aquaculture industry. The future use of these ω3 LC-PUFA rich

oils will be dependant on the cost and availability of fish oil as a commodity. Single cell

biomass with high amounts of ω3 LC-PUFA will provide an option, but the present high

production cost is limiting their immediate use. New ω3 LC-PUFA oils from GM land plants

are still in development, with trials including field planting, fish and animal feeding, toxicity

and other assessments required before the large-scale consideration of their use by aquafeed

companies. The short term forecast for aquafeeds rests in the careful management of the use of

fish oil from wild fisheries and the use of vegetable and other oils in blends. This will be

continuously evaluated as new technologies become available to prevent impacts on wild fish

stocks, and to increase resource security and market feasibility. With the mounting evidence

present in the greater scientific community, aquaculture will need to increase efforts to find new

sources of ω3 LC-PUFA, in particular for intensive carnivorous aquaculture species. Whether replacement sources will be derived from single cell biomass, oil from GM land plants or a combination of the two is yet to be determined, but will ultimately depend on scientific developments, social acceptance, community needs, and governmental policy. The main contribution made by this thesis to the future of a sustainable aquaculture industry is the assessment of the only secure and sustainable ω3 LC-PUFA oil options for the aquaculture industry that are not based on the capture of fish. It is foreseen that the data presented in this thesis will contribute to and aid the aquaculture industry in producing sustainable and higher quality salmon in regards to the ω3 LC-PUFA content for increased health benefits to the consumer and reduced ecological impact to the environment.

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