UNIVERSITY OF STIRLING

BIOACTIVE FATTY ACIDS AS DIETARY SUPPLEMENTS FOR FARMED FISH:

EFFECTS ON GROWTH PERFORMANCE, LIPID METABOLISM, GENE EXPRESSION AND IMMUNE PARAMETERS.

Thesis submitted for the degree of Doctor of Philosophy

by

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DECLARATION

I hereby declare that this thesis has been composed entirely by myself and is a result of my own investigations. It has neither been accepted nor submitted for any other degree. All sources of information have been dully acknowledged.

.....

S. R. Kennedy 2007

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I'd like to thank a number of people whom without which I am certain I would have been unable to have completed this research. Firstly, I'd like to thank my supervisor Dr. Douglas Tocher for contributing many hours of guidance and support, some of which were entirely unrelated to academic endeavours. Secondly, I'd like to thank all my friends past and present at Stirling who made some of the darker days just that little bit brighter (you all know who you are!!). Thanks to BioMar and in particular Ralph Bickerdike for help and support throughout the project. Special thanks to the lipid group and in particular Dr. Jim Henderson, Mr. James Dick and Mrs. Fiona Strachan for advise on all matters technical and some hilarious memories. Finally, I'd like to dedicate this thesis to my family: my mother and father, Susie and Laura whose love and support throughout has been boundless.

ABSTRACT

Current feed formulations within the aquaculture industry have tended to rely on high dietary lipid thus offsetting relatively expensive protein as a source of energy. In this way, protein can be 'spared' for synthesis of new tissue and the high lipid content can also fulfil both fish and consumer essential fatty acid (EFA) requirements. However, the main disadvantage of feeding high lipid levels to farmed fish is a surplus of fat deposition in the flesh and other important tissues, which can detrimentally impact on quality characteristics central to the human consumer. However, based on previous work in other animal models, it is entirely feasible that supplementation of the diet with bioactive fatty acids such as conjugated linoleic acid (CLA) and tetradecylthioacetic acid (TTA) may mitigate the deleterious effects of feeding farmed fish high fat diets by reducing fat deposition in particular.

The general objective of this research work was to test the hypothesis that CLA and/or TTA could augment growth, reduce fat deposition and enhance fatty acid composition via incorporation of these bioactive fatty acids, and increase n-3 highly unsaturated fatty acid (HUFA) levels in the flesh of commercially important fish species such as Atlantic salmon (*Salmo salar)*, Atlantic cod (*Gadus morhua* L.) and rainbow trout (*Oncorhynchus mykiss*). This project also considered the influence of CLA and TTA on enzymes and transcription factors thought to be pivotal in lipid metabolism and fatty acid oxidation in particular. A subsidiary aim of this research work was to investigate the immunological impact of dietary CLA and TTA administration in these fish.

The results of this project have revealed that the hypothesis was only partly proved. There was no effect in growth or biometry after either CLA or TTA supplementation in any of the fish species investigated. Additionally, there were few physiologically significant effects on fat levels on fish as a result of TTA or CLA administration. However, there were a number of effects on fatty acid metabolism including inhibition of steroyl coenzyme desaturase (SCD) in cod and trout in particular and also enhancement of hepatic n-3 HUFA levels in trout. Importantly, it was determined that both TTA and CLA could be incorporated into the flesh thus providing a vehicle through which these bioactive fatty acids can be delivered to the consumer. There were also a number of beneficial effects on activity and gene expression of a number of enzymes and transcription factors thought to be fundamental to the modulation of fatty acid oxidation in particular. However, the effects on gene transcription and biochemistry had little impact at the whole body level. This research work also showed that there were no detrimental effects on immune status after supplementation with dietary CLA or TTA. Conclusively, this thesis has contributed to the overall understanding of the influence of dietary CLA and TTA in farmed fish.

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LIST OF ABBREVIATIONS

CLA – Conjugated Linoleic Acid

TTA – Teteradecylthioacetic Acid

MUFA – Monounsaturated Fatty Acid

PUFA – Polyunsaturated Fatty Acid

HUFA – Highly Unsaturated Fatty Acid

FCR – Feed Converstion Ratio

SGR – Specific Growth Rate

HSI – Hepatosomatic Index

VSI – Visero-somatic Index

ACC – Acetlyl-CoA Caroboxylase

FAS – Fatty Acid Synthetase

EFA- Essential Fatty Acid

SCD – Stearoyl-CoA Desaturase

TAG - Triacylglyceride

CPT – Carnitine Palmitoyl Transferase

FFA – Free Fatty Acid

PPAR – Peroxisome Proliferator-Activated Receptors

ACO – Acyl Coenzyme-A Oxidase

FABP – Fatty Acid Binding Protein

LPL – Lipoprotein Lipase

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CHAPTER 1. GENERAL INTRODUCTION

1.1 Fatty Acid Metabolism

1.1.1 Structure and Nomenclature

Lipids constitute a large and diverse group of oils and fats that are organic in nature and characteristically are soluble in organic solvents but only sparingly soluble in aqueous solvents. Fatty acids are a family of lipids, which are generally aliphatic monocarboxylic acids that have the ability to be liberated via hydrolysis from naturally occurring fats and oils. Fatty acids can be broadly classed into three structurally and ergo, functionally, diverse groups based on the presence or absence of carbon to carbon double bonds within the hydrocarbon chain of the molecule. A fatty acid is referred to as saturated when it contains no carbon to carbon double bonds. Conversely, monounsaturated fatty acids (MUFA) contain one double bond whilst fatty acids with two or more double bonds are termed polyunsaturated fatty acids (PUFA). Methylene-interrupted (non-conjugated) bonds constitute the most common structural configuration of double bonds. PUFA consisting of non-conjugated unsaturation can be of *cis* (Z) or *cis* (Z) and/or *trans* (E) configuration, which are terms used to denote geometry of the double bonds in this instance (Gunstone *et al*., 1994). Highly unsaturated fatty acids (HUFA) are a subgroup of PUFA distinguished by having chain lengths of $\geq C_{20}$ and with ≥ 3 double bonds; an abbreviation used frequently in aquaculture nutrition (Sargent *et al*., 2002).

Fatty acid nomenclatures have to define chain length, degree of unsaturation and double bond position. There are two accepted nomenclature systems used within lipid biochemistry. Both systems are similar in view of the fact that a numerical value denotes

chain length followed by a colon, which in turn is followed by the number denoting the double bonds. However, the two nomenclatures differ in how they represent double bond position. The delta configuration (Δ) uses numerical values in order to detail the position of each double bond in relation to the carboxyl terminus of the chain whilst the omega/*n* system designates the location of the first double bond from the methyl end of the fatty acid. For example, arachidonic acid (ARA), 20:4 $\Delta^{5, 8, 11, 14}$ has a carbon chain length of 20 and contains 4 double bonds, which are situated at carbons 5, 8, 11 and 14 from the carboxylic end of the fatty acid, respectively. Using the *n* nomenclature, ARA is presented as 20:4n-6, indicating the first double bond is situated 6 carbons from the methyl moiety. In view of the fact that the majority of PUFA within organisms contain methylene-interrupted double bonds, specifying only the position of the first double bond is usually sufficient. Some key fatty acids denoted by their trivial and IUPAC names and their respective nomenclatures are illustrated in Table 1.1.

Table 1.1 Structure and nomenclature of common long chain fatty acids.

n-designation	Δ-designation	Systematic Name	Common Name
Saturated			
12:0	12:0	dodecanoic	lauric acid
14:0	14:0	tetradeconoic	myristic acid
16:0	16:0	hexadecanoic	palmitic acid
18:0	18:0	octadecanoic	stearic acid
20:0	20:0	eicosanioc	arachidic acid
Unsaturated			
16:1n-7	$16:1^{\Delta 9}$	9-hexadecenoic	palmitoleic acid
18:2n-6	$18:2^{\Delta 9,12}$	9,12-octodecadienoic	linoleic acid
18:3n-3	$18:3^{\Delta 9,12,15}$	9,12,15-octadecatrienoic	α-linolenic acid
20:4n-6	$20:4^{\Delta 5,8,11,14}$	5,8,11,14-eicosatetraenoic	arachidonic acid
20:5n-3	$20:5^{\Delta 5,8,11,14,17}$	5,8,11,14,17-eicosapentaenoic	EPA
22:6n-3	$22:6^{\Delta 4,7,10,13,16,19}$	4,7,10,13,16,19,-docosahexanenoic	DHA

1.1.2 Biosynthesis

All vertebrates share a similar fatty acid biosynthesis pathway, which principally begins with mitochondrial acetyl-CoA production via oxidative decarboxylation of pyruvate or mitochondrial β-oxidation. Two cytoplasmic, multi-enzymatic polypeptide structures named acetyl-CoA carboxylase (ACC) and fatty acid synthetase (FAS) are responsible for fatty acid biosynthesis in animals, with energy from NADPH oxidation principally driving the reaction (Wakil *et al*., 1983). After transfer from the mitochondria to the cytoplasm, acetyl-CoA is carboxylated to form malonyl-CoA via ACC, which is the first and rate-limiting step of fatty acid synthesis.

Regulation of ACC is achieved via a combination of mechanisms including hormone-mediated phosphorylation, allosteric binding and feedback inhibition by local metabolites. In this way, the activated polymeric form of ACC is elevated through allosteric binding of citrate whilst inhibition results from feedback inhibition via long chain fatty acyl-CoAs. Acetyl-CoA and malonyl-CoA both bind to FAS and then undergo a series of cyclic condensation, reduction and dehydration processes each facilitated in turn by four enzymatic subcomponents of FAS, namely beta-keto-acyl carrier protein (ACP) synthase, beta-keto-ACP reductase, 3-OH acyl-ACP dehydratase and enoyl-CoA reductase. With each cycle, two successive saturated carbon atoms from malonyl-CoA are added to the original acetyl-CoA moiety, ultimately forming 16:0 (palmitic acid) and to a lesser extent, 18:0 (stearic acid). These fatty acids are then able to undergo separate elongation and/or desaturation reactions in order to yield longer and/or unsaturated fatty acids respectively.

1.1.3 Desaturation and Elongation

Fatty acid desaturation and elongation pathways are a necessity particularly when dietary long chain fatty acids are inadequately supplied. There are two main eukaryotic fatty acid elongation pathways localised to two different organelles within the cell. Mitochondrial fatty acid elongation predominates when fatty acyl CoA substrates are primarily shorter than 16 carbons. This pathway is essentially the reversal of the β-oxidation pathway described in section 1.1.4 except that one NADPH and one NADH are required (β-oxidation yields two NADH). The second and most active pathway is confined to the endoplasmic reticulum (ER) and involves separate, ER-specific enzymes that are functionally similar to the enzymatic sub-domains of FAS. Fatty acids sequestered for elongation must be activated to CoA derivatives; thus providing energy for attachment of the donor group to a growing fatty acyl chain. A subsequent condensation reaction between malonyl-CoA and fatty acyl-CoA results in the addition of a two carbon moiety, thus the chain is lengthened. It is noteworthy that the enzyme responsible for this first condensation step in the elongation process is aptly termed 'elongase' and furthermore, is rate limiting and determines fatty acyl specificity (www.medlib.med.utah.edu/Netbiochem/fattyacids/6_2a.html).

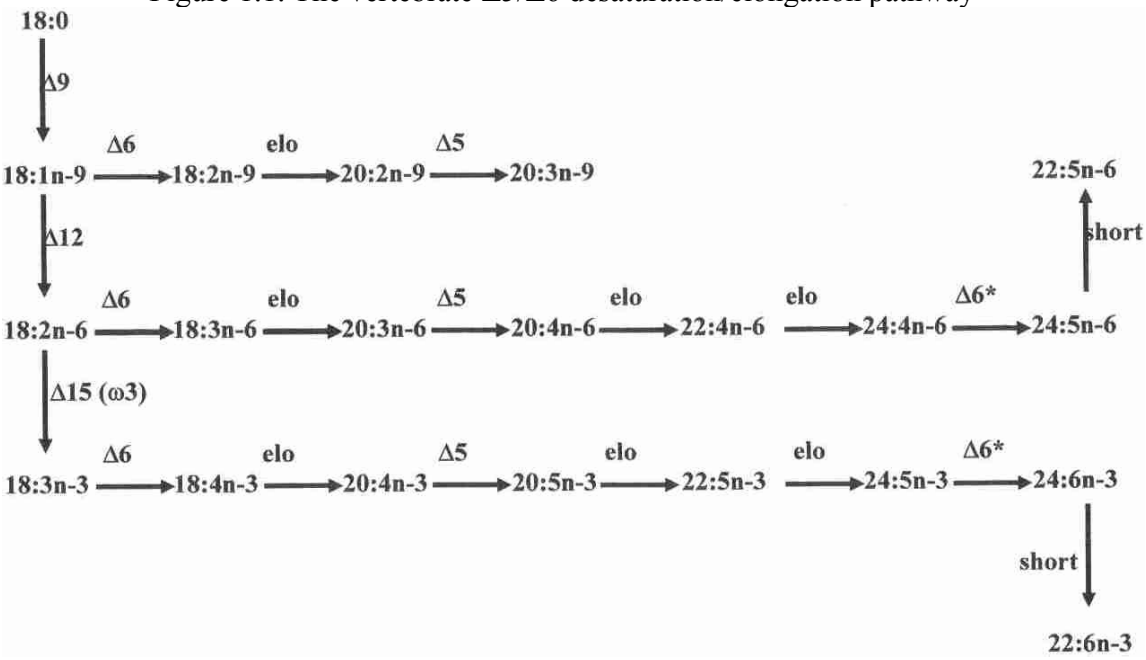
Further modification involves the addition of carbon-carbon double bonds in order to produce unsaturated fatty acids. The family of enzymes that facilitate the introduction of double bonds between defined carbons of fatty acyl chains are named 'desaturases'. Delta (Δ) desaturases are nonheme iron-containing enzymes that display broad chain length specificity and share the ability to create a double bond at a fixed position generally counted from the carboxyl end of the fatty acid. The acyl-CoA desaturation pathway is localised to the ER of particular tissues and is an aerobic process involving the reduction of

CoA-linked substrates via a multi-component electron transport system comprising cytochrome *b5* and cytochrome *b5* reductase together with a terminal desaturase.

All animals have the capacity to introduce double bonds at positions Δ5, Δ6 and Δ9 of a fatty acid. Monounsaturated fatty acids are synthesised from saturated fatty acids via Δ9 desaturase; otherwise known as stearoyl-CoA desaturase (SCD). Broad chain length specificity allows SCD to catalyse the desaturation of fatty acyl-CoA with 12 to 19 carbons, including 16:0 and 18:0, thus producing 16:1Δ9 (16:1n-7 or palmitoleic acid) and 18:1Δ9 (18:1n-9 or oleic acid) respectively (Nakamura and Nara, 2004). Δ9 desaturase is of particular physiological significance given that regulation of cell membrane viscosity is achieved via introduction of 16:1n-7 and 18:1n-9 to constituent phosphoglycerides imbedded in the phospholipid bilayer (Tocher, 2003).

In most animals, HUFA are synthesised from dietary PUFA via trans-membrane Δ6 and Δ5 desaturases. Higher animals, including all vertebrates and fish, lack desaturases with the ability to introduce double bonds between the Δ9 position and the methyl end of the carbon chain, which can only be facilitated by Δ12 and Δ15 (or n-3) desaturases generally found only in plants and insects (Tocher, 2003). Thus, the n-3 and n-6 fatty acid HUFA precursors (18:3n-3 and 18:2n-6), synthesised by Δ12 and Δ15 desaturases, are termed essential fatty acids (EFA) since most animals are unable to manufacture these *de novo* and therefore are reliant on dietary sources. Furthermore, both Δ5 and Δ6 desaturase exhibit affinity towards EFA (18:3n-3 > 18:2n-6) over MUFA (Sargent *et al*., 2002). It is therefore apparent that EFA, especially n-3 fatty acids in the case of fish, are nutritionally of major importance and as such are discussed in section 1.2. The desaturation/elongation pathway outlined in Figure 1.1 appears to be the same, at least qualitatively, for rainbow trout (*Oncorhynchus mykiss*) (Buzzi *et al*., 1996, 1997) as in rats (Voss *et al*., 1991).

Figure 1.1. The vertebrate Δ5/Δ6 desaturation/elongation pathway



1.1.4 Cellular Transport, Esterification and Oxidation

Outer membrane transport and cytosolic solubilisation of fatty acids are facilitated by a family of proteins collectively termed fatty acid binding proteins (FABP). Specifically, FABPs are responsible for fatty acid desorption into the cytosol and cytosolic transport itself (Chmurzynska, 2006) and are present in both mammalian and non-mammalian organisms including fish (Londraville, 1996). Once transported into the cytosol, fatty acids are usually esterified into different lipid classes or oxidised as a source of energy. It is common for fatty acids to be assimilated into triacylglycerol (TAG) and stored in lipid droplets, particularly in adipose tissue, when energy supply exceeds expenditure. Fatty acids that enter the cell can also be incorporated into phospholipids (PL) that form the lipid bilayer of biological membranes. A small quantity of fatty acids can be esterified to cholesterol to form cholesteryl ester, which is the storage form of cholesterol in cells (Buzzi, 1996). Esterification into PL, TAG and cholesteryl esters

proceeds following fatty acid activation in order to produce fatty acyl-CoA. The formation of fatty acyl-CoA is catalysed by acyl-CoA synthetase, which is active in different subcellular compartments depending on tissue type (Buzzi, 1996). Acyltranferase enzymes subsequently facilitate the incorporation of fatty acyl-CoA into PL and TAG. However, esterification is a selective process in fish, by which PUFA are preferentially incorporated into PL or TAG based on degree of unsaturation. For example, 18:3n-3 and 18:2n-6 may be more esterified into TAG, whilst eicosapentaenioc acid (20:5n-3), docosahexaenoic acid (22:6n-3) and arachidonic acid (20:4n-6) are found to a higher concentration in PL (Henderson and Tocher, 1987). Early studies utilising rainbow trout hepatocytes showed that exogenous C_{18} fatty acids are retained in TAG for subsequent utilisation as desaturation and elongation substrates, which are later incorporated into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Sellner and Hazel, 1982a; 1982b). The pathways of *de novo* glyceride synthesis are essentially the same in fish as in higher vertebrates (Sargent *et al.*, 1989).

In a process analogous to fatty acid esterification, fatty acids destined for mitochondrial oxidation must first be modified via the addition of a CoA moiety in order to aid translocation through the outer mitochondrial membranes. The fatty acyl CoA binds to carnitine in a reaction facilitated by a specific acyltransferase named carnitine palmitoyl transferase-1 (CPT-1). The subsequent acyl carnitine esters are therefore able to transverse the inner mitochondrial membrane. CPT-1 activation is rate limiting and the enzyme is allosterically inhibited by malonyl-CoA, thus preventing simultaneous cellular synthesis and degradation of fatty acids. Once fatty acyl carnitine enters the mitochondrial matrix it interacts with CoA in a reaction facilitated by carnitine palmitoyl transferase-2 (CPT-2) to yield carnitine and fatty acyl-CoA. Long chain fatty acyl-CoA can then be utilised in

mitochondrial β-oxidation. Unlike cytosolic fatty acid synthesis, the β-oxidation pathway is compartmentalised to specific organelles, namely mitochondria and peroxisomes. Mitochondrial β-oxidation involves cyclic dehydration, hydration, second dehydration and cleavage of long chain fatty acyl-CoA in order to produce acetyl-CoA along with $FADH_2$ and NADH, the latter of which is oxidised by the mitochondrial electron transport system to yield cellular energy in the form of ATP (Lehninger, 1975). Moreover, acetyl-CoA can then be metabolised via the tricaboxylic cycle to produce additional NADH. Peroxisomal β-oxidation produces the same two carbon acetyl-CoA, however it produces hydrogen peroxide in place of $FADH_2$ and has a different fatty acid specificity compared with mitochondrial β-oxidation since there is no membrane transport system required for very long chain fatty acids to enter peroxisomes, thus they are able to freely diffuse. Many of the enzymes involved in both mitochondrial and peroxisomal β-oxidation are genetically different but share common functionality. Piscine β-oxidation is prevalent in liver, heart, white and red muscle. High levels of peroxisomal β-oxidation have been observed in red muscle and liver of Atlantic salmon (*salmo salar*) (Frøyland *et al.,* 2000) and haddock respectively (Nanton *et al*., 2000).

1.2 Nutritional Aspects of Essential Fatty Acids

1.2.1 EFA in Human Nutrition

Fundamentally, essential fatty acids (EFA) are fatty acids that an organism must obtain through dietary means as the enzymatic processes required for *de novo* synthesis are not present. Humans like all vertebrates and most animals, display an absolute dietary requirement for 18:2n-6 and α-linolenic acid (18:3n-3). However, as a consequence of

modern civilisation, the human diet has significantly changed principally in the amount and type of fat consumed. It is now apparent that saturated fat and n-6 PUFA constitute the majority of fat consumed in 'western' countries with an n-6/n-3 PUFA ratio estimated to be 10:1 to 25:1, averaging around 15:1 whilst the purported optimum value should be closer to 5:1 (Simopoulos, 1999b). This is noteworthy considering n-3 and n-6 PUFA are not metabolically interconvertable and have different physiological functions or efficacies. Indeed, the observed changes in dietary fat intake have been linked with the occurrence of many pathologies and health disorders common in the modern industrialised world, which now extends to Asia (Janus *et al*., 1996; Okuyama *et al*., 1997; Bulliyya, 2000). Nonetheless, n-6 PUFA are still of physiological importance in humans. For instance, 18:2n-6 deficiency is known to interfere with growth, reproduction and skin function in mammals (Burr, 1942; Holman, 1968; Hansen and Jensen, 1985). 18:3n-3 is also responsible for maintaining normal growth and skin function (Burr, 1942; Fu and Sinclair, 2000). HUFA such as 20:4n-6, 20:5n-3 and 22:6n-3 derived from 18:2n-6 and 18:3n-3 are also physiologically important. 22:6n-3 is found largely in brain, retina and other neural tissues (Uauy *et al*., 1999) and is a requirement for normal cognitive and visual development particularly in foetal and newly born infants (Hornstra *et al*., 1995; Laurtizen *et al*., 2001). There is also evidence to suggest that adult brain disorders such as schizophrenia, Alzheimer's disease and clinical depression are associated with relatively low levels of n-3 HUFA, particularly 22:6n-3, in brain and adipose tissue (Mamalakis *at al*., 2002; Conquer and Holub, 1997)

Eicosanoids are essentially a family of active fatty acid metabolites that include prostaglandins, leukotrienes and thromboxanes, which can collectively mediate many humoral and cellular immune functions and are derived primarily from the C_{20} HUFA,

20:3n-6, 20:4n-6 and 20:5n-3 (Fischer, 1989). Moreover, increased consumption of n-3 HUFA results in the prevention or attenuation of many inflammatory conditions that are prevalent in the developed world, including rheumatoid arthritis, atopic illness, psoriasis, multiple sclerosis, bronchial asthma and type I diabetes among others (Leaf and Weber, 1988; Harris, 1989; Kinsella *et al*., 1990; Calder, 1997; de Deckere *et al*., 1998; Simopoulos, 1990a; Connor, 2000). There is also evidence to suggest that consumption of fish containing high levels of 20:5n-3 and 22:6n-3 helps prevent coronary heart disease (Dyerberg *et al*., 1975,1978; Stansby, 1990; Kelly, 1991), and dietary HUFA have been speculated to lower the risk of atherosclerotic plaque formation, colon and breast cancer (Bougnoux *et al*., 1994; Caygill *et al*., 1995). Fish are the most abundant natural source of n-3 HUFA in the human food basket. Therefore, increasing the amount of oily fish in the human diet will help rebalance the n-6/n-3 ratio and reduce many of the abovementioned diseases associated with n-3 HUFA deficiencies (British Nutrition Foundation, 1999).

1.2.2 EFA in Fish Nutrition

Fish, like other vertebrates, must obtain dietary sources of non-synthesisable fatty acids in order to sustain optimum health. A lack of dietary EFA is known to interfere with growth, and reproduction, and in severe cases results in death through various pathologies (Castell *et al.,* 1972; Watanabe, 1982; Sargent *et al.,* 2002). Moreover, lipids and their constituent fatty acids are also very important as a source of energy in fish, especially carnivorous species, which do not rely heavily on carbohydrate due to its low abundance in natural diets. As aforementioned, vertebrates including fish lack an inherent ability to synthesise 18:3n-3 and 18:2n-6 and must therefore rely on dietary sources. It is interesting to note that both 18:3n-3 and 18:2n-6 play no direct functional role in fish and rather serve

only as precursors of the functionally bioactive HUFA; 20:4n-6, 20:5n-3 and 22:6n-3 (Tocher, 2003). 20:4n-6 is a component of PL contributing to the structural integrity of membranes and together with 20:5n-3, is the primary precursor of eicosanoids such as prostaglandins and leukotrienes (Cook and McMaster, 2002). The most abundant n-3 fatty acids in fish are 20:5n-3 and 22:6n-3, which contribute the same basic structural functions in cell membrane phospholipids in fish as 20:4n-6 in terrestrial mammals (Sargent *et al*., 1995). Visual and neural impairment have also purported to be caused by dietary n-3 HUFA deficiencies in juvenile herring (*Clupea harengus*) (Bell *et al*., 1995). Thus, it is probable that n-3 HUFA share similar physiological roles in fish and humans.

The fundamental EFA requirements of fish vary depending on species and habitat. Generally, freshwater/herbivorous fish can survive by converting dietary C_{18} PUFA to HUFA whereas marine/carnivorous fish rely on preformed dietary HUFA. Thus, HUFA can be regarded as EFA in certain instances, particularly when considering the dietary requirements of marine and carnivorous species. The habitual inadequacy of marine fish to convert C_{18} PUFA to HUFA is believed to be caused by enzymatic insufficiency within the desaturation/elongation pathway, specifically impairment of $\Delta 5$ desaturase and/or $C_{18\text{-}20}$ elongase (Owen *et al*., 1975, Tocher *et al*., 1989). Anadromous fish such as Atlantic salmon display the EFA requirements of freshwater fish prior to parr-smolt transformation and, during adaptation prior to migration to seawater, develop fatty acid profiles similar to marine species. Studies have suggested that the changes observed in the fatty acid profiles of anadromous fish are not solely dictated by dietary changes as a result of environment but instead may be due to an innate ability to alter desaturase activity (Bell *et al.,* 1989). It is clear that an important aspect of fish nutrition is the provision of sufficient amounts of the

correct EFA to satisfy growth and development by taking into account particular species specificities.

1.3 Issues in Aquaculture

1.3.1 High energy Diets

With the continuing decline of wild fish stocks the consumer market has become increasingly reliant on the aquaculture industry to provide farmed fish that mirror the nutritional qualities of wild fish. High lipid levels, traditionally provided by fish oil (FO), are utilised in commercial aquaculture diets in order to offset relatively expensive protein as a source of energy. In this way, protein can be 'spared' for synthesis of new tissue (Wilson, 1989; Bell, 1998) and the high lipid content can also fulfil both fish and consumer EFA requirements. Paradoxically, the aquaculture industry relies on wild fisheries to provide FO and fish meal (FM) required to satisfy the dietary EFA requirements of cultured fish. It is noteworthy that the recent average yield of FO from industrial fisheries, circa 1.4 million tonnes in 2000 (Sargent and Tacon, 1999) is unlikely to be exceeded in the future and climatic phenomena such as El Niño can significantly reduce this tonnage. Furthermore, 57% of the total world supply of FO in 2000 was used in aquaculture, with farmed salmon and trout consuming over 60% of that total (Sargent and Tacon, 1999). Global aquaculture has grown at 11.6% per annum compound growth since 1984 (Tacon, 1996) and this rate shows no sign of abating greatly. Thus, it is estimated that around 98% of the total world supply of FO will be required for aquafeeds by 2010 (Barlow, 2000). Compounding this issue is the growing consumer perception that pollutants such as dioxins, polychlorinated biphenyls (PCBs) and flame retardants in FOs have now reached unacceptable levels in farmed fish. In addition, high energy diets can promote excessive fat

deposition, particularly in the liver and flesh, which can compromise the overall market quality of the fish through various mechanisms including reduced pigment visualisation and smoking performance in the case of salmon (Sheehan *et al*., 1996; Johansen and Jobling, 1998). Although early nutritional studies elucidated that dietary HUFA derived from FO is a requisite for optimum fish health (March, 1992), it has now been concluded that generally, levels of 20:5n-3 and 22:6n-3 in current aquafeeds are well in excess of the minimum n-3 EFA requirements (Sargent *et al*., 2002). Despite this, technical advances in extruded feed production have enabled pelleted diets to continue an upward trend in dietary lipid quantity, particularly in the case of Atlantic salmon where diets containing up to 40% lipid have been used (Tocher, 2003). Thus, research into feed formulation is necessary to address many of the aforementioned issues.

1.3.2 FO Replacement

It is apparent that alternatives to FO must be sought in order to increase current global production levels of farmed fish. Although more judicious use of fish trimmings, offal and discards could increase FO output, it is a temporary solution given that the wild fish catch will ultimately decline. It has been suggested that marine crustaceans such as krill and copepods could be harvested for FO however concerns have been raised over the potential impact on ecosystems reliant on such organisms. Probably the most viable sustainable alternative involves the partial or complete replacement of FO with vegetable/plant derived, vegetable oils (VO). However, plant derived oils are rich in C_{18} PUFA such as 18:2n-6 and 18:3n-3 but are devoid of n-3 HUFA abundant in FO. Nevertheless, it has been elucidated that some species of fish including Atlantic salmon display limited ability to convert 18:2n-6 to 20:4n-6, and 18:3n-3 to 20:5n-3 and 22:6n-3.

Therefore, current research has focussed on partial inclusion of VO in aquafeeds in order to test the hypothesis that some fish, such as Atlantic salmon, may be able to endogenously convert 18:3n-3 to n-3 HUFA. To date, there is an extensive body of literature encompassing the biochemical and physiological effects of VO replacement on cultured fish; a review of which is beyond the scope of this discussion. However, a number of studies have elucidated that substitution of FO by VOs is feasible (Torstensen *et al*., 2000; Bell *et al*., 2001, 2002; Rosenlund *et al*., 2001; Tocher *et al*., 2002). Obviously, it is imperative that any replacement should not compromise fish health, welfare or growth performance, which in turn may affect health benefits, taste and other quality characteristics important to consumers. Some of the key findings have shown that in salmon and trout, replacement of FO with VO up to 100% did not influence growth and feed conversion and that flesh lipid content was unaffected by dietary lipid source (Bell *et al*., 2004). Thus, a diet rich in VO can provide the same energy for growth as one composed purely of FO. Furthermore, it has been reported that a high level of dietary VO shows little to no effect on the organoleptic properties of salmon and trout, and organic pollutants such as PCBs and dioxins are also significantly reduced (Berntssen *et al*., 2005). However, a number of studies have also elucidated that flesh 22:6n-3 and 20:5n-3 concentrations are significantly reduced in salmonids fed high levels of VO indicating that desaturation/elongation of 18:3n-3 is not sufficient in providing the same quantity of n-3 HUFA found in FO (Bell *et al*., 2005). Therefore, the obvious implications are that consumers cannot obtain the same quantity of n-3 HUFA from fish fed VO as opposed to FO but this has, to some extent been resolved by the introduction of FO 'finishing' diets. Although flesh lipid content is unaffected by VO, salmon fed linseed oil exhibit a higher incidence of 'fatty liver', which in severe cases has been known to result in increased risk

of mortality (Bell *et al*., 2004). Investigations into the immunological consequences of VO replacement concluded that 20:4n-6 derived prostaglandin production is reduced whilst incidences of cataract in salmon were significantly increased, raising potential fish welfare issues (Waagbo *et al*., 2004) Linseed oil supplementation also altered a number of basic immune parameters including haematocrit, leukocyte numbers and macrophage respiratory burst (Good, 2004). It is possible that some of these concerns can be resolved through VO and VO/FO blending however, the research described in this thesis investigating the physiological and biochemical effects of prospective dietary 'bioactive' fatty acids, is highly pertinent to this area as it was hypothesised that they may minimise detrimental effects of feeding cultured fish a diet high in lipid or VO.

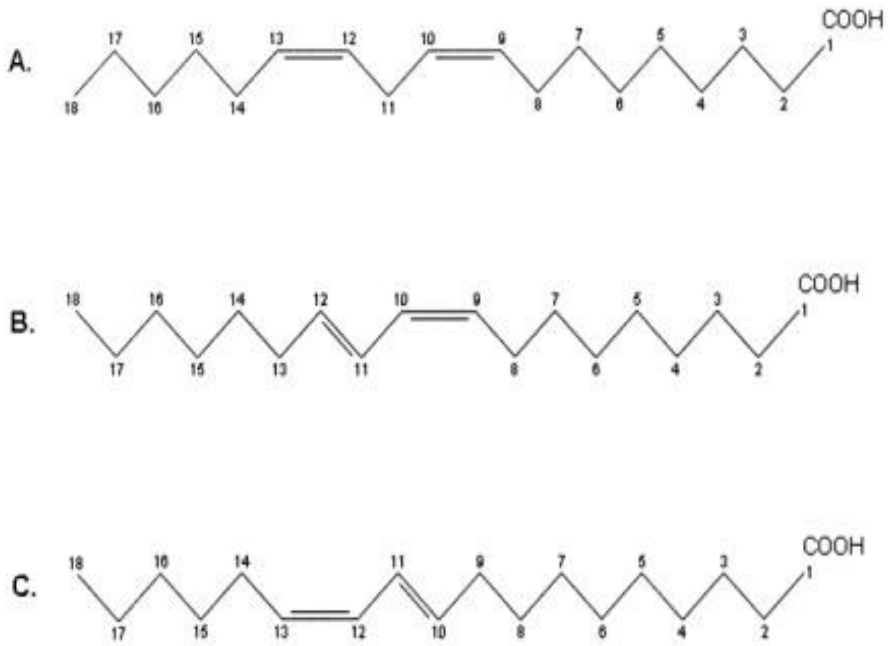
1.4 Conjugated Linoleic Acid (CLA)

1.4.1 Introduction

One prospective 'functional' fatty acid that has garnered a lot of interest within nutritional research is conjugated linoleic acid (CLA). CLA was probably first identified in 1935 after ultraviolet analysis established the presence of conjugated fatty acids in cow milk fat (Booth *et al.*, 1935). However, the nutritional potential of CLA was not realised until 1978 when Michael Pariza and colleagues identified a 'beef-derived mutagenesis factor' when studying the effects of temperature and time on mutagen formation in grilled ground beef (Pariza, *et al*., 1979). Subsequent studies indicated that the mutagen displayed anti-carcinogenetic properties and was in fact a conjugated derivative of linoleic acid (Pariza and Hargaves, 1985; Ha *et al*., 1987). Since that time, a vast amount of work concerning the structural and functional aspects of CLA has been conducted. It is now accepted that conjugated linoleic acids represent a mixture of positional and geometric

isomers of octadecadienoic acid with 'non-methylene-interrupted' double bonds. The range of potential health benefits associated with CLA in experimental models now include anti-obesity, anti-atherogenic and immunomodulatory effects however only two isomers (cis-9, trans-11 and trans-10, cis-12) are thought to exhibit biological activity (Pariza *et al*., 2001). Both isomers are illustrated in Figure 1.2 below.

Figure 1.2 The two principal biological isomers of conjugated linoleic acid (CLA) in comparison with linoleic acid



A. Linoleic Acid. B. c9t11 CLA. C. t10c12 CLA.

CLA are produced naturally mainly by bacterial biohydrogenation of linoleic acid (18:2n-6) in the ruminant gut by a variety of species including *Butryrivibrio fibrosolvens* (Kepler, 1966 in Riserus 2003; Griinari *et al*., 2000; Ogawa *et al*., 2006). As a consequence, food products derived from ruminants are the major source of CLA in the human diet; with the cis-9, trans-11 (c9t11) and trans-10, cis-12 (t10c12) isomers

comprising 80 – 90 % and 3 – 5 % of the total, respectively (Khanal and Dhiman, 2004). However, recent studies have ascertained that c9t11 CLA can also be endogenously synthesised via Δ9 desaturation of trans-vaccenic acid in the mammary glands of cows, and monogastrics such as mice and humans (Adolf *et al.*, 2000; Santora *et* al., 2000; Turpeinen *et* al., 2002; Mosley, *et al*., 2006a, b). In addition, 1:1 mixtures of the two bioactive CLA isomers are being produced commercially via alkaline isomerisation or partial hydrogenation of either linoleic acid itself or, more usually, sunflower or safflower oils that are rich in linoleic acid (Banni, 2002), and currently CLA is being marketed as a dietary supplement in amounts up to 1000 mg/capsule. This is around five times the average purported natural human daily intake of CLA of 200 mg per day for men and 150 mg per day for women (Ritzenthaler *et al*., 2001). It is likely that the current natural daily intake of CLA will not increase and perhaps may even decrease given that the amount of red meat and dairy produce consumed is declining as a consequence of the consumer understanding that excessive saturated fatty acids are promoters of chronic disease.

The goal of increasing the quality of animal-derived food has, and continues to be, of major importance in the agricultural sector. As a result, additional focus has been given to designing and enhancing foods with components that have beneficial effects on human health. Thus, the majority of current CLA research is focused on the adjustment of animal diets in order to raise the CLA levels found in milk, meat and other agricultural foodstuffs. By introducing CLA into aquafeeds it is hoped that the reported physiological changes, particularly the anti-adipogenic and immunomodulatory effects (see following sections) established in other animals, could also be mirrored in fish.

1.4.2 CLA and Body Composition in Experimental Animals

Most studies to date have explored the influence of mixed isomer preparations of dietary CLA (referred to as CLA for the remainder of this chapter). Reductions in body weight and/or fat deposition in animals supplemented with CLA have been the most commonly reported effect thus far. A decrease in body fat mass, an increase in lean body mass and growth rate, and improvements in feed efficiency were initially described in Fischer rats and ICR (Institute of Cancer Research) mice supplemented with 0.5 – 1 % CLA (Chin *et al*., 1994; Park *et al*., 1997). In fact, recent studies have shown that dietary CLA concentrations as little as 0.4 % of total diet have decreased fat mass and increased lean mass in BALB/C male mice fed over a 14 week period (Bhattacharya *et al*., 2006). A reduction in fat deposition in brown and/or white adipose tissue has been particularly evident in C57BL/6J mice fed ≥ 1 % CLA (West *et al.*, 1998; Tsuboyama-Kasaoka *et al.*, 2000; Whigham *et al.*, 2000; Takahashi *et al.*, 2002). Similarly, 1 % CLA significantly reduced the weight of epididymal and perirenal adipose tissues in ICR mice (Akahoshi *et al.*, 2002). Studies investigating the interaction between dietary CLA and fat level found that concentrations of 0.5 – 1 % reduced fat deposition equivalently in AKR/J mice fed either a high-fat (45 % of calories) or a low-low fat (15 % of calories) diet (Delany *et al.*, 1999). Thus, it seems likely that the attenuation of body fat evidenced through CLA supplementation is independent of dietary fat intake, at least in mice. It is evident that the type of dietary lipid used does influence the anti-adipogenic capability of CLA however. It has recently been shown that CLA, when fed in combination with coconut or soybean oil, can decrease body mass and epididymal fat mass in mice. Mice fed CLA in combination with FO showed no effect on adiposity however (Hargrave *et al*., 2005).

The observed changes in body composition caused by dietary CLA are most obvious in mice, however doses as low as 0.25 % of total diet have also been shown to reduce retroperitoneal and parametrial fat pad weights without effecting growth rate or food intake in Sprague-Dawley rats (Azain *et al.*, 2000). Similar studies have also elucidated that 1.5 % dietary CLA can decrease white adipose tissue weight and fat pad mass in male Sprague-Dawley and ZDF rats, respectively (Ryder *et al.*, 2001; Yamasaki *et al.*, 2003). Conversely, recent studies have shown that 2 % dietary CLA has no effect on body composition in adult male Wister rats (Mirand *et al.,* 2004), and long term feeding of 1 % CLA to Fischer rats for 18 months has shown no effect on body composition (Park *et al.,* 2005). Studies evaluating the impact of dietary fat on the anti-adiposity effects of CLA have shown that 1.5 % of a mixed isomer preparation had no effect in Sprague-Dawley rats given alongside diets rich in either coconut oil (EFA deficient) or corn oil (Kloss *et al*., 2005). It has been suggested that the effects of CLA on body composition may be limited to growing, lean animals. Feeding lean and obese rats diets containing 0.5 % CLA for 5 weeks induced a reduction in fat mass of growing rats, but not in rats established with obesity (Sisk *et al.*, 2001). Taken together, it seems likely that the effects of CLA on fat deposition are based on dose, age, length of treatment, dietary lipid content and/or genotype variability in rats.

Improved growth rates and feed efficiencies together with reductions in carcass fat have been observed in the majority of studies analysing the influence of mixed isomer preparations of CLA in pigs (Dugan *et al*., 2004). Preliminary studies suggest that low concentrations of dietary CLA have no effect on adiposity in cats or dogs however (Jewell *et al*., 2006; Leray *et al*., 2006). Advances in the manufacture of individual CLA isomers have made possible the independent analysis of t10c12 and c9t11 CLA. A number of

studies have now indicated that t10c12 CLA is responsible for the attenuation of fat deposition *in vitro* and *in vivo* (Park *et al.,*1999; Gavino *et* al., 2000; Choi *et al.*, 2001 Ryder *et al.*, 2001; Navarro *et al*., 2003; Wargent *et al*., 2005; Simon *et al.*, 2006).

1.4.3 CLA and Body Composition in Humans

There are relatively few papers reporting the effects of CLA or its individual isomers in humans and current information available suggests the results are inconclusive. A 12 week study in which normal-weight exercising subjects were administered 1.8 g/d of a CLA mixture showed a 4 % decrease in body fat with no affect on body weight compared to that of the control group fed a hydrogel placebo (Thom *et al*., 2001). An earlier study carried out by the same laboratory administered 1.7, 3.4, 5.1, and 6.8 g/day CLA to exercising obese humans concluding that a dose of 3.4 g/day was enough to significantly decrease body fat without affecting body weight (Blankson *et al.*, 2000). Interestingly, exercising overweight subjects initially fed a fat-reducing diet and subsequently 1.8 to 3.6 g/day CLA, increased their resting metabolic rate and lean mass without affecting body weight regain (Kamphuis *et al*., 2003). The abovementioned studies seem to indicate that CLA has the ability to decrease fat and improve lean mass in humans who lead a physically active lifestyle in particular. Another study revealed that 4.2 g CLA/day for 4 weeks was enough to reduce sagittal abdominal diameter without effecting body weight in middle-aged obese men with signs of metabolic syndrome (Riserus *et al*., 2001). Thus, it may be concluded that dietary CLA can decrease fat mass without affecting cardiovascular health. Additionally, healthy individuals fed 0.7 g/day for 4 weeks and thereafter 1.4 g/day CLA for 4 weeks decreased in fat mass relative to control subjects (Kreider *et al*., 2002).

In contrast, a 64 day investigation into the effects of 3 g/d dietary CLA in sedentary and exercising healthy women found that lean body mass, body weight, energy expenditure, fat oxidation, rate of lypolysis and FA esterification were not altered (Zambell *et al.*, 2000). A similar trial involving 2.1 g/day CLA administered to sedentary women had no effect on body composition (Petridou *et al*., 2003). Two studies have evaluated the impact of CLA administered in the form of dairy products. The first study analysed the effects of purified isomers at 1.5 and 3 g/day for 18 weeks supplemented as a drinkable dairy product and found that there were no differences in body composition in middle-aged overweight subjects (Malpuech-Brugere *et al.*, 2004). Similarly, abdominal and subcutaneous adipose tissue did not change in overweight and obese men fed butter fat comprising 4.2 % CLA (Desroches *et al*., 2005).

The long-term effects of CLA on body composition have been addressed in two trials. In the first study, subjects of average body mass index were administered either CLA as a free fatty acid (CLA-FFA) or CLA in the form of TAG (CLA-TAG) for 12 months. Both forms of CLA decreased body fat mass significantly and a higher lean body mass was evidenced in subjects fed CLA-FFA (Gaullier *et al*., 2004). A follow-up study purported that subjects administered 3.4 g/day CLA-TAG for 12 months could maintain a decrease in fat mass over a period of 24 months (Gaullier *et al*., 2005). At present, the presumption that CLA can elicit an effect on body composition in humans is questionable considering the inconsistencies between studies. The dramatic effects seen in animal studies, especially in mice, have not been reflected in human studies. This may be attributed to lower CLA dosage in humans in comparison with experimental animal studies. In humans, Isomer-specific effects of t10c12 CLA have not been investigated, which is believed to be the causative agent of reduction in body composition in animal studies. Age seems to be a

critical factor in determining CLA-specific effects. Experiments using rodents have been conducted with growing animals and there is little evidence of CLA effectiveness in adult animals. Conversely, human studies have been performed solely with adults. However, preliminary evidence suggests that long-term supplementation of CLA can induce a fat-lowering effect in humans. Moreover, it would seem that dietary CLA administered in conjunction with regular exercise helps promote fat loss and improve lean body mass in humans.

1.4.4 CLA and Immunomodulation

The immune response in fish, like all other vertebrates, consists of two closely related defence mechanisms – the innate and adaptive immune system. The adaptive immune system allows for specific cellular responses (such as antibody production) that are tailored to specific pathogens or pathogen-infected cells; a review of which is beyond the scope of this project. The innate immune response is the first line of defence against pathogens and consists of a multitude of cells, proteins and peptides in tissue and body fluids (Robertson, 1999). Skin, gills, mucus, macrophages, eicosanoids, cytokines, phagocytes and neutrophils all contribute to the innate immune system in fish.

Macrophage activation, increased eicosanoid and cytokine production, increased phagocytosis, greater lymphocyte numbers and enhanced lysozyme activity are indicators of an elevated innate immune response (Sakai, 1999; Grannam and Schrock, 2001). This non-specific defence system is routinely reported to be modulated by dietary supplementation with immuno-stimulants. There is increasing evidence to suggest that CLA confers an immumo-modulatory effect in animals when fed as a dietary supplement (O'Shea *et al*., 2003). For instance, CLA has been shown to inhibit cytokine (specifically

TNF-α) production in rat macrophages (Yang and Cook, 2003), ICR mice (Akahoshi *et al*., 2002), and Sprague-Dawley rats (Yamasaki *et al*., 2003), and reduce prostaglandin synthesis in a variety of studies (Liu *et al*., 1998; Kavanaugh *et al*., 1999; Shen *et al*., 2004; Lai *et al*., 2005), suggesting this bioactive fatty acid may act as an anti-inflammatory agent. However, the majority of current evidence suggests that the inflammatory response is unaffected by dietary CLA in adult humans (Albers *et al.*, 2003; Nugent *et al*., 2005; Gaullier *et al.*, 2007; Mullen *et al*., 2007). Recently, *in vitro* studies have revealed that t10c12 CLA increased phagocytosis in porcine blood cells (Kang *et al*., 2007). Moreover, dietary CLA increased serum lysozyme activity and enhanced phagocytic ability in broiler chicks (Zhang *et al*., 2005). It is therefore plausible that dietary CLA may modulate the immune system, in part by enhancing the non-specific immune response in selective species. Thus, dietary manipulation through provision of CLA may prove to be one of the optimal ways of improving fish health.

1.4.5 Dietary CLA in Fish

Another aspect of CLA research concerns the beneficial effects dietary CLA may elicit on cultured animals, including fish. For example, it is hoped that some of the immuno-modulatory effects of CLA may have application in aquaculture as an alternative to feed antibiotics, by enabling increased stocking density or as a means of improving the response to vaccination and conferring disease resistance. Of equal importance is the impact dietary CLA may have on feed efficiency, body composition and growth, which have all been improved in previous mammalian studies.

There are very few papers reporting the effects of CLA in fish. On commencement of the project, only four papers had been published in the peer-reviewed literature. The papers focussed in particular on the influence of dietary CLA on growth and biometry and in some instances the hepatic and muscle fatty acid compositions of freshwater species. Species studied included hybrid striped bass (*Morone cyhrysops x M. saxatilus)*, yellow perch (*Perca flavescens)*, channel catfish (*Ictalurus punctatus*), Nile tilapia (*Oreochromis niloticus*), rockfish (*Sebastes schlegeli*) and common carp (*Cyprinus carpio*) (Choi *et al*., 1999; Twibell *et al*., 2000, 2001; Twibell and Wilson, 2003). All these studies used dietary fat levels (< 10 % of total diet), which were arguably too low to adequately test the proposed protein sparing effect of dietary CLA. Indeed, dietary CLA did not influence growth in yellow perch or channel catfish. However, tilapia and rockfish fed CLA at 2.5 – 10 % of total diet and carp fed 10 % CLA exhibited significantly lower weight gain and feed efficiencies compared with fish fed no CLA, suggesting a growth-inhibiting and possibly toxicological response when CLA was fed at extremely high levels. In contrast, dietary CLA concentrations of up to 1 % of total diet significantly increased weight gain in carp (Choi *et al*., 1999) and improved feed efficiency in hybrid striped bass (Twibell *et al*., 2000). Additionally, total liver lipid concentration decreased significantly in hybrid striped bass and yellow perch whilst HSI increased in bass fed 1 % CLA. Hepatic and muscle fatty acid compositions have been assessed in yellow perch and hybrid striped bass fed CLA. Of note is that flesh concentrations of 20:5n-3 and 22:6n-3 were significantly decreased in hybrid striped bass supplemented with 1 % CLA, but this was not reported in any other trial. In addition, all the aforementioned trials were carried out using juvenile fish and so the effect of CLA on adult fish that were actively depositing lipid in the tissues had not been investigated. Arguably the main and most consistent finding is that CLA

deposition occurs in fish muscle and liver and the amount of CLA retained in these tissues after supplementation can be higher than deposited in meat or milk fat derived from natural ruminant sources. Thus, consumption of CLA-supplemented fish may be an effective means of increasing human intake of these fatty acids (Wahle *et al*., 2004). In summary, it has been shown that the effects of CLA in fish are species-specific and dose-dependant. It is also clear from other animal studies that experimental differences in CLA isomer content, dietary lipid source and/or amount, and age of fish may be of importance when detailing the physiological influence of dietary CLA.

1.4.6 Prospective Biochemical Mechanisms of Action

CLA have been reported to increase both the activity and gene expression of enzymes implicated in fatty acid catabolism in a number of animal studies. Arguably the best gauge of mitochondrial fatty acid oxidation capacity is the measurement of carnitine palmitoyl transferase-1 (CPT-1) activity. A number of studies are in agreement that t10c12 CLA is implicated in enhancing mitochondrial fatty acid oxidation through increasing CPT-1 activity (Martin *et al*., 2000; Degrace *et al*., 2004; Marcarulla *et al*., 2005; Zabala *et al*., 2006) and gene expression (Wang *et al*., 2005; LaRosa *et al*., 2006) particularly in liver and skeletal muscle. Furthermore, elevated peroxisomal β-oxidation capacity has been reported to be caused by increased enzyme activity and/or transcript levels of hepatic acyl coenzyme-A oxidase (ACO) in animals fed CLA (Moya-Camarena *et al*., 1999; Yamasaki *et al*., 2001; Choi *et al*., 2004; Degrace *et al*., 2004).

Transcriptional regulation of a number of enzymes involved in hepatic β-oxidation (including CPT-1 and ACO) is mediated, in part by peroxisome proliferator-activated receptor alpha (PPARα). PPARα is known to instigate rapid increases in the size and

number of hepatic peroxisomes since it increases the activity of enzymes required for β-oxidation (Ruyter et al., 1997). CLA may arbitrate its effects via this transcription factor; indeed both c9t11 and t10c12 isomers are ligands for all three isoforms of PPAR (α,β,γ) (Moya-Camarena *et al*., 1999; Clement *et al*., 2002;).

Other direct effects of CLA on lipid metabolism extend to encompass an increase in lipolysis and apoptosis, principally in adipocytes (Park *et al*., 1997; Tsuboyama-Kasaoka *et al*., 2000; Miner *et al*., 2001; Evans *et al*., 2002). Studies exploring the effects of individual CLA isomers on primary cultures of stromal vascular cells from human adipose tissue identified t10c12 as the only lypolytic agent (Brown *et al*., 2001) and the resultant reduction in TAG content is associated with the differential localisation and expression of lipid droplet-associated proteins (Chung *et al*., 2005). However, recent studies have argued that the fat lowering effects of t10c12 CLA is not due to increased apoptosis (Xu *et al*., 2003) but rather reduced preadipocyte differentiation into mature adipocytes (Brodie *et al*., 1999; Satory and Smith., 1999; Evans *et al*., 2000; Simon *et al*., 2005). Indeed, t10c12 CLA did not appear to cause apoptosis in human adipocytes (Brown *et al*., 2003a, 2004).

Modulation of preadipocyte differentiation and adipogenesis has been suggested to be mediated in part by PPARγ. Activation of PPARγ is known to increase adipogenesis (Desvergne *et al*., 2006). Both *in vitro* studies in human and 3T3-L1 adipocytes (Evans *et al*., 2000; Kang *et al*., 2003; Brown *et al*., 2003a, 2004; Granlund *et al.*, 2003, 2005) and *in vivo* work in mice (Tsuboyama-Kasaoka *et al*., 2000; Takahasi *et al*., 2002; Kang *et al*., 2004) hamsters (Zabala *et al.*, 2006) and pigs (Brandebourg and Hu, 2005) have identified that upon CLA supplementation, with the t10c12 isomer in particular, there is a decrease of PPARγ expression. Consistent with this, down-regulation of PPARγ downstream targets

aP2, perilipin-A and acyl-CoA-binding protein (ACBP) have been observed in rodents fed CLA (Houseknecht *et al*., 1998; Belury, 2002).

Inhibition of lipogenesis is well documented in studies detailing the mechanisms behind the anti-obesity effects of CLA. Indeed, reductions in TAG content after CLA supplementation have been verified *in vitro* and *in vivo* (Azain *et al*., 2000; Evans *et al*., 2000; Tsuboyama-Kasaoka *et al*., 2000; Poulos *et al*., 2001; Rahman *et al.*, 2002). This has been purported to be caused by reductions in expression of adipocyte ACC (Tsuboyama-Kasaoka *et al*., 2000; Brown *et al*., 2003a; Peterson *et al*., 2003; Lin *et al*., 2004; Zabala *et al.*, 2006) and FAS (Tsuboyama-Kasaoka *et al*., 2000; Kang *et al*., 2003; Peterson *et al*., 2003; Lin *et al*., 2004; Zabala *et al.*, 2006). Moreover, evidence suggests that the glucose transporter GLUT 4 gene expression is down-regulated in human preadipocytes, white and brown adipose tissue during CLA supplementation (Takahishi *et al*., 2002; Brown *et al*., 2003a), and in 3T3-L1 adipocytes treated with 25 μm t10c12 CLA (Granlund *et al*., 2005). Indeed, significantly higher levels of glucose and fatty acid have been found in serum, plasma, liver and surrounding tissues after CLA supplementation (Baumgard *et al*., 2002; Brown *et al*., 2003a). The inhibitory effect of CLA on lipogenesis is also correlated with inhibition of uptake and transport of fatty acids as evidenced by decreased lipoprotein lipase (LPL) and FABP levels (Bretillion *et al*., 1999; Park *et al*., 1999) and is probably due to the t10c12 isomer in particular (Peterson *et al*., 2003). The fact that LPL and FABP are regulated via PPARγ only serves to strengthen the notion that CLA mediates its anti-lipogenic effects through this transcription factor in mammals (Khan and Vanden-Heuvel, 2003). *In vivo* evidence of a TAG-lowering effect after CLA supplementation has also arisen from decreased gene expression levels of enzymes directly responsible for TAG synthesis (Baumgard *et al*., 2002). Thus, the reduction in uptake of

glucose and fatty acids together with the inhibition of *de novo* fatty acid and TAG synthesis is likely to account for the reduction in the TAG content of adipocytes in this instance.

Lipid-droplet size has also been shown to decrease in 3T3-L1 adipocyte cells treated with t10c12 CLA and has been attributed to reduced amounts of palmitoleic and oleic acid (Choi *et al*., 2000). *In vivo* experiments with rats (Sebedio *et al*., 2001), mice (Lee *et al*., 1998) and pigs (Gatlin *et al*., 2002), and *in vitro* studies with HepG2 cells (Eder *et al*., 2002) and human preadipocytes (Brown *et al.*, 2003a) have confirmed an increase in the ratio of saturated fatty acids to monounsaturated fatty acids in particular. Decreases in adipocyte size have also been noted in mice (Tsuboyama *et al.*, 2000) and rats (Azain *et al.*, 2000; Ohnuki *et al.*, 2001; Poulos *et al.*, 2001). In support of these findings, a number of studies have determined that SCD gene expression and/or activity is significantly decreased in mice, pigs and poultry fed CLA, particularly the t10c12 isomer (Lee *et al.*, 1998; Choi *et al*., 2001; Park *et al.*, 2001; Smith *et al*., 2002; Shang *et al*., 2005). Thus, there is evidence to suggest that decreased lipogenesis and therefore cellular TAG content is as a result of decreased lipogenic enzyme activity/expression in studies involving t10c12 CLA. However, it is also likely that increased fatty acid oxidation could contribute to the TAG lowering effect in adipocytes.

1.5 Tetradecylthioacetic Acid (TTA)

Tetradecylthioacetic acid (TTA) belongs to a family of 3-thia fatty acids that contain a sulphur atom at the third position from the carboxyl terminus. Hence, TTA cannot be catabolised via the β-oxidation pathway and instead is processed via ω-oxidation (Skrede *et al.*, 1997). The chemical structure of TTA is illustrated in Figure 1.3 below.

Figure 1.3 The chemical structure of tetradecylthiocacetic acid (TTA).

Although structurally very different from CLA, dietary TTA is responsible for a number of similar physiological and biochemical responses. For instance, TTA fed in combination with a high fat diet is known to decrease epididymal adipose tissue mass in Wister rats (Madsen *et al*., 2002). Furthermore, TTA has been shown to stimulate both hepatic peroxisomal and mitochondrial β-oxidation of fatty acids (Berge and Hvattum, 1994) and increase the activity and up-regulate the gene expression of a number enzymes associated with both metabolic pathways. For example, short term dosing of TTA *in vitro* resulted in up-regulation and increased enzyme activity of ACO in addition to an increase in activity of both CPT-I and CPT-II (Vaagenes *et al.*, 1998). Similar studies have also noted an increase in both gene expression and enzyme activity of CPT-II and ACO in isolated hepatocytes of rats fed 150 mg/kg body weight TTA for 10 days (Willumsen *et al*., 1997). Conversely, CPT-II, but not CPT-1, transcript level and associated activity increased in hepatocyte mitochondria after chronic treatment of rats with dietary TTA (Madsen and Berge, 1999; Madsen *et al.*, 1999). Both hepatic TAG synthesis rate and level are suppressed in rats supplemented with TTA (Berge *et al*., 2005) and preliminary evidence suggests that TTA can also modulate the immune response by acting as an anti-inflammatory agent in peripheral blood mononuclear cells (Aukrust *et al*., 2003) and patients infected with HIV (Fredriksen *et al*., 2004)

Although dietary CLA and TTA share a similar influence on lipid metabolism and both induce an anti-inflammatory response, TTA also elicits a number of effects which

have not been documented in trials involving CLA. For example, TTA lowers serum lipid levels (Asiedu *et al*., 1996; Frøyland *et al*., 1997), increases the flux of plasma free fatty acids to the liver ready for catabolism, (Berge *et al.*, 2005) and increases proliferation of both peroxisomes and mitochondria (Berge *et al*., 1989; Bremer, 2001) which are all likely to contribute to the overall anti-adipogenic and hypolipidemic effects associated with this bioactive fatty acid (Berge *et al*., 2005).

The hypolipidemic and anti-inflammatory effect has been speculated to be caused by the ability of TTA to activate PPARα (Berge *et al*., 2001; Dyroy *et al.*, 2005). Indeed, like CLA, TTA is a potent ligand for all three PPAR isoforms (α,β,γ) (Gotticher *et al*., 1993; Forman *et al*., 1996; Berge *et al*., 1999) in both human and murine cell lines (Berge *et al*., 2001; Westergaard *et al*., 2001). Interestingly, mitochondria from TTA-treated rats were found to have a lower electrochemical potential and an increased UCP-2 expression (Grav *et al*., 2003) indicative of an enhancement in energy expenditure.

1.6 General Objectives

To summarise, there is a considerable body of evidence to suggest TTA and, particularly, CLA bestow a wide variety of biological effects in mammals. These effects extend to encompass beneficial alterations in body composition via reduction in body fat mass, enhanced growth rate and immune function and anticarcinogenetic properties. Nevertheless. it has been evidenced in this chapter that the effects of CLA are variable in animals and not fully investigated in fish.

Prior to this project no published data was available determining the influence of dietary CLA in salmonid or marine species. In addition, all of the previous studies on freshwater species utilised juvenile fish fed low lipid diets and focussed primarily on

potential alterations in growth and fatty acid compositional changes as a consequence of CLA supplementation. The potential influence of CLA on lipid metabolism and the mechanisms underpinning these effects had only previously been explored in mammals. Moreover, no effects of dietary TTA in cultured fish had been recorded prior to commencement of this study.

It is clear that the physiological effects of dietary CLA and TTA described in other studies could have the potential for application in aquaculture, particularly in relation to issues pertaining to high fat or FO-substituted diets, fish welfare and human health benefits. The general hypothesis tested by this project was that CLA and TTA could elicit physiological and biochemical effects in fish leading, but not limited, to augmentation of growth, reduction in fat deposition and enhancement of fatty acid composition via incorporation of these bioactive fatty acids, and increased n-3 HUFA levels in the flesh of fish. Additionally, it was also important to consider the impact of feeding CLA and TTA on fish health and welfare considering the reported immunomodulatory effects in other animal models.

In detail, the specific aims of the present project were:-

1. To determine the effects of dietary CLA on growth, biometry, lipid and fatty acid metabolism, and selected innate immune function parameters of Atlantic salmon, rainbow trout and Atlantic cod.
2. To investigate the influence of dietary CLA in Atlantic salmon in response to dietary lipid level.

3. To determine the effects of dietary CLA and TTA on the gene expression of proteins and enzymes involved in lipid homeostasis and fatty acid oxidation in salmonids.
4. To develop suitable analytical methods that would allow an accurate representation of the effects of dietary CLA and TTA on gene expression and fatty acid composition in fish.

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

[1-^{14}C] Palmitoyl CoA (50-55 mCi/mmol) and [methyl-^{3}H] L-carnitine (60-86 Ci/mmol) were obtained from Amersham Biosciences (Little Chalfont, Bucks, U.K.). [1-^{14}C]18:3n-3 (50-55 mCi/mmol), was obtained from NEN Brand Radiochemicals (Perkin Elmer Life and Analytical Sciences (U.K.) Ltd., Beaconsfield, Buckinghamshire, U.K.). Adenosine triphosphate (ATP), aminotriazole, butylated hydroxytoluene (BHT), carnitine, coenzyme A, dichlorofluorescein diacetate, dimethylformamide, dithiothreitol, EDTA, fatty acid free–bovine serum albumin (FAF-BSA), glutathione, horseradish peroxidase, leuco-2, 7-dichlorofluorescein (DCF), magnesium chloride, *N*-acetylcysteine, NADP, nicotinamide, palmitoyl–CoA, potassium chloride, potassium fluoride, silver nitrate, sodium dodecyl sulphate (SDS), TriReagent and Triton X-100 were obtained from Sigma Chemical Co. (Poole, U.K.). TLC (20 cm × 20 cm × 0.25 mm) and HPTLC (10 cm × 10 cm × 0.15 mm) plates, precoated with silica gel 60 (without fluorescent indicator) were obtained from Merck (Darmstadt, Germany). All solvents were HPLC grade and were obtained from Fisher Scientific UK, Loughborough, England. All compressed gases were obtained from the British Oxygen Company (BOC, Glasgow, UK).

2.2 Experimental Fish and Diet Formulations

The fish that were used in this project are described in the relevant experimental chapters. The experimental diets were formulated to satisfy the nutritional requirements for the species used in each experiment (U.S. National Research Council, 1993), and were

formulated and manufactured by Biomar A/S, Brande, Denmark. The specific dietary formulations for each dietary trial are described respectively in the appropriate chapters.

2.2.1 Determination of Growth Parameters

Growth parameters were measured using the following formulae.

Feed Conversion Ratio:

$$\text{FCR} = \frac{\text{feed intake (g)}}{\text{wet weight gain (g)}}$$

Specific Growth Rate:

$$\text{SGR, \%/day} = 100 \times \frac{\ln(W_1) - \ln(W_o)}{\text{number of days}}$$

where W_o and W_1 represent initial and final body weight, respectively.

Thermal Growth Coefficient:

$$\text{TGC} = 1000 \times \frac{\sqrt[3]{W_1} - \sqrt[3]{W_0}}{\text{days} \times \text{Temp.}(^{o}\text{C})}$$

Hepato-somatic index:

HSI = 100 x liver weight x body weight^{-1}

Viscero-somatic index:

VSI = 100 x viscera weight x body weight^{-1}

2.3 Lipid Metabolism

2.3.1 Preparation of Tissue Homogenates and Liver Microsomes

Portions of tissues (liver, white and red muscle) were weighed and homogenised to 10 % (w/v) in sucrose buffer, which was 0.25 M sucrose in 40 mM phosphate or HEPES buffers pH 7.4 containing 1 mM EDTA, 0.15 M KCl, 40 mM KF and 1 mM N-acetyl cysteine. The homogenates were centrifuged at 1880 x g for 10 min at 4˚C, the floating fat layer aspirated and the post-nuclear fractions collected, and 100 µl taken for protein determination according to Lowry et al. (1951) after incubation with 400 µl of 0.25% (w/v) SDS/1M NaOH for 45 min at 60 ^{o}C as described in section 2.3.6. The homogenates were used directly for assay of fatty acid oxidation, and associated enzyme assays. To prepare microsomes for HUFA synthesis assays, the liver homogenate was centrifuged at 25,000 g for 15 min at 4 ^{o}C, the floating fat layer removed by aspiration and the remaining supernatant centrifuged at 105,000 g for 60 min at 4 ^{o}C. The floating fat layer was aspirated and the supernatant decanted. The pelleted microsomal fraction was resuspended in 1 ml of sucrose buffer and 50 µl taken for protein determination as described above.

2.3.2 Determination of HUFA Synthesis

The assay mixture, in sucrose buffer pH 7.4, contained 5 mM $MgCl_2$, 1.5 mM glutathione, 0.5 mM nicotinamide, 1 mM NADH, 100 µM coenzyme A, and 5 mM ATP in a total volume of 0.75 ml. Fifty µl of [1-^{14}C]18:3n-3 (0.25 µCi, 5 µM final concentration), as a FAF-BSA complex (Ghioni *et al*., 1997), was added and the reaction initiated by the addition of 200 µl of microsomes. Incubation continued for 1 h at 20 ^{o}C and the reaction was stopped by the addition of 5 ml of chloroform/methanol (2:1, v/v) containing 0.01%

BHT, and total lipid extracted, transmethylated and FAME prepared according to section 2.3.9. The extracted methyl esters were redissolved in 100 µl isohexane containing 0.01 % BHT and applied as 2.5 cm streaks to TLC plates impregnated by spraying with 2 g silver nitrate in 20 ml acetonitrile and pre-activated at 110 ^{o}C for 30 min. Plates were fully developed in toluene/acetonitrile (95:5, v/v) (Wilson and Sargent, 1992). Autoradiography was performed with Kodak MR2 film for 6 days at room temperature. Areas of silica containing individual PUFA were scraped into scintillation mini-vials containing 2.5 ml of scintillation fluid (Ecoscint A, National Diagnostics, Atlanta, Georgia) and radioactivity determined in a TRI-CARB 2000CA scintillation counter (United Technologies Packard, U.K.). Results were corrected for counting efficiency and quenching of ^{14}C under exactly these conditions.

2.3.3 Assay of Fatty Acyl Oxidation Activity in Selected Tissues.

Fatty acyl β-oxidation in selected tissue homogenates was estimated by determination of acid-soluble radioactivity as described previously (Frøyland *et al*., 1996). Briefly, 30 µl of liver or red muscle or 300 µl of white muscle homogenate in sucrose buffer (prepared as described in section 2.3.1) was added to 250 µl of an assay master mix containing 16.5 mM $MgCl_2$, 82.5 mM KCl, 13.2 mM DTT, 6.6 mM ADP, 0.2 mM NAD+, 0.7 mM EDTA and 1.2 mM carnitine. The tubes were left at room temperature to equilabrate for 2 min. 10 µl of [1-^{14}C]palmitoyl-CoA substrate (0.1 µCi/100 µM) was subsequently added and the tubes left to incubate at room temperature for precisely 10 min. The reaction was stopped by the addition of 150 µl of 1.5 M KOH and 25 µl FAF-BSA (100 mg/ml) added as carrier and to bind any unreacted labelled fatty acyl substrate. After briefly vortexing, 500 µl of ice-cold 4 M $HClO_4$ (perchloric acid) was added to precipitate

protein. The tubes were then centrifuged at 1880 g for 10 min and 500 µl of the resultant supernatant was measured for radioactivity as described in section 2.3.2

2.3.4 Assay of Carnitine Palmitoyl Transferase-I Activity

CPT-1 activity in tissue homogenates was determined according to the method described by Saggerson and Carpenter (1986). Briefly, 90 µl of an assay master mix comprising 100 mM Tris buffer pH 7.4, 5 mg/ml BSA, 1 mM DTT, 4 mM $MgCl_2$, 15 mM KCl, 1 mM KCN, 2 mM carnitine and 1 µl ^{3}H-carnitine was added to 10 µl of tissue homogenate (prepared previously as described in section 2.3.1). Five µl of a 100 µM palmitoyl-CoA substrate (in sucrose buffer) was then added to initiate the reactions. After 15 min at room temperature, the reactions were terminated by the addition of 60 µl of 1 M HCl followed by the addition of 500 µl of water-saturated butanol. The samples were vortexed for 30 s and centrifuged for 5 min at 5000 g in a microfuge to accelerate phase separation after which 300 µl of the upper butanol layer was recovered. The recovered phase was back extracted by vigorous mixing with 1 ml of water for 30 s before centrifugation was repeated. One hundred µl of the resultant upper butanol phase was transferred to a scintillation mini-vial and radioactivity determined as described in section 2.3.2.

2.3.5 Assay of Acyl CoA Oxidase Activity

The spectrophotometric determination of ACO utilised the method devised by Small *et al*. (1985). The assay is based on determination of H_2O_2 production, which is coupled to the oxidation of leuco-2, 7-DCF (leuco-DCF) in a reaction catalysed by exogenous peroxidase. Precisely, 1 ml of peroxidase buffer comprising 0.08 mg/ml

horseradish peroxidase in 20 mM phosphate buffer pH 7.4, containing 40 mM aminotriazole and 0.02% (v/v) Triton X-100, was added to 30 µl of DCF reagent (1.6 mM 2',7' dichlorofluorescein diacetate in NN-dimethylformamide/0.01 M NaOH, 1 : 9 by volume), and the appropriate volume of tissue homogenate (50 µl for red muscle and liver, 500 µl for white muscle), with the final assay volume adjusted to 1.5 ml with 20 mM phosphate buffer pH 7.4. This mixture was preincubated at room temperature in the dark for 5 min before being measured spectrophotometrically (RSA Cecil 2021 spectrophotometer) at an absorbance of 502 nm for 2 min to determine autoxidation rate. The reaction was then initiated with the addition of 30 µM palmitoyl-CoA and the enzymatic reaction rate was determined. Rates were then corrected for the substrate blank.

2.3.6 Protein Determination

Protein concentration in tissue homogenates or liver microsomes was determined according to the method of Lowry *et al*. (1951) after digestion. In summary, 400- 450 µl of 1 M NaOH/0.25 % (w/v) SDS were added to appropriate volumes of tissue homogenates or microsome preparations (see section 2.3.1). Samples were incubated at 60 ^{o}C for 45 min in a water bath in order for solubilisation of cellular components to occur. A standard curve of 0 – 100 µg protein in increments of 20 µg was prepared by addition of bovine serum albumin (1 mg/ml) to individual plastic test tubes and volumes adjusted to 100 µl with distilled water. One percent (w/v) $CuSO_4$ and 2% (w/v) sodium potassium tartrate was added to 2% (w/v) Na_2CO_3 (at a ratio of 1:1:100 respectively), and 2.5 ml of this solution was added to standards and samples. After 15 min incubation at room temperature, 250 µl of Folin-Ciocaulteau phenol reagent, diluted 1:1 with water, was added to the standards and samples. After vigorous mixing, the absorbance at a wavelength of 660 nm was read on a

spectrometer (RSA Cecil 2021) 30 min later. Protein concentration (in mg/ml) was calculated from the resultant standard curve.

2.3.7 Total Lipid Extraction and Quantification

Total lipids of liver, flesh and diet were extracted by homogenisation in chloroform/methanol (2:1, v/v) containing 0.01 % (w/v) BHT as an antioxidant, according to the method of Folch *et al*. (1957). Principally, 1 g of tissue or diet was homogenised using an Ultraturrax^{TM} tissue disrupter in quickfit boiling tubes (50 ml) with 16 ml of ice-cold 2:1 chloroform/methanol and left for 1 h on ice. After incubation, non-lipid impurities were removed by the addition of 4 ml aqueous 0.88 % (w/v) KCl and thorough mixing. After a further 5 min the tubes were centrifuged (Jouan C312, France) at 400 g_{ave} (1500 rpm) for 2 - 3 min and the resultant upper aqueous phase containing non-lipid impurities was drawn off by aspiration and discarded. The lower organic layer was transferred into a clean, pre-weighed 10 ml test tube, through a pre-washed (with chloroform/methanol, 2:1) 9 cm filter paper (Whatman No.1). Solvent was evaporated under a stream of oxygen-free nitrogen (OFN) on an N-Evap evaporator (Organomation Associates, Inc. USA). The weight of lipid was determined gravimetrically after overnight desiccation *in vacuo* prior to reconstitution in chloroform/methanol (2:1) at a concentration of 10 mg/ml and stored under nitrogen at -20 ^{o}C for future analyses.

2.3.8 Determination of Lipid Class Composition

Separation of lipid classes was performed by single-dimension double-development high performance thin-layer chromatography (HPTLC). A 10 x 10 cm HPTLC plate was washed and 'activated' by fully developing in chloroform/methanol (2:1, v/v) and then

drying in an oven at 110 ^{o}C for 15 min. Approximately 20 µg (2 µl of a 10 mg/ml lipid solution) of each total lipid sample was applied in 2 mm streaks 1 cm up from the bottom of the plate. Plates were developed up to 5 cm in methyl acetate/isopropanol/ chloroform/methanol/0.25% KCl (25:25:25:10:9, by vol). The plate was then briefly desiccated *in vacuo* prior to being fully developed in isohexane/diethyl ether/acetic acid (85:15:1, by vol). The lipid classes were visualised by charring at 160 ^{o}C for 15 min after spraying with 3 % (w/v) cupric acetate in 8 % phosphoric acid and quantified by densitometry using a Camag 3 TLC Scanner (Camag, Muttenz, Switzerland) and winCATS software (Henderson and Tocher, 1987). The identities of individual lipid classes were confirmed by comparison with reference to Rf values of authentic standards run alongside samples on HPTLC plates developed in the above solvent solutions.

2.3.9 Analysis of Fatty Acid Composition

Total lipids were subjected to acid-catalysed transesterification based on a standard method described by Christie (2003). However, this basic method was modified as necessary to take account of problems associated with the quantitative preparation of fatty acid methyl esters (FAME) from CLA and TTA. Thus, FAME were produced under conditions specific to each dietary trial, which are specified in the relevant sections of the experimental chapters. Irrespective of the detailed transmethylation procedure, after methylation, FAME were extracted using 5ml isohexane/diethyl ether (1:1, v/v) with BHT (0.01%) (w/v) and centrifugation at 350-400g_{ave} (1500rpm) for 2 min. The upper organic layer was transferred to a clean test tube. Five ml of isohexane/diethyl ether (1:1, v/v), without BHT, was added to the original tube before mixing and centrifuging for a second time. Again the upper organic layer was transferred to the tube containing the organic

portion of the previous extraction and the aqueous layer discarded. Solvent was then evaporated under a stream of OFN on an N-Evap and the resultant FAME resuspended in 100 µl of isohexane. Methyl esters were purified prior to gas chromatography. Briefly, FAME solutions were applied to 20 x 20 cm TLC plates using microsyringes (Hamilton) onto previously marked 1 cm origins and the plates developed using isohexane/diethyl ether/acetic acid (90:10:1 v/v) as the developing solvent as described previously (Tocher and Harvie, 1988). After separation, the plate was partially sprayed with 1 % (w/v) iodine in chloroform to visualise the FAMEs. The bands were scraped from the TLC plate into test tubes using a straight edged scalpel. FAME were eluted from the silca with 10 ml isohexane/diethyl ether (1:1, v/v) containing 0.01 % BHT, then vortexed and centrifuged at 350-400g_{ave} (1500rpm) for 2 min in order to precipitate the silica. The solvent was carefully decanted into a 15 ml glass quickfit tube and evaporated under OFN. The recovered FAME were dissolved in an appropriate volume of isohexane containing 0.01 % (w/v) BHT to make a 1-2 mg/ml solution of methyl esters. FAME were quantified by gas-liquid chromatography using a Fisons GC8600 gas chromatograph (Fisons Ltd., Crawley, U.K.) equipped with on-column injection and a 60 m x 0.32 mm i.d. wall-coated capillary column (CP wax 52CB; Chrompak Ltd., London, U.K). Hydrogen was used as a carrier gas and temperature programming was from 50 ^{o}C to 150 ^{o}C at 40 ^{o}C/min and then to 225 ^{o}C at 2 ^{o}C/min. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman, 1980). Data were collected and processed using Chromcard for Windows, Version 1.19 computer package (Thermoquest Italia S.p.A., Italy)

2.3.10 Whole Body Proximate Composition

Fish were weighed to determine wet weight of whole fish. Fish were cut into pieces and placed in a pre-weighed dish. Moisture content of whole fish was determined after drying in an oven at 80 ^{o}C for a minimum of 72 h. The dishes were allowed to cool in a desiccator, reweighed and the dry sample weight determined and hence the percentage moisture rate determined by the formula:-

$$\% \text{ Moisture} = \frac{\text{Wet Sample weight (g)} - \text{Dried Sample Weight (g)} \times 100}{\text{Wet Sample Weight (g)}}$$

The dried fish samples were then blended into a homogeneous crumble/meal and used for determination of percent protein, lipid and ash content.

Lipid content of dried fish crumb was determined using the Soxhlet method of extraction (Avanti Soxtec 2050 Auto Extraction apparatus; Foss, Warrington, UK). Briefly, 1 g of fish crumb was accurately weighed (W_1) and placed in an extraction thimble along with some glass beads, and the thimble then sealed with cotton wool. The Soxtec extraction unit was switched on and the oil bath heated to 115 ^{o}C, the cold water supply to the condensers opened and the sample thimbles fixed into the unit. Approximately 50 ml of petroleum ether (Sigma-Aldrich, Surrey, UK) was added to the extraction collection cups, which were subsequently attached to the Soxtec apparatus. The samples were boiled for 20 min, then rinsed for 2 h before the solvent was evaporated for 15 min. The cups were removed, dried at 100 ^{o}C for 1 h, and cooled in a desiccator before being reweighed (W_2). The percentage lipid was thus calculated using the formula:-

$$\% \text{ Lipid} = \frac{W_2 \text{ (g)} \times 100}{W_1 \text{ (g)}}$$

Protein content was determined in the fish crumble using the automated Kjeldahl method (Tecator Kjeltec Auto 1030 Analyser, Foss, Warrington, UK). Two hundred mg of fish crumble was accurately weighed into a digestion tube and 2 mercury kjeltabs with 5 ml concentrated sulphuric acid added. The samples were subsequently digested at 400 ^{o}C for 1 h and then allowed to cool for 20 mins. Then 20 ml of de-ionised water and 5 ml of sodium thiosulphate solution were added to the tubes prior to distillation and titration. Fifty mg of urea was used as a positive standard and background titre effects were taken into consideration by measuring tubes containing no sample. Percent protein/nitrogen was calculated as follows:-

$$*\% \text{ Nitrogen in the standards} = \frac{(\text{standard titre} - \text{blank titre}) \times 280.14}{\text{Standard weight (mg)}}$$

$$**\% \text{ Protein in the samples} = \frac{(\text{sample titre} - \text{blank titre}) \times 1750.875}{\text{Sample weight (mg)}}$$

* Derived from 14.007 (N_2) x 0.2 (acid molarity) x 100 (%)

** Derived from 14.007 x 0.2 x 100 x 6.25 (protein factor)

Percent ash content of samples was determined by placing 1 g of dried crumb into a porcelain crucible, which was subsequently heated to 600 ^{o}C overnight in a muffle furnace. The samples were allowed to cool and then reweighed. Percentage ash composition was determined using the formula:-

$$\% \text{ Ash} = \frac{\text{Ash weight (g)} \times 100}{\text{Sample weight (g)}}$$

2.4 Basic Haematology and Non-Specific Immune Response Analyses

2.4.1 Extraction and Preparation of Blood

Fish were bled by caudal venepuncture using 1 ml syringes pre-coated with lithium heparin (2500 U/ml in Leibovitz L-15 medium) with varying needle gauge sizes depending on size of fish (Terumo Europe N.V. Belgium). As much blood as possible was removed from the fish before samples were placed into microcentrifuge tubes (ThermoLife Sciences, Basingstoke, Hampshire, UK) after collection. Whole blood samples were stored at 4 ^{o}C. Plasma was extracted by centrifuging whole blood samples at 7000 rpm for 10 min at 4 ^{o}C (IEC micromax centrifuge, International Equipment Co. MA, USA). The plasma supernatant was extracted and frozen at -20 ^{o}C for further analyses.

2.4.2 Blood Cell Counts

From whole blood samples, 10 µl was removed and mixed with 990 µl of 0.2 M phosphate buffered saline (PBS, 0.2 M $NaH_2PO_4.2H_2O$, 0.2 M $NaHPO_4.1H_2O$, pH 5.8) and a drop of the 1:100 dilution placed on a Neubauer haemocytometer (Hawksley and Son, England). Round and refractile cells were counted as white blood cells (WBC) under a phase contrast microscope at 100 x magnification. The counts were expressed as WBC per ml of blood.

For red blood cell measurement, 100 µl of the 1:100 dilution prepared previously was added to 900 µl of PBS (1:1000 dilution) Red blood cells (RBC) were then counted as described above and quantified per ml of blood.

2.4.3 Total Packed Cell Volume

A heparinised 25 µl capillary tube was inserted into the blood sample and the tube allowed to fill via capillary action until almost full. One end of the tube was sealed with Critaseal (BDH) and then centrifuged at 800 g using a Hawksley Micro-haematocrit centrifuge (Hawksley and Son, England, UK). The haematocrit was calculated by expressing the red blood cell layer as a percentage of the total sample volume.

2.4.4 Lyzozyme Activity

Lysozyme activity was measured turbidimetrically using a modified 96 multiwell plate method (Peddie *et al.*, 2002). Lyophilised *Micrococcus lysodiekticus* was added to 0.04 M sodium phosphate buffer, pH 5.8 (0.2 M $NaH_2PO_4.2H_2O$, 0.2 M $Na_2HPO_4.H_2O$) to a concentration of 0.2 mg of bacteria/ml and incubated at 25 ^oC for 20 min. To four replicate wells of a non-coated 96 multiwell plate (NUNC, BDH), 10 µl of plasma were added, with exception to the last column which contained only buffer as a negative control. To the plasma samples, 190 µl of bacterial suspension was quickly added to all except control well. After 1 min the sample absorbance was read at 540 nm and again at 5 min, using a Dynex MRX II plate reader (Dynex Technologies, USA). Lysozyme activity was expressed as the amount of sample causing a decrease in absorbance of 0.001 min^{-1} (U min/ml).

2.4.5 Phagocytosis by Head Kidney Macrophages

A field technique developed by Crumlish *et al*. (2000) was utilised in order to isolate head kidney macrophages from fish due to the large number of samples required. Using aseptic technique, the kidney was dissected and placed on to sterile 100 µm nylon mesh. The kidney sample was then teased through the mesh using the flat end of a sterile syringe into 5 ml of Leibovitz-15 medium (L-15, Sigma, Dorset, UK) containing 10 U/ml of heparin. The resulting kidney suspension was then transferred to a sterile bijoux ready for analysis.

Microscope slides were prepared by dipping in ethanol and then 2 wells drawn on the slide using a PAP pen (AGAR Scientific). One hundred µl of kidney suspension was then placed onto each well and incubated for 1 h at room temperature to allow the macrophages to attach to the slide. After this time, any non-adhered cells were washed off by rinsing with ice-cold L-15 medium. Fifty µl of a 5 mg/ml yeast (*Saccharomyces cerevisiae*) suspension in L-15 medium were added to one of the wells containing the adhered macrophages and after 1-2 hours incubation at room temperature, subsequently washed off using L-15 media. Slides were then fixed with 70 % ethanol and allowed to air dry before being stained using Rapi-diff staining kit (Sigma, Dorset, UK). Slides were examined using oil immersion x1000 magnification and the number of yeast particles engulfed by 100 macrophages determined. Phagocytic index (PI), which indicates the average number of particles engulfed per macrophage and also phagocytic ratio (PR), which is the ratio of phagocytic cells in the population (equation) were measured.

$$PI = \frac{\text{Total number of ingested particles}}{\text{Total number of macrophages counted}}$$

$$PR = \frac{\text{No. of macrophages with 1 or more ingested particles x 100}}{\text{Total number of macrophages counted}}$$

2.4.6 Respiratory Burst of Head Kidney Macrophages

Head kidney macrophages were isolated and prepared as described in section 2.4.5. A modified method adapted from Rook *et al.* (1985) was used to determine respiratory burst. One hundred µl aliquots of head kidney suspension were placed into 8 replicate wells of a 96-well microtitre plate (NUNC) and left for 4 - 5 hours at 4 ^{o}C to allow the macrophages to adhere. The non-adhered cells were then washed off by rinsing with L-15 medium. The 96-well plate was divided into three separate sections. One hundred µl of 1mg/ml nitrobluetetrazolium (NBT) in L-15 medium was added in triplicate to one section of the plate. To separate wells on the same plate, 100 µl of L-15 medium containing 100 µl of 1 mg/ml NBT solution and 1 µl/ml of phorbol 12-myristste 13-acetate (PMA, phorbol ester) was added in triplicate. One hundred µl of lysis buffer (0.1 M citric acid, 1 % (v/v) Tween 20, 0.05 % (w/v) crystal violet) was added to the remaining two columns of the plate. The plate was then left for 1 h at room temperature after which all wells containing the NBT and NBT + PMA had the solution removed and 100 µl of methanol was added and incubated for 5 min at room temperature to stop the reaction. The wells previously containing the NBT and NBT + PMA were rinsed three times with 70 % methanol and allowed to air dry. One hundred and twenty µl of dimethylsulphoxide (DMSO) and 140 µl of 2 M potassium hydroxide were added to each of the washed wells to dissolve the blue formazan residue. The absorbance of each well was then read at 610 nm (Dynex MRXII ELISA plate reader). The number of attached cells was determined using a Neubeuar haemocytometer by counting released nuclei in wells containing lysis buffer. The

absorbance and thus the respiratory burst activity in each well was adjusted to represent that from 1 x 10^4 cells.

2.5 Gene Expression Analyses

2.5.1 RNA Extraction

Approximately 50-100 mg tissue was homogenised with 1 ml of TRIzol® in a 1.5 ml microcentrufuge tube using an Ultramax TS S8N5G (IKA Labortechnik, Staufen, Germany). The homogenised sample was then incubated at room temperature for 5 min before the addition of 200 µl of chloroform (Sigma-Aldrich, Dorset, UK). The tube was then lightly mixed by inversion and then shaken vigorously for 15 s, incubated for a further 3 min at room temperature and then centrifuged in a refrigerated centrifuge (4 ^oC) at 12,000 g for 15 min (Sigma 4K15 centrifuge). The aqueous phase was transferred to a clean 1.5 ml microcentrifuge tube and 500 µl of isopropanol (Sigma-Aldrich, Dorset, UK) added in order to precipitate the RNA. The mixture was subsequently incubated for 10 min at room temperature after which it was centrifuged at 12,000 g for 10 min at 4 ^oC. The resultant supernatant was discarded and the pellet washed with 1 ml of 75 % ethanol (v/v). The ethanol was aspirated and the pellet was allowed to air dry until it began to turn transparent and thereafter 50-100 µl of ultrapure H_2O was added (volume dependant on pellet size) in order to resuspend the RNA. The integrity of the RNA was verified by running a 1.2 % agarose gel with 1 x TBE buffer including 10 ng ethidium bromide (see appendix 1) and the ratio of absorbances at 260/280 nm determined spectrophotometrically (NanoDrop ND-1000, Spectrophotometer, Sussex, U.K.). The RNA solution was then stored at -70 ^oC for further analyses.

2.5.2 First Strand cDNA Synthesis

cDNA was prepared by adding 5 µg (in the case of salmon) or 2 µg (in the case of cod and trout) RNA (diluted with ultrapure H_2O) to 1 µg Oligo (dT) primer (Promega, Madison, WI, USA) in a sterile RNase-free 0.2 ml PCR tube, making a total volume of 14 µl. The mixture was then heated to 70 ^oC for 5 min in a PTC-100 thermocycler PCR machine (MJ Research, MA, USA) and then cooled on ice for 5 min. 5 µl of M-MLV (molony-murine leukaemia virus) reverse transcriptase (RT) reaction buffer (250 mM Tris-HCl, pH 8.3, containing 375 mM KCl, 15 mM $MgCl_2$ and 50 mM DTT), (Promega, Madison, WI, USA) was added to the mixture together with 1.25 µl of 10 mM dNTP (Promega, Madison, WI, USA), 1 µl of 200 U/µl M-MLV RT (H-) and brought to 11 µl with ultrapure H_2O. The tube was then mixed gently and incubated at 40 ^oC for 10 min, then 48 ^oC for 50 min and finally the RT enzyme was inactivated by heating for 15 min at 70 ^oC according to the manufacturer's protocol. The resultant cDNA was diluted 1 in 5 with ultrapure H_2O and stored at -20 ^oC for future analyses.

2.5.3 Preparation of Amplicons

Sequences of interest (target and reference genes) were selected from the NCBI database and appropriate primers were designed for each gene using Primer 3 HTML applet. PCR was performed on cDNA from liver in order to isolate the genes of interest. Briefly, the PCR consisted of 10 µl of 2x PCR master mix (50 U/ml Taq Polymerase, 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$ (pH 8.5)) and 1 µl/primer and 3 µl H_2O. The generic PCR reaction procedure consisted of 45 cycles of 15 s at 95 ^oC, 15 s at 50 - 60 ^oC (depending upon gene) and 30 s at 72 ^oC. An optimal annealing temperature was selected for all genes after temperature gradient analysis.

One µl of the PCR product was visualised on a 1.2 % agarose gel containg 1x TBE buffer and 10 ng of ethidium bromide using a UV transilluminator (UVP, San Gabriel, CA, USA) in order to verify amplification. The remainder of the PCR product was subsequently purified using a GFX^{TM} PCR DNA Purification Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK), according to the manufacturers protocol (see appendix 2).

2.5.4 Production of Recombinant DNA

A pBluescript II KS (+/-) vector was first linearised at the polycloning site by digesting 4.4 µl of 1.1 µg/µl vector with 2 µl EcoR V (20 U/µl) enzyme (NewEngland Biolabs., MA, USA), 5 µl of 10X NEB Buffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9) (NewEngland Biolabs, MA, USA), 5 µl of 10X BSA (NewEngland Biolabs., MA, USA) and the final volume brought to 50 µl with ultrapure H_2O in a sterile 0.2 ml PCR tube. The mixture was incubated at 37 ^{o}C for 1 h and then purified using a GFX^{TM} column (Amersham Pharmacia Biotech, Uppsala, Sweden) (see appendix 2).

TA overhangs were added by incubating 1.25 µl of digested vector with 0.05 µl Thermoprime plus polymerase (Abgene, Surrey, UK), 1 µl of the corresponding buffer (750 mM Tris-HCl, pH 8.8, containing 200 mM $(NH_4)_2SO_4$ and 0.1% v/v Tween 20), 0.6 µl 25 mM of $MgCl_2$, 0.5µl of dTTP's (5mM) and made up to a total volume of 10 µl with ultrapure H_2O in a 0.2 ml sterile PCR tube. The mixture was incubated for 30 - 40 min at 72 ^{o}C and then put on ice to await ligation with PCR product.

One µl of the previously isolated PCR product (section 2.5.3) was added to a ligation mixture consisting of 1.25 µl TA-vector, 1 µl of T4 ligase (Promega, Madison, WI, USA), 1µl of 10X ligation buffer (10 mM HCl (pH 7.0), 50 mM KCl, 1 mM DTT, 0.1 mM

EDTA and 50 % glycerol) and the final volume brought to 10 µl with ultrapure H_2O. The ligation mixture was gently mixed and left at 14 ^oC overnight in order for the reaction to take place. Ligation of the PCR product to the TA-vector was verified against known standards using gel electrophoresis.

For each transformation, 2 µl of the ligation reaction was added to 50 µl of competent TOP10F' *E.coli* freshly thawed on ice. The tubes were mixed gently and then incubated on ice for 30 min before being heat shocked at 42 ^oC for precisely 40 s. The tubes were then incubated on ice for a further 2 min after which 250 µl of LB broth (appendix I) was added and the tubes shaken horizontally at 37 ^oC for 1 h at 225 rpm in a rotary shaking incubator (Gallenkamp, Loughbourgh, UK). 125 µl of the resultant cell suspension was spread onto a Petri dish containing LB agar/X-gal/IPTG/ampicillin (appendix I) The plates were inverted and placed in a 37 ^oC incubator for 48 h (Gallenkamp, Loughborough, UK).

Recombinant (white) colonies were picked from plates and grown overnight in 3 ml LB broth containing 50 mg/ml ampicillin (appendix) at 37 ^oC in a shaking incubator (Gallenkamp, Loughbourgh, UK) before being harvested and the plasmids extracted from the cells using the GFX^{TM} miscroplasmid preparation kit (appendix). The integrity of recombinant DNA was determined by adding 1 µl of a 1 in 50 dilution of plasmid to a 0.2 ml microcentrifuge tube along with PCR reagents and appropriate primers at reaction conditions identical to that detailed in section 1.6.3. The subsequent PCR product was then visualised and verified using gel electrophoresis. The plasmid was linearised using an appropriate endonuclease determined using Restriction Mapper software. One µl of endonuclase (type dependent on gene) was added to 31 µl of uncut plasmid DNA together with 4 µl of BSA and 4 µl of endonuclease-specific buffer and incubated at 37 ^oC for 2 h.

The digestion was deactivated by heating to 65 ^{o}C for 15 min. The cut plasmid was visualised once again using gel electrophoresis to verify linearity and to ensure that the plasmid had only been cut once. The recombinant plasmid was then diluted to 1 x 10^{9} copies/µl and subsequently serially diluted to produce a standard curve of known copy number.

2.5.5 Quantitative Real-Time Polymerase Chain Reaction Analysis

Measurement of gene expression was carried out using quantitative real-time polymerase chain reaction (qPCR) on a Rotor-Gene™ 3000 (Corbett Research, Sydney, Australia). Fifteen µl of a master mix containing 10 µl 10X SYBR-green (Qiagen, Crawley, UK), 3 µl of molecular grade water, 1µl (10 pmol) of each primer (forward and reverse) was added to a 0.2 ml Rotor-Gene™ tube together with 5 µl of an appropriately diluted cDNA sample. Amplification of cDNA samples was carried out using the QuantiTect® SYBR Green PCR Kit (Qiagen, Crawley, UK) with the following conditions: 15 min at 95 ^{o}C to activate the HotStarTaq DNA polymerase, followed by 45 cycles of 15 s at 95 ^{o}C, 15 s at 56 ^{o}C and 30 s at 72 ^{o}C, followed by product melting to check purity of PCR product. Thermal cycling and fluorescence detection were conducted using the Rotor-Gene 3000 system (Corbett Research, Cambridge, UK). Expression of genes was normalised and expressed relative to total RNA or selected flat-liner (housekeeping genes) as described in detail in the appropriate chapters.

CHAPTER 3. INFLUENCE OF DIETARY OIL CONTENT AND CLA ON GROWTH AND LIPID DEPOSITION IN ATLANTIC SALMON

3.1 Introduction

Atlantic salmon is an anadromous fish mainly found in the temperate and Arctic regions of the northern hemisphere. The culture of Atlantic salmon has been practised in northern Europe, the North America and Chile since the latter half of the 20th century (FAO, 2004) and is presently the most intensively farmed fish in the western world, with production in 2004 reaching more than 1.2 million tonnes, estimated to be worth around 4 billion US$ (FAO, 2007). This boom in production has helped maintain market supply of Atlantic salmon in a climate where wild stocks have been steadily decreasing and, in some instances, decimated by over fishing, climatic changes and pollution. Aside from the many environmental concerns voiced by opponents of the aquaculture industry, many believe that cultured Atlantic salmon does not provide the same nutritional quality as wild salmon. This is partly due to current dietary formulations, which have tended to see an upward trend in dietary lipid as the principal source of energy, to enhance protein sparing, improve feed conversion and minimise fish waste (Tocher, 2003). The corollary of maintaining fish on high oil level diets is that despite beneficial increases in growth, potentially detrimental effects including deposition of excess lipid in tissues, specifically flesh in the case of salmon, can reduce nutritional quality (Sargent *et al*., 2002; Tocher, 2003). As well as dietary lipid levels, lipid deposition can be related to a number of factors including size, season and disease (Sargent *et al*., 2002). Consequently, it is important to gain a clearer

understanding of the physiological mechanisms that control energy metabolism, and determine lipid and fatty acid homeostasis in fish.

As described previously, CLA is a bioactive fatty acid that has been shown to bestow several beneficial effects on lipid metabolism in mammals (DeLany and West, 2000), particularly in relation to body composition (Wang and Jones, 2004). Specifically, CLA decreased body fat and increased lean body mass in mice (Ohnuki *et al.*, 2001; Terpstra *et al*., 2002), rats (Yamasaki *et al*., 2003) and pigs (Thiel-Cooper *et al*., 2001; Tischendorf *et al*., 2002). Decreased body fat has also been observed in human studies (Riserus *et al*., 2001; Smedman and Vessby, 2001; Thom *et al.*, 2001) although the effect was much less than that observed with mice (Terpstra, 2004). CLA is also known to affect activity and gene expression of mammalian stearoyl CoA Δ9 desaturase (SCD) (Choi *et al*., 2001, 2002) and may also influence Δ6 and Δ5 desaturases (Chuang *et al.*, 2001a; Eder *et al.,* 2002) and fatty acid elongase (Chuang *et al.,* 2001b). Further studies have suggested that CLA may enhance growth and feed efficiency in young rodents although this has not been consistently observed (Pariza *et al*., 2001).

At the initiation of this studentship, there were no reports in the literature on the effects of dietary CLA in salmonids. However, recently, some studies investigating the physiological and biochemical influences of dietary CLA on lipid and fatty acid metabolism in farmed salmonids have appeared in the literature. A 12 week study feeding Atlantic salmon fry graded levels of CLA up to 2 % of the diet did not increase growth rate or influence body proximate composition (Berge *et al*., 2004). However, fatty acid composition of the fish was strongly affected by dietary CLA such that levels of saturates including 14:0, 16:0 and 18:0 were increased while monoenes 16:1 and 18:1 decreased. In

a similar trial with rainbow trout juveniles fed graded levels of CLA up to 2 % of the diet over a 12 week period, feed conversion, body composition and growth rates were not influenced by dietary treatment (Figueiredo-Silva *et al*., 2005). Moreover, hepatic enzyme activity of various lipogenic enzymes including malic enzyme and fatty acid synthase were also shown to be unaffected in rainbow trout. A subsequent study performed by the same workers using an analogous dosing regime concluded that growth performance, FCR, HSI and VSI were unaffected in rainbow trout juveniles fed CLA (Bandarra *et al*., 2006). This study also found that total monoene levels were decreased while total saturate levels significantly increased. In a very recent study, graded levels of CLA up to and including 1 % dietary CLA in rainbow trout, reported increased 18:0 and decreased 18:1n-9 levels, but there was no influence growth performance, feed conversion, nutrient or energy utilisation or body composition (Valente *et al*., 2007a). Of equal importance was the implication in all the aforementioned trials that CLA can be incorporated into the diet without adversely impacting fish quality characteristics.

These other trials studying the effects of dietary CLA in salmonids (although, as noted above, none were published at the initiation of the present study) generally used small, young fish (fry and juvenile) fed diets with correspondingly low lipid contents. However, it would appear obvious that to fully test the possible efficacy of CLA in diets for salmonids, fish consuming higher dietary oil levels and at a developmental stage where lipid deposition is more likely to occur should be investigated. Only by matching developmental stage of the fish and dietary oil level could a possible protein-sparing effect of CLA in salmonids be determined. Therefore, the trial described in the next two chapters

was designed to study dietary CLA in salmon smolts in seawater consuming diets with much higher lipid contents.

Therefore, the aim of the present trial was to determine the effects of CLA on lipid and fatty acid composition in Atlantic salmon smolts. The overall objective being to test the hypotheses that CLA has beneficial effects in Atlantic salmon including growth enhancement, improved flesh quality through decreased adiposity and lipid deposition thereby minimising detrimental effects of feeding high fat diets, and increased nutritional quality through increased levels of beneficial fatty acids including n-3 HUFA and CLA itself. A further specific aim of this trial was to determine if absolute content of CLA in the diet (as percentage of total diet) or the level of CLA relative to other fatty acids in the diets were more important in eliciting effects, and thus the diets were specifically formulated to test this. A subsidiary aim of this trial was to determine the minimum period required to obtain the maximum amount of CLA deposition in the muscle of Atlantic salmon smolts. In the trial described, salmon smolts were fed diets containing two levels of dietary lipid (FO at 17 and 34 %) supplemented with three levels of CLA (0, 1 and 2 % of diet) for 3 months and the effects on growth performance, liver and flesh lipid contents and fatty acid composition determined.

3.2 Materials and Methods

3.2.1 Experimental Fish

Photoperiod manipulated Atlantic salmon (*Salmo salar L.*) smolts ($S^{1/2}$) were obtained from a commercial salmonid farm (Howietoun Fish Farm, Sauchieburn, Scotland) in late October 2003 and transported to the Machrihanish Marine Environmental Research

Laboratory, Machrihanish, Scotland. The fish were maintained in stock tanks for 3 weeks at ambient temperature of 10 - 11 ^{o}C to acclimatise during which time the fish were fed standard salmon diet, before being randomly distributed between eighteen indoor, round, conical tanks of 1.5 m^3 volume (1.72 m diameter). The initial stocking density was 100 fish of average fish weight 87.5 ± 1.6 g per tank (5.8 kg/m^3). Water temperature was maintained at 11 ^{o}C (± 1 ^{o}C) throughout the trial, with a light regime of 12L:12D.

3.2.2 Experimental Diets

Salmon were fed diets formulated in order to satisfy the nutritional requirements of salmonid fish (U.S National Research Council, 1993) and were manufactured by BioMar Ltd, Brande, Denmark. Dietary formulations and proximate compositions are given in Table 3.1.

Table 3.1Formulations (percentage of dry ingredients) and proximate compositions (percentage of total diet) of experimental diets

Component	L0	L1	L2	H0	H1	H2
Fishmeal	44	44	44	50	50	50
Sunflower meal	15	15	15	4	4	4
Corn Gluten	8	8	8	8	8	8
Legume seeds	9	9	9	9	9	9
Cereal grains	10	10	10	0	0	0
Micronutrients	3	3	3	2	2	2
FO	11	9.3	7.6	27	25.3	23.6
CLA	0	1.7	3.4	0	1.7	3.4
Moisture	8.0 ± 0.3	8.7 ± 0.1	8.9 ± 0.1	3.1 ± 0.1	4.2 ± 0.4	4.4 ± 0.1
Lipid	18.2 ± 0.3	17.4 ± 0.1	16.4 ± 0.5	33.2 ± 0.6	32.4 ± 1.2	32.6 ± 1.6
Protein	44.8 ± 0.1	44.8 ± 0.2	45.8 ± 0.5	47.0 ± 0.3	47.0 ± 0.3	47.0 ± 0.3
Ash	7.8 ± 0.1	7.8 ± 0.1	7.9 ± 0.1	8.2 ± 0.1	8.1 ± 0.0	8.0 ± 0.0

Results for proximate compositions are means ± SD (n=3). Micronutrients, incl. essential amino acids (methionine and lysine), vitamins, minerals and astaxanthin (Carophyll pink®), BioMar A/S, Brande, Denmark. H0, H1, H2, diets containing FO at 34 % and supplemented with 0 %, 1 % and 2 % CLA; L0, L1 and L2, diets containing FO at 17 % and supplemented with 0 %, 1 % and 2 % CLA.

The trial had a 3 x 2 factorial design with CLA added at three concentrations to diets with two oil contents (low and high). Thus diets were produced with 0, 1 and 2 % CLA replacing standard northern hemisphere FO in smolt feeds containing either 17 or 34 % total lipid. The diets were thus formulated to investigate whether effects of CLA were more dependent upon absolute content of CLA in the diet (as percentage of total diet) or the relative level of CLA to other fatty acids. Diets were formulated to be isonitrogenous and so protein content was constant between diets of different oil content (Table 3.1).

Diets within the low or high oil groups were identical in formulation other than fatty acid composition with CLA (LUTA-CLA^{TM} 60, containing 60 % CLA methyl esters as a 50:50 mixture of c9t11 and t10c12 isomers; BASF AG, Ludwigshafen, Germany) balanced by FO (capelin oil, Norsemeal Ltd., London, UK). The fatty acid compositions of the diets are presented in Table 3.2.

Table 3.2. Fatty acid composition (percentage of weight) of experimental diets containing CLA fed to Atlantic salmon

	L0	L1	L2	H0	H1	H2
14:0	5.6 ± 0.2	4.7 ± 0.1	4.3 ± 0.3	6.8 ± 0.3	6.0 ± 0.2	5.8 ± 0.1
16:0	19.6 ± 0.5	18.1 ± 0.7	18.2 ± 0.7	19.9 ± 0.2	19.2 ± 0.2	19.2 ± 0.3
18:0	4.5 ± 0.2	4.5 ± 0.1	5.0 ± 0.4	4.3 ± 0.0	4.4 ± 0.1	4.6 ± 0.1
Total saturated[1]	30.7 ± 1.0	28.6 ± 1.1	28.9 ± 1.4	31.9 ± 0.4	30.6 ± 0.7	31.0 ± 0.8
16:1n-7[2]	6.7 ± 0.1	5.7 ± 0.2	5.3 ± 0.5	7.5 ± 0.1	6.9 ± 0.1	6.7 ± 0.1
18:1n-9	13.4 ± 0.2	15.1 ± 0.2	17.7 ± 0.6	12.0 ± 0.2	13.1 ± 0.1	14.2 ± 0.0
18:1n-7	2.9 ± 0.1	2.6 ± 0.0	2.5 ± 0.1	3.0 ± 0.1	2.9 ± 0.1	2.9 ± 0.2
20:1n-9[3]	3.0 ± 0.1	2.9 ± 0.3	2.5 ± 0.1	3.2 ± 0.1	2.8 ± 0.0	2.9 ± 0.0
22:1n-11[4]	3.4 ± 0.0	3.1 ± 0.0	2.4 ± 0.1	3.2 ± 0.0	2.9 ± 0.0	2.7 ± 0.0
24:1n-9	0.7 ± 0.1	0.7 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
Total monoenes	30.1 ± 0.7	30.0 ± 0.7	31.0 ± 0.9	29.8 ± 0.6	29.3 ± 0.4	30.2 ± 0.5
18:2n-6	6.4 ± 0.2	6.6 ± 0.1	6.8 ± 0.2	3.1 ± 0.1	3.3 ± 0.0	3.2 ± 0.0
20:4n-6	0.9 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	1.0 ± 0.1	0.9 ± 0.0	0.8 ± 0.0
CLA (9c,11t)	0.0 ± 0.0	2.9 ± 0.5	4.8 ± 1.6	0.0 ± 0.0	1.6 ± 0.2	2.9 ± 0.7
CLA (10t,12c)	0.0 ± 0.0	3.0 ± 0.4	4.6 ± 1.7	0.0 ± 0.0	1.7 ± 0.4	2.9 ± 0.8
Total n-6 PUFA[5]	8.4 ± 0.1	8.2 ± 0.1	8.2 ± 0.2	5.1 ± 0.1	4.2 ± 0.0	4.0 ± 0.0
18:3n-3	1.2 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	1.1 ± 0.0
18:4n-3	2.5 ± 0.1	2.1 ± 0.0	1.8 ± 0.0	2.7 ± 0.0	2.6 ± 0.0	2.3 ± 0.0
20:4n-3	0.7 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.7 ± 0.0
20:5n-3	11.8 ± 0.7	10.2 ± 0.2	8.4 ± 0.5	13.6 ± 0.1	13.0 ± 0.1	11.5 ± 0.1
22:5n-3	1.8 ± 0.1	1.6 ± 0.0	1.3 ± 0.1	2.0 ± 0.0	1.9 ± 0.0	1.7 ± 0.0
22:6n-3	12.7 ± 0.9	11.5 ± 0.5	9.5 ± 0.1	12.9 ± 0.0	12.4 ± 0.2	10.9 ± 0.1
Total n-3 PUFA[6]	30.8 ± 1.8	27.3 ± 0.8	22.6 ± 0.7	33.2 ± 0.3	31.8 ± 0.4	28.2 ± 0.2

Values are means ± SD of 3 samples. [1], contains 15:0, 20:0 and 22:0, present in some samples at up to 0.6 %; [2]contains n-9 isomer; [3] contains n-7 isomer; [4], contains n-9 isomer; [5], totals include 18:3n-6, 20:2n-6 and 22:5n-6 present in some samples at up to 0.4 %; [6], contains 20:3n-3 present at up to 0.2 %. H0, H1 and H2, diets containing FO at 34 % and supplemented with 0, 1 and 2 % CLA; L0, L1 and L2, diets containing FO at 17 % and supplemented with 0, 1 and 2 % CLA.

3.2.3 Sampling and Analyses

At the initiation and termination of the trial, all the fish in each tank were anaesthetised with benzocaine (50 mg/L), and bulk weighed. At termination, 24 fish per dietary treatment (8/tank) were individually weighed and sampled for analyses, with 6 whole fish (2/tank) frozen immediately on dry ice for whole body compositional (proximate) analyses as described in section 2.3.10. The remaining sampled fish were eviscerated and used for biometric determinations (hepato-, and viscero-somatic indices) (see section 2.2.1) and for tissue lipid analyses. Flesh samples (Norwegian quality cut) and livers were taken from each fish, pooled in six samples of 3 fish each, and frozen immediately in liquid nitrogen (livers) or dry ice (flesh). All samples were subsequently stored at –20 ^{o}C prior to analyses. Six fish per treatment (2/tank) were also collected after 1 and 2 months for tissue lipid analyses.

3.2.4 Analyses and Transmethylation Procedure

Total lipids of liver, muscle and diet were extracted and quantified as described in section 2.3.7. Lipid class analysis was carried out as detailed in section 2.3.8.

There has been debate as to which methylation procedure is ideal for producing FAME in lipid samples containing CLA. It has been suggested that base-catalysed tranesterfication using sodium methoxide is favourable over acid catalysis since the former does not produce allylic methoxy artefacts or isomerisation of CLA, which has been evidenced in the latter (Krammer *et al*., 1997). More recently, methylation with boron triflouride has been shown to efficiently methylate the free fatty acid form of CLA by completely supressing artificial isomerisation (Igarashi, *et* al., 2004). However, it is known that the majority of lipid in tissue is esterified, either stored as TAG or as membrane PL,

and therefore samples must initially be saponified before methylation can take place with boron trifluoride, incurring more time in sample preparation. This may become crucial particularly when high throughput analysis is required. In addition, impurities or artefacts in gas chromatograms have been detected as a result of both sodium methoxide and boron triflouride-catalysed methylation of CLA (Park *et al.*, 2001). Indeed, it has been conceded that no single method or combination of methods could adequately prepare FAME for all lipid classes within a sample (Krammer *et al*., 1997). In this study investigating only CLA (not TTA), acid-catalysed transesterification, essentially as routinely utilised in this laboratory was employed for preparing FAME from diets and tissue total lipid. Thus, FAME were prepared from total lipid by incubating at 50 ^{o}C overnight in the presence of 2 ml 1 % (v/v) H_2SO_4 in methanol together with 1 ml of toluene under nitrogen essentially as described by Christie (2003). FAME were subsequently extracted, purified and quantified as described in section 2.3.8. The fatty acid compositions obtained for the experimental diets justifies the chosen methodology since the amount of CLA as a percentage of the total diet was accurately reflected in values of CLA obtained in the FAME as a percentage of total fatty acid. It was also noteworthy that the data in Table 3.2 showed a 1:1 ratio of c9t11 and t10c12 CLA, concurring with the manufacturers formulation and thus suggesting that little to no isomersation had taken place.

3.2.5 Statistical Analysis

All data presented are as means ± SD (n value as stated). The effects of dietary CLA and oil content and any interaction thereof were determined by two-way analysis of variance (ANOVA) with Bonferroni post-tests to determine significance of differences (Prism 3, Graphpad Software, Inc., San Diego, USA). Percentage data and data that were

identified as non-homogenous were subjected to arcsine transformation before analysis. Differences were regarded as significant when $P < 0.05$ (Zar, 1984).

3.3 Results

3.3.1 Diets

There was no significant difference in the proximate composition of the experimental diets (Table 3.1). Inclusion of CLA in the low oil diets resulted in levels of total CLA of 5.9 % and 9.5 % of total fatty acids at the 1 % and 2 % inclusion levels, respectively (Table 3.2). The high oil diets reduced the amount of total CLA at the 1 % and 2 % inclusion levels, to 3.3 % and 5.8 % of total fatty acids, respectively. The levels of CLA in relative terms were identical in the L1 and H2 diets with an overall rank order for CLA content of L2 > L1 = H2 > H1 > L0/H0.

3.3.2 Growth and Biometry

There were no effects of diet on growth parameters, with no significant effects of CLA or oil content on final weights, specific growth rates (SGR) or feed efficiency as measured by feed conversion ratio (FCR) (Table 3.3). Both the viscero-somatic (VSI) and hepato-somatic (HSI) indices were significantly increased in fish fed high fat diets, however dietary CLA had no effect on these parameters (Table 3.3).

Table 3.3 Growth and biometric parameters for Atlantic salmon fed the experimental diets for 3 months

	L0	L1	L2	H0	H1	H2	ANOVA		
							CLA	Oil	Interact
Initial weight (g)[1]	87.80 ± 1.50	87.4 ± 0.5	87.9 ± 1.5	85.5 ± 1.5	89.3 ± 0.9	86.9 ± 0.6	0.1301	0.4740	0.0506
Final weight (g)[1]	203.2 ± 7.10	199.7 ± 1.8	195.1 ± 12.3	196.1 ± 4.7	201.0 ± 10.1	199.5 ± 1.8	0.7631	0.8964	0.4108
SGR[2]	1.02 ± 0.10	1.01 ± 0.01	0.97 ± 0.05	1.01 ± 0.05	0.99 ± 0.05	1.01 ± 0.02	0.6236	0.8749	0.4712
FCR[2]	1.24 ± 0.10	1.26 ± 0.03	1.32 ± 0.13	1.27 ± 0.07	1.29 ± 0.10	1.28 ± 0.03	0.6547	0.8681	0.7100
VSI[4]	9.20 ± 0.61	9.20 ± 0.80	9.42 ± 0.43	10.87 ± 0.33	10.66 ± 0.52	10.63 ± 0.48	0.9356	0.0001	0.7722
HSI[4]	1.09 ± 0.09	1.12 ± 0.09	1.11 ± 0.06	1.13 ± 0.05	1.20 ± 0.03	1.2 ± 0.02	0.3543	0.0372	0.7742

Data are presented as means ± SD (n=3). FCR= feed conversion ratio; HSI = hepato-somatic index; SGR = specific growth rate; VSI = viscero-somatic index; L0, L1 and L2, low lipid diets with 0 %, 1 % and 2 % CLA; H0, H1 and H2, high lipid diets with 0 %, 1 % and 2 % CLA. Significance (P values) of effects of CLA, oil content and their interaction were determined by two-way ANOVA as described in the Materials and methods.

3.3.3 Whole Body Proximate Compositions

Whole body proximate composition was analysed at 1, 2 and 3 (end point) months post trial initiation. Whole body proximate composition of the fish was unaffected by CLA content at both 1 and 2 % inclusion levels at all time points analysed. Oil content significantly affected the proximate composition of the fish at all time points, with high oil diets significantly increasing whole body lipid levels and, as such, decreasing the protein, moisture and ash contents (Tables 3.4 - 3.6)

Table 3.4 Proximate composition of Atlantic salmon after 1 month of dietary treatment.

Diet	Moisture	Protein	Lipid	Ash
L0	70.9 ± 1.5	61.1 ± 1.5	29.6 ± 1.6	7.3 ± 0.2
L1	71.2 ± 0.9	62.5 ± 1.4	29.8 ± 1.0	7.5 ± 0.4
L2	70.9 ± 1.1	60.1 ± 2.3	29.3 ± 1.8	7.6 ± 0.5
H0	69.6 ± 1.0	56.8 ± 1.7	33.8 ± 1.3	7.1 ± 0.2
H1	69.8 ± 1.2	56.3 ± 1.7	34.0 ± 2.3	7.1 ± 0.3
H2	69.0 ± 2.2	55.3 ± 3.9	34.3 ± 3.8	7.4 ± 0.6
ANOVA				
CLA	0.7931	0.1775	0.9748	0.2770
Oil	0.0371	<0.0001	<0.0001	0.0303
Interact.	0.9231	0.5701	0.8733	0.8267

Values are means ± SD of 12 fish. Significance (P values) of effects of CLA, oil content and their interaction were determined by two-way ANOVA as described in the Materials and methods. H0, H1 and H2, diets containing FO at 34 % and supplemented with 0 %, 1 % and 2 % CLA; L0, L1 and L2, diets containing FO at 17 % and supplemented with 0 %, 1 % and 2 % CLA.

Table 3.5 Proximate composition of whole Atlantic salmon after 2 months of dietary treatment

Diet	Moisture	Protein	Lipid	Ash
L0	71.4 ± 1.3	61.3 ± 3.7	28.7 ± 2.7	7.4 ± 0.2
L1	71.2 ± 0.7	60.7 ± 3.7	29.4 ± 1.5	7.3 ± 0.3
L2	70.7 ± 0.5	59.7 ± 1.6	31.8 ± 1.5	7.4 ± 0.4
H0	69.4 ± 0.2	53.7 ± 1.1	36.2 ± 1.1	6.8 ± 0.6
H1	69.4 ± 0.8	54.7 ± 2.4	36.4 ± 1.6	7.2 ± 0.7
H2	69.1 ± 1.7	54.5 ± 1.0	35.7 ± 2.0	7.1 ± 0.9
ANOVA				
CLA	0.6663	0.8646	0.2178	0.7600
Oil	0.0024	<0.0001	<0.0001	0.0896
Interact.	0.9420	0.3824	0.0430	0.5635

Values are means ± SD of 12 fish. Significance (P values) of effects of CLA, oil content and their interaction were determined by two-way ANOVA as described in the Materials and methods. H0, H1 and H2, diets containing FO at 34 % and supplemented with 0 %, 1 % and 2 % CLA; L0, L1 and L2, diets containing FO at 17 % and supplemented with 0 %, 1 % and 2 % CLA.

Table 3.6 Proximate composition of whole Atlantic salmon after 3 months of dietary treatment (end of trial).

Diet	Moisture	Protein	Lipid	Ash	P:L ratio
L0	69.8 ± 1.1	61.2 ± 2.0	29.7 ± 3.1	7.3 ± 0.6	2.06 ± 0.20
L1	70.0 ± 0.6	62.1 ± 2.1	29.4 ± 1.9	7.3 ± 0.7	2.11 ± 0.16
L2	69.9 ± 1.1	61.5 ± 1.7	29.4 ± 2.7	7.2 ± 0.7	2.09 ± 0.17
H0	68.1 ± 0.9	54.9 ± 1.7	35.1 ± 2.1	6.4 ± 0.6	1.56 ± 0.15
H2	68.0 ± 1.3	54.0 ± 1.8	37.3 ± 2.2	6.7 ± 0.9	1.45 ± 0.16
H3	67.5 ± 0.7	53.4 ± 1.4	37.8 ± 1.3	5.9 ± 0.5	1.41 ± 0.11
ANOVA					
CLA	0.5281	0.4150	0.1676	0.0799	0.4372
Oil	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Interact.	0.4674	0.1428	0.0589	0.2215	0.1118

Values are means ± SD of 12 fish. Significance (P values) of effects of CLA, oil content and their interaction were determined by two-way ANOVA as described in the Materials and methods. P:L, protein:lipid ratio; H0, H1 and H2, diets containing FO at 34 % and supplemented with 0 %, 1 % and 2 % CLA; L0, L1 and L2, diets containing FO at 17 % and supplemented with 0 %, 1 % and 2 % CLA.

3.3.4 Lipid Contents and Class Compositions of Liver and Flesh

Neither CLA nor dietary oil content influenced lipid class composition in liver (Table 3.7) or muscle (Table 3.8) after 1 month of dietary treatment. However, total neutral lipid and, particularly, TAG levels, increased over time in both liver (Table 3.9) and muscle (Table 3.10) in fish fed the high fat diets in comparison with fish fed the low fat diets. These changes in TAG, and total neutral lipids reached statistical significance after 2 months of

dietary treatment (Table 3.9 & 3.10). Similarly, after 3 months, liver and flesh (muscle) lipid contents were significantly increased in fish fed the diets containing the higher oil content (Tables 3.11 & 3.12). The increased lipid contents in fish fed the high oil diets were reflected in increased proportions of TAG and total neutral lipids in both the liver (Table 3.11) and flesh (Table 3.12). Dietary CLA also had an effect on the lipid content of liver and flesh with increasing CLA leading to increased lipid content (Table 3.11). This effect was more pronounced in fish fed the high oil diets and was significant in the case of flesh although it was just short of statistical significance in the liver (Table 3.11). The increased lipid content in the liver in response to dietary CLA was also reflected in increased proportions of TAG and total neutral lipids particularly in the high oil diets. Similarly, increased lipid contents in the flesh in response to dietary CLA were reflected in increased proportions of TAG and total neutral lipids (Table 3.12). The effects of CLA and oil content on liver and flesh lipid contents and class compositions as described above were statistically significant except for TAG levels in the liver (Table 3.11).

Table 3.7 Lipid content (percentage of tissue wet weight) and class composition (percentage of total lipid) of liver of Atlantic salmon fed CLA for 1 month.

	L0	L1	L2	H0	H1	H2	ANOVA		
							CLA	Oil	Interact.
Lipid Content	3.4 ± 0.4	3.4 ± 0.6	3.7 ± 0.1	3.8 ± 0.4	3.8 ± 0.4	4.3 ± 1.5	0.5546	0.1936	0.9621
PC	27.0 ± 0.4	27.6 ± 1.3	27.2 ± 0.7	25.5 ± 1.2	23.9 ± 1.1	26.2 ± 2.2	0.4608	0.005	0.1935
PE	15.5 ± 0.3	16.2 ± 0.4	16.0 ± 0.6	16.4 ± 0.3	15.8 ± 1.0	16.0 ± 0.6	0.9856	0.5575	0.1866
PS	3.7 ± 0.3	3.9 ± 0.4	3.8 ± 0.4	3.6 ± 0.3	3.9 ± 0.6	3.7 ± 0.2	0.5477	0.7214	0.9673
PI	5.5 ± 0.3	5.7 ± 0.9	5.6 ± 0.8	5.8 ± 0.9	5.3 ± 0.3	6.0 ± 0.5	0.7454	0.7568	0.5465
PG/CL	3.7 ± 0.0	3.9 ± 0.1	3.6 ± 0.1	3.3 ± 1.0	3.5 ± 0.2	3.2 ± 0.9	0.6484	0.1544	1.0000
Sphingomyelin	3.9 ± 0.4	3.9 ± 0.5	4.3 ± 0.3	3.0 ± 0.5	4.0 ± 1.0	3.2 ± 0.3	0.3242	0.032	0.1751
Lyso-PC	1.8 ± 0.3	1.7 ± 0.4	1.9 ± 0.4	2.1 ± 0.3	2.4 ± 0.4	1.9 ± 0.0	0.7334	0.0544	0.2271
Total Polar	61.2 ± 1.4	63.0 ± 2.5	62.4 ± 0.8	59.8 ± 1.4	58.8 ± 1.6	60.2 ± 3.8	0.8158	0.0249	0.5284
Total Neutral	38.8 ± 1.4	37.0 ± 2.5	37.6 ± 0.8	40.2 ± 1.4	41.2 ± 1.6	39.8 ± 3.8	0.8158	0.0249	0.5284
Cholesterol	17.1 ± 0.9	17.1 ± 0.3	16.6 ± 0.2	16.9 ± 0.6	17.7 ± 1.0	16.8 ± 2.1	0.5338	0.6953	0.8099
Triacylglycerol	15.9 ± 1.9	14.4 ± 0.7	14.1 ± 0.3	17.9 ± 1.4	18.5 ± 1.3	16.1 ± 0.7	0.0529	0.0004	0.2444
Free fatty acid	5.8 ± 1.3	5.6 ± 2.7	6.9 ± 0.9	5.4 ± 0.6	4.9 ± 1.2	7.0 ± 5.2	0.4928	0.7852	0.9628
Steryl ester	tr	tr	tr	tr	tr	tr	-	-	-

Values are means ± SD of 6 samples. CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; tr, trace < 0.5%.

Table 3.8 Lipid content (percentage of tissue wet weight) and class composition (percentage of total lipid) of flesh of Atlantic salmon fed CLA for 1 month.

	L0	L1	L2	H0	H1	H2	ANOVA		
							CLA	Oil	Interact.
Lipid Content	3.5 ± 0.9	2.1 ± 0.9	3.8 ± 0.7	3.2 ± 1.3	2.8 ± 0.3	3.6 ± 1.1	0.0917	0.8806	0.5988
PC	12.6 ± 2.6	14.3 ± 1.7	11.4 ± 2.3	12.3 ± 3.5	14.0 ± 1.7	12.3 ± 1.1	0.2351	0.9275	0.8724
PE	8.8 ± 1.0	9.9 ± 1.7	7.9 ± 1.8	7.9 ± 2.0	9.3 ± 0.8	7.4 ± 1.3	0.1145	0.3637	0.9715
PS	0.9 ± 0.2	1.2 ± 0.5	0.6 ± 0.2	0.7 ± 0.3	0.7 ± 0.1	0.6 ± 0.3	0.1610	0.1185	0.3654
PI	2.5 ± 0.3	3.0 ± 0.5	2.2 ± 0.6	2.2 ± 0.8	2.3 ± 0.2	1.8 ± 0.5	0.1389	0.0818	0.7909
PG/CL	2.2 ± 0.0	2.2 ± 0.2	2.0 ± 0.1	1.9 ± 0.5	2.1 ± 0.3	1.6 ± 0.3	0.1293	0.0687	0.6555
Sphingomyelin	0.9 ± 0.3	1.2 ± 0.2	0.7 ± 0.2	0.6 ± 0.3	0.7 ± 0.1	0.6 ± 0.2	0.1069	0.0161	0.3459
Lyso-PC	0.5 ± 0.2	1.0 ± 0.4	0.5 ± 0.2	0.6 ± 0.5	0.4 ± 0.0	0.4 ± 0.1	0.3521	0.1674	0.1386
Total Polar	28.3 ± 4.3	32.8 ± 4.7	25.3 ± 5.2	26.1 ± 7.6	29.5 ± 2.4	24.7 ± 3.4	0.1282	0.3938	0.8912
Total Neutral	71.7 ± 4.3	67.2 ± 4.7	74.7 ± 5.2	73.9 ± 7.6	70.5 ± 2.4	75.3 ± 3.4	0.1282	0.3938	0.8912
Cholesterol	10.7 ± 1.0	11.6 ± 1.5	10.1 ± 0.8	9.8 ± 0.7	10.4 ± 0.3	9.2 ± 1.2	0.1014	0.0538	0.9555
Triacylglycerol	55.1 ± 5.9	48.5 ± 8.8	58 ± 4.6	58 ± 7.8	54.1 ± 3.1	60.8 ± 6.6	0.0480	0.1404	0.8642
Free fatty acid	5.9 ± 1.2	7.0 ± 2.6	6.7 ± 1.1	5.8 ± 0.7	6.0 ± 0.6	5.3 ± 2.3	0.7692	0.2937	0.7778

Values are means ± SD of 6 samples. CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

Table 3.9 Lipid content (percentage of tissue wet weight) and class composition (percentage of total lipid) of liver of Atlantic salmon fed CLA for 2 months.

	L0	L1	L2	H0	H1	H2	ANOVA		
							CLA	Oil	Interact.
Lipid Content	3.5 ± 0.3	3.6 ± 0.1	3.3 ± 0.3	3.3 ± 0.2	3.7 ± 0.3	3.7 ± 0.5	0.3966	0.5044	0.2793
PC	26.8 ± 1.2	24.9 ± 2.4	24.9 ± 1.1	26.1 ± 0.6	23.4 ± 0.7	25.3 ± 0.2	0.0240	0.3268	0.4397
PE	16.2 ± 0.7	16.0 ± 0.9	15.2 ± 0.7	16.5 ± 1.3	13.7 ± 0.0	16.0 ± 0.7	0.0249	0.3174	0.0136
PS	3.7 ± 0.1	3.7 ± 0.7	3.9 ± 0.7	4.1 ± 0.3	2.9 ± 0.3	4.0 ± 0.3	0.0547	0.6517	0.1014
PI	5.8 ± 0.5	5.8 ± 0.6	5.4 ± 2.0	6.0 ± 0.9	4.5 ± 0.7	5.4 ± 0.9	0.4798	0.4765	0.4367
PG/CL	3.8 ± 0.6	3.6 ± 0.2	3.9 ± 1.8	4.3 ± 0.8	3.0 ± 0.3	3.8 ± 0.3	0.3301	0.8724	0.5583
Sphingomyelin	3.8 ± 0.8	3.2 ± 0.9	4.2 ± 1.1	4.0 ± 1.0	2.6 ± 0.0	3.9 ± 0.5	0.0597	0.5512	0.6944
Lyso-PC	1.3 ± 0.2	1.3 ± 0.4	1.5 ± 0.8	1.5 ± 0.3	1.6 ± 0.8	1.6 ± 0.2	0.8789	0.4287	0.9459
Total Polar	61.5 ± 1.6	58.5 ± 4.7	58.8 ± 8.3	62.5 ± 4.2	51.7 ± 0.7	60.0 ± 2.3	0.0547	0.4755	0.2418
Total Neutral	38.5 ± 1.6	41.5 ± 4.7	41.2 ± 8.3	37.5 ± 4.2	48.3 ± 0.7	40.0 ± 2.3	0.0547	0.4755	0.2418
Cholesterol	17.6 ± 1.8	17.3 ± 3.0	16.8 ± 2.9	17.2 ± 2.0	16.4 ± 0.7	17.8 ± 1.6	0.8954	0.9230	0.7355
Triacylglycerol	14.2 ± 0.8	13.4 ± 2.6	14.3 ± 1.3	14.5 ± 2.2	15.5 ± 2.0	15.3 ± 2.9	0.9270	0.2736	0.7596
Free fatty acid	6.7 ± 0.8	10.7 ± 9.3	10.0 ± 4.2	5.8 ± 0.9	16.4 ± 3.4	6.8 ± 2.2	0.0426	0.8061	0.2470
Steryl ester	tr	tr	tr	tr	tr	tr	-	-	-

Values are means ± SD of 6 samples. CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine. tr, trace < 0.5%.

Table 3.10 Lipid content (percentage of tissue wet weight) and class composition (percentage of total lipid) of flesh of Atlantic salmon fed CLA for 2 months

	L0	L1	L2	H0	H1	H2	ANOVA		
							CLA	Oil	Interact.
Lipid Content	3.9 ± 0.2	4.5 ± 0.0	8.1 ± 1.9	5.2 ± 0.7	6.2 ± 0.7	7.3 ± 0.9	0.0002	0.1134	0.0796
PC	12.0 ± 0.1	11.3 ± 0.0	8.9 ± 1.7	10.9 ± 1.8	10.0 ± 0.2	8.0 ± 0.1	0.0007	0.0404	0.9438
PE	7.5 ± 0.2	6.5 ± 0.3	5.0 ± 1.3	6.3 ± 1.4	5.7 ± 0.1	5.0 ± 0.5	0.0059	0.1104	0.4589
PS	0.6 ± 0.1	0.8 ± 0.0	0.5 ± 0.1	0.6 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	0.2621	0.1902	0.1878
PI	2.0 ± 0.3	1.8 ± 0.2	1.6 ± 0.7	2.0 ± 0.6	1.3 ± 0.2	1.5 ± 0.2	0.1435	0.3327	0.5674
PG/CL	1.8 ± 0.1	0.9 ± 0.5	0.8 ± 0.4	1.0 ± 0.7	1.1 ± 0.2	1.1 ± 0.1	0.1447	0.6056	0.0647
Sphingomyelin	0.4 ± 0.1	0.6 ± 0.2	0.4 ± 0.0	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2621	0.0911	0.0348
Lyso-PC	0.6 ± 0.1	0.4 ± 0.2	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.5 ± 0.1	0.0282	1.0000	0.0687
Total Polar	24.9 ± 0.4	22.3 ± 0.3	17.5 ± 4.3	21.8 ± 4.5	19.3 ± 0.1	16.8 ± 0.7	0.0043	0.0854	0.6663
Total Neutral	75.1 ± 0.4	77.7 ± 0.3	82.5 ± 4.3	78.2 ± 4.5	80.7 ± 0.1	83.2 ± 0.7	0.0043	0.0854	0.6663
Cholesterol	3.4 ± 0.5	2.7 ± 0.9	3.1 ± 0.8	3.6 ± 0.5	2.1 ± 0.7	3.9 ± 0.6	0.0238	0.6861	0.2449
Triacylglycerol	62.2 ± 0.5	65.0 ± 0.4	70.9 ± 3.8	66.0 ± 5.0	70.7 ± 0.7	71.5 ± 1.2	0.002	0.0191	0.2771
Free fatty acid	9.5 ± 0.4	10.0 ± 1.5	8.5 ± 0.6	8.6 ± 0.3	7.9 ± 0.1	7.8 ± 0.1	0.0854	0.0026	0.2085

Values are means ± SD of 6 samples. CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

Table 3.11 Lipid content (percentage of tissue wet weight) and class composition (percentage of total lipid) of liver of Atlantic salmon fed CLA for 3 months

	L0	L1	L2	H0	H1	H2	ANOVA		
							CLA	Oil	Interact.
Lipid Content	3.3 ± 0.3	3.4 ± 0.1	3.5 ± 0.2	3.5 ± 0.2	3.6 ± 0.2	3.8 ± 0.2	0.0636	0.0008	0.8762
PC	29.8 ± 0.9	28.2 ± 3.8	30.1 ± 2.4	28.2 ± 2.1	25.3 ± 2.2	24.7 ± 1.2	0.0625	0.0002	0.1379
PE	17.1 ± 0.4	17.7 ± 1.0	17.5 ± 0.9	17.5 ± 0.9	15.0 ± 0.6	14.4 ± 0.9	0.0008	<0.0001	<0.0001
PS	4.1 ± 0.5	4.1 ± 0.8	4.1 ± 0.8	3.6 ± 0.5	3.2 ± 0.2	3.2 ± 0.1	0.5971	0.0002	0.5971
PI	5.8 ± 0.8	6.0 ± 0.7	5.7 ± 0.8	5.6 ± 0.4	5.4 ± 0.3	4.9 ± 0.6	0.2160	0.0165	0.5017
PG/CL	4.7 ± 0.2	4.8 ± 0.4	4.5 ± 0.4	4.5 ± 0.2	3.9 ± 0.3	3.5 ± 0.3	0.0002	<0.0001	0.0069
Sphingomyelin	4.8 ± 0.8	4.7 ± 0.9	4.8 ± 0.8	4.2 ± 0.6	3.7 ± 0.3	3.8 ± 0.3	0.5350	0.0005	0.6972
Lyso-PC	0.9 ± 0.6	0.7 ± 0.3	0.8 ± 0.2	0.9 ± 0.4	0.7 ± 0.2	0.7 ± 0.1	0.3414	0.7717	0.9181
Total Polar	67.2 ± 3.9	66.2 ± 6.6	67.4 ± 5.8	64.5 ± 3.4	57.2 ± 3.2	55.3 ± 2.2	0.0341	<0.0001	0.0441
Total Neutral	32.8 ± 3.9	33.8 ± 6.6	32.6 ± 5.8	35.5 ± 3.4	42.8 ± 3.3	44.7 ± 2.2	0.0341	<0.0001	0.0441
Cholesterol	18.3 ± 0.9	18.2 ± 1.5	18.8 ± 0.3	18.0 ± 0.6	15.4 ± 1.8	15.1 ± 1.2	0.0154	<0.0001	0.0036
Triacylglycerol	12.3 ± 4.7	13.4 ± 8.5	11.9 ± 5.8	15.4 ± 3.8	25.7 ± 4.7	27.3 ± 3.8	0.0212	<0.0001	0.0263
Free fatty acid	2.2 ± 0.6	2.1 ± 0.5	1.9 ± 1.0	2.1 ± 1.2	1.7 ± 0.6	2.3 ± 0.7	0.7264	0.9021	0.4793
Steryl ester	tr	tr	tr	tr	tr	tr	-	-	-

Values are means ± SD of 6 samples pooled from 3 fish each. CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; tr, trace, < 0.5%.

Table 3.12 Lipid content (percentage of tissue wet weight) and class composition (percentage of total lipid) of flesh of Atlantic salmon fed CLA for 3 months

	L0	L1	L2	H0	H1	H2	ANOVA		
							CLA	Oil	Interact.
Lipid Content	3.1 ± 0.4	3.2 ± 0.5	3.2 ± 0.4	3.9 ± 0.8	4.5 ± 0.5	5.0 ± 0.2	0.0231	<0.0001	0.0676
PC	8.6 ± 1.0	8.2 ± 0.6	8.1 ± 1.0	8.0 ± 0.5	7.4 ± 0.5	7.3 ± 0.9	0.1493	0.0086	0.9368
PE	6.9 ± 0.9	6.5 ± 0.9	6.0 ± 0.6	5.6 ± 0.7	5.3 ± 0.5	5.0 ± 0.5	0.0461	<0.0001	0.8687
PS	0.8 ± 0.2	0.7 ± 0.2	0.6 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.2	0.7192	0.0072	0.1143
PI	3.4 ± 0.5	3.0 ± 0.5	2.7 ± 0.6	2.2 ± 0.3	2.0 ± 0.2	1.8 ± 0.2	0.0107	<0.0001	0.6688
PG/CL	1.4 ± 0.4	1.5 ± 0.5	1.4 ± 0.3	1.2 ± 0.2	1.1 ± 0.2	1.1 ± 0.1	0.9036	0.0075	0.7393
Sphingomyelin	0.6 ± 0.2	0.6 ± 0.2	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.1	0.5852	0.0009	0.5852
Lyso-PC	2.6 ± 0.4	1.7 ± 0.4	1.6 ± 0.5	1.4 ± 0.4	1.1 ± 0.3	1.0 ± 0.1	0.0001	<0.0001	0.0907
Total Polar	24.3 ± 2.7	22.3 ± 2.4	20.9 ± 2.8	19.3 ± 1.7	17.6 ± 1.4	17.2 ± 1.7	0.0139	<0.0001	0.7494
Total Neutral	75.7 ± 2.7	77.7 ± 2.4	79.1 ± 2.8	80.7 ± 1.7	82.4 ± 1.4	82.8 ± 1.7	0.0139	<0.0001	0.7494
Cholesterol	9.3 ± 0.3	8.8 ± 0.5	8.7 ± 0.5	8.5 ± 0.7	8.4 ± 0.4	8.3 ± 0.3	0.1131	0.0019	0.4941
Triacylglycerol	53.4 ± 3.0	56.4 ± 3.0	59.5 ± 3.9	62.0 ± 3.4	65.4 ± 2.5	66.0 ± 2.0	0.0012	<0.0001	0.5606
Free fatty acid	13.0 ± 1.3	12.5 ± 1.1	11.0 ± 1.1	10.2 ± 2.8	8.6 ± 0.8	8.5 ± 0.4	0.0148	<0.0001	0.4733

Values are means ± SD of 6 samples pooled from 3 fish each. CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

3.3.5 Fatty Acid Compositions of Liver and Flesh

Similar to the lipid class data, the effects of diet on fatty acid compositions changed over time (see Tables 3.13 – 3.18). Therefore, the amount of CLA deposited in both liver and flesh of salmon increased with duration of feeding, and after 2 months, 22:6n-3 and total n-3 PUFA levels began to be significantly depressed in muscle particularly in fish fed 2 % dietary CLA incorporated into both low and high fat diets (Table 3.16). The details of dietary effects on fatty acid compositions are described in relation to the data at the end of the trial, after the fish had been fed the diets for 3 months. After 3 months, the fatty acid compositions of total lipid from liver (Table 3.17) and flesh (Table 3.18) reflected the fatty acid composition of the diets. Dietary CLA resulted in the deposition of CLA in both tissues with the rank order for total CLA in both liver and flesh being L2 > H2 > L1 > H1 > L0/H0 (Tables 3.17 & 3.18). Accumulation of CLA was 2-fold higher in the flesh, with total CLA in fish fed the L2 diet reaching 3.4 % in the liver (Table 3.17), and 6.9 % in the flesh (Table 3.18). CLA deposition in the liver was not at the expense of any one particular fatty acid, but tended to be spread across several saturated and monounsaturated fatty acids (Table 3.17). In contrast, in the flesh, CLA deposition appeared to be primarily at the expense of 20:5n-3 and 22:6n-3 rather than saturated and monounsaturated fatty acids (Table 3.18). It was noteworthy that dietary CLA had no significant effect on the proportions of n-6 fatty acids 18:2n-6 or 20:4n-6 in either liver or flesh (Tables 3.17 & 3.18). Oil content also had an effect on tissue fatty acid compositions with most fatty acids being significantly affected, with fatty acids derived from the plant (meal) sources, such as 18:0, 18:1n-9 and 18:2n-6, being reduced and generally all the other fatty acids being increased in liver and

flesh of fish fed the high oil diets (Tables 3.17 & 3.18). The statistical significance of dietary oil level and CLA is shown in Tables 3.19 – 3.21.

Table 3.13 Fatty acid composition (percentage of weight) of total lipid from liver of Atlantic salmon fed CLA for 1 month.

	L0	L1	L2	H0	H1	H2
18:0	6.6 ± 0.9	7.2 ± 0.3	7.0 ± 0.8	6.5 ± 1.3	6.8 ± 0.5	6.6 ± 0.9
Total saturated[1]	30.0 ± 1.5	29.3 ± 1.2	28.0 ± 0.8	28.8 ± 1.4	28.8 ± 1.2	30.5 ± 0.4
18:1n-9	8.9 ± 0.4	8.9 ± 0.2	8.4 ± 0.5	9.4 ± 0.5	7.7 ± 0.1	8.6 ± 1.1
Total monoenes	16.5 ± 0.6	16.4 ± 1.4	14.6 ± 0.2	18.9 ± 1.8	15.4 ± 0.1	16.0 ± 0.1
CLA (9c,11t)	0.0 ± 0.0	0.4 ± 0.3	1.3 ± 0.3	0.0 ± 0.0	0.2 ± 0.2	0.3 ± 0.1
CLA (10t,12c)	0.0 ± 0.0	0.3 ± 0.3	1.2 ± 0.2	0.0 ± 0.0	0.3 ± 0.0	0.2 ± 0.1
Total n-6 PUFA[2]	7.1 ± 1.0	7.3 ± 0.5	7.9 ± 0.8	6.7 ± 0.7	6.5 ± 0.2	6.7 ± 0.5
20:5n-3	7.4 ± 1.2	7.0 ± 1.3	6.6 ± 0.3	8.4 ± 1.0	10.0 ± 0.4	6.6 ± 1.4
22:6n-3	35.7 ± 2.0	35.5 ± 2.7	36.4 ± 2.5	32.8 ± 1.0	34.4 ± 0.3	34.2 ± 0.1
Total n-3 PUFA[3]	46.4 ± 2.1	45.7 ± 1.9	46.4 ± 1.9	45.6 ± 0.8	48.8 ± 0.9	45.2 ± 3.7

Values are means ± SD of 6 samples. [1], contains 15:0, 20:0 and 22:0, present in some samples at up to 0.4 % [2], totals include 18:3n-6, 20:2n-6, 20:3n-6 and 22:4n-6 and 22:5n-6 present in some samples at up to 0.6 % [3], contains 20:3n-3 present at up to 0.1 %. H0, H1 and H2, diets containing FO at 34 % and supplemented with 0, 1 and 2 % CLA; L0, L1 and L2, diets containing FO at 17 % and supplemented with 0, 1 and 2 % CLA.

Table 3.14 Fatty acid composition (percentage of weight) of total lipid from muscle of Atlantic salmon fed CLA for1 month

	L0	L1	L2	H0	H1	H2
18:0	3.9 ± 0.4	4.0 ± 0.2	4.0 ± 0.3	3.9 ± 0.9	3.7 ± 0.2	3.8 ± 0.1
Total saturated[1]	25.3 ± 1.7	24.9 ± 1.5	24.5 ± 1.6	25.4 ± 3.7	24.7 ± 0.5	25.2 ± 0.2
18:1n-9	14.7 ± 0.9	14.2 ± 0.8	15.5 ± 0.8	14.3 ± 1.0	13.5 ± 1.1	14.6 ± 1.2
Total monoenes	37.9 ± 3.3	36.0 ± 1.7	38.7 ± 2.3	36.5 ± 3.7	35.3 ± 2.5	37.1 ± 3.8
CLA (9c,11t)	0.0 ± 0.0	0.5 ± 0.3	1.5 ± 0.3	0.0 ± 0.0	0.5 ± 0.1	1.0 ± 0.5
CLA (10t,12c)	0.0 ± 0.0	0.4 ± 0.2	1.3 ± 0.3	0.0 ± 0.0	0.4 ± 0.1	0.9 ± 0.4
Total n-6 PUFA[2]	5.7 ± 0.5	5.5 ± 0.4	5.9 ± 0.2	5.1 ± 0.5	5.0 ± 0.2	5.1 ± 0.2
20:5n-3	6.2 ± 0.9	6.5 ± 0.9	5.5 ± 0.1	6.8 ± 0.7	7.1 ± 0.5	6.4 ± 0.5
22:6n-3	18.8 ± 1.1	20.8 ± 3.3	16.8 ± 2.0	20.1 ± 0.1	20.8 ± 2.6	18.4 ± 2.9
Total n-3 PUFA[3]	31.1 ± 1.7	32.6 ± 3.7	28.1 ± 1.8	33.1 ± 0.6	34.2 ± 2.9	30.8 ± 3.2

Values are means ± SD of 6 samples. [1], contains 15:0, 20:0 and 22:0, present in some samples at up to 0.4 % [2], totals include 18:3n-6, 20:2n-6, 20:3n-6 and 22:4n-6 and 22:5n-6 present in some samples at up to 0.6 % [3], contains 20:3n-3 present at up to 0.1 %. H0, H1 and H2, diets containing FO at 34 % and supplemented with 0, 1 and 2% CLA; L0, L1 and L2, diets containing FO at 17 % and supplemented with 0, 1 and 2 % CLA.

Table 3.15 Fatty acid composition (percentage of weight) of total lipid from liver of Atlantic salmon fed CLA for 2 months

	L0	L1	L2	H0	H1	H2
18:0	6.9 ± 0.5	6.1 ± 1.3	8.7 ± 1.5	5.2 ± 0.6	6.0 ± 0.7	5.5 ± 1.0
Total saturated[1]	27.0 ± 0.5	27.1 ± 1.0	28.1 ± 1.1	27.9 ± 1.5	27.6 ± 0.5	27.1 ± 2.8
18:1n-9	9.9 ± 1.5	9.5 ± 1.7	8.8 ± 0.9	8.4 ± 0.3	8.5 ± 0.8	8.9 ± 2.1
Total monoenes	17.8 ± 1.9	18.8 ± 4.6	15.8 ± 2.2	16.2 ± 0.8	16.8 ± 2.4	17.8 ± 4.5
CLA (9c,11t)	0.0 ± 0.0	0.8 ± 0.3	1.6 ± 0.2	0.0 ± 0.0	0.6 ± 0.1	1.1 ± 0.3
CLA (10t,12c)	0.0 ± 0.0	0.5 ± 0.3	1.4 ± 0.2	0.0 ± 0.0	0.4 ± 0.1	0.8 ± 0.1
Total n-6 PUFA[2]	7.8 ± 0.3	7.0 ± 1.6	7.8 ± 0.4	6.2 ± 0.2	6.1 ± 0.2	6.3 ± 0.5
20:5n-3	9.1 ± 0.5	8.2 ± 0.9	7.5 ± 0.8	9.9 ± 0.6	9.8 ± 0.3	10.2 ± 0.9
22:6n-3	34.2 ± 2.0	33.6 ± 1.5	34.8 ± 2.5	35.5 ± 2.2	34.4 ± 2.9	32.2 ± 2.1
Total n-3 PUFA[3]	47.4 ± 2.3	45.8 ± 2.1	45.3 ± 2.5	49.5 ± 2.5	48.3 ± 2.4	46.9 ± 2.1

Values are means ± SD of 6 samples. [1], contains 15:0, 20:0 and 22:0, present in some samples at up to 0.4 % [2], totals include 18:3n-6, 20:2n-6, 20:3n-6 and 22:4n-6 and 22:5n-6 present in some samples at up to 0.6 % [3], contains 20:3n-3 present at up to 0.1 %. H0, H1 and H2, diets containing FO at 34 % and supplemented with 0, 1 and 2 % CLA; L0, L1 and L2, diets containing FO at 17 % and supplemented with 0, 1 and 2 % CLA..

Table 3.16 Fatty acid composition (percentage of weight) of total lipid from muscle of Atlantic salmon fed CLA for 2 months

	L0	L1	L2	H0	H1	H2
18:0	4.2 ± 0.1	4.2 ± 0.3	5.0 ± 0.9	3.7 ± 0.3	4.0 ± 0.3	4.1 ± 0.2
Total saturated[1]	26.1 ± 0.6	24.5 ± 1.4	26.3 ± 1.2	25.0 ± 1.1	25.2 ± 0.9	25.9 ± 1.4
18:1n-9	15.3 ± 0.8	15.2 ± 1.0	16.7 ± 0.4	14.9 ± 1.0	14.8 ± 0.6	15.5 ± 1.3
Total monoenes	37.1 ± 1.7	37.3 ± 4.2	37.3 ± 5.0	38.1 ± 2.8	36.3 ± 1.8	37.8 ± 2.1
CLA (9c,11t)	0.0 ± 0.0	0.9 ± 0.3	2.9 ± 0.3	0.0 ± 0.0	0.7 ± 0.2	1.7 ± 0.1
CLA (10t,12c)	0.0 ± 0.0	0.8 ± 0.3	2.6 ± 0.2	0.0 ± 0.0	0.6 ± 0.2	1.6 ± 0.1
Total n-6 PUFA[2]	6.7 ± 0.3	6.1 ± 0.3	6.2 ± 0.9	5.1 ± 0.1	5.1 ± 0.2	5.1 ± 0.2
20:5n-3	6.8 ± 0.5	6.7 ± 0.8	5.4 ± 0.6	6.6 ± 0.7	7.0 ± 0.4	6.9 ± 1.3
22:6n-3	16.0 ± 1.9	17.5 ± 1.6	13.8 ± 1.1	18.2 ± 1.5	18.1 ± 1.0	14.2 ± 1.9
Total n-3 PUFA[3]	29.5 ± 1.6	30.5 ± 2.0	24.8 ± 1.0	31.6 ± 2.3	32.1 ± 1.1	27.8 ± 3.8

Values are means ± SD of 6 samples. [1], contains 15:0, 20:0 and 22:0, present in some samples at up to 0.4 % [2], totals include 18:3n-6, 20:2n-6, 20:3n-6 and 22:4n-6 and 22:5n-6 present in some samples at up to 0.6 % [3], contains 20:3n-3 present at up to 0.1 %. H0, H1 and H2, diets containing FO at 34 % and supplemented with 0, 1 and 2% CLA; L0, L1 and L2, diets containing FO at 17 % and supplemented with 0, 1 and 2 % CLA

Table 3.17 Fatty acid composition (percentage of weight) of total lipid from liver of Atlantic salmon fed CLA for 3 months

	L0	L1	L2	H0	H1	H2
14:0	1.6 ± 0.1	1.5 ± 0.1	1.3 ± 0.2	2.3 ± 0.5	1.8 ± 0.2	1.9 ± 0.2
16:0	18.5 ± 1.3	17.8 ± 0.2	17.1 ± 0.9	19.4 ± 0.9	18.7 ± 0.9	18.4 ± 0.7
18:0	8.3 ± 1.0	8.8 ± 1.1	8.9 ± 0.8	6.3 ± 0.3	7.1 ± 0.4	7.4 ± 0.5
Total saturated[1]	29.0 ± 1.3	28.8 ± 1.3	27.8 ± 0.7	28.6 ± 1.1	28.2 ± 1.2	28.3 ± 1.2
16:1n-7[2]	2.3 ± 0.1	2.2 ± 0.3	2.0 ± 0.2	3.2 ± 0.5	2.6 ± 0.3	2.6 ± 0.3
18:1n-9	9.6 ± 1.0	8.8 ± 0.4	9.4 ± 0.6	9.4 ± 1.0	8.2 ± 0.8	8.9 ± 0.8
18:1n-7	2.4 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.6 ± 0.2	2.5 ± 0.2	2.5 ± 0.1
20:1n-9[3]	1.8 ± 0.2	1.8 ± 0.1	1.6 ± 0.2	2.1 ± 0.4	1.6 ± 0.3	2.0 ± 0.2
22:1n-11[4]	0.5 ± 0.1	0.4 ± 0.3	0.6 ± 0.2	1.0 ± 0.4	0.7 ± 0.2	8.0 ± 0.2
24:1n-9	1.1 ± 0.1	1.0 ± 0.2	0.8 ± 0.1	1.1 ± 0.2	0.9 ± 0.0	0.9 ± 0.1
Total monoenes	17.7 ± 0.5	16.7 ± 1.0	16.6 ± 1.4	19.3 ± 2.4	16.4 ± 1.0	17.6 ± 1.6
18:2n-6	2.8 ± 0.2	2.9 ± 0.2	3.0 ± 0.2	1.9 ± 0.2	1.7 ± 0.2	1.8 ± 0.1
20:4n-6	3.6 ± 0.4	3.7 ± 0.2	3.7 ± 0.2	3.5 ± 0.4	3.9 ± 0.3	3.8 ± 0.3
CLA (9c,11t)	0.0 ± 0.0	0.9 ± 0.2	1.8 ± 0.2	0.0 ± 0.0	0.6 ± 0.1	1.2 ± 0.2
CLA (10t,12c)	0.0 ± 0.0	0.7 ± 0.1	1.6 ± 0.2	0.0 ± 0.0	0.5 ± 0.1	1.0 ± 0.2
Total n-6 PUFA[5]	7.9 ± 0.4	8.3 ± 0.3	8.3 ± 0.4	6.5 ± 0.3	6.7 ± 0.3	6.7 ± 0.3
18:3n-3	0.4 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.0
18:4n-3	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.2	0.3 ± 0.1	0.3 ± 0.1
20:4n-3	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1
20:5n-3	8.4 ± 0.7	8.3 ± 0.6	7.9 ± 0.3	10.2 ± 0.6	10.6 ± 0.3	10.0 ± 0.5
22:5n-3	2.7 ± 0.2	2.5 ± 0.3	2.4 ± 0.1	3.0 ± 0.2	3.1 ± 0.2	2.8 ± 0.1
22:6n-3	33.0 ± 1.3	32.6 ± 0.8	32.5 ± 1.3	30.0 ± 2.3	31.9 ± 1.9	30.7 ± 0.1
Total n-3 PUFA[6]	45.4 ± 2.0	44.7 ± 0.9	43.9 ± 1.3	45.6 ± 2.5	47.6 ± 1.6	45.3 ± 0.3

Values are means ± SD of 6 samples. [1], contains 15:0, 20:0 and 22:0, present in some samples at up to 0.4 %; [2], contains n-9 isomer; [3],contains n-7 isomer; [4], contains n-9 isomer; [5], totals include 18:3n-6, 20:2n-6, 20:3n-6 and 22:4n-6 and 22:5n-6 present in some samples at up to 0.6 %; [6], contains 20:3n-3 present at up to 0.1 %. H0, H1 and H2, diets containing FO at 34 % and supplemented with 0, 1 and 2 % CLA; L0, L1 and L2, diets containing FO at 17 % and supplemented with 0, 1 and 2 % CLA.

Table 3.18 Fatty acid composition (percentage of weight) of total lipid from muscle of Atlantic salmon fed CLA for 3 months.

	L0	L1	L2	H0	H1	H2
14:0	3.8 ± 0.2	3.7 ± 0.6	3.3 ± 0.2	4.2 ± 0.3	4.3 ± 0.3	4.2 ± 0.2
16:0	18.4 ± 0.3	18.1 ± 1.4	16,7 ± 0.4	17.3 ± 0.6	17.5 ± 0.7	16.7 ± 0.5
18:0	5.2 ± 0.2	5.4 ± 0.5	5.3 ± 0.2	4.1 ± 0.2	4.4 ± 0.2	4.3 ± 0.1
Total saturated[1]	28.2 ± 0.5	28.1 ± 2.5	26.2 ± 0.7	26.3 ± 0.9	26.9 ± 1.1	25.9 ± 0.7
16:1n-7[2]	5.2 ± 0.2	5.2 ± 0.3	4.7 ± 0.1	6.3 ± 0.2	6.2 ± 0.2	6.0 ± 0.2
18:1n-9	14.9 ± 0.4	15.6 ± 0.7	16.0 ± 0.4	14.2 ± 0.8	14.4 ± 0.4	15.0 ± 0.4
18:1n-7	3.3 ± 0.1	3.3 ± 0.2	3.0 ± 0.0	3.4 ± 0.1	3.4 ± 0.2	3.3 ± 0.1
20:1n-9[3]	4.6 ± 0.4	4.8 ± 0.5	4.4 ± 0.2	4.8 ± 0.3	4.5 ± 0.4	4.8 ± 0.5
22:1n-11[4]	3.9 ± 0.4	4.2 ± 0.4	3.8 ± 0.2	4.1 ± 0.3	3.9 ± 0.2	4.0 ± 0.3
24:1n-9	0.7 ± 0.2	0.7 ± 0.2	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
Total monoenes	32.7 ± 1.5	34.0 ± 2.2	32.6 ± 0.8	33.7 ± 1.5	33.1 ± 1.3	33.6 ± 1.1
18:2n-6	5.2 ± 0.2	5.2 ± 0.2	4.9 ± 0.2	3.4 ± 0.1	3.3 ± 0.1	3.4 ± 0.0
20:4n-6	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.0	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.2
CLA (9c,11t)	0.0 ± 0.0	1.9 ± 0.2	3.7 ± 0.4	0.0 ± 0.0	1.1 ± 0.1	2.2 ± 0.2
CLA (10t,12c)	0.0 ± 0.0	1.6 ± 0.2	3.2 ± 0.4	0.0 ± 0.0	1.0 ± 0.1	2.0 ± 0.2
Total n-6 PUFA[5]	7.2 ± 0.3	7.0 ± 0.7	6.7 ± 0.3	5.2 ± 0.3	5.2 ± 0.4	5.3 ± 0.3
18:3n-3	1.0 ± 0.0	0.9 ± 0.1	0.8 ± 0.0	1.1 ± 0.0	1.0 ± 0.1	1.0 ± 0.1
18:4n-3	1.4 ± 0.0	1.3 ± 0.1	1.2 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.6 ± 0.0
20:4n-3	1.1 ± 0.1	0.9 ± 0.1	0.9 ± 0.0	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.0
20:5n-3	7.2 ± 0.3	6.1 ± 1.0	5.9 ± 0.1	8.5 ± 0.5	8.3 ± 0.4	7.6 ± 0.3
22:5n-3	0.8 ± 0.1	2.3 ± 0.4	2.4 ± 0.1	3.2 ± 0.2	3.1 ± 0.2	2.9 ± 0.1
22:6n-3	18.2 ± 1.4	15.5 ± 0.5	16.0 ± 1.3	18.6 ± 1.5	17.0 ± 1.2	16.6 ± 0.5
Total n-3 PUFA[6]	31.9 ± 1.8	27.4 ± 4.1	27.5 ± 1.2	34.8 ± 2.2	32.6 ± 1.6	31.0 ± 0.8

Values are means ± SD of 6 samples. [1], contains 15:0, 20:0 and 22:0, present in some samples at up to 0.5 %; [2], contains n-9 isomer; [3],contains n-7 isomer; [4], contains n-9 isomer; [5], totals include 18:3n-6, 20:2n-6, 20:3n-6 and 22:4n-6 and 22:5n-6 present in some samples at up to 0.5 %; [6], contains 20:3n-3 present at up to 0.5 %. H0, H1 and H2, diets containing FO at 34 % and supplemented with 0, 1 and 2 % CLA; L0, L1 and L2, diets containing FO at 17 % and supplemented with 0, 1 and 2 % CLA.

Table 3.19 Significance (P values) of effects of dietary conjugated linoleic acid (CLA) and oil content (Oil), and their interaction (Interact.) on tissue fatty acid compositions after feeding the diets for 1 month, as determined by two-way ANOVA.

	Liver			Muscle		
	CLA	Oil	Interact	CLA	Oil	Interact
18:0	0.6623	0.4662	0.9393	0.9743	0.4351	0.8354
Total saturated	0.8702	0.6307	0.0367	0.8593	0.8271	0.9171
18:1n-9	0.0559	0.5437	0.0508	0.1466	0.1738	0.9062
Total monoenes	0.0028	0.0633	0.0280	0.4350	0.3979	0.9632
CLA (9c,11t)	<0.0001	<0.0001	0.0005	<0.0001	0.2162	0.2231
CLA (10t,12c)	<0.0001	<0.0001	<0.0001	<0.0001	0.2299	0.2421
Total n-6 PUFA	0.5070	0.0257	0.5966	0.5018	0.0029	0.7685
20:5n-3	0.0217	0.0176	0.0715	0.1191	0.0442	0.9029
22:6n-3	0.5897	0.0286	0.6805	0.0758	<0.0001	0.0020
Total n-3 PUFA	0.4594	0.7191	0.1923	0.0541	0.1054	0.9311

Table 3.20 Significance (P values) of effects of dietary conjugated linoleic acid (CLA) and oil content (Oil), and their interaction (Interact.) on tissue fatty acid compositions after feeding the diets for 2 months, as determined by two-way ANOVA.

	Liver			Muscle		
	CLA	Oil	Interact	CLA	Oil	Interact
18:0	0.1546	0.0042	0.0603	0.0818	0.0229	0.4026
Total saturated	0.9566	0.8497	0.5133	0.2031	0.6274	0.4114
18:1n-9	0.9300	0.2357	0.5942	0.1042	0.1417	0.6816
Total monoenes	0.8237	0.7052	0.4500	0.8893	0.9136	0.8541
CLA (9c,11t)	<0.0001	0.0265	0.1256	<0.0001	0.0003	0.0004
CLA (10t,12c)	<0.0001	0.0087	0.0141	<0.0001	0.0004	0.0007
Total n-6 PUFA	0.4440	0.0021	0.6721	0.4472	<0.0001	0.4472
20:5n-3	0.2821	0.0002	0.1028	0.2930	0.1693	0.1900
22:6n-3	0.5876	0.8773	0.2974	0.0025	0.1676	0.5568
Total n-3 PUFA	0.2511	0.0835	0.9453	0.0038	0.0505	0.8548

Table 3.21 Significance (P values) of the effects of dietary conjugated linoleic acid (CLA) and oil content (Oil), and their interaction (Interact.) on tissue fatty acid compositions after feeding the diets for 3 months, as determined by two-way ANOVA.

	Liver			Muscle		
	CLA	Oil	Interact	CLA	Oil	Interact
14:0	0.0042	<0.0001	0.1530	0.1205	<0.0001	0.1950
16:0	0.0224	0.0043	0.8450	0.0008	0.0293	0.2091
18:0	0.0238	<0.0001	0.7143	0.0825	<0.0001	0.8675
Total saturated	0.2904	0.6673	0.4691	0.0187	0.0112	0.3098
16:1n-7	0.0031	<0.0001	0.1530	<0.0001	<0.0001	0.0649
18:1n-9	0.0146	0.1128	0.8156	0.0008	<0.0001	0.5323
18:1n-7	0.1530	0.0002	1.0000	0.0010	0.0009	0.1304
20:1n-9	0.0649	0.0561	0.0145	0.8284	0.4568	0.1022
22:1n-11	0.1460	0.0004	0.3442	0.1296	0.0979	0.0061
24:1n-9	<0.0001	1.0000	0.1229	0.2394	1.0000	0.2394
Total monoenes	0.0206	0.1747	0.3679	0.7342	0.4586	0.2031
18:2n-6	0.4345	<0.0001	0.0932	0.0649	<0.0001	0.0088
20:4n-6	0.1315	0.5249	0.4930	0.4810	0.0935	0.4810
CLA (9c,11t)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
CLA (10t,12c)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Total n-6 PUFA	0.0561	<0.0001	0.7055	0.4984	<0.0001	0.2065
18:3n-3	0.0195	<0.0001	0.2394	<0.0001	<0.0001	0.1530
18:4n-3	0.0859	0.0003	0.0859	<0.0001	<0.0001	0.2394
20:4n-3	0.3798	<0.0001	0.3798	0.0003	<0.0001	0.0195
20:5n-3	0.0851	<0.0001	0.5323	<0.0001	<0.0001	0.1190
22:5n-3	0.1785	<0.0001	0.1785	0.0006	<0.0001	0.0712
22:6n-3	0.5863	0.0046	0.3651	0.0018	0.1102	0.6440
Total n-3 PUFA	0.1380	0.0214	0.2188	0.0002	<0.0001	0.4302

3.4 Discussion

The principal aim of this trial was to determine if dietary CLA had any important effects on lipid metabolism and growth parameters in Atlantic salmon. Collectively there was little evidence from preceding studies that dietary CLA can influence proximate composition, growth, feed efficiency or biometry of salmonids (Berge *et al*, 2004; Figueiredo-Silva *et al*., 2005; Bandarra *et al*., 2006; Valente *et al*, 2007a). This is in agreement with the results of the present study, which also show that dietary CLA, at levels up to 2 % of total diet, does not influence SGR, FCR, VSI and HSI. However, dietary CLA was shown to significantly increase HSI in striped bass (Twibell *et al*., 2000) and yellow perch (Twibell *et al*., 2001). Although HSI was not influenced by dietary CLA in the present trial, it was increased in fish fed high dietary oil. The increased HSI was associated with increased lipid and TAG content in the liver, whereas in the studies on yellow perch and tilapia, increased HSI was not associated with increased lipid content and, indeed, liver lipid content was reduced by CLA in striped bass (Twibell *et al*., 2001; Yasmin *et al*., 2004). The effects of dietary CLA on lipid content and composition were more prevalent towards the end of the study, with significant increases in flesh TAG associated with significant increases in lipid content. These data are as expected of course, and are consistent with the changes in lipid content, composition and fatty acid composition in salmon fed CLA being gradual and progressive with time.

Dietary CLA has been shown to have some beneficial effects on body composition in mammals, with decreased body fat and increased lean body mass having been shown in mice, rats and pigs (Ohnuki *et al*., 2001; Thiel-Cooper *et al*., 2001;

Terpstra *et al*., 2002; Tischendorf *et al*., 2002; Yamasaki *et al*., 2003). CLA also decreased whole body TAG accumulation in hamsters (Bouthegourd *et al*., 2002) and reduced hepatic TAG levels in rats (Rahman *et al*., 2002). In contrast, similar effects have rarely been observed in fish. No significant effects of CLA on carcass lipid, intra-peritoneal fat or liver lipid content were reported in catfish (Twibell and Wilson, 2003), or on tissue lipid contents in tilapia (Yasmin *et al*., 2004). However, intra-peritoneal fat and liver lipid content were decreased by dietary CLA in striped bass (Twibell *et al*., 2001). Whole body proximate composition remained unchanged throughout the course of the trial, even when fed in combination with high fat diets, in line with previous studies on salmon and trout fed CLA (Berge *et* al., 2004; Figuredo-Silva *et* al., 2005; Valente *et al*., 2007a). Thus, in this instance, high dietary oil level does not greatly enhance the effects of dietary CLA in Atlantic salmon as hypothesised. Indeed, overall the data suggest that dietary CLA does not have any beneficial effects on growth performance or body composition in salmon smolts that can tolerate higher levels of lipid in their diet. However, as expected dietary oil level significantly increased the amount of lipid in salmon as a percentage of total carcass composition consistent with higher dietary oil leading to higher deposition of fat in the tissues of fish in general (Sargent *et al*., 2002; Tocher, 2003).

In the present trial we also aimed to determine if absolute content of CLA in the diet or the level of CLA relative to other fatty acids in the diets were more important in eliciting effects, and the diets were specifically formulated to this end. The appropriate formulations were largely achieved and the diets provided a graded amount of CLA relative to total fatty acids with diets L1 and H2 supplying around the same amount of CLA relative to other fatty acids, but at two levels of dietary inclusion, 1 % and 2 %. The

few significant effects observed made it difficult to be conclusive on the comparative importance of absolute or relative amount of dietary CLA. However, the effects of dietary CLA were generally more pronounced in salmon fed the high oil diets suggesting that oil content in the diet was important in determining some of the effects of CLA although, as alluded to above, probably not to a level that would be physiologically significant.

Taking all these studies together, it is becoming increasingly appreciated that dietary CLA has few effects on lipid homeostasis in salmonids. However, the effects are not consistent and may vary with species, dependant on the pattern of lipid metabolism which itself varies with species (Tocher, 2003). In addition, patterns of lipid metabolism may also vary with developmental stage of the fish so this may also be important (Tocher, 2003). Thus, as the salmon used in the present trial were smolts in seawater, this is arguably more relevant than the earlier published trial on salmon that used fry in freshwater (Berge *et al*., 2004). However, ideally, even larger and older Atlantic salmon that are known to actively deposit lipid at a much greater rate would have been the preferred candidates for study in this instance. It is possible that the results obtained may also be dependant upon other dietary factors such as CLA isomer mix, and duration of the feeding trial. Levels of oil used in the trials investigating the effects of CLA in fish have varied between 5 % and 12 % of diet for all species other than salmon, or up to 24 % and 34 % (the present trial) of the diet in salmon. The diets have generally been based on FO although the exact type has varied, and two trials also used diets including a mix of FO and VOs such as corn and soybean oil (Twibell *et al*., 2000, 2001; Twibell and Wilson, 2003; Berge *et al*., 2004; Yasmin *et al*., 2004). A diet containing exclusively VO could possibly be interesting to investigate, to determine if CLA could have more pronounced

effects when the basal diet had such a substantial difference in dietary fatty acid composition that a diet rich in VO would have. Although the influence of individual CLA isomer preparations as dietary supplements have yet to be investigated in fish, it can be surmised from studies in rodents that each isomer may have different metabolic effects in salmon and that c9t11 and t10c12 CLA may even have acted antagonistically (Brown *et al*., 2003a; Brandebourg and Hu, 2005). Many of the previous trials in fish also failed to define the precise chemical from of the CLA (free fatty acid, methyl esters or TAG). Thus, the effects of dietary CLA on lipid metabolism in the present trial may have been masked by the type of CLA preparation used.

In mammals, CLA is also known to decrease the activity and gene expression of SCD and may also suppress Δ5 and Δ6 desaturases, and elongase (Choi *et al*., 2001, 2002; Chuang *et al*., 2001a,b; Eder *et al*., 2002). Dietary CLA increased saturated fatty acids and decreased 18:1 and C_{18} PUFA in pig muscle and fat (Ramsay *et al*., 2001), indicating a suppression in SCD activity. In chicken, dietary CLA decreased 22:6n-3 in all tissues (Yang *et al*., 2003) and increased saturates and decreased monoenes and PUFA in eggs (Szymczyk and Pisulewski, 2003). The tissue fatty acid composition data in the present study indicated that dietary CLA might have some similar effects in salmon. In support of the above findings, dietary CLA has also been reported to significantly alter the ratio of 18:0/18:1n-9 in tissues of related studies involving rainbow trout (see section 6.3.4; Bandarra *et al*., 2006; Valente *et al*., 2007a), striped bass (Twibell *et al*., 2000), yellow perch (Twibell *et al*., 2001), salmon (Berge *et al*., 2004) and sea bass (Valente *et al*., 2007b). Dietary CLA has had rather diverse effects on tissue fatty acid compositions in fish. For example, lower 16:1n-7 was reported in striped bass, and lower monoenes were reported in liver and muscle of yellow perch and in tilapia tissues (Twibell *et al*.,

2000, 2001; Yasmin *et al*., 2004). In striped bass, CLA increased PUFA, including 20:5n-3, 22:6n-3 and 18:2n-6, in liver, but decreased PUFA in muscle (Twibell *et al*., 2000). Total n-3 PUFA, especially 22:6n-3, were increased in salmon fry fed CLA (Berge *et al*., 2004), however 22:6n-3 was significantly decreased in muscle of rainbow trout fed up to 2 % CLA (Bandarra *et al*., 2006). No effect on 22:6n-3 levels were reported in a similar trial by the same laboratory using larger sized trout (Valente *et* al., 2007a). In the present study, it was noteworthy that deposition of CLA in flesh was at the expense of n-3 HUFA whereas in liver it was at the expense of saturated and monounsaturated fatty acids. Clearly, the benefits of CLA-fed fish would be greater if this was the other way around and CLA replaced saturated and monounsaturated fatty in the flesh. One potential consequence of feeding dietary CLA to oily fish such as salmon, is that they can incorporate an appreciable quantity of the bioactive fatty acid in their flesh, thus potentially providing a source of CLA to the human diet. In line with previous studies in salmonids, results indicate that CLA is preferentially incorporated into flesh tissue with levels reaching approximately 7 % of the two CLA isomers, c9t11 is retained in both liver and flesh somewhat more than t10c12 CLA. This finding may have commercial impact if CLA is ever to be incorporated into mainstream aquafeeds considering the potential physiological differences each isomer elicits in other animal models. This trial was unable to elucidate at which point maximal deposition of CLA was attained in flesh. This was due to the fact that levels of this bioactive fatty acid continued to increase steadily in both liver and flesh during the entire duration of the trial. However, it is possible this time could be reduced if relatively larger and older fish, actively depositing lipid, were fed dietary CLA although this has to be balanced with the fact that it can take longer to effect changes in tissue fatty acid compositions in larger

animals. Indeed, near market-sized rainbow trout incorporated a maximum CLA level of 2.4 % of total fatty acids after only six weeks of a twelve week trial in fish diets containing 1 % CLA (Ramos *et al*., 2006).

In summary, CLA had no effect on growth parameters, but there was a clear but gradual trend of increased total lipid and TAG contents in both liver and flesh in fish fed CLA, particularly in fish fed high oil diets. CLA was incorporated into tissue lipids, with levels in flesh being 2-fold higher than in liver, but importantly, incorporation in flesh was at the expense of n-3 HUFA. In conclusion however, the results of the present study generally do not support the hypothesis that dietary CLA elicits beneficial changes in Atlantic salmon smolts in terms of enhanced growth parameters or improved body composition and flesh quality through decreased adiposity or lipid deposition. However, salmon fed CLA could be beneficial in the human diet through provision of CLA with minimal effect on n-3 HUFA levels.

CHAPTER 4. INFLUENCE OF DIETARY CLA ON LIPID METABOLISM AND IMMUNE FUNCTION IN ATLANTIC SALMON

4.1 Introduction

Evidence suggests that CLA could affect lipid accumulation both by decreasing synthesis and increasing oxidation in mammals (Pariza *et al*., 2001). Although the exact biochemical mechanism of action of CLA is yet to be elucidated, CLA has been proven to alter the gene expression of an array of transcription factors known to be pivotal in the control of lipid metabolism. The most extensively studied of these with regard to studies involving CLA in mammals includes the family of nuclear transcription factors named peroxisome proliferator-activated receptors or PPARs. As outlined previously, there are three main isoforms of PPAR in mammals named PPARα, PPARβ and PPARγ. CLA isomers are ligands and activators of PPARα/PPARβ (Moya-Camarena; *et al*., 1999a,b) and PPARγ (Belury, 2002) that in mammals are, in turn, known to regulate the expression of genes of fatty acid oxidation and lipid deposition in liver/skeletal muscle (PPARα/PPARβ) and adipose tissue (PPARγ), respectively. Generally, increased expression and activity of PPARα stimulates fatty acid oxidation through activation of a comprehensive set of target genes, including CPT-1 and ACO, primarily in liver (Desvergne *et al*., 2006). Similarly, activation of PPARβ leads to increased fatty acid oxidation, however, principally in muscle (Tanaka *et al.*, 2003). Activation of PPARγ is known to enhance adipogenenis and steatosis in murine models (Desvergne *et al*., 2006) and deletion of this transcription factor results in reduced lipid distribution in muscle

(Norris *et al*., 2003). However, to date only gross effects have been measured in fish administered dietary CLA, and the biochemical pathways have not been directly studied.

The influence of PPARα is not restricted to effects on fatty acid catabolism given that this transcription factor is also implicated in the regulation of fatty acid desaturation. Indeed, it has been elucidated that PPARα can directly activate Δ6 desaturase gene transcription in rodents (Nakamura and Nara, 2004). Moreover, PPARs may also be implicated in immunological function and have been shown to mediate the expression of various genes involved in proliferation of lymphocytes, macrophages, apoptosis and inflammation (O'Shea *et al*., 2003). In addition, other studies have also recently shown that CLA confers an anti-inflammatory response in human vascular smooth cells (Ringseis *et al*., 2006) and decreases production of pro-inflammatory products in macrophages (Yu *et al*., 2002), via a PPARγ-dependant mechanism.

Accordingly, the overall objective was to test the hypothesis that CLA has beneficial effects on the biochemistry and physiology of Atlantic salmon through affecting lipid and fatty acid metabolism as well as immune function. The specific aims of the present study were to determine the effects of CLA on some key pathways of fatty acid metabolism including fatty acid oxidation and HUFA synthesis via fatty acyl desaturation and elongation, and in addition, to determine the influence of dietary CLA on the innate immunological response of Atlantic salmon. To this end, salmon smolts were fed diets containing two levels of FO (low, ~17 % and high, ~34 %) containing three levels of CLA (a 1:1 mixture of the two main isomers present in nature, c9t11 and t10c12 at 0, 1 and 2 % of diet) for 3 months. The effects of dietary CLA on the expression of key genes of fatty acid oxidation and HUFA synthesis, and on the enzymic activities of the respective pathways were determined. In addition, the effect of CLA on

the expression of the potentially important transcription factors, PPARs, was determined in selected tissues. Finally, the influence of dietary CLA on some basic haematology and non-specific immune response parameters were measured.

4.2 Materials and Methods

4.2.1 Experimental Fish and Diets

This experiment was an extension of the work described in Chapter 3. Photoperiod adapted Atlantic Salmon smolts ($S^{1/2}$) were obtained from a commercial salmonid farm (Howietoun Fish Farm, Sauchieburn, Scotland) in late October 2003 and transported to the Machrihanish, Marine Environmental Research Laboratory, Machrihanish, Scotland. General fish husbandry was followed as described in section 3.2.2. The formulations and fatty acid compositions of the diets are also presented in section 3.2.2.

4.2.2 Sampling and Analysis

Sampling of the fish was carried out essentially as described in section 3.2.3. In addition though, 0.5 g samples of liver, white and red muscle, intended for molecular analysis, were rapidly dissected from six fish (two per tank) and immediately frozen in liquid nitrogen prior to storage at -80 ^{o}C . Samples of 1-2 g of liver, white and red muscle for biochemical analyses were also collected and immediately frozen in liquid nitrogen prior to storage at -80 ^{o}C. Blood and head kidney was extracted from the remaining sampled fish prior to being assayed for basic haematology and non-specific immune response parameters as detailed in section 2.4**.**

For biochemical analyses (enzyme and pathway assays), homogenates of liver, red and white muscle, and liver microsomes were prepared as described in sections 2.3.1 and 2.3.2. Peroxisomal β-oxidation capacity and CPT-1 activity were measured in homogenates of liver, red and white muscle as described in sections 2.3.3 and 2.3.4, respectively. HUFA synthesis in liver microsomes was determined as described in detail in section 2.3.2.

For gene expression studies, the PCR primers were designed according to the salmon cDNA sequences for Δ6 desaturase (accession no. AY458652), Δ5 desaturase (accession no: AF478472), CPT-1 and PPARα (M.J. Leaver, personal communication), PPARβ1 (accession no: AJ416953) and PPARγ (accession no: AJ416951). Primer sequences and PCR product sizes are given in Table 4.1.

Table 4.1 Forward (sense) and reverse (antisense) primers used for QPCR.

Gene	PCR product length	Forward primer	Reverse primer
Δ5 Desaturase	192	5'-GTGAATGGGGATCCATAGCA-3'	5'-AAACGAACGGACAACCAGA-3'.
Δ6 Desaturase	181	5'-CCCCAGACGTTTGTGTCAG-3'	5'-CCTGGATTGTTGCTTTGGAT-3'
CPT-1	161	5'-GAGAGAGCTGCGACTGAAAC-3'	5'-GACAGCACCTCTTTGAGGAA-3'
PPARα	204	5'-ATCTTCCACTGCTGCCAGTGC-3'	5'-GATGAAGCCCGATCCGTAGGCCACCAGG-3'
PPARβ1	517	5'-TACCGCTGCCAGTGCACCACGGTG-3'	5'-TTCTGGACCAAGCTGGCGTTCTCA-3'
PPARγ	266	5'-TATCTCCCCTCTCTAGAGTA-3'	5'-AGGGCTTATCGTTTACTGAACCTTGATACACGC-3'

The linearised plasmid DNA containing the target sequence for each gene was quantified to generate a standard curve of known copy number as described in section 2.5.4. Amplification and quantification of cDNA samples and DNA standards was carried out by quantitative real-time PCR (Q-PCR) essentially as described in section 2.5.5, with the modifications noted below. Expression of target genes was normalised to total cDNA (i.e. tissue RNA) using Quant-iTTM High Sensitivity DNA Assay kit

containing PicoGreen reagent, (Molecular Probes, Rijinsburgerweg, The Netherlands) using a modified version of the manufacturers protocol as reported previously (Whelan *et al*., 2003). Twelve µl of the total cDNA from each of the reverse transcribed tissue RNA samples was added to individual wells of a 96-well plate along with 200 µl of a reaction mix containing a 1:200 dilution of Quant-iTTM DNA HS reagent in Quant-iTTM DNA HS Buffer. A lid was fixed and the plate vortexed for three bursts of 1 s before flicking the plate downward to force the liquid to the bottom of the wells. The plate was wrapped in aluminium foil to prevent light degradation of the PicoGreen Reagent and left for 30 mins at room temperature. All reactions were carried out in duplicate. The plate was read using a Wallac Victor2 1420 Multilabel Counter (PerkinElmerTM) using an absorbance spectrum for PicoGreen standard of 480 nm for excitation with measurement at 520 nm. A linear standard curve was drawn, plotting the average absorbance values obtained for M13 cDNA standard values against the standard DNA concentration.

4.2.3 Statistical Analysis

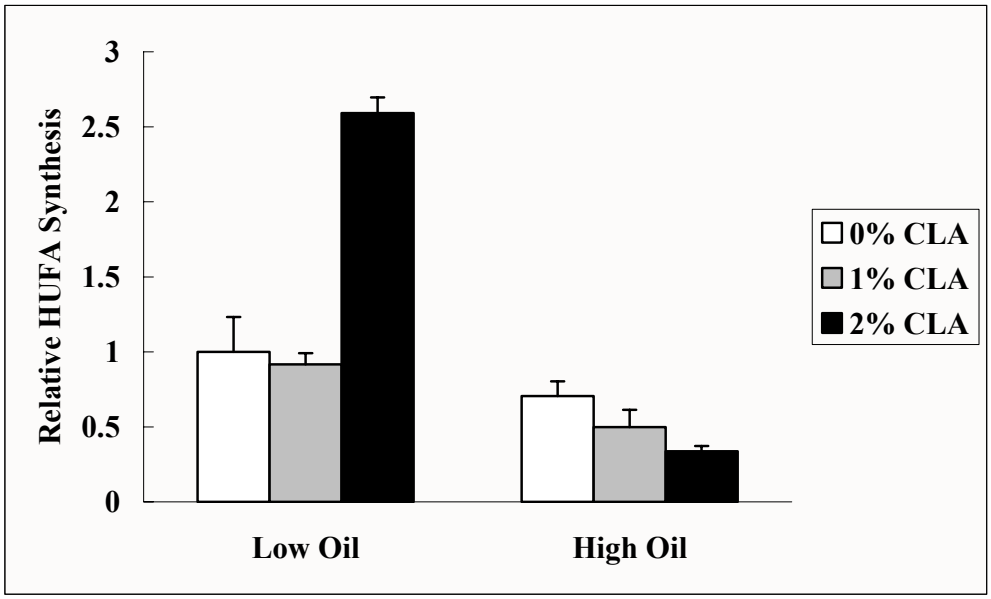
All data presented are as means ± SD (n value as stated). Percentage data and data that were identified as non-homogenous were subjected to arcsine transformation before analysis. The effects of dietary CLA and oil content and any interaction thereof was determined by two-way analysis of variance (ANOVA) with Bonferroni post-tests to determine significance of differences (Prism 3, Graphpad Software, Inc., San Diego, USA). Differences were regarded as significant when $P < 0.05$ (Zar, 1984).

4.3 Results

4.3.1 HUFA Synthesis, Peroxisomal β-oxidation Capacity and CPT-1 Activity

Microsomal HUFA synthesis, measured as the sum of desaturated and elongated products of radiolabelled precursor, 18:3n-3, is presented in Figure 4.1. HUFA synthesis was significantly affected by both dietary oil and CLA content as determined by 2-way ANOVA (Table 4.2). However, there was significant interaction between the variables. Dietary CLA at the 2 % inclusion level fed in combination with the low oil diet increased total HUFA synthesis 3-fold compared to fish fed FO alone whereas CLA decreased HUFA synthesis at the high dietary oil level. Supplementation with high dietary oil resulted in a significant decrease in HUFA synthesis.

Figure 4.1 Effects of dietary oil content and conjugated linoleic acid (CLA) on highly unsaturated fatty acid (HUFA) synthesis in the liver microsomes.



Specific activities are presented as means ± SD (n = 3) relative to Low Oil / 0 % CLA (i.e. diet L0) = 1.

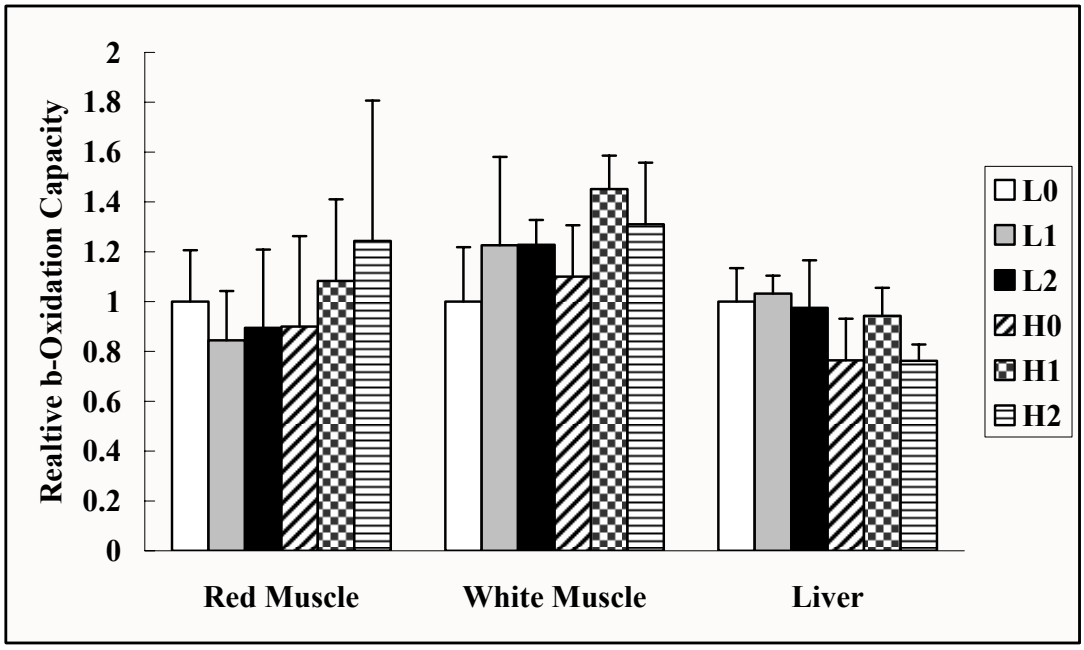
Table 4.2 Significance (P values) of effects of dietary conjugated linoeic acid (CLA) and oil content (Oil) and there interaction (Interact.) as determined by ANOVA.

	P Value		
	CLA	Oil	Interact.
Liver			
HUFA synthesis activity	**< 0.0001**	**< 0.0001**	**< 0.0001**
Δ6 desaturase expression	**0.0009**	**<0.0001**	**0.0156**
Δ5 desaturase expression	**0.0031**	**0.0009**	0.5247
β-oxidation activity	0.2757	**0.0136**	0.5943
CPT-1 activity	0.8506	0.5573	0.8676
CPT-1 expression	**0.0027**	**0.001**	**0.0483**
PPARα expression	**0.0001**	**0.0347**	0.1144
PPARβ expression	**0.0004**	**0.0445**	0.1649
PPARγ expression	**0.0449**	0.2061	0.237
Red muscle			
β-oxidation activity	0.8133	0.3457	0.5257
CPT-1 activity	**0.0355**	0.1511	0.4857
CPT-1 expression	**0.0022**	0.7695	0.3469
PPARα expression	0.2603	**0.0172**	**0.0034**
PPARβ expression	0.1182	**<0.0001**	**0.046**
White muscle			
β-oxidation activity	0.0795	0.1473	0.8623
CPT-1 activity	**0.0075**	**0.0001**	0.1409
CPT-1 expression	**0.0077**	0.7394	0.2716
PPARα expression	**0.0292**	0.0518	0.0836
PPARβ expression	**0.0032**	**0.0006**	0.0822

CPT-1. carnitine palmitoyltransferase-1, HUFA, highly unsaturated fatty acids; PPAR, peroxisome proliferator activated receptor; PUFA, polyunsaturated fatty acid.

Peroxisomal β-oxidation, estimated by measuring the recovery of radioactivity in acid-soluble products of radiolabelled palmitate, was not significantly changed in red or white muscle of fish by either dietary treatment, oil content or CLA (Table 4.2). However, there appeared to be a trend that indicated CLA may enhance peroxisomal β-oxidation in white muscle tissue although the high variability made the observed incremental increase non-significant. In contrast, hepatic peroxisomal β-oxidation was significantly reduced at the high dietary oil inclusion level however it was unaffected by CLA administration (Figure 4.2).

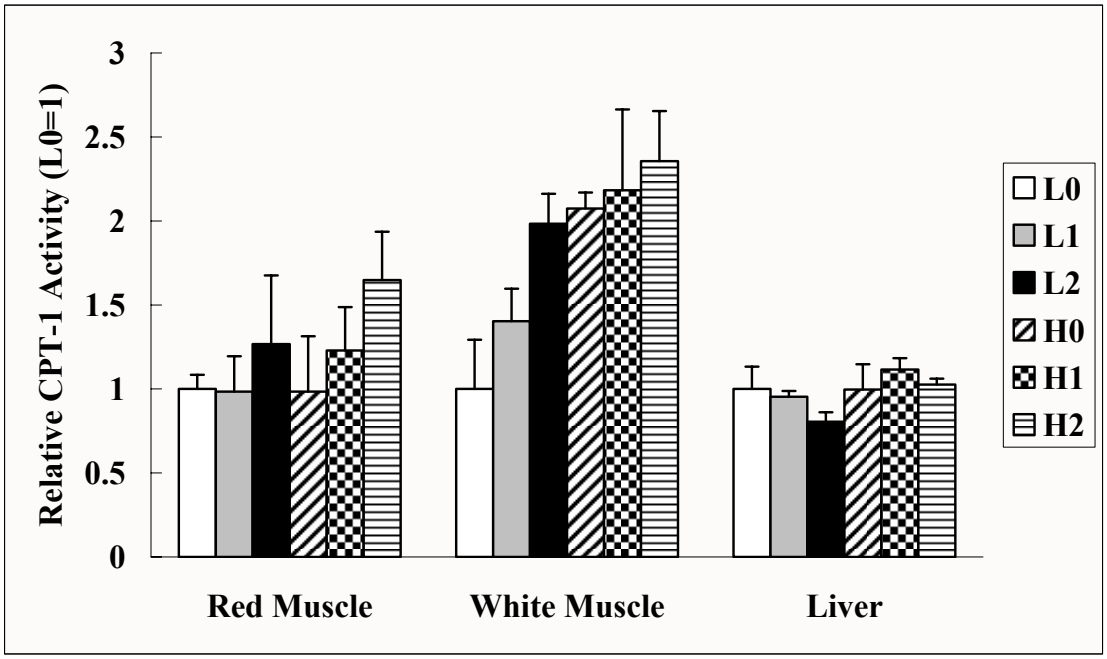
Figure 4.2 Effects of dietary oil content and conjugated linoleic acid (CLA) on peroxisomal β-oxidation activities in tissue homogenates of red and white muscle, and liver.



Data are presented as means ± SD (n = 3) relative to diet L0 = 1.

The activity of CPT-1, a key enzyme of mitochondrial β-oxidation, was significantly increased in white muscle of salmon fed high oil diets (Figure. 4.3, Table 4.2). Conversely, dietary oil level did not influence CPT-1 activity in liver or red muscle of fish. CLA significantly increased the CPT-1 activity of both red and white muscle, particularly when administered in combination with high dietary oil, however the observed incremental trend was not mirrored in liver. Although not significant, there was a clear trend that fish administered CLA decreased hepatic CPT-1 activity when fed in conjunction with low fat diets (Figure 4.3).

Figure 4.3. Effects of dietary oil content and conjugated linoleic acid (CLA) on carnitine palmitoyl acyltransferase-1 (CPT-1) activities in tissue homogenates of red and white muscle, and liver.

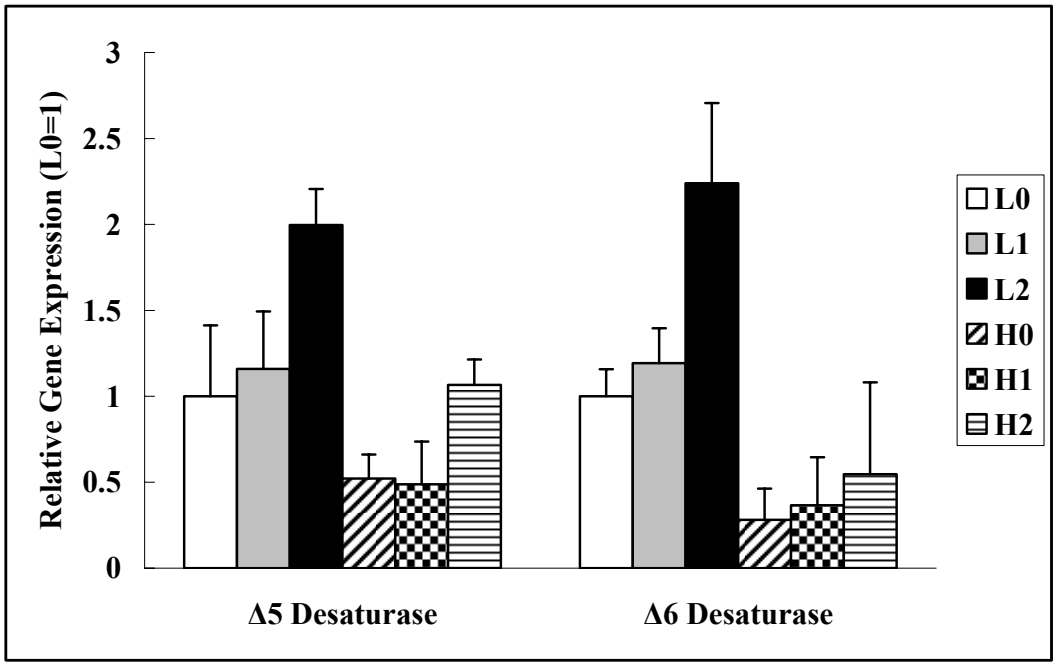


Data are presented as means ± SD (n = 3) relative to diet L0 = 1.

4.3.2 Tissue Expression of CPT-1, Fatty Acyl Desaturases and PPARs

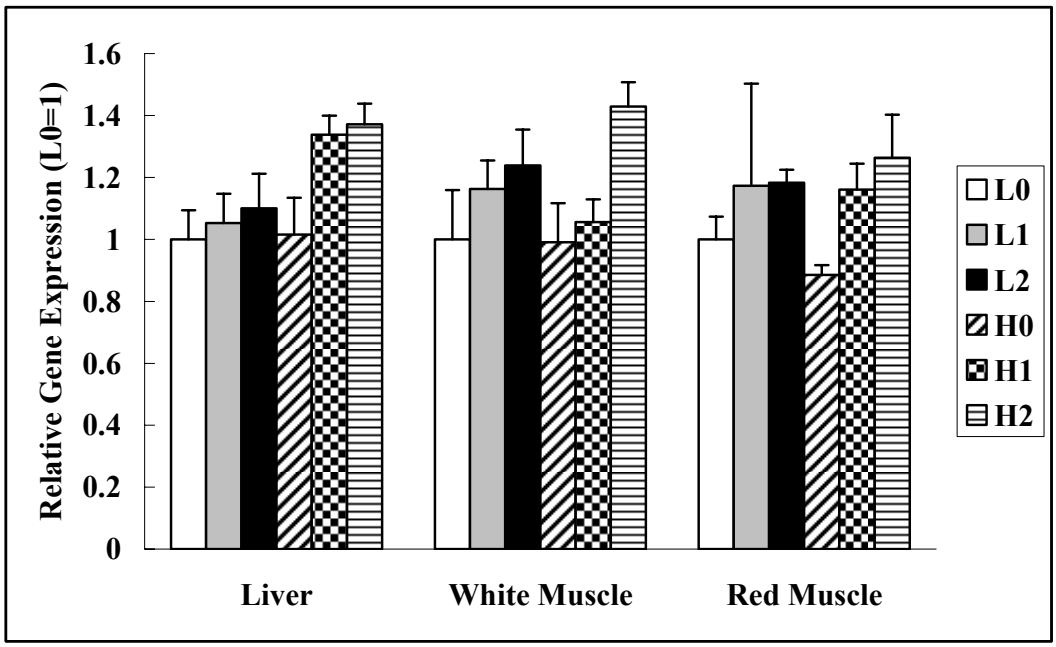
Both dietary oil and CLA affected Δ5 and Δ6 gene expression (Figure. 4.4). Specifically, gene expression was significantly decreased in response to high dietary oil whereas dietary CLA significantly increased both dietary Δ5 and Δ6 desaturase transcript levels (Table 4.2). Dietary CLA significantly increased the expression of CPT-1 in liver, red and white muscle (Figure 4.5; Table 4.2). CPT-1 expression was significantly increased in liver of fish fed high dietary oil, at least when fed in combination with CLA. In contrast, CPT-1 expression was not affected by dietary oil in either muscle tissue examined.

Figure 4.4 Effects of dietary oil content and conjugated linoleic acid (CLA) on the expression of Δ6 and Δ5 fatty acid desaturase genes in liver.



Genes were determined by Q-PCR and normalised relative to total RNA, determined by fluorescent assay, as described in Materials and Methods. Results are presented as means and SD (n = 3) relative to diet L0 = 1.

Figure 4.5 Effects of dietary oil content and conjugated linoleic acid (CLA) on the expression of carnitine palmitoyl transferase-I (CPT-I) in the liver, red and white muscle.

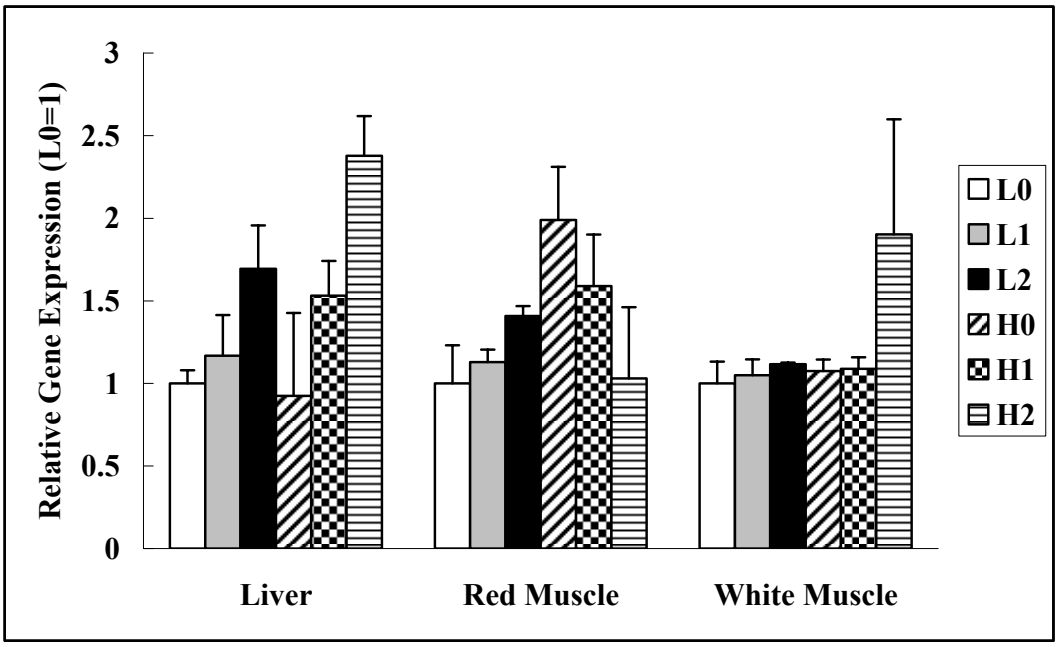


Genes were determined by Q-PCR and normalised relative to total RNA, determined by fluorescent assay, as described in Materials and Methods. Results are presented as means and SD (n = 3) relative to diet L0 = 1.

The expression of all PPAR subtypes was significantly affected by both dietary CLA and oil content (Table 4.2). PPARα expression was increased by dietary CLA in liver and, to a lesser extent, white muscle (Figure 4.6). In red muscle, there was a strong interaction between dietary CLA and oil content such that CLA increased expression at low oil contents, but the opposite at high dietary oil content. High oil content may have induced increased expression of PPARα in red muscle although the interaction also made this difficult to interpret. PPARβ expression in the muscle tissues was similar to that for PPARα in these tissues. Thus the expression of PPARβ was increased by dietary CLA in white muscle whereas there was a significant interaction between CLA and dietary oil in red muscle, which made the effects of CLA difficult to interpret (Table 4.2). However,

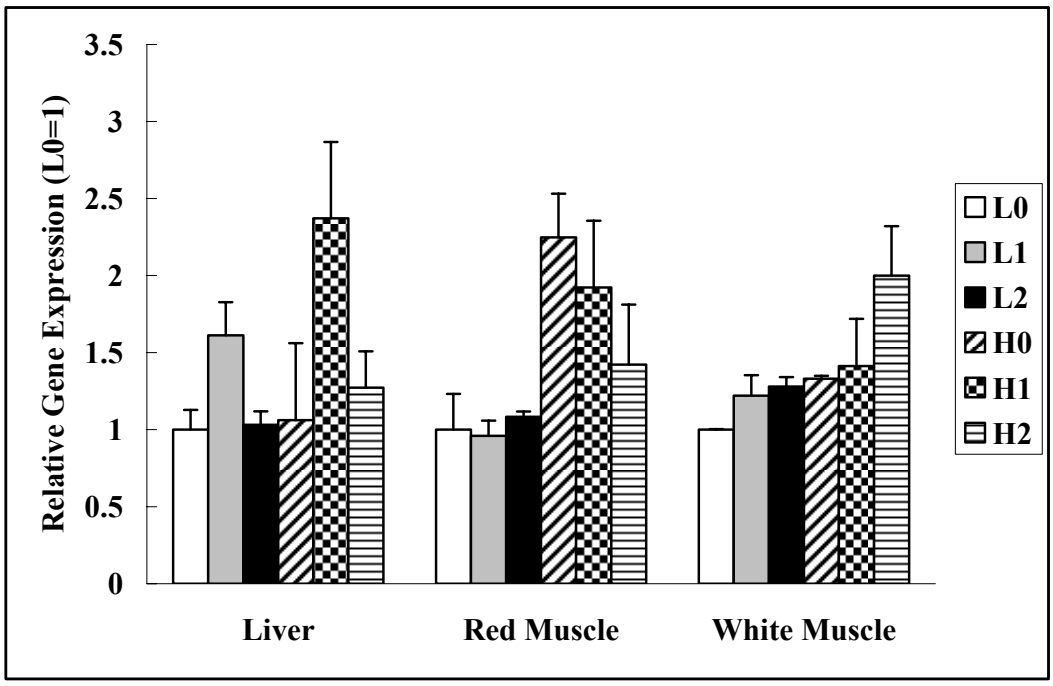
PPARβ expression was increased by high dietary oil, but dietary CLA decreased PPARβ expression in red muscle in fish fed high dietary oil (Figure 4.7). The expression of PPARβ in liver was greatest in fish fed 1 % CLA (Table 4.2, Figure 4.7). Expression of PPARγ in liver was significantly increased by dietary CLA, but dietary oil content had no significant effect (Table 4.2, Figure 4.8). PPARγ expression in muscle tissues was too low to be reliably determined.

Figure 4.6 Effects of dietary oil content and conjugated linoleic acid (CLA) on the expression of PPARα in the liver, red and white muscle.



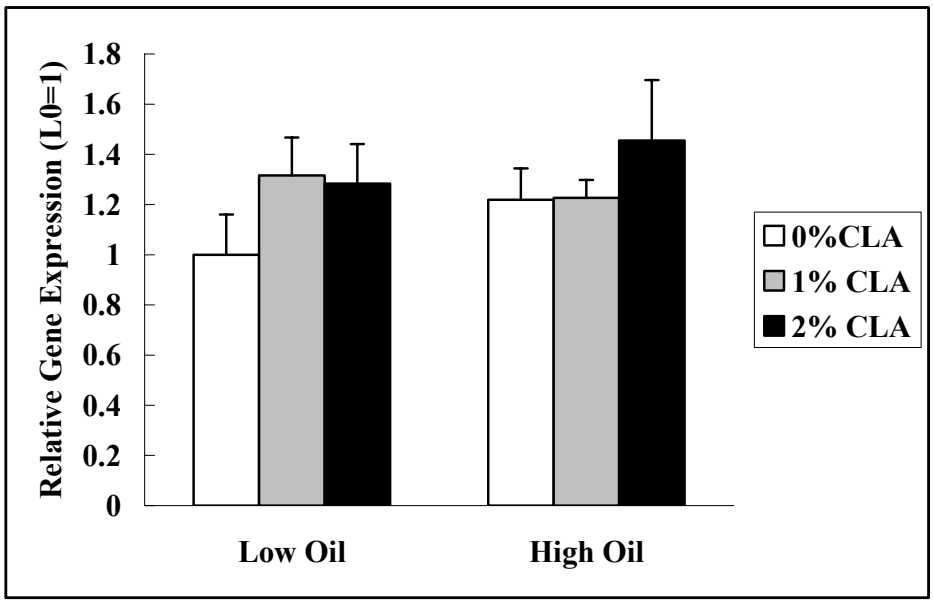
Genes were determined by Q-PCR and normalised relative to total RNA, determined by fluorescent assay, as described in Materials and Methods. Results are presented as means and SD (n = 3) relative to diet L0 = 1.

Figure 4.7 Effects of dietary oil content and conjugated linoleic acid (CLA) on the expression of PPARβ in the liver, red and white muscle.



Genes were determined by Q-PCR and normalised relative to total RNA, determined by fluorescent assay, as described in Materials and Methods. Results are presented as means and SD (n = 3) relative to diet L0 = 1.

Figure 4.8 Effects of dietary oil content and conjugated linoleic acid (CLA) on the expression of PPARγ in the liver.



Genes were determined by Q-PCR and normalised relative to total RNA, determined by fluorescent assay, as described in Materials and Methods. Results are presented as means and SD (n = 3) relative to diet L0 = 1.

4.3.3 Basic Haematology and Selected Innate Immunological Measurements

There were no significant differences in blood cell counts in Atlantic salmon fed CLA at either 1 or 2 % dietary inclusion levels compared with controls (Table 4.2). In contrast, red blood cell counts were significantly decreased in fish fed the high oil diets. However, haematocrit value was unaffected by either dietary treatment. There was a tendency for the phagocytic ability of head kidney macrophages, as measured by the phagocytic index and ratio, to increase in fish fed dietary CLA, particularly when fed in combination with low oil diets however this trend was not significant (Table 4.3). Respiratory burst as measured by the reduction of NBT by head kidney macrophages of Atlantic salmon in the presence of PMA was not influenced by either CLA or dietary oil

level (Table 4.3). Additionally, lysozyme activity was not altered in response to dietary treatment (Table 4.3).

Table 4.3 Effects of dietary oil content and conjugated linoleic acid (CLA) on basic haematology and selected immunological parameters of Atlantic salmon.

Diet	RBC (L0 =1)	WBC (L0 =1)	PCV (%)	PI	PR (%)	RB A_{610} (10^4 cells)	Lysozyme (U/min/ml)
L0	1.00 ± 0.43	1.00 ± 0.19	62.9 ± 10.6	0.19 ± 0.09	11.43 ± 5.10	2.32 ± 1.01	1503.0 ± 378.4
L1	1.03 ± 0.25	1.07 ± 0.21	61.4 ± 4.7	0.23 ± 0.10	12.12 ± 2.45	2.22 ± 0.67	1456.2 ± 389.2
L2	1.13 ± 0.48	0.89 ± 0.19	61.6 ± 10.4	0.24 ± 0.08	12.70 ± 4.81	2.39 ± 0.15	1477.3 ± 409.9
H0	0.96 ± 0.31	1.24 ± 0.16	57.3 ± 10.6	0.18 ± 0.01	12.96 ± 4.21	2.92 ± 0.33	1402.3 ± 299.8
H1	0.98 ± 0.21	1.02 ± 0.25	57.5 ± 7.8	0.17 ± 0.03	14.11 ± 2.99	2.19 ± 1.10	1555.5 ± 329.0
H2	0.92 ± 0.23	1.15 ± 0.17	67.3 ± 9.0	0.19 ± 0.11	13.23 ± 2.52	2.52 ± 0.34	1493.0 ± 190.2
ANOVA							
CLA	0.4232	0.9412	0.3571	0.6539	0.8206	0.3543	0.9286
Oil	0.0298	0.3836	0.6822	0.14	0.2992	0.3242	0.9668
Interact.	0.1225	0.7923	0.2727	0.7179	0.8929	0.5244	0.7723

RBC, red blood cell count (relative to L0 diet); WBC, white blood cell count (relative to L0 diet); PCV, packed cell volume; PI, phagocytic index; PR, phagocytic ratio; RB, respiratory burst.

4.4 Discussion

CLA has the capacity to exert both agonistic and antagonistic effects on a wide variety of lipid metabolic factors. These effects are entirely dependent upon the pattern of lipid metabolism (Tocher, 2003), which itself varies with species, tissue type, age, dietary factors such as lipid content and oil composition, CLA isomer composition and duration of supplementation. Thus, it is hard to define a specific biochemical mechanism of action. However, the view that CLA can affect lipid accumulation both by decreasing *de novo*

fatty acid synthesis and increasing oxidation is well established, at least in mammals. For example, CLA has been shown to inhibit fatty acid synthetase activity in rat liver (Oku *et al*., 2003), and can also suppress TAG accumulation and can increase fatty acid oxidation in 3T3-L1 adipocytes (Evans *et al*., 2002). It is hypothesised that CLA exert such effects on lipid metabolism at the transcriptional level by altering gene expression of key regulatory proteins and enzymes mediated, in part, by PPARs. However, it was unclear whether CLA have the ability to alter lipid metabolism in fish in a manner similar to that of mammalian models (Berge *et al*., 2004).

In the present study, salmon fed CLA exhibited increased expression of Δ5 and Δ6 fatty acyl desaturases in liver; particularly evident in the low oil diet, which displayed around a 2-fold increase in expression of Δ5 and Δ6 desaturase at 2 % inclusion of CLA relative to the control. This was at least partly reflected in HUFA synthesis activity, which increased in fish fed 2 % CLA at the low dietary oil content. Analogously, Δ5 and Δ6 fatty acid desaturase expression was shown to increase in mice fed CLA (Takaheshi *et al*., 2002), while the amount of the HUFA, 20:5n-3 and 22:6n-3, increased in hybrid striped bass in response to dietary CLA (Twibell *et al*., 2000). One explanation may be that transcription factors equivalent to mammalian PPARα and sterol response element binding protein-1c (SREBP-1c, a transcription factor involved in regulating adipogenesis) are directly involved in regulating Δ6 and Δ5 fatty acyl desaturases in fish via a feedback mechanism. A similar mechanism for the regulation of fatty acyl desaturase gene expression has been proposed in mammals whereby Δ6 desaturase gene expression is partly regulated by PPARα (Nakamura and Nara, 2004). Indeed, it has been shown that ligand activation of PPARα via peroxisome proliferators can result in up-regulation of Δ5 and Δ6 desaturases, while high PUFA feeding down-regulated these genes in a SREBP-1-

dependent mechanism (Nakamura and Nara, 2004). In salmon liver, CLA feeding was associated with increases in Δ5 and Δ6 fatty acyl desaturase expression and HUFA synthesis in low oil treatments, which might be indicative of a role for ligand activation of PPARα by CLA. In contrast, in high oil diets, Δ5 and Δ6 expression and HUFA synthesis was repressed, even in the presence of similar amounts of CLA, possibly indicating a role for SREBP-1 proteins. Interestingly, CLA feeding also increased the levels of PPARα, providing some further support for this mechanism of regulation in salmon liver. As previously elucidated however, dietary CLA had no significant effect on either liver or flesh (muscle) fatty acid compositions in salmon (Chapter 3), thus it appears that the increased fatty acyl desaturase expression and HUFA synthesis activity does not have a major physiological consequence in terms of gross fatty acid compositions. That alterations in desaturase gene expression and HUFA synthesis activity in liver can have relatively little effect on tissue fatty acid compositions has been demonstrated previously in several trials in which salmon have been fed VOs (Bell *et al*., 2001, 2002). The data from the present trial also show a clear correlation between desaturase expression and HUFA synthesis activity, and dietary oil content, with diets containing high oil conferring significantly lower levels of desaturase expression and HUFA synthesis activity compared to low oil diets. In addition to effects on HUFA synthesis, it is fairly well established that there is a reduction in fatty acid synthesis (lipogenesis) as a result of high dietary oil levels, as has previously been demonstrated in Atlantic salmon and other fish species (Tocher, 2003).

Beta-oxidation is the principal means of fatty acid catabolism in vertebrates and the basic mechanism of the pathway, and possibly regulation, appear to be highly conserved in mammals and fish (Nanton *et al*., 2003). Unfortunately, in the present trial,

it was not possible to measure mitochondrial and hence total β-oxidation as this would require assay of fresh tissue and it was not possible to do radioactive work outwith the University laboratory. Therefore assays had to be performed on frozen tissue samples. To estimate mitochondrial β-oxidation in these frozen samples, the activity of a key enzyme in mitochondrial β-oxidation, CPT-1, was measured. However, peroxisomal β-oxidation can still be measured in tissue samples previously frozen as the peroxisomal membranes are not damaged by freezing as the mitochondrial membranes are. Nevertheless, in the present trial, peroxisomal β-oxidation capacity was not significantly affected in red or white muscle tissue of fish fed dietary CLA. In contrast, CLA significantly increased both CPT-1 expression and activity in these tissues. It has been previously suggested that in rats, CLA can increase mitochondrial β-oxidation in a variety of tissues via an increase in CPT-1 activity (Rahman *et al*., 2001). Mitochondria are the major site of β-oxidation in salmonid muscle and thus are more likely to influence total fatty acid catabolism in comparison to peroxisomal β-oxidation in this tissue (Frøyland *et al*., 2000). Obviously, it would have been advantageous to have measured mitochondrial β-oxidation in order to offer a more unequivocal interpretation of these results; however the preliminary evidence suggests that CLA may enhance total fatty acid catabolism in muscle. Although red muscle was the site of higher peroxisomal β-oxidation activity in the present study, in terms of total activity, white muscle actually represents the site of the largest proportion of fatty acid oxidation in salmon due to the large size of the tissue (Frøyland *et al*., 2000). There are few studies in fish species to compare the present data with. However, dietary lipid level did not alter β-oxidation activity in the muscle of haddock *Melanogrammus aeglefinus L.* fed graded levels of oil (Nanton *et al*., 2003). In the present study, dietary oil content had no major effect on

peroxisomal β-oxidation activity or CPT-1 mRNA levels in either red or white muscle, or CPT-1 activity in red muscle, although CPT-1 activity was increased by dietary oil content in white muscle.

Previous studies in rodents showed that CPT-1 mRNA level was elevated in liver in response to dietary CLA (Degrace *et al*., 2004). In the present study, and similar to results in muscle, CPT-1 gene expression was found to be up-regulated in salmon liver in response to dietary CLA supplementation, although this was not accompanied by increased CPT-1 activity. Furthermore, CPT-1 expression was increased in salmon liver in fish fed the high oil diet, at least when fed in combination with CLA. Consistent with this it appeared that in fish fed CLA, CPT-1 activity was also higher in fish fed the high oil diet compared with fish fed the low oil. Previously, it was shown that the *in vivo* rate of fatty acid oxidation was associated with *in vitro* CPT-I activity (Rasmussen and Wolfe, 1999). However, studies determining changes in energy metabolism in hamsters fed CLA found disparities between the activity of CPT-1 and lipid oxidation in the liver (Bouthegourd *et al*., 2002). Pertinent to this lack of correlation between CPT-1 expression and activity in liver is the fact that, with Atlantic salmon of similar age and size, catabolism of fatty acids in liver was reported to be principally due to peroxisomal, rather than mitochondrial, β-oxidation (Frøyland *et al*., 2000). Consequently, CPT-1 may not have a prominent role as a regulatory element of fatty acid oxidation in salmon smolt liver, considering it is primarily involved in mitochondrial metabolism (Eaton *et al*., 1996). This may also be relevant in the present study as it provides a mechanism whereby changes in CPT-1 expression and/or activity could be unrelated to total β-oxidation activity in salmon liver. Clearly, it would have been desirable to have been able to measure mitochondrial β-oxidation, and also to have measured ACO activity, the

rate limiting enzyme of peroxisomal β-oxidation, in the present study. However, the data that were obtained suggest that the major fatty acid oxidation pathway (peroxisomal) in salmon liver was generally unaffected by dietary CLA, and actually decreased by high dietary oil. In comparison, graded levels of dietary lipid showed no significant differences in total β-oxidation activity in liver of haddock (Nanton *et al*., 2003).

There is consolidating evidence that lipid homeostasis is, at least in part, modulated through the PPAR transcription factors in mammals. Recent studies suggest that CLA could mediate this activation by acting as high-affinity ligands for a number of PPAR isotypes, particularly PPARα (Moya-Camarena *et al*., 1999b,c). PPARα is intimately involved in the regulation of genes involved in mitochondrial fatty acid oxidative processes in mammals (Gulick *et al.,* 1994), whereas PPARγ is primarily involved in lipid deposition via preadipocyte differentiation and lipogenesis (Gregoire *et al*., 1998). Very recent work has elucidated that marine fish also share homologous gene sequences, with similar phylogenetic characteristics, to the mammalian PPAR counterparts, possibly also suggesting similar molecular roles (Boukouvala *et al*., 2004; Leaver *et al*., 2005, 2007). However, Atlantic salmon may possibly contain up to five PPAR genes, as opposed to three in mammals, and therefore the precise roles of piscine PPARs have not been conclusively defined (Andersen *et al*., 2000; Leaver *et al*., 2005).

The effects of CLA on mammalian PPAR gene expression are ambiguous and seem to be dependant partly on species, partly on tissue type, and partly on CLA isomer. The majority of studies describe a decrease in PPARγ mRNA in isolated adipocytes, or in adipose tissue from mice treated with t10c12 CLA or a 1:1 mixture of the two predominant CLA isomers (Brown *et al*., 2003a; Granlund *et al*., 2003; Kang *et al*., 2003). Conversely, CLA has also been reported to increase expression of PPARγ in liver

of mice, and in white adipose tissue in rats (Clement *et al*., 2002; Zhou *et al*., 2004). T10c12 CLA down-regulated PPARα in mice (Warren *et al*., 2003), however there was no change in PPARα expression in liver of hamsters fed diets containing t10c12 or c9t11 CLA (Macarulla *et al.*, 2005). In the present study, there was a trend for PPARα expression to be increased by dietary CLA in liver, red and white muscle of fish fed the low oil diets. In addition, up-regulation of PPARα in response to dietary CLA was also observed in white muscle and liver in fish fed the high oil diets. As mentioned above, activation of PPARα (via peroxisome proliferators) can induce β-oxidation through up-regulation of key enzymes such as CPT-1 in mammals (Nakamura and Nara, 2004). The data in the present trial suggest that PPARα may also be implicated in the action of CLA. In general CLA increased the levels of both CPT-1 mRNA and PPARα and, in some cases, PPARβ mRNA. The exceptions to this were PPARα and PPARβ expression in red muscle from fish fed the high oil diets, which tended to decrease with dietary CLA inclusion and at high CLA inclusion liver PPARβ was decreased. It has been shown in PPARα-knockout mice that the effects of CLA on body fat distribution and mitochondrial lipid catabolism genes are not mediated by PPARα, but that the peroxisomal β-oxidation gene ACO is mediated by a PPARα-dependent mechanism (Peters *et al*., 2001). If this is true in fish then it is possible that effects on mitochondrial lipid catabolic genes such as CPT-1 could be mediated through PPARβ subtypes. However, PPARα expression exceeds that of PPARβ1 by an order of magnitude in muscle tissues suggesting PPARα would have the predominant regulatory role. Of the tissues investigated in salmon, PPARγ expression was only detected in liver. However, in agreement with work carried out on mice (Clement *et al*., 2002), PPARγ mRNA levels increased in liver of salmon fed CLA, and CLA has been reported to cause 'fatty liver' in

mice (Tsuboyama-Kasaoka *et al*., 2000; Clement *et al*., 2002). However, fish PPARγ does not appear to share the same ligand activation profile as in mammals (Leaver *et al*., 2005). Fish PPARγ does not respond to fatty acids and has specific amino acid differences compared to the mammalian form which may explain the lack of activation, suggesting that is unlikely, despite increases in liver, that CLA would mediate its effects through this transcription factor. Indeed, neither liver size nor lipid content was increased in salmon fed CLA (Chapter 3).

The extent to which the effects of CLA on immunological status could be investigated in the present study was rather limited but the data obtained showed that CLA had very little effect on the few parameters measured. Therefore, the data in the present study suggest that dietary CLA does not confer a non-humoral immunological response in Atlantic salmon, which is in agreement with similar studies in rainbow trout (Clarke, 2003; Marshall, 2003).

In summary, gene expression and activity of various lipid metabolic factors were altered in response to graded levels of CLA and/or dietary oil content in Atlantic salmon smolts. Specifically, some association was observed between dietary CLA, liver HUFA synthesis and desaturase gene expression, and liver PPARα expression although this varied with dietary oil content. In addition, some association between dietary CLA, CPT-1 expression and activity, and PPARα expression was observed in muscle tissues. However, the magnitude of the changes in fatty acid metabolism observed were not sufficient to bring about major changes in the whole body lipid and fatty acid composition of the fish (Chapter 3). Additionally, dietary CLA does not appear to significantly influence the immunological response in Atlantic salmon. In conclusion, this study has presented evidence that dietary CLA may have some effects on fatty acid

metabolism in Atlantic salmon, but that there is little evidence of a direct mechanism involving PPAR transcription factors. However, considering the importance of dietary lipid in aquaculture, further work is required to assess the potential of CLA as a dietary supplement, and the role of PPARs in the regulation of lipid metabolism in fish.

CHAPTER 5. INFLUENCE OF DIETARY CLA AND TTA IN ATLANTIC COD

5.1 Introduction

Aquaculture is the fastest growing animal-based agricultural food production sector, expanding at more than 9 % per year, and currently contributes over one third of all the fish in the human food basket (Tacon, 2003). Farming of finfish in seawater is dominated by Atlantic salmon (*Salmo salar* L.), but other marine species are becoming increasingly important, including temperate species such as gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*) and turbot (*Psetta maximus*) and, in colder waters, halibut (*Hippoglossus hippoglossus*). Recently, declining catches and high market prices have seen Atlantic cod (*Gadus morhua* L.) emerge as the most promising species for culture in the northern Atlantic area (Brown and Puvanendran, 2002; Brown *et al*., 2003b) with production expanding in Norway and Scotland. A major factor influencing the commercial success of cod culture will be the development of diets and feeding strategies to maximise growth and feed conversion efficiencies (Morais *et al.*, 2001; Lall and Nanton, 2002; Hemre *et al*., 2003, 2004; Rosenlund *et al*., 2004). One major issue pertaining to this is that cod store lipid in the liver and, in early studies, farmed fish were reported to have enlarged livers and display a HSI of over 12 % compared to values of 2 – 6 % in wild fish (Jobling, 1988). In early feeding trials with captive cod, the HSI increased from 9.5 % to an average of 13 %, and liver fat increased from around 55 % to 67 – 70 % in cod fed various dietary oils (Lie *et al*., 1986). Further studies have consistently shown that farmed cod have high liver lipid levels that can exceed 70 % of wet weight, and have higher HSI and condition factor (K) than their wild

counterparts (Dos Santos *et al*., 1993; Shahidi and Dunajski, 1994; Grant *et al*., 1998; Gildberg, 2004). Dietary formulations in aquaculture have tended to exhibit an upward trend in lipid as this has successfully increased weight gains, but several studies have shown that a potential detrimental side-effect of high fat diets is the deposition of excess lipid in tissues (Sargent *et al.*, 2002; Tocher, 2003). This may exacerbate the enlarged fatty liver problem in farmed cod and so it is important to gain a clearer understanding of the mechanisms determining lipid and fatty acid homeostasis and deposition.

Excess lipid in cod liver is undesirable since in most cases it is not utilised as a food or supplement source in mainstream aquaculture. Hence, lipid that is deposited in the liver of cod is in effect wasted energy that can be used for growth. However, it is noteworthy that unlike salmon, there is no effect on edible muscle lipid content. It is therefore important to investigate the possibility of incorporating micronutrients, such as CLA and TTA, as additives in aquafeeds so that they may be able to counteract any of the abovementioned deleterious consequences.

Although there are a number of studies detailing the effect of TTA in animal models (Berge *et al*., 2005) prior to the initiation of this study there were no documented reports of this bioactive fatty acid being assessed as a dietary supplement in the context of aquaculture nutrition. However, recently four studies have been published, which largely investigated the effects of dietary TTA on Atlantic salmon lipid metabolism both *in vitro* and *in vivo*. Specifically, the first study analysed the effects of dietary TTA on feed intake, growth, tissue fatty acid composition and β-oxidation (Moya-Falcon *et al*., 2004). This study concluded that administration of TTA altered the fatty acid composition of several tissues and increased hepatic mitochondrial β-oxidation but also increased mortality and depressed the growth of the fish. A subsequent study

investigated the effect of 0.75 mM TTA on TAG accumulation and secretion by salmon hepatocytes in culture (Vegusdal *et al*., 2005). The results indicated that hepatocytes incubated with TTA secreted less TAG than when incubated with no fatty acid. It was also suggested the TAG formation was retarded in response to dietary TTA inclusion. A third paper investigated the influence of 0.8 mM TTA and temperature on the lipid metabolism of cultured Atlantic salmon hepatocytes (Moya-Falcon *et al.*, 2006); concluding that ACO activity was not altered in response to TTA administration. This study also elucidated the effect of feeding Atlantic salmon 0.6 % TTA on cellular uptake and oxidation of [1-^{14}C]18:1n-9 in hepatocytes. Although not significant, there was a higher production of oxaloacetate and malate in hepatocytes from TTA-fed fish, which was purportedly due to increased mitochondrial β-oxidation. The fourth study examined the consequence of feeding dietary TTA to Atlantic salmon smolts, focussing specifically on lipid metabolism and gene expression of a number of enzymes implicated in lipid homeostasis in muscle and liver (Kleveland *et al*., 2006). This study found that there was significantly higher mortality and lower growth rate in fish fed 0.6 % TTA compared with fish fed FO diets alone. However, ACO mRNA level and enzyme activity were unaffected by dietary TTA suggesting that peroxisomal β-oxidation was not altered. Additionally, TTA increased the HSI whilst PPARα and ApoA1 transcripts were decreased in liver of salmon. This study also reported that there were no significant effects on the fatty acid composition, lipid content or gene expression profiles of enzymes involved in lipid metabolism of muscle, implying that the liver is more responsive to the effects of dietary TTA.

The aims of the present study were to determine the effects of CLA and TTA on growth performance, lipid content, composition and metabolism, and immunomodulation

in Atlantic cod. The overall objective being to test the hypotheses that CLA and TTA have beneficial effects in cod culture including decreased liver size and proportion through decreased lipid content, positive health benefits through improved immune status, and increased nutritional quality through effects on fatty acid compositions including accumulation of bioactive fatty acids, CLA and TTA, in flesh. Juvenile cod were fed for three months on FM and FO diets of basically commercial formulation, but containing either 0.5 % or 1 % CLA, or 0.5 % TTA. The effects of the functional fatty acids on growth, feed efficiency, body proximate composition, liver weight and lipid composition, fatty acid compositions of flesh and liver, key enzymes of fatty acid oxidation, and some immune parameters were determined.

5.2 Materials and Methods

5.2.1 Experimental Fish

The dietary trial was performed at Viking Fish Farms, Ardtoe Marine Laboratory, Ardnamurchan, Scotland, between October 2005 and January 2006. Hatchery reared Atlantic cod of the 2004 year class were randomly distributed between twelve indoor, round tanks of 1.5 m^3 volume (1.72 m diameter) supplied with filtered seawater. The initial stocking density was 50 fish of average weight 127 ± 15 g per tank (5.8 kg/m^3), with 25 fish per tank individually PIT tagged (Passive Induced Transponder, Fish Eagle, Gloucestershire, England) prior to stocking. Water temperature was maintained at 12 ^oC (± 1 ^oC) throughout the trial, with a light regime of 12L:12D.

5.2.2 Experimental Diets

Four experimental diets were fed to triplicate tanks for three months, with feeding supplied to appetite manually in one morning feed over a period of 1 h. Waste feed pellets were collected and counted 1 h later. The experimental diets were formulated in order to satisfy the nutritional requirements of marine fish (National Research Council, 1993), and were formulated and manufactured by BioMar A/S, Brande, Denmark. The dietary formulations are presented in Table 5.1.

Table 5.1 Formulations (percentage of dry ingredients) and proximate compositions (percentage of total diet) of experimental diets.

	FO	CLA1	CLA2	TTA
Fishmeal	53	53	53	53
Sunflower meal	17	17	17	17
Wheat gluten	5	5	5	5
Legume seeds	17	17	17	17
Micronutrients	0.4	0.4	0.4	0.4
Fish oil	8.8	8.0	7.1	8.3
CLA	0.0	0.8	1.7	0.0
TTA	0.0	0.0	0.0	0.5
Moisture	7.5 ± 0.1 [ab]	7.3 ± 0.3 [b]	8.0 ± 0.2 [a]	8.0 ± 0.2 [a]
Lipid	14.8 ± 0.3	15.4 ± 0.8	15.4 ± 0.7	15.4 ± 0.5
Protein	49.9 ± 0.4	50.2 ± 0.1	49.7 ± 0.4	49.5 ± 0.5
Ash	10.6 ± 0.0 [ab]	10.7 ± 0.1 [a]	10.5 ± 0.0 [b]	10.5 ± 0.0 [b]

Results for proximate compositions are means ± S.D. (n=3). Micronutrients, includes essential amino acids (methionine and lysine), vitamins, minerals. FO, control diet containing fish oil alone, CLA1 and CLA2, diets supplemented with 0.5 and 1 % CLA; TTA, diet supplemented with 0.5 % TTA.

Isonitrogenous diets were based on FM and standard Northern hemisphere FO with the latter being replaced partially by 0.5 and 1 % CLA, and 0.5 % TTA (as a percentage of the total diet). Diets were identical in formulation other than fatty acid composition with CLA (LUTA-CLA^{TM} 60, containing 60 % CLA methyl esters as a 50:50 mixture of c9t11 and t10c12 isomers; BASF AG, Ludwigshafen, Germany) and TTA (supplied by Dr Rolf Berge, Thia Medica A.S., Bergen, Norway) balanced by FO (capelin oil, Norsemeal Ltd, London, UK). The fatty acid compositions of the diets are presented in Table 5.2.

Table 5.2 Fatty acid compositions (percentage of weight) of experimental diets containing CLA and TTA.

	FO	CLA1	CLA2	TTA
14:0	7.2 ± 0.1 [a]	6.8 ± 0.2 [b]	6.2 ± 0.1 [c]	6.2 ± 0.0 [c]
15:0	0.6 ± 0.0	0.5 ± 0.0 [b]	0.5 ± 0.0 [b]	0.5 ± 0.0 [b]
16:0	19.7 ± 0.2 [a]	18.9 ± 0.1 [b]	18.2 ± 0.0 [c]	18.7 ± 0.1 [b]
18:0	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0	3.9 ± 0.0
Total saturated[1]	31.8 ± 0.3 [a]	30.5 ± 0.3 [b]	29.1 ± 0.1 [c]	29.3 ± 0.1 [c]
16:1n-7	7.8 ± 0.1 [a]	7.4 ± 0.1 [b]	7.0 ± 0.2 [c]	7.4 ± 0.0 [b]
18:1n-9	9.9 ± 0.1 [c]	10.8 ± 0.0 [b]	11.8 ± 0.2 [a]	9.8 ± 0.1 [c]
18:1n-7	3.2 ± 0.0 [a]	3.1 ± 0.1 [a]	2.8 ± 0.0 [b]	3.1 ± 0.0 [a]
20:1n-9	2.3 ± 0.0	2.2 ± 0.0	2.3 ± 0.2	2.3 ± 0.0
22:1n-11	3.2 ± 0.0 [a]	3.1 ± 0.0 [ab]	3.0 ± 0.0 [b]	3.1 ± 0.0 [a]
24:1n-9	0.6 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.0
Total monoenes[2]	27.3 ± 0.2 [a]	27.4 ± 0.2 [a]	27.6 ± 0.1 [a]	26.5 ± 0.0 [b]
CLA (9c,11t)	0.0 ± 0.0 [c]	1.8 ± 0.1 [b]	3.2 ± 0.2 [a]	0.0 ± 0.0 [c]
CLA (10t,12c)	0.0 ± 0.0 [c]	1.7 ± 0.1 [b]	3.1 ± 0.2 [a]	0.0 ± 0.0 [c]
TTA	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	2.8 ± 0.2 [a]
18:2n-6	5.7 ± 0.1 [b]	5.6 ± 0.0 [b]	5.9 ± 0.0 [a]	5.6 ± 0.1 [b]
20:4n-6	1.1 ± 0.0 [a]	1.0 ± 0.0 [b]	0.9 ± 0.0 [b]	1.1 ± 0.0 [a]
Total n-6 PUFA[3]	7.8 ± 0.0 [a]	7.6 ± 0.1 [b]	7.7 ± 0.1 [ab]	7.6 ± 0.1 [b]
18:3n-3	1.0 ± 0.0 [a]	1.0 ± 0.0 [a]	0.9 ± 0.0 [b]	1.1 ± 0.0 [a]
18:4n-3	2.5 ± 0.0 [a]	2.4 ± 0.0 [b]	2.2 ± 0.0 [c]	2.5 ± 0.0 [a]
20:4n-3	0.7 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.7 ± 0.0
20:5n-3	14.0 ± 0.0 [a]	13.2 ± 0.2 [b]	12.3 ± 0.1 [c]	14.2 ± 0.1 [a]
22:5n-3	1.9 ± 0.5	1.5 ± 0.0	1.4 ± 0.0	1.7 ± 0.0
22:6n-3	12.8 ± 0.0 [b]	12.3 ± 0.1 [c]	11.9 ± 0.1 [d]	13.4 ± 0.1 [a]
Total n-3 PUFA[4]	33.1 ± 0.5 [b]	31.1 ± 0.3 [c]	29.3 ± 0.2 [d]	33.9 ± 0.2 [a]
Total PUFA	40.9 ± 0.4 [a]	38.7 ± 0.3 [b]	37.0 ± 0.3 [c]	41.5 ± 0.3 [a]
n-3/n-6	4.2 ± 0.1 [b]	4.1 ± 0.0 [b]	3.8 ± 0.0 [c]	4.5 ± 0.0 [a]

Values are means ± S.D. (n=3). Superscript letters denote significant differences between the diets. [1], includes 20:0, present at up to 0.3 %. [2], includes 20:1n-7 present at up to 0.3 %. [3], includes 18:3n-6, 20:2n-6, 20:3n-6 and 22:4n-6 present at up to 0.4 %. [4], includes 20:3n-3, present up to 0.2 %.

5.2.3 Sampling and Analysis

At the initiation of the trial, all fish were anaesthetised with Metomidate (50 mg/L), identified by PIT tag if present, and individually weighed and fork length recorded. At the end of the trial, 9 fish per tank (27 per dietary treatment) were killed by a blow to the cranium then sampled for compositional analyses, with 3 whole fish/tank frozen immediately at -20 ^{o}C for whole body proximate compositional (proximate) analysis. Blood and head kidney for immunological studies were removed from the remaining fish as described in 2.4.1 and 2.4.5 respectively. The fish were then eviscerated and used for biometric determinations (hepato-, and viscero-somatic indices) and for tissue lipid analyses. Flesh samples were excised from the epaxial myotomes anterior to the first dorsal fin ray (Flesh Quality Cut) and livers were taken from six fish, pooled in two samples of 3 fish each, and frozen immediately in liquid nitrogen (livers) or dry ice (flesh). Samples of 1-2 g of liver, white and red muscle for biochemical analyses were also collected and immediately frozen in liquid nitrogen. Samples were subsequently stored at -80 ^{o}C prior to biochemical analysis.

Blood cell counts and haematocrit measurements were carried out as described in sections 2.4.2 and 2.4.3 respectively. Total lipids of liver, muscle and diet were extracted and quantified as described in section 2.3.7 and lipid class analysis was carried out as detailed in section 2.3.8. The method previously employed to produce fatty acid methyl ester (FAME) in samples containing CLA described in section 3.2.3, was unsuccessful in quantitatively methylating TTA in this trial since degradation of TTA was observed upon GC analysis. It is probable that the breakdown products formed using the abovementioned methylation procedure are in fact sulphoxide or sulphone derivatives, which are commonly formed during strong acidic hydrolytic conditions (Pavol Bohov,

Personal Communication). A small experiment was conducted to deduce the optimal time and temperature at which TTA could successfully be methylated with little to no artefact formation. It was also imperative that the experimental conditions facilitated complete methylation of all components including CLA and with little to no residual degradation of other fatty acids within the sample. By varying the time (duration) and temperature of the methylation procedure, it was concluded that the optimal reaction conditions using acid-catalysed transesterification and facilitating the complete methylation of TTA, CLA and all other fatty acids were incubation at 80 ^{o}C for 3 h in the presence of 2 ml 1 % (v/v) H_2SO_4 in methanol together with 1 ml of toluene under a nitrogen atmosphere. FAME were subsequently extracted, purified and quantified as described in section 2.3.9. Charring of TLC plates after FAME purification allowed visualisation of any non-methylated total lipid. Since, after 3 h of incubation, there were no bands corresponding to non-methylated total lipid on the TLC plates, it was deduced that all the fatty acids in the samples had been successfully methylated. Indeed. the levels of CLA and TTA obtained in Table 5.2 expressed as a percentage of total dietary lipid show quantitative recovery of these bioactive fatty acids after using the above described methylation procedure. Some key activities associated with fatty acid oxidation were determined in liver, white and red muscle. Peroxisomal β-oxidation capacity was estimated as described in section 2.3.3. CPT-1 and ACO activities were determined as described in sections 2.3.4 and 2.3.5, respectively.

5.3 Results

5.3.1 Dietary Formulation

The control FO diet contained 41 % total PUFA, including 14 % 20:5n-3, 13 % 22:6n-3 and almost 6 % 18:2n-6, 32 % total saturates, mainly 16:0, and 27 % total monoenes, mainly 18:1n-9 and 16:1n-7 (Table 5.2). Inclusion of CLA in the diets resulted in levels of total CLA of 3.5 % and 6.3 % of total fatty acids at the 0.5 % and 1 % dietary inclusion levels, respectively. CLA inclusion resulted in lower levels of 14:0, 16:0, 20:5n-3 and 22:6n-3, but 18:1n-9 and 18:2n-6 (the other main components of the CLA mixture) increased (Table 5.2). Inclusion of 0.5 % TTA resulted in TTA at a level of 2.8 % of total fatty acids in the diet, together with lower levels of 14:0 and 18:0.

5.3.2 Growth, Biometry and Whole Body Proximate Compositions

Growth and biometry of Atlantic cod fed the different dietary treatments are presented in Table 5.3 below. There were no significant differences in final weights, growth rates (SGR), or thermal growth coeffeficients in fish fed dietary CLA or TTA. There were statistically significant effects on VSI such that it decreased in fish fed 0.5% CLA however remained unchanged at the 1% inclusion level. FCR levels increased as a result of 0.5% CLA administration but were unaffected in fish fed 1% CLA and 0.5% TTA. HSI was significantly lower in fish fed TTA however was not affected in fish fed CLA.

Table 5.3 Growth and biometric parameters of Atlantic cod fed diets containing CLA and TTA.

	FO	CLA1	CLA2	TTA
Initial weight (g)[1]	125 ± 14 [b]	130 ± 15 [a]	125 ± 15 [b]	127 ± 15 [ab]
Final weight (g)[1]	307 ± 50	305 ± 47	309 ± 57	303 ± 44
FCR[2]	0.80 ± 0.01 [b]	0.84 ± 0.02 [a]	0.80 ± 0.02 [b]	0.83 ± 0.00 [ab]
SGR[2]	1.03 ± 0.04	0.98 ± 0.05	1.04 ± 0.02	1.00 ± 0.02
TGC[2]	1.93 ± 0.08	1.84 ± 0.10	1.95 ± 0.04	1.87 ± 0.04
Gutted weight (%)[3]	81.5 ± 0.2 [c]	81.7 ± 0.3 [bc]	82.2 ± 0.4 [a]	81.8 ± 0.2 [b]
Condition factor (K)[3]	1.14 ± 0.04 [a]	1.12 ± 0.01 [b]	1.14 ± 0.03 [a]	1.12 ± 0.01 [b]
HSI[3]	11.0 ± 0.5 [b]	11.3 ± 0.1 [a]	10.9 ± 0.3 [b]	10.6 ± 0.1 [c]
VSI[3]	9.3 ± 1.2 [a]	8.0 ± 0.7 [b]	8.9 ± 0.2 [a]	8.8 ± 0.2 [a]
Mortality	1	2	1	4

Data are presented as means ± S.D, [1]n = 146 - 150, [2]n = 3, [3]n = 27
Condition factor (K) = (wet weight in g) x 100)/(length in mm^3) x1000; FCR, feed converstion ratio, TGC, thermal growth coefficient = 100 x (final $weight^{1/3}$ – initial $weight^{1/3}$)/sum day degrees; HSI, hepato-somatic index; SGR, specific growth rate; VSI, viscero-somatic index; FO, control diet containing fish oil alone, CLA1 and CLA2, diets supplemented with 0.5 and 1 % CLA; TTA, diet supplemented with 0.5 % TTA.

There were no significant differences in the whole body proximate composition (moisture, lipid, protein and ash) of the fish fed either CLA or TTA compared with fish fed FO alone as determined by 1-way ANOVA (Table 5.4)

Table 5.4 The whole body proximate composition of Atlantic cod fed diets containing CLA and TTA.

	FO	CLA1	CLA2	TTA
Moisture	73.6 ± 0.6	73.3 ± 0.6	73.8 ± 0.6	73.9 ± 0.7
Protein	60.4 ± 1.4	59.7 ± 1.7	59.1 ± 1.7	60.6 ± 1.4
Lipid	26.2 ± 1.9	28.4 ± 2.2	27.9 ± 2.1	26.7 ± 1.8
Ash	9.6 ± 0.7	9.3 ± 0.5	9.5 ± 0.6	9.9 ± 0.4

Values are means ± SD of 9 fish. There were no significant differences between dietary treatment as determined by one-way ANOVA. FO, control diet containing fish oil alone, CLA1 and CLA2, diets supplemented with 0.5 and 1 % CLA; TTA, diet supplemented with 0.5 % TTA.

5.3.3 Lipid Contents and Class Compositions of Liver and Flesh

Table 5.5 illustrates the lipid contents and class compositions of liver. The lipid content of the livers, which varied between 50 and 55 % of the wet weight, was significantly decreased in response to 0.5 % CLA however was unaltered in fish treated with 1 % CLA or TTA. Neutral lipid, comprising mainly of TAG, was the predominant lipid fraction in liver accounting for around 97 % of the total. There was a trend that indicated that fish fed dietary CLA reduced their total neutral lipid and TAG content, however this only became significant at the 1 % dietary inclusion level. Neither dietary CLA nor TTA altered the lipid content of cod flesh, which was constant at around 0.8 % of wet weight (Table 5.6). Polar lipids, mainly PC and PE, predominated in flesh accounting for 57 to 61 % of total lipid, with neutral lipids accounting for 39 to 43 %. FFA and steryl esters were highest in fish fed 0.5 % CLA, whereas flesh TAG was slightly, but significantly, higher in fish fed TTA.

Table 5.5 Lipid content (percentage of weight) and lipid class compositions (percentage of total lipid) of liver of Atlantic cod fed CLA or TTA.

	FO	CLA 1	CLA2	TTA
Lipid content	53.8 ± 2.6 ^{ab}	50.0 ± 2.1 ^{b}	55.2 ± 2.0 ^{a}	55.2 ± 2.0 ^{a}
PC	1.5 ± 0.2 ^{b}	1.8 ± 0.1 ^{ab}	2.0 ± 0.4 ^{a}	1.5 ± 0.1 ^{b}
PE	1.3 ± 0.1 ^{b}	1.3 ± 0.2 ^{b}	1.7 ± 0.3 ^{a}	0.9 ± 0.3 ^{c}
Total polar	2.8 ± 0.3 ^{b}	3.1 ± 0.3 ^{ab}	3.7 ± 0.7 ^{a}	2.4 ± 0.3 ^{b}
Total neutral	97.2 ± 0.3 ^{a}	96.9 ± 0.3 ^{ab}	96.3 ± 0.7 ^{b}	97.6 ± 0.3 ^{a}
Cholesterol	4.4 ± 0.6 ^{ab}	4.9 ± 1.4 ^{ab}	5.4 ± 0.3 ^{a}	3.9 ± 0.6 ^{b}
Triacylglycerol	92.7 ± 0.8 ^{a}	92.0 ± 1.6 ^{ab}	90.9 ± 0.8 ^{b}	93.6 ± 0.8 ^{a}
Free Fatty Acid	tr	tr	tr	tr
Steryl ester	tr	tr	tr	tr

Values are means ± SD of 6 samples each of tissue pooled from 3 fish. Superscript letters denote significant differences between dietary treatments as determined by ANOVA. PC, phosphatidylcholine; PE, phosphatidylethanolamine; tr, trace < 0.5%; FO, control diet containing fish oil alone; CLA1 and CLA2, diets supplemented with 0.5 and 1 % CLA; TTA, diet supplemented with 0.5 % TTA.

Table 5.6 Lipid content (percentage of weight) and lipid class compositions (percentage of total lipid) of flesh of Atlantic cod CLA or TTA.

	FO	CLA 1	CLA2	TTA
Lipid content	0.8 ± 0.0	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.0
PC	30.7 ± 1.8 [a]	27.9 ± 1.6 [b]	30.8 ± 1.5 [a]	28.7 ± 1.2 [ab]
PE	17.9 ± 1.2 [a]	15.6 ± 0.7 [b]	17.1 ± 0.6 [a]	17.6 ± 0.5 [a]
PS	2.5 ± 0.6	2.8 ± 0.4	2.7 ± 0.4	3.2 ± 0.5
PI	3.8 ± 0.6	4.1 ± 0.6	3.8 ± 0.7	4.3 ± 0.5
PG/CL	2.9 ± 0.7 [b]	2.8 ± 0.3 [b]	2.8 ± 0.4 [b]	3.7 ± 0.2 [a]
Sphingomyelin	1.5 ± 0.4	1.7 ± 0.3	1.6 ± 0.2	1.6 ± 0.6
Lyso-PC	1.8 ± 0.3 [bc]	2.2 ± 0.2 [a]	2.0 ± 0.2 [ab]	1.5 ± 0.2 [c]
Total polar	61.2 ± 2.1 [a]	57.2 ± 1.6 [b]	60.8 ± 1.2 [a]	60.5 ± 2.1 [a]
Total neutral	38.8 ± 2.1 [b]	42.8 ± 1.6 [a]	39.2 ± 1.3 [b]	39.5 ± 2.1 [b]
Cholesterol	16.1 ± 0.9	15.6 ± 0.3	15.2 ± 1.8	15.7 ± 0.4
Triacylglycerol	6.9 ± 1.2 [b]	6.3 ± 1.6 [b]	5.7 ± 1.1 [b]	9.2 ± 0.8 [a]
Free fatty acid	11.9 ± 1.2 [b]	15.1 ± 1.6 [a]	13.0 ± 1.7 [ab]	11.5 ± 1.4 [b]
Steryl ester	3.9 ± 0.4 [b]	5.9 ± 0.9 [a]	5.4 ± 0.4 [a]	3.2 ± 0.6 [b]

Values are means ± S.D of 6 samples each of tissue pooled from 3 fish. Superscript letters denote significant differences between dietary treatments as determined by ANOVA. CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidyletholamine; PG, phosphatidylglycerol; phosphatidylinositol; PS, phosphatidylserine; FO, control diet containing fish oil alone; CLA1 and CLA2, diets supplemented with 0.5 and 1 % CLA; TTA, diet supplemented with 0.5 % TTA.

5.3.4 Fatty Acid Compositions of the Liver and Flesh

Dietary CLA and TTA were both incorporated into tissue lipids, but it was noteworthy that CLA was deposited to a greater extent in liver lipids (Table 5.7), whereas TTA was deposited to a greater extent in flesh (Table 5.8). Thus, CLA accounted for 1.5 and 2.9 % of total fatty acids in livers of fish fed 0.5 and 1 % CLA, respectively, whereas TTA accounted for 0.6 % of liver fatty acids in fish fed 0.5 % TTA (Table 5.7). In contrast, TTA accounted for 1.6 % of flesh fatty acids in TTA-fed fish, compared to 0.8 and 1.9 % CLA in fish fed the lower and higher CLA, respectively (Table 5.8). Dietary CLA had no significant effect on the proportions n-3 or n-6 PUFA in either liver or flesh, but in fish fed TTA there were decreased percentages of monoenes, n-6 PUFA and 20:5n-3, but an increased proportion of 22:6n-3 in the flesh (Tables 5.7 and 5.8). Dietary CLA resulted in increased percentages of 18:0 and decreased percentages of 18:1n-9 and total monoenes in both tissues, but especially liver.

Table 5.7 Fatty acid composition (percentage of weight) of total lipid from liver of Atlantic cod fed CLA or TTA.

	FO	CLA1	CLA2	TTA
14:0	2.9 ± 0.3 [b]	3.4 ± 0.1 [a]	3.3 ± 0.2 [a]	2.8 ± 0.1 [b]
16:0	15.7 ± 0.5 [a]	15.6 ± 0.2 [a]	15.0 ± 0.7 [ab]	14.7 ± 0.6 [b]
18:0	5.6 ± 0.2 [b]	8.3 ± 0.5 [a]	8.7 ± 0.4 [a]	4.9 ± 0.2 [c]
Total saturated[1]	24.5 ± 0.7 [b]	27.7 ± 0.6 [a]	27.3 ± 1.0	22.6 ± 0.9 [c]
16:1n-7	6.6 ± 0.2 [a]	6.2 ± 0.1 [b]	5.9 ± 0.2 [c]	6.7 ± 0.2 [a]
18:1n-9	18.8 ± 0.6 [a]	15.7 ± 0.3 [b]	15.6 ± 0.3 [b]	19.6 ± 0.7 [a]
18:1n-7	5.3 ± 0.2 [a]	4.8 ± 0.2 [b]	4.8 ± 0.1 [b]	5.7 ± 0.4 [a]
20:1n-9	5.2 ± 0.1	5.1 ± 0.2	5.1 ± 0.3	5.1 ± 0.2
22:1n-11	3.3 ± 0.2 [a]	3.2 ± 0.1 [ab]	2.9 ± 0.3 [b]	3.3 ± 0.3 [a]
24:1n-9	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.1
Total monoenes[2]	40.0 ± 1.1 [a]	35.6 ± 0.6 [b]	34.9 ± 0.5 [b]	41.0 ± 1.1 [a]
CLA (9c,11t)	0.0 ± 0.0 [c]	0.9 ± 0.2 [b]	1.6 ± 0.5 [a]	0.0 ± 0.0 [c]
CLA (10t,12c)	0.0 ± 0.0 [c]	0.6 ± 0.1 [b]	1.3 ± 0.4 [a]	0.0 ± 0.0 [c]
TTA	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	0.6 ± 0.1 [a]
18:2n-6	5.4 ± 0.1	5.5 ± 0.1	5.5 ± 0.0	5.5 ± 0.2
20:4n-6	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0
Total n-6 PUFA[3]	7.2 ± 0.2	7.2 ± 0.1	7.2 ± 0.0	7.0 ± 0.3
18:3n-3	1.0 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	1.0 ± 0.0
18:4n-3	2.0 ± 0.1	2.0 ± 0.1	1.9 ± 0.0	2.0 ± 0.1
20:4n-3	0.7 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.7 ± 0.0
20:5n-3	11.5 ± 0.6	11.2 ± 0.2	11.2 ± 0.3	11.7 ± 0.5
22:5n-3	1.5 ± 0.1	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.1
22:6n-3	11.5 ± 0.8	11.6 ± 0.3	11.5 ± 0.4	11.8 ± 0.7
Total n-3 PUFA[4]	28.3 ± 1.6	28.0 ± 0.6	27.6 ± 0.8	28.8 ± 1.4
Total PUFA	35.5 ± 1.7	35.2 ± 0.6	34.8 ± 0.8	35.9 ± 1.6
n-3/n-6	3.9 ± 0.2	3.9 ± 0.1	3.8 ± 0.1	4.1 ± 0.1

Values are means ± SD of 6 samples each of tissue pooled from 3 fish. Superscript letters denote significant differences between dietary treatments as determined by ANOVA.
[1], includes 20:1n-7 present up to 0.3 %; [2], includes 20:2n-6, 20:3n-6 and 22:4n-6 present up to 0.3 %; [3], includes 20:3n-3 present up to 0.3 %; FO, control diet containing fish oil alone; CLA1 and CLA2, diets supplemented with 0.5 and 1 % CLA; TTA, diet supplemented with 0.5 % TTA.

Table 5.8 Fatty acid composition (percentage of weight) of total lipid from flesh of Atlantic cod fed CLA or TTA.

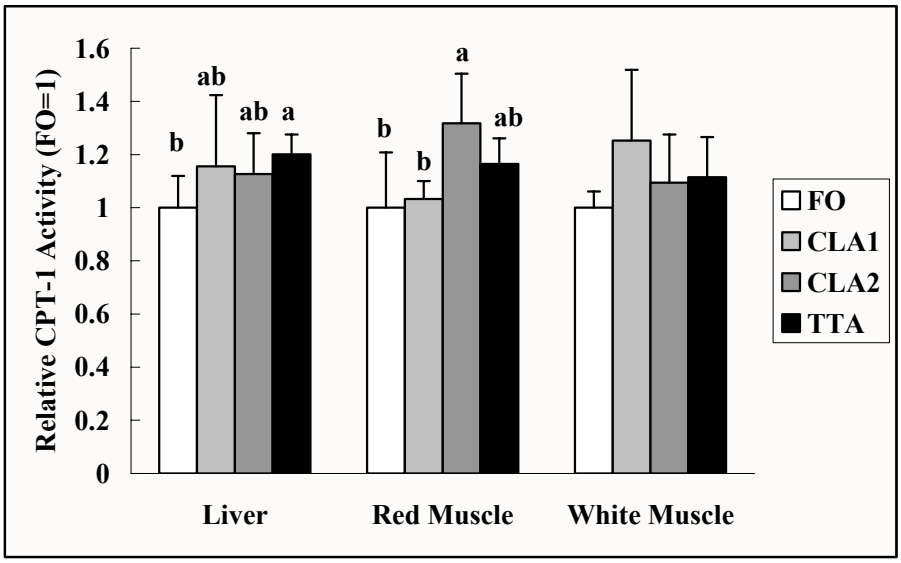
	FO	CLA1	CLA2	TTA
14:0	1.6 ± 0.2 [a]	1.3 ± 0.1 [b]	1.3 ± 0.1 [b]	1.0 ± 0.0 [c]
16:0	19.3 ± 0.8 [ab]	19.2 ± 0.8 [ab]	18.6 ± 0.5 [b]	20.1 ± 0.4 [a]
18:0	3.2 ± 0.1 [b]	4.0 ± 0.1 [a]	3.9 ± 0.2 [a]	3.4 ± 0.1 [b]
Total saturated[1]	24.4 ± 0.9	24.8 ± 0.8	24.2 ± 0.3	24.8 ± 0.5
16:1n-7	2.9 ± 0.3 [a]	2.6 ± 0.1 [ab]	2.4 ± 0.2 [b]	2.3 ± 0.2 [b]
18:1n-9	9.2 ± 0.1 [a]	8.8 ± 0.2 [ab]	8.5 ± 0.5 [b]	8.7 ± 0.4 [ab]
18:1n-7	2.9 ± 0.0 [a]	2.8 ± 0.1 [ab]	2.7 ± 0.1 [b]	2.7 ± 0.1 [b]
20:1n-9	1.0 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	1.0 ± 0.2
22:1n-11	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0
24:1n-9	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.1
Total monoenes[2]	16.7 ± 0.4 [a]	15.9 ± 0.3 [ab]	15.2 ± 0.8 [b]	15.4 ± 0.6 [b]
CLA (9c,11t)	0.0 ± 0.0 [c]	0.3 ± 0.1 [b]	0.7 ± 0.0 [a]	0.0 ± 0.0 [c]
CLA (10t,12c)	0.0 ± 0.0 [c]	0.5 ± 0.1 [b]	1.2 ± 0.1 [a]	0.0 ± 0.0 [c]
TTA	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	1.6 ± 0.3 [a]
18:2n-6	4.1 ± 0.1	4.0 ± 0.4	4.1 ± 0.3	3.9 ± 0.1
20:4n-6	1.9 ± 0.0 [a]	1.9 ± 0.1 [a]	1.9 ± 0.0 [a]	1.8 ± 0.0 [b]
22:5n-6	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0
Total n-6 PUFA[3]	7.1 ± 0.2 [a]	7.2 ± 0.3 [a]	7.0 ± 0.2 [a]	6.5 ± 0.1 [b]
18:3n-3	0.6 ± 0.0 [a]	0.6 ± 0.1 [a]	0.6 ± 0.0 [a]	0.5 ± 0.0 [b]
18:4n-3	0.9 ± 0.0 [a]	0.8 ± 0.1 [ab]	0.8 ± 0.1 [ab]	0.7 ± 0.0 [b]
20:4n-3	0.7 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.7 ± 0.0
20:5n-3	18.6 ± 0.3 [a]	18.0 ± 0.9 [a]	18.2 ± 0.5 [a]	16.4 ± 0.2 [b]
22:5n-3	2.2 ± 0.0 [a]	2.2 ± 0.1 [a]	2.2 ± 0.0 [a]	2.1 ± 0.0 [b]
22:6n-3	28.6 ± 0.6 [b]	28.9 ± 0.8 [b]	29.4 ± 1.3 [b]	31.0 ± 0.7 [a]
Total n-3 PUFA[4]	51.7 ± 0.9	51.3 ± 0.5	51.7 ± 0.7	51.4 ± 0.8
Total PUFA	58.9 ± 0.9	58.5 ± 0.8	58.7 ± 0.5	58.3 ± 0.6
n-3/n-6	7.2 ± 0.2 [b]	7.1 ± 0.2 [b]	7.4 ± 0.3 [b]	7.8 ± 0.2 [a]

Values are means ± SD of 6 samples each of tissue pooled from 3 fish. Superscript letters denote significant differences between dietary treatments as determined by ANOVA.
[1], includes 20:1n-7 present up to 0.3 %; [2], includes 20:2n-6, 20:3n-6 and 22:4n-6 present up to 0.3 %; [3], includes 20:3n-3 present up to 0.3%; FO, control diet containing fish oil alone; CLA1 and CLA2, diets supplemented with 0.5 and 1 % CLA; TTA, diet supplemented with 0.5 % TTA.

5.3.5 CPT-1 and ACO Activities, and Peroxisomal β-Oxidation Capacity

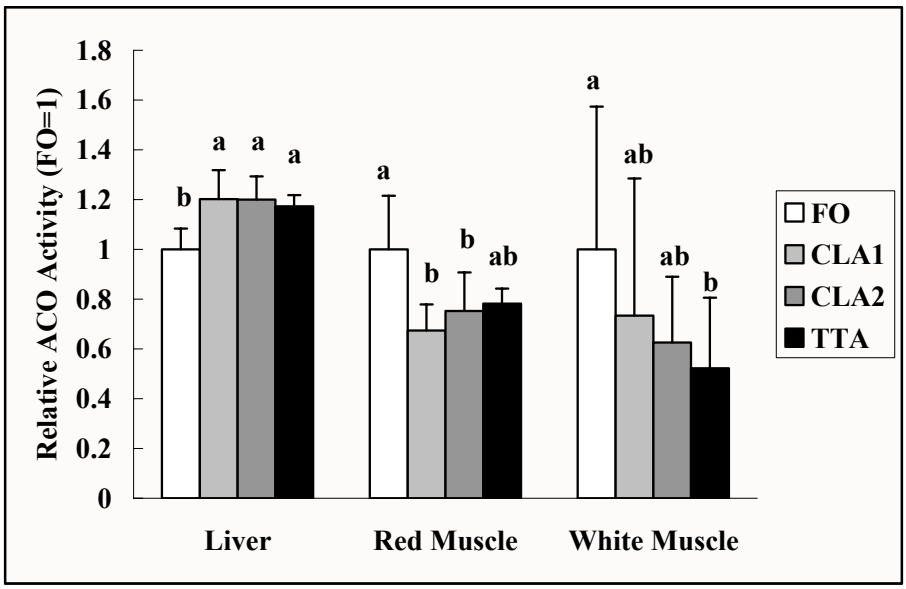
There was a trend for CPT-I activity in liver to be increased by dietary CLA and TTA, although it was only significant in the case of TTA (Figure 5.1). In red muscle, a dietary level of 1 % CLA resulted in increased CPT-1 activity, but 0.5 % dietary CLA or TTA had no effect. In contrast, CPT-1 activity in white muscle was not affected by any dietary treatment. ACO activity in liver was significantly increased by both dietary CLA and TTA (Figure 5.2). Conversely, ACO activity in both red and white muscle was decreased by CLA and TTA, with the effects being significant for CLA in red muscle and TTA in white muscle. In liver, there was a trend that indicated increased peroxisomal β-oxidation in cod fed dietary CLA, however this increase was not statistically significant. Dietary TTA significantly increased the capacity for hepatic peroxisomal β-oxidation (Figure 5.3). In muscle, the peroxisomal β-oxidation capacity of Atlantic cod was unaffected by either dietary CLA or TTA (Figure 5.3).

Figure 5.1 Effects of CLA or TTA on CPT-1 activity in tissue homogenates of liver, red and white muscle of Atlantic cod.



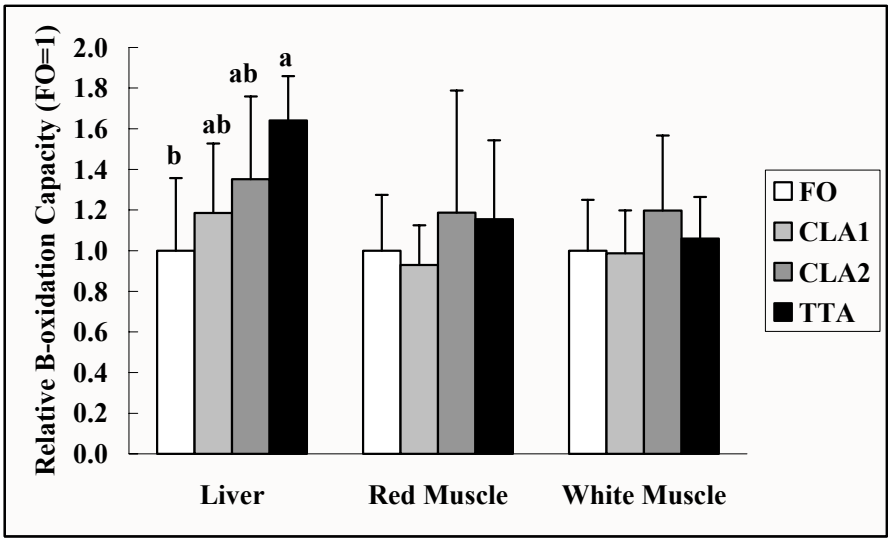
Results are presented relative to the activity in fish fed FO for each tissue and are means ± SD (n = 6). Different letters denote significant differences between dietary treatments within each tissue as determined by ANOVA.

Figure 5.2 Effects of CLA or TTA on ACO activity in tissue homogenates of liver, red and white muscle of Atlantic cod.



Results are presented relative to the activity in fish fed FO for each tissue and are means ± SD (n = 6). Different letters denote significant differences between dietary treatments within each tissue as determined by ANOVA.

Figure 5.3 Effects of CLA or TTA on peroxisomal β-oxidation capacity in tissue homogenates of liver, red and white muscle of Atlantic cod.



Results are presented relative to the activity in fish fed FO for each tissue and are means ± SD (n = 6). Different letters denote significant differences between dietary treatments within each tissue as determined by ANOVA.

5.3.6 Basic Haematology

There were no statistically significant differences in the red or white blood cell counts of fish fed either dietary CLA or TTA compared to fish fed FO alone (Table 5.9). However, there was a clear trend suggesting decreased blood cell counts with both dietary CLA and TTA, but the high variation between samples made this trend non-significant. The PCV was reduced in response to dietary TTA and CLA, significantly so in the former.

Table 5.9 Effects of conjugated linoleic acid (CLA) and tetradecylthioacetic acid (TTA) on basic haematology of Atlantic cod.

Diet	RBC (FO =1)	WBC (FO =1)	PCV (%)	
FO	1.00 ± 0.10	1.00 ± 0.80	30.6 ± 4.4	a
CLA1	0.76 ± 0.10	0.62 ± 0.29	25.7 ± 4.8	a
CLA2	0.81 ± 0.19	0.88 ± 0.43	24.8 ± 2.2	a
TTA	0.81 ± 0.27	0.80 ± 0.36	21.8 ± 4.2	b

RBC, red blood counts (relative to FO diet), WBC, white blood counts (relative to FO diet), PVC, packed cell volume.

5.4 Discussion

Atlantic cod deposit a larger proportion of lipid in liver compared to salmonids even when the former are fed a lower lipid level. This is a problem to cod farmers since deposition of fat in the liver represents diet that is "going to waste" by being deposited in a portion of the fish not eaten by the consumer, at least, not in the UK. Excessive fat deposition in liver may also lead to fatty liver syndrome and thus may affect the health of the fish. In cod, the liver is the primary excess fat store that sequesters lipid which is not immediately utilised as energy. Once lipid levels have reached a satisfactory level in cod flesh the remaining lipid is then directed to the liver. Thus, high fat diets rich in n-3HUFA may not be utilised fully. The biological implications of feeding animals dietary CLA and TTA have been discerned in both *in vitro* and *in vivo* work in mammalian models (Berge *et al*., 2002; Bhattacharya *et al*., 2006). Less clear is the effect these bioactive fatty acids have on piscine species, particularly of marine origin, and the present study is the first to investigate TTA in a marine fish. Some recent studies

involving CLA-supplemented rodents have elucidated that hepatic steatosis is decreased (Wang *et al*., 2005; Purushotham *et al*., 2007). Reductions in liver TAG content after CLA supplementation have been verified in rats (Rahman *et al*, 2002). In addition, the hypolipidaemic effect of TTA has been purported to be due in part, to decreased hepatic TAG formation (Willumsen *et al.*, 1997; Berge *et al*., 1999). Thus, the primary aim of the present trial was to test the hypothesis that dietary CLA or TTA could have beneficial effects on lipid metabolism in Atlantic cod, specifically that these bioactive fatty acids could lower liver lipid levels, and liver size. The results clearly show that the hypothesis was not proved and that neither of these bioactive fatty acids had a major effect on liver lipid content or relative liver size as determined by HSI in cod. It can be reasoned that, although there was a statistically significant decrease in HSI of cod fed TTA, this reduction was of relatively little physiological consequence considering the index was still over 10 % and there was no change in liver lipid content compared to fish fed FO alone. Conversely, Atlantic salmon smolts fed TTA at 0.6 % of the diet showed a slight, but significant, increase in HSI in addition to a higher liver lipid content, although the latter was not significant (Moya-Falcon *et al*., 2004). In the present trial, CLA inclusion did not effect HSI in the cod in agreement with other trials which showed that CLA at up to 1 % had no effect on HSI or liver lipid content in juvenile channel catfish (Twibell and Wilson, 2003), and HSI was unaffected by dietary CLA at up to 2 % in rainbow trout juveniles (Figueiredo-Silva *et al*., 2005). Conversely, increased HSI in response to feeding CLA had been previously reported in hybrid striped bass (Twibell *et al*., 2000), yellow perch (Twibell *et al*., 2001) and tilapia (Yasmin *et al*., 2004). Perhaps surprisingly, liver lipid content was reduced by dietary CLA in striped bass and yellow perch despite increased HSI (Twibell *et al*., 2000, 2001). In Atlantic salmon smolts there

were trends of increasing HSI and liver lipid in fish fed CLA at 1 and 2 % of the diet, although the data were not statistically significant (section 3.3.2). Interestingly, liver TAG content was significantly decreased in cod fed 1 % CLA in the present trial, however it is unlikely that a reduction in TAG had any bearing on liver physiology considering there was no change in liver lipid level or HSI.

As discussed in detail in section 1.4.2, dietary CLA can have beneficial effects on body composition by decreasing body fat and, in some cases, increasing lean body mass in mice, rats and pigs in particular. It would seem that these effects have not been reciprocated in the present study as evidenced through the lack of influence on whole body proximate composition in cod fed dietary CLA compared with cod fed FO alone. However, whole body proximate composition was also unaffected by dietary CLA in salmon fry (Berge *et al*., 2004) and smolts (section 3.3.3), and rainbow trout juveniles (Figueiredo-Silva *et al*., 2005). Similarly, dietary CLA had no effect on tissue lipid contents in tilapia (Yasmin *et al*., 2004) or carcass lipid and intraperitoneal fat in catfish (Twibell and Wilson, 2003). Furthermore, VSI was unaffected by dietary CLA up to 2 % in both Atlantic salmon smolts (section 3.3.2) and rainbow trout (Figueiredo-Silva *et al*., 2005). However, intraperitoneal fat was decreased by dietary CLA in hybrid striped bass (Twibell *et al*., 2000) and, in the present trial, visceral fat may have been reduced in cod fed 0.5 % CLA as VSI was lower in this group along with a relatively higher HSI, which may suggest some redistribution of fat. However, fat redistribution has not been apparent in other trials with fish. The present trial is the first to record the effects of dietary TTA on fish whole body composition. These data suggest that TTA does not beneficially influence body composition to any appreciable physiological extent. However, dietary TTA had been shown to prevent high fat diet induced adiposity in rats (Madsen *et al*.,

2002). Interestingly, the levels of TAG within flesh significantly increased in cod fed TTA in the present trial. It has been proposed that animals fed TTA may instigate a greater flux of TAG from serum to surrounding tissue thus providing additional fatty acids for catabolism and this may be the case in the cod (Berge *et al*., 2004).

An additional objective of this study was to determine whether CLA or TTA could influence fatty acid oxidation. Unfortunately, mitochondrial β-oxidation could not be analysed since assays using radioactive isotopes on fresh tissue were not possible at the commercial farm site. Thus, freezing and thawing of the samples disrupted the mitochondrial membranes such that only peroxisomal β-oxidation capacity could be measured. Nevertheless, it is noteworthy that fatty acid β-oxidation activity was recently measured in a related gadoid, haddock, and it was shown that peroxisomal β-oxidation predominates over mitochondrial β-oxidation in liver (Nanton *et al*., 2003). Similarly, fatty acid oxidation in Atlantic salmon liver is principally due to peroxisomal, rather than mitochondrial, β-oxidation (Frøyland *et al*., 2000). In the present study, hepatic peroxisomal β-oxidation capacity was significantly elevated in cod fed dietary TTA. Furthermore, both CPT-1 and ACO activities were significantly increased as a result of TTA supplementation. Taken together, these results strongly suggest that hepatic fatty acid oxidation is elevated in response to dietary TTA in Atlantic cod. This does not correlate with previous studies of TTA in fish, which revealed that both ACO gene expression and activity in liver of salmon were unaffected (Kleveland *et al*., 2006; Moya-Falcon *et al*., 2006). Contrary to the abovementioned results, CPT-1 activity in cod liver was unaffected by dietary CLA, suggesting that mitochondrial β-oxidation would not be enhanced. Perhaps surprisingly, an increase in ACO activity in liver of cod fed dietary CLA was not concomitant with elevated peroxisomal β-oxidation capacity. This lack of

association between peroxisomal β-oxidation capacity and ACO activity was also observed in muscle of cod fed either CLA or TTA ergo it is difficult to interpret these results. However, it can be assumed that both ACO activity and peroxisomal β-oxidation capacity are relatively inconsequential in gadoid muscle considering it is likely that fatty acid oxidation in the muscle predominantly occurs in mitochondria (Nanton *et al*., 2003). Previous studies have reported that dietary CLA can increase CPT-1 activity in a variety of murine tissues (Rahman *et al.*, 2001; Degrace *et al*., 2004). The CPT-1 activity in red muscle of cod fed 1 % CLA was significantly increased in the present study; indicative of an enhanced mitochondrial β-oxidation capacity. However, it is known that red muscle comprises only a small proportion of total flesh, the remainder of which is composed mostly of white muscle. Thus, red muscle may not influence lipid composition to any appreciable extent. Indeed, these data seem to indicate that white muscle CPT-1 activity, peroxisomal β-oxidation, and almost certainly overall fatty acid oxidation is not significantly influenced by dietary CLA or TTA in cod muscle.

A further aim of the present trial was to determine if dietary CLA or TTA had beneficial effects on fatty acid compositions in Atlantic cod. This part of the study revealed a very interesting result with TTA accumulating in flesh to a greater extent than the equivalent dietary level of CLA and also to a greater extent than in liver. In contrast, CLA was incorporated to a greater extent in liver lipids compared to flesh. TAG predominates in liver of cod whereas polar lipids predominate in flesh, and so these data suggest that TTA may be deposited to a greater extent in PL and CLA to a greater extent in TAG. In Atlantic salmon fed TTA, flesh fatty acid compositions were not reported but, of the tissues investigated, the highest incorporation was found in gills with approximately equal percentages, 0.8 % and 0.7 % of total fatty acids in PL and TAG,

respectively, in fish fed TTA at 0.6 % of total diet (Moya-Falcon *et al*., 2004). Furthermore, TTA in both heart and liver was only recovered in PL and not TAG. Consistent with the above, preferential uptake of TTA into PL was also evidenced in rat hepatocytes (Grav *et al.*, 1994; Madsen *et al.*, 1997). Thus it appears that bioactive fatty acids similar to, and including, TTA could be efficiently accumulated in 'lean" fish such as cod with low flesh oil contents. It remains to be established whether fatty acid analogues such as TTA are feasible or, indeed, appropriate as supplements for the human diet (Berge *et al*., 2002). However, CLA may be better delivered to humans via oily fish with the level accumulating in salmon and trout fed CLA at 2 % of diet by weight reaching 7 % of total fatty acids in flesh (dietary lipid 16 – 17 %), (section 3.3.5; Bandarra *et al*., 2006), or 4 % in flesh of salmon smolts (dietary lipid 34 %) (section 3.3.5), and 7 % in whole salmon fry fed 2 % CLA (24 % dietary lipid) (Berge *et al*., 2004). Similarly, striped bass with high flesh lipid (> 15 %) accumulated CLA to over 7 % of total fatty acids in fish fed CLA at 1 % of diet, whereas the levels of CLA accumulated in yellow perch with only 3 % lipid in the flesh were much lower (Twibell *et al*., 2000, 2001). Consistent with the above, the incorporation of CLA into neutral lipids was around 10-fold higher than incorporation into polar lipids in both muscle and liver in tilapia (Yasmin *et al*., 2004).

There is evidence to suggest that dietary CLA can suppress SCD activity and gene expression in mammals (Lee *et al.*, 1998; Choi *et al*., 2001, 2002; Park *et al.*, 2001; Smith *et al*., 2002; Shang *et al*., 2005). In support of the above findings, dietary CLA has also been reported to increase the 18:0/18:1n-9 ratio; a proxy for the measurement of SCD activity, in liver and flesh of rainbow trout (section 6.3.4), striped bass (Twibell *et al*., 2000), yellow perch (Twibell *et al*., 2001), salmon (Berge *et al*., 2004; section 3.3.5)

and sea bass (Valente *et al*., 2007b). Furthermore, dietary CLA increased the proportions of 18:0 and decreased percentages of 18:1 in liver, muscle and viscera of rainbow trout juveniles (Bandarra *et al*., 2006). In the present study, increased proportions of 18:0 and decreased 18:1n-9 was observed in flesh and, especially, liver of cod fed CLA suggesting inhibition of Δ9 desaturation. Purportedly, suppression of SCD may lead to a lower lipid droplet size in cells, which in turn could account in part for the anti-obesity effect exhibited in animals supplemented with CLA. However, experiments utilising SCD-null mice have shown that this is not how CLA arbitrates its fat lowering effect (Kang *et al*., 2004). Conversely, the data in the present trial indicate that TTA appears to have had no effect on SCD activity in Atlantic cod. Previously, dietary TTA was shown to have no major effects on 18:0/18:1n-9 levels in salmon liver, gill, heart (Moya-Falcon *et al*., 2004) or flesh lipids (Kleveland *et al*., 2006). However, *in vitro* studies utilising hepatic murine cell lines have recorded increased SCD expression after administration of TTA (Madsen *et al*., 1997; Vaagenes *et al*., 1998).

CLA suppresses PUFA desaturases and elongase in cell systems (Chuang *et al*., 2001a,b; Eder *et al*., 2002), and CLA decreased C_{18} PUFA in pig muscle and fat (Ramsay *et al*., 2001), and 22:6n-3 in chicken tissues (Yang *et al*., 2003). Additionally, there is some evidence to suggest that the influence of dietary CLA on n-3 PUFA distribution is tissue specific in fish. For example, dietary CLA at up to 2 % inclusion was at the expense of 20:5n-3 and 22:6n-3 in flesh of salmon smolts and hybrid striped bass (section 3.3.5; Twibell *et al.*, 2000; Leaver *et al*., 2006) and whole body (comprising mainly of flesh) in salmon fry (Berge *et al*., 2004). Similarly, 22:6n-3 levels were also depressed in muscle of juvenile rainbow trout fed 1 – 2 % CLA (Bandarra *et al*., 2005). Conversely, 20:5n-3 and 22:6n-3 levels have been shown to rise in liver of rainbow trout (section

6.3.4) and hybrid striped bass (Twibell *et al.*, 2000) supplemented with CLA. In the present study, CLA had no effect on PUFA levels in either liver or flesh of cod. This was perhaps not unexpected as the activity of the PUFA desaturation/elongation pathway is very low in cod (Bell *et al*., 2006). In contrast, however, dietary TTA significantly increased the percentage of 22:6n-3, and decreased the proportions of 20:5n-3 and total n-6 PUFA in flesh of cod in the present study, a phenomena also noted in Atlantic salmon liver after TTA supplementation (Moya-Falcon *et al*., 2004). This could be a result of increased conversion of 20:5n-3 to 22:6n-3, but there is no evidence from previous studies to support TTA having an effect on fatty acid Δ5/Δ6 desaturation or elongation (Berge *et al*., 2002). Therefore, it may be more likely due to specificity of β-oxidation, with 22:6n-3 being more resistant to oxidation than 20:5n-3 (Tocher, 2003). Indeed, reduction of hepatic 20:5n-3 levels in rats fed dietary TTA has been suggested to be due to selectively increased β-oxidation of this n-3 HUFA (Frøyland *et al*., 1997).

Although not consistently observed, some studies had suggested that CLA might enhance growth and feed efficiency in young rodents (Pariza *et al*., 2001). Thus, a further aim of the present trial was to determine if dietary CLA or TTA had beneficial effects on growth parameters in Atlantic cod. However, neither CLA nor TTA had any effect on growth (SGR, TGC) or feed efficiency (FCR) in the present trial. In recent studies on salmonids, no effects of dietary CLA on growth rates or FCR were observed in Atlantic salmon fry (Berge *et al*., 2004), or smolts (section 3.3.2), or in rainbow trout (Figueiredo-Silva *et al*., 2005) fed diets containing up to 2 % CLA. Similarly, no effects on weight gain or feed efficiency were noted in juvenile yellow perch or catfish fed diets containing up to 1 % CLA (Twibell *et al*., 2001; Twibell and Wilson, 2003), or in juvenile tilapia fed CLA at up to 5 % of diet (Yasmin *et al*., 2004). However, growth of

tilapia was inhibited by 10 % CLA, as was growth of carp and rockfish at both 5 and 10 % CLA (Choi *et al*., 1999). Therefore, the data are consistent in suggesting that dietary CLA does not have any beneficial effects on growth performance in a variety of fish species, and can inhibit growth at high inclusion levels. In contrast, dietary TTA inhibited growth in salmon smolts as evidenced by decreased final weights, SGRs and TGCs, although FCR was unaffected (Moya-Falcon *et al*., 2004).

An important result in the present study was that there were no mortalities in cod fed the TTA treatment. Previously, in addition to a reduction of growth (Moya-Falcon *et al*., 2004), TTA was also reported to cause an increase in mortality in salmon (Moya-Falcon *et al.*, 2004; Kleveland *et al*., 2006). Therefore, it was hoped that the present study would help to elucidate whether the observed rise in mortality in previous studies involving fish was due, in part, to a modulation of the innate immune response. An attempt was made to measure the rate of phagocytosis from isolated head kidney macrophages (section 2.4.5). Unfortunately the assay, which had to be completed entirely on-site at the commercial farm, did not work as the yeast failed to adhere to the slides, possibly due to the low ambient temperature in the laboratory in January. Moreover, lysozyme activity failed to produce a result using the methodology outlined in section 2.4.4, possibly for similar reasons. Thus, only basic haematological parameters were able to be tested. Nevertheless, the results proved interesting given that TTA decreased both red and white blood cell counts, and packed cell volume, significantly so in the latter. This may have implications on fish health as it implies that TTA may act as an immunodepressor. Obviously, a more thorough examiniation of the immunological aspects of dietary TTA with regards to Atlantic cod health is required before any conclusive judgment can be made.

In conclusion, the results of the present study only partially supported the hypotheses that were tested. CLA and TTA at the levels used had few beneficial effects in Atlantic cod and did not enhance growth parameters, or improve feed conversion or potential yield through decreased adiposity or liver lipid deposition. However, nutritional quality could be enhanced, and cod fed CLA and/or TTA could be beneficial in the human diet, through provision of bioactive fatty acids with no detrimental effects on n-3 PUFA levels.

CHAPTER 6. INFLUENCE OF DIETARY CLA AND TTA IN RAINBOW TROUT

6.1 Introduction

Rainbow trout are a fast growing, robust fish that can tolerate a wide variety of environmental conditions and can be easily weaned on to an artificial diet. Hence, this species has been an ideal candidate for aquaculture. Being intensively farmed since the latter half of the 20th century, culture of rainbow trout is expected to reach global production levels of circa 500,000 tonnes in the near future (FAO, 2007), making it one of the most intensively farmed diadromous fishes on the planet. As of 2002, rainbow trout aquaculture was practised in 64 countries, with the majority of production output located in Europe, Japan and North America (FAO, 2007). An integral part of the ethos of fish culture is the ability to be recognised as a consumer friendly alternative to wild fish. Research and development over the past few decades has seen this ideal largely achieved, however more work is still required. One of the most important facets of aquaculture research is the optimisation of feed formulations in order to improve both fish welfare and consumer satisfaction.

As previously highlighted in section 1.3.1, high lipid levels are utilised in commercial aquaculture diets in order to offset relatively expensive protein as a source of energy. In this way, protein can be 'spared' for synthesis of new tissue (Wilson, 1989; Bell, 1998). Although high lipid diets provide an invaluable energy source, they may also promote excessive fat deposition in tissues particularly, in the case of rainbow trout, in the flesh, which can affect the overall market quality of the fish (Sargent *et al*., 2002). Furthermore, an increase in the production of farmed fish over the last decade, juxtaposed

with a decline in finite sources of FO has prompted research into alternative sustainable feed formulations, primarily examining the potential for plant-derived oil replacement (Sargent *et al*., 2002; Tacon, 2003). However, it has been suggested that VO replacements may also impact on consumer welfare by reducing the amount of human health promoting n-3 HUFA found in oily fish such as rainbow trout (Tocher, 2003). The physiological and biochemical influences of potential dietary "functional fatty acids" are currently being assessed in an attempt to alleviate the deleterious consequences of feeding fish high lipid levels or alternative oil diets. Two of these prospective "functional" dietary supplements are the bioactive fatty acids, CLA and TTA.

Since the initiation of this project, only a handful of studies have been published detailing the influence of dietary CLA or TTA in salmonids, all of which focussed on relatively small sized fish (Moya-Falcon *et al*., 2004; Figueirdo-Silva *et al*., 2005; Kleveland *et al*., 2006; Leaver *et al*., 2006; Valente *et al*., 2007a). What can generally be concluded from these studies was that no appreciable increase in growth or decrease in lipid deposition (attributes associated with both CLA or TTA supplementation in studies involving mammalian models) have been observed after dietary supplementation with these fatty acids in salmonids. In a commercial context, one valuable result in studies involving dietary CLA or TTA supplementation in fish has been the deposition of these fatty acids in flesh, thus potentially providing a consumer friendly route through which provision of these bioactive fatty acids could be attained for humans. However, it has yet to be determined whether feeding either dietary CLA or TTA to fish at such an early juncture in their life-cycle would be economically viable. Furthermore, and most importantly, the effects of CLA or TTA on large rainbow trout, which are actively depositing lipid, have hitherto not been investigated and therefore it is unknown if these

bioactive fatty acids can influence growth or trout flesh lipid at an arguably more commercially important life-cycle stage.

The present study aimed to test the hypothesis that feeding dietary CLA or TTA to rainbow trout of near market size and weight could increase growth, decrease lipid deposition, and enhance fatty acid composition via an increase in n-3 HUFA levels and provision of CLA or TTA in flesh lipid in particular. Additionally, to examine the effects of CLA and TTA on lipid metabolism, in the expectation that one of the main biochemical *modus operandi* thought to be responsible for reductions in lipid deposition in similar studies investigating the effect of these fatty acids in mammals, namely an increase in fatty acid oxidation, would also be elicited in trout. Currently, there is no evidence for the mechanism of the previously observed increased mortality in salmon supplemented with TTA (Moya-Falcon *et al.*, 2004; Kleveland *et al.*, 2006;), or any information about whether it is as a result of an immunomodulatory response. Similarly, it is unclear whether dietary CLA can influence the immune response in rainbow trout of this size. Thus, an ancillary objective of this study was to determine the effect of CLA or TTA on some key non-specific immune parameters.

Thus, this study reports the influence of CLA and, for the first time, TTA, on growth and lipid composition and metabolism in large, near market-size rainbow trout grown in seawater. Trout of almost 440 g were fed for eight weeks on FM and FO diets containing either 0.5 % or 1 % CLA, or 0.5 % TTA and growth, feed efficiency, tissue lipid content, class and fatty acid compositions determined, along with activities of HUFA synthesis and key enzymes of fatty acid oxidation, and the expression of associated genes. A number of immune parameters including blood cell counts and lysozyme activity were also investigated.

6.2 Materials and Methods

6.2.1 Experimental Fish and Husbandry

The dietary trial was performed at BioMar ForsøgsStation, Hirtshals, Denmark, between March and May 2006. Stock, unpigmented rainbow trout (Skinnerup, Moeldrup, Denmark) acclimated to 20 ppt salinity, were randomly distributed between twelve indoor, round tanks of 1 m^3 volume (1.72 m diameter). The initial stocking density was 25 fish of average weight 437 ± 56 g (< 13 % CV) per tank (~10.5 kg/m^3). Water temperature was maintained at 15.5 ± 0.2 ^oC throughout the trial, with a light regime of 14L:10D. Four experimental diets were fed to triplicate tanks for 55 days, with the feeding regime based on restricted feeding at 100 % of BioMar recommended feeding tables. The calculated feed ration was offered to the fish for 8 h each day using automatic individual belt feeding units. In order to facilitate accurate calculations of feed intake and FCR, feed waste was collected daily via a lift-up system.

6.2.2 Experimental Diets

The experimental diets were formulated to satisfy the nutritional requirements of salmonid fish (National Research Council, 1993), and were formulated and manufactured by BioMar A/S, Brande, Denmark (Table 6.1).

Table 6.1 Formulations (percentage of dry ingredients) and proximate compositions (percentage of total diet) of experimental diets.

Component	FO	CLA1	CLA2	TTA
Fishmeal	41	41	41	41
Sunflower meal	10.7	10.7	10.7	10.7
Wheat Gluten	10	10	10	10
Legume seeds	14	14	14	14
Soya	4	4	4	4
Micronutrients	0.4	0.4	0.4	0.4
Fish Oil	22.5	21.7	20.8	22
CLA	0	0.8	1.7	0
TTA	0	0.0	0	0.5
Moisture	7.3 ± 0.2	7.5 ± 0.1	7.6 ± 0.1	7.5 ± 0.2
Lipid	27.7 ± 1.1	25.7 ± 0.5	24.8 ± 0.7	24.7 ± 1.9
Protein	43.5 ± 0.6	42.1 ± 0.2	42.6 ± 1.1	42.6 ± 0.6
Ash	8.4 ± 0.3	8.4 ± 0.0	8.4 ± 0.3	8.5 ± 0.1

Results for proximate compositions are means ± S.D. (n = 3). FO, control diet containing fish oil alone; CLA1 and CLA2, diets supplemented with 0.5 and 1 % CLA; TTA, diet supplemented with 0.5 % TTA. There were no significant differences in proximate compositions between the diets. Micronutrients, incudes essential amino acids (methionine and lysine), vitamins, minerals, and astaxanthin (LucantinØ Pink, BASF), Biomar A/S, Brande, Denmark

Moisture, oil, protein and ash contents of diets were determined by standard methods (AOAC, 2000). The isonitrogenous diets were based on FM and standard Northern hemisphere FO with CLA and TTA added to 0, 0.5 and 1 % (CLA), and 0.5 % (TTA) as percentages of total diet. Diets were identical in formulation other than fatty acid composition with CLA (LUTA-CLA^{TM} 60, containing 60 % CLA methyl esters as a 50:50 mixture of c9t11 and t10c12 isomers; BASF AG, Ludwigshafen, Germany) and TTA (supplied by Dr Rolf Berge, Thia Medica A.S., Bergen, Norway) balanced by FO (capelin oil, Norsemeal Ltd., London, UK). The fatty acid compositions of the diets are presented in Table 6.2.

Table 6.2 Fatty acid compositions (percentage of weight) of experimental diets containing CLA and TTA.

	FO	CLA1	CLA2	TTA
14:0	7.8 ± 0.2	7.7 ± 0.2	7.4 ± 0.2	7.9 ± 0.3
15:0	0.7 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.7 ± 0.0
16:0	20.2 ± 0.1	20.2 ± 0.3	19.6 ± 0.4	20.4 ± 0.5
18:0	4.2 ± 0.1	4.2 ± 0.1	4.2 ± 0.1	4.2 ± 0.1
Total saturated[1]	33.3 ± 0.3	33.1 ± 0.6	32.2 ± 0.8	35.5 ± 0.9
16:1n-7	8.3 ± 0.2 [a]	8.1 ± 0.0 [a]	7.8 ± 0.0 [b]	8.2 ± 0.1 [a]
18:1n-9	8.3 ± 0.0 [c]	8.8 ± 0.1 [b]	9.4 ± 0.0 [a]	8.1 ± 0.1 [d]
18:1n-7	3.2 ± 0.1	3.2 ± 0.0	3.1 ± 0.1	3.3 ± 0.0
20:1n-9	1.5 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.1
22:1n-11	1.7 ± 0.2	1.7 ± 0.1	1.7 ± 0.0	1.7 ± 0.1
24:1n-9	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
Total monoenes[2]	23.9 ± 0.4	23.9 ± 0.3	24.2 ± 0.1	23.5 ± 0.3
CLA (9c,11t)	0.0 ± 0.0 [c]	0.9 ± 0.1 [b]	2.0 ± 0.2 [a]	0.0 ± 0.0 [c]
CLA (10t,12c)	0.0 ± 0.0 [c]	0.9 ± 0.1 [b]	2.0 ± 0.2 [a]	0.0 ± 0.0 [c]
TTA	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	1.9 ± 0.1 [a]
18:2n-6	3.7 ± 0.1	3.6 ± 0.0	3.8 ± 0.0	3.6 ± 0.0
20:4n-6	1.3 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0
Total n-6 PUFA[3]	6.3 ± 0.2	6.0 ± 0.1	6.0 ± 0.0	5.8 ± 0.1
18:3n-3	1.1 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.1 ± 0.0
18:4n-3	2.8 ± 0.0	2.6 ± 0.0	2.6 ± 0.0	2.7 ± 0.0
20:4n-3	0.9 ± 0.1	1.0 ± 0.3	0.7 ± 0.0	0.7 ± 0.0
20:5n-3	15.3 ± 0.3 [a]	14.9 ± 0.1 [ab]	14.4 ± 0.1 [b]	15.2 ± 0.2 [a]
22:5n-3	1.8 ± 0.0	1.7 ± 0.0	1.6 ± 0.0	1.7 ± 0.0
22:6n-3	14.2 ± 0.2 [a]	13.6 ± 0.2 [bc]	13.2 ± 0.2 [c]	13.8 ± 0.3 [ab]
Total n-3 PUFA[4]	36.4 ± 0.3 [a]	35.1 ± 0.1 [b]	33.7 ± 0.3 [c]	35.3 ± 0.5 [b]
Total PUFA	42.7 ± 0.3 [a]	41.1 ± 0.1 [b]	39.7 ± 0.3 [c]	41.1 ± 0.6 [b]
n-3/n-6	5.8 ± 0.2 [bc]	5.9 ± 0.1 [ab]	5.6 ± 0.0 [c]	6.1 ± 0.0 [a]

Values are ± S.D. (n = 3). Superscript letters denote significant differences between the diets as determined by ANOVA. [1], includes 20:0 present at up to 0.3 %; [2], includes 20:1n-7 present at up to 0. 3%; [3], includes 18:3n-6, 20:2n-6, 20:3n-6 and 22:4n-6 present at up to 0.4 %; [4], includes 20:3n-3 present up to 0.2 %.

6.2.3 Sampling and Analysis

At the initiation and termination of the trial, all the fish in each tank were anaesthetised with benzocaine (50 mg/L), and weighed individually. At the end of the trial, 8 fish per tank (24 per dietary treatment) were sampled, eviscerated and biometric parameters (hepato-, and viscero-somatic indices) determined. Before the samples were eviscerated and used for growth and biometric analyses, blood was removed from the fish as described in section 2.4.1. Liver and flesh samples (Flesh Quality Cut) were taken from six fish per tank, pooled in two samples of 3 fish each, and frozen immediately in liquid nitrogen (livers) or dry ice (flesh). Samples of 0.5 g of liver and red and white muscle from the epaxial myotomes anterior to the first dorsal fin ray were rapidly dissected from the remaining six fish (two per tank) and immediately frozen in liquid nitrogen for molecular analyses. In addition, samples of 1 - 2 g of liver, white and red muscle for biochemical analyses were collected from the same fish and immediately frozen in liquid nitrogen. All samples were subsequently stored at –80 ^{o}C prior to analyses

Blood cell counts and haematocrit measurements were carried out as described in sections 2.4.2 and 2.4.3 respectively. Lysozyme activity was measured as detailed in section 2.4.4. Total lipids of liver, muscle and diet extracted and quantified as described in section 2.3.7 and lipid class analysis was carried out as detailed in section 2.3.8. Samples taken for biochemical analysis were used to estimate peroxisomal β-oxidation capacity (section 2.3.3). CPT-1 and ACO activities were also determined as described in sections 2.3.4 and 2.3.5, respectively.

Total RNA from liver, red and white muscles was isolated, DNase treated and reverse transcribed into cDNA as described previously in sections 2.5.1 and 2.5.2. PCR

primers were designed according to the trout cDNA sequences for CPT-I (accession no. AF327058), fatty acyl Δ6 desaturase (accession no. AF301910) and fatty acyl elongase (accession no. AY605100) as outlined in Table 6.3. Three reference genes, elongation factor-1α (EF1α), β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were used to normalise the expression data. Primer sequences and PCR product sizes are given in Table 6.3.

Table 6.3 Sequences of Primers and PCR product sizes.

Gene	Accession No.	Primer	Sequence	Product size (bp)
CPT-I	AF327058	Forward	GCGCTATTCGACCAAAAAGA	133
		Reverse	CTAGTCGTGACCAGCCGTTT	
Desaturase	AF301910	Forward	ACCTAGTGGCTCCTCTGGTC	119
		Reverse	CAGATCCCCTGACTTCTTCA	
Elongase	AY605100	Forward	GAACAGCTTCATCCATGTCC	149
		Reverse	TGACTGCACATATCGTCTGG	
Elongation factor 1α	AF498320	Forward	GAATTCTCCTCCCACAGGAT	119
		Reverse	ACGATGGGTTTTAATCAGCA	
Actin	AJ438158	Forward	CAAGCAGGAGTACGACGAGT	110
		Reverse	CTGAAGTGGTAGTCGGGTGT	
GAPDH	AB066373	Forward	GTCTCAGTGGTGGACCTGAC	149
		Reverse	GCCGTTGAAGTCTGAAGAGA	

All genes are from rainbow trout.
CPT-I, carnitine palmitoyltransferase-I; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Quantitative real-time PCR was subsequently carried out essentially as described in section 2.5.5. The suitability of all three reference genes was determined via pair-wise correlation analysis using the BestKeeper© software (Pfaffl *et al*., 2004). Sample specific PCR efficiency (E) was determined using comparative quantitation analysis as part of the Rotor-Gene software. This negates the need for a serial dilution curve of pooled template

cDNA and provides a more robust representation of the overall PCR efficiency. The data were subjected to a pair-wise fixed reallocation randomization test© (10000 randomisations) in order to determine significance between treatments as facilitated by Relative Expression Software Tool – Rotor-Gene (REST-RG© - version 3) (Pfaffl, *et al*., 2002).

6.2.4 Statistical Analysis

All data are presented as means ± S.D (n value as stated). Percentage data and data which were identified as non-homogeneous (Bartlett's test) were subjected to arcsine transformation before analysis. Other than for gene expression (see above), the effects of dietary CLA and TTA were determined by one-way analysis of variance (ANOVA) with Tukey's post-tests to determine significance of differences due to functional fatty acids. Differences were regarded as significant when $P < 0.05$ (Zar, 1999).

6.3 Results

6.3.1 Dietary Inclusions

The control FO diet contained around 43 % total PUFA including 15 % 20:5n-3, 14 % 22:6n-3 and almost 4 % 18:2n-6, 33 % total saturates, mainly 16:0, and 24 % total monoenes, mainly 18:1n-9 and 16:1n-7 (Table 6.2). Inclusion of CLA in the diets resulted in levels of total CLA of 1.8 % and 4.0 % of total fatty acids at the 0.5 and 1 % inclusion levels, respectively, and inclusion of 0.5 % TTA resulted in TTA at a level of 1.9 % of total fatty acids in the diet. CLA and TTA inclusion resulted in lower levels of n-3 and total PUFA and increased levels of 18:1n-9. In addition, inclusion of 1 % dietary CLA also decreased the proportions of 20:5n-3, 22:6n-3 and 16:1n-7.

6.3.2 Growth and Biometry

There were no significant differences in final weight, growth rate (SGR), feed efficiency (FCR), gutted weight and condition factor in trout fed CLA or TTA compared to fish fed the control diet (Table 6.4). Similarly, viscero- and hepato-somatic indices were unaffected by dietary treatment. Pigmentation, measured using the Minolta light meter system, was also unaltered in response to dietary CLA or TTA treatment (Table 6.4).

Table 6.4 Growth and biometric parameters of rainbow trout fed diets containing CLA and TTA.

	FO	CLA1	CLA2	TTA
Initial weight (g)	429 ± 17	437 ± 2	450 ± 8	433 ± 12
Final weight (g)	755 ± 46	760 ± 7	777 ± 15	762 ± 21
SGR	1.06 ± 0.07	1.08 ± 0.07	1.02 ± 0.02	1.05 ± 0.03
FCR	0.99 ± 0.06	0.95 ± 0.08	1.03 ± 0.02	0.98 ± 0.01
Gutted weight (%)	83.7 ± 0.8	84.3 ± 0.7	84.6 ± 0.7	83.3 ± 0.6
Condition factor (K)	1.58 ± 0.02	1.58 ± 0.02	1.62 ± 0.04	1.58 ± 0.03
HSI	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.5 ± 0.1
VSI	17.0 ± 1.1	15.7 ± 0.7	15.4 ± 0.7	16.7 ± 0.6
Pigmentation	12.6 ± 0.4	12.8 ± 0.5	12.8 ± 0.3	12.6 ± 0.4
Mortality (n)	0	0	0	0

All data are means ± S.D. with triplicate tanks being experimental units (n=3). There were no significant effects of dietary treatment as measured by ANOVA. Condition factor (K) = (wet weight in g) x 100)/(length in mm)^3 x 1000; FCR, feed conversion ratio; TGC, thermal growth coefficient; HSI, hepato-somatic index; SGR, specific growth rate; VSI, viscero-somatic index.

6.3.3 Lipid Content and Class Composition

The hepatic lipid content of trout was significantly decreased in response to dietary CLA, but was unaffected in fish fed dietary TTA (Table 6.5). There were no significant effects on lipid class composition in either liver or flesh of trout fed CLA or TTA compared to FO alone. However, there was a trend indicating a decrease in total neutral lipids in liver of trout fed CLA. In addition, flesh lipid content did not change in response to dietary treatment, as determined by 1-way ANOVA (Table 6.5).

Table 6.5 Lipid content (percentage of weight) and polar/neutral lipid compositions (percentage of total lipid) of liver and flesh of rainbow trout fed CLA or TTA.

Lipid class	FO	CLA 1	CLA2	TTA
Liver				
Lipid content	4.5 ± 0.1 [a]	3.7 ± 0.3 [c]	3.9 ± 0.4 [bc]	4.3 ± 0.4 [ab]
Total polar	37.6 ± 2.0	40.2 ± 3.7	41.3 ± 3.0	42.3 ± 2.6
Total neutral	62.4 ± 2.0	59.8 ± 3.7	58.7 ± 3.0	57.7 ± 2.6
Flesh				
Lipid content	7.2 ± 0.6	7.2 ± 0.3	7.7 ± 0.5	8.2 ± 1.0
Total polar	12.4 ± 3.3	12.3 ± 1.9	11.2 ± 0.9	9.5 ± 1.1
Total neutral	87.6 ± 3.3	87.7 ± 1.9	88.8 ± 0.9	90.5 ± 1.1

Values are means ± S.D of 6 samples each of tissue pooled from 3 fish. Superscript letters denote significant differences between dietary treatments as determined by ANOVA.

6.3.4 Tissue Fatty Acid Composition

Dietary CLA and TTA were incorporated into both liver and flesh of rainbow trout with the bioactive fatty acids deposited at higher levels in flesh compared to liver (Tables 6.6 & 6.7). The total levels of CLA deposited in liver were 0.6 and 1 % of the total fatty acids at inclusion levels of 0.5 % and 1 % of the diet, respectively, whilst accumulation of TTA reached 0.6 % of total fatty acids at 0.5% dry weight of the diet (Table 6.7). In flesh, levels of total CLA accounted for 1.1 and 1.6 % of the total fatty acids in fish fed 0.5 and 1 %, respectively (Table 6.6). The proportion of TTA as a percentage of total fatty acid reached 1.2 % in flesh of fish fed 0.5 % dietary TTA. Both CLA and TTA significantly increased the proportions of hepatic n-3 PUFA, total PUFA and 20:4n-6 in liver (Table 6.7). Both 20:5n-3 and 22:6n-3 tended to be higher in fish fed CLA or TTA, however this only reached statistical significance with 20:5n-3 in fish fed 1 % CLA. Total saturated fatty acids and 18:0 were increased by CLA whereas total monoenes, particularly 18:1n-9 and 16:1n-7, were reduced in livers of fish fed both CLA and TTA (Table 6.6). Similarly in flesh, 18:0 was significantly increased and 18:1n-9 decreased in fish fed dietary CLA in particular (Table 6.7). It is noteworthy that in flesh, neither CLA nor TTA were deposited at the expense of n-3 HUFA.

Table 6.6 Fatty acid composition (percentage of weight) of total lipid from flesh of rainbow trout fed CLA or TTA.

	FO	CLA1	CLA2	TTA
14:0	5.9 ± 0.4	5.6 ± 0.3	5.8 ± 0.2	5.9 ± 0.4
15:0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0
16:0	19.1 ± 0.6	18.9 ± 0.4	18.9 ± 0.4	18.9 ± 0.4
18:0	4.4 ± 0.3 [b]	5.3 ± 0.2 [a]	5.0 ± 0.5 [a]	4.4 ± 0.1 [b]
Total saturated	30.0 ± 1.2	30.2 ± 0.7	30.2 ± 0.8	29.8 ± 0.7
16:1n-7	7.2 ± 0.4	6.8 ± 0.1	6.9 ± 0.2	7.0 ± 0.2
18:1n-9	13.4 ± 0.6 [a]	12.4 ± 0.4 [b]	12.7 ± 0.2 [b]	12.8 ± 0.6 [ab]
18:1n-7	3.5 ± 0.1	3.5 ± 0.1	3.4 ± 0.1	3.6 ± 0.1
20:1n-9	2.8 ± 0.3 [ab]	2.5 ± 0.3 [b]	3.1 ± 0.2 [a]	2.8 ± 0.3 [ab]
22:1n-11	2.5 ± 0.4 [ab]	2.2 ± 0.3 [b]	2.9 ± 0.2 [a]	2.4 ± 0.4 [ab]
24:1n-9	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1
Total monoenes[1]	30.1 ± 0.5 [a]	28.1 ± 0.9 [b]	29.7 ± 0.5 [a]	29.3 ± 0.8 [a]
CLA (9c,11t)	0.0 ± 0.0 [c]	0.6 ± 0.1 [b]	0.9 ± 0.2 [a]	0.0 ± 0.0 [c]
CLA (10t,12c)	0.0 ± 0.0 [c]	0.5 ± 0.1 [b]	0.7 ± 0.1 [a]	0.0 ± 0.0 [c]
TTA	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	1.2 ± 0.1 [a]
18:2n-6	4.8 ± 0.8	5.5 ± 0.7	4.6 ± 0.3	5.4 ± 0.9
20:4n-6	0.9 ± 0.0	1.0 ± 0.0	0.9 ± 0.0	0.9 ± 0.0
Total n-6 PUFA[2]	6.7 ± 0.8	7.5 ± 0.7	6.4 ± 0.3	7.0 ± 0.9
18:3n-3	1.2 ± 0.2	1.2 ± 0.0	1.2 ± 0.1	1.3 ± 0.1
18:4n-3	1.5 ± 0.1	1.4 ± 0.1	1.6 ± 0.1	1.5 ± 0.1
20:4n-3	1.3 ± 0.1	1.3 ± 0.1	1.1 ± 0.2	1.4 ± 0.2
20:5n-3	8.7 ± 0.3	8.7 ± 0.2	8.4 ± 0.2	8.5 ± 0.2
22:5n-3	2.9 ± 0.1	2.9 ± 0.0	2.7 ± 0.1	3.0 ± 0.2
22:6n-3	17.3 ± 1.1	17.5 ± 0.4	16.8 ± 0.5	16.8 ± 0.6
Total n-3 PUFA[3]	33.2 ± 1.2	33.2 ± 0.3	32.0 ± 0.8	32.8 ± 0.7
Total PUFA	39.9 ± 1.1 [a]	40.7 ± 0.8 [a]	38.5 ± 0.5 [b]	39.7 ± 0.7 [ab]
n-3/n-6	5.0 ± 0.7	4.5 ± 0.4	5.0 ± 0.3	4.8 ± 0.6

Values are means ± SD of 6 samples each of tissue pooled from 3 fish.
[1], includes 20:1n-7 present up to 0.3 %; [2], includes 20:2n-6, 20:3n-6 and 22:4n-6 present up to 0.3 %; [3], includes 20:3n-3 present up to 0.3 %.

Table 6.7 Fatty acid composition (percentage of weight) of total lipid from liver of rainbow trout fed CLA or TTA.

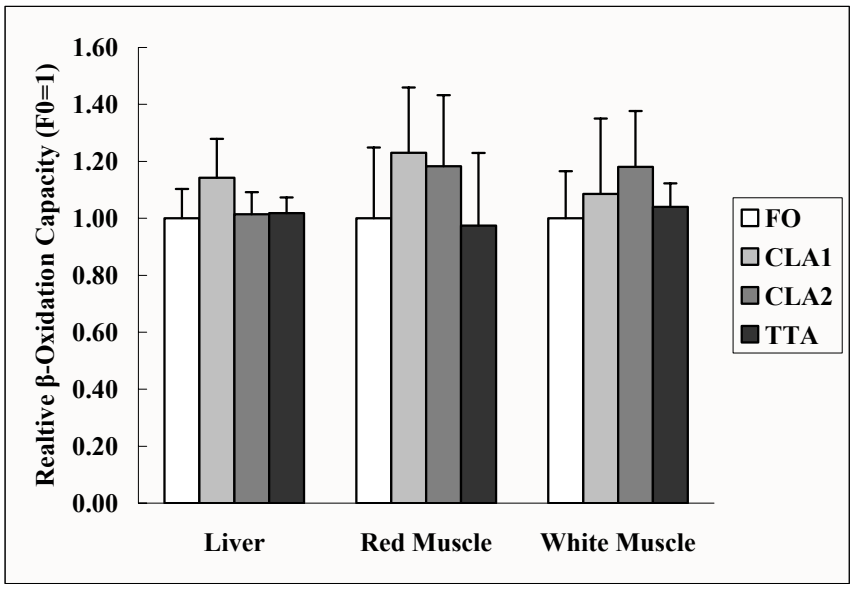
	FO	CLA1	CLA2	TTA
14:0	2.0 ± 0.2	1.8 ± 0.2	1.8 ± 0.1	1.8 ± 0.1
16:0	13.1 ± 1.1 [a]	12.6 ± 1.0 [a]	12.2 ± 0.5 [ab]	10.8 ± 1.1 [b]
18:0	8.2 ± 0.3 [d]	13.4 ± 0.5 [b]	14.8 ± 0.5 [a]	9.6 ± 0.4 [c]
Total saturated[1]	23.7 ± 1.3 [b]	28.3 ± 1.1 [a]	29.3 ± 0.5 [a]	22.6 ± 1.4 [b]
16:1n-7	4.7 ± 0.4 [a]	2.8 ± 0.3 [bc]	2.6 ± 0.2 [c]	3.2 ± 0.3 [b]
18:1n-9	17.7 ± 2.0 [a]	10.8 ± 1.8 [bc]	9.0 ± 0.9 [c]	12.2 ± 1.6 [b]
18:1n-7	3.4 ± 0.1 [a]	2.7 ± 0.2 [b]	2.6 ± 0.1 [b]	3.5 ± 0.2 [a]
20:1n-9	2.3 ± 0.3 [a]	1.9 ± 0.3 [b]	1.5 ± 0.1 [c]	2.6 ± 0.2 [a]
24:1n-9	0.4 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
Total monoenes[2]	29.0 ± 2.2 [a]	19.1 ± 2.2 [c]	16.8 ± 1.2 [c]	22.5 ± 2.2 [b]
CLA (9c,11t)	0.0 ± 0.0 [c]	0.4 ± 0.1 [b]	0.7 ± 0.0 [a]	0.0 ± 0.0 [c]
CLA (10t,12c)	0.0 ± 0.0 [c]	0.2 ± 0.0 [b]	0.4 ± 0.0 [a]	0.0 ± 0.0 [c]
TTA	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	0.9 ± 0.1 [a]
18:2n-6	1.7 ± 0.1 [ab]	1.5 ± 0.1 [c]	1.6 ± 0.1 [bc]	1.8 ± 0.1 [a]
20:4n-6	2.2 ± 0.2 [b]	3.0 ± 0.4 [a]	2.9 ± 0.2 [a]	3.1 ± 0.4 [a]
22:4n-6	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
Total n-6 PUFA[3]	5.2 ± 0.4 [b]	6.0 ± 0.5 [a]	5.8 ± 0.3 [ab]	5.8 ± 0.3 [ab]
18:3n-3	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.1	0.6 ± 0.0
20:4n-3	0.6 ± 0.1 [b]	0.6 ± 0.0 [b]	0.7 ± 0.1 [b]	0.9 ± 0.2 [a]
20:5n-3	9.4 ± 0.4 [b]	10.2 ± 0.7 [ab]	10.5 ± 0.5 [a]	9.6 ± 0.5 [b]
22:5n-3	3.7 ± 0.4 [b]	3.6 ± 0.4 [b]	3.6 ± 0.3 [b]	4.9 ± 0.7 [a]
22:6n-3	27.7 ± 1.2	31.0 ± 1.8	31.5 ± 1.6	31.8 ± 1.0
Total n-3 PUFA[4]	42.2 ± 1.8 [b]	46.1 ± 1.9 [a]	47.0 ± 1.4 [a]	48.2 ± 1.0 [a]
Total PUFA	47.3 ± 2.1 [b]	52.0 ± 1.9 [a]	52.9 ± 1.5 [a]	53.9 ± 1.1 [a]
n-3/n-6	8.2 ± 0.4	7.8 ± 0.7	8.1 ± 0.3	8.4 ± 0.5

Values are means ± S.D. of 6 samples each of tissue pooled from 3 fish. Superscript letters denote significant differences between dietary treatments as determined by ANOVA.[1], includes 15:0 and 20:0 present is some samples at up to 0.2 %; [2], includes 20:1n-7 and 22:1n-11 present in some samples at up to 0.3 %; [3], includes 20:2n-6 and 20:3n-6 present in some samples at up to 0.4 %; [4], includes 18:4n-3 and 20:3n-3 present in some samples at up to 0.2 %.

6.3.5 Peroxisomal β-Oxidation Capacity, CPT-1 and ACO Activities

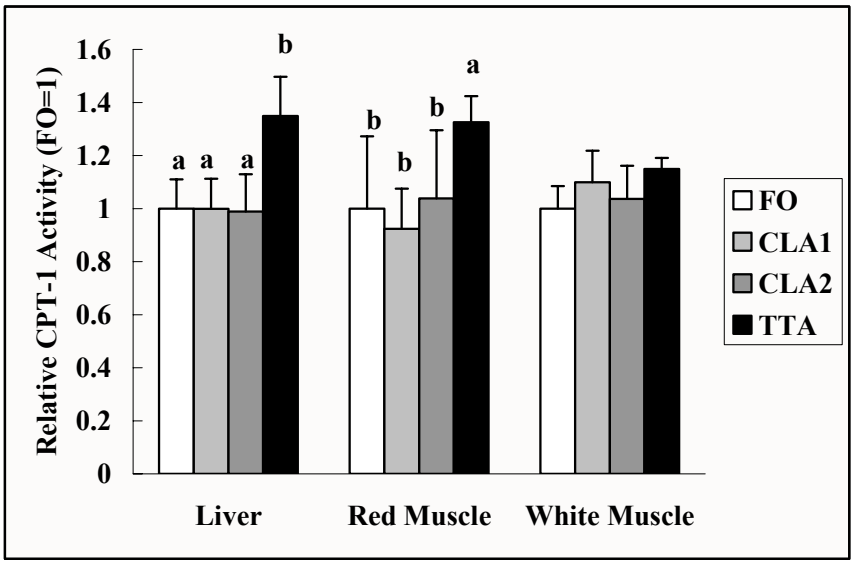
Peroxisomal fatty acid β-oxidation capacity was not affected in fish fed CLA or TTA (Figure 6.1). There was a trend that suggested an increase in metabolic flux of palmitate in muscle of fish fed CLA, however the deviation between samples made the pattern non-significant. Dietary CLA had no effect on CPT-1 activity in any of the three tissues, liver, white and red muscle, relative to fish fed the control FO diet (Figure 6.2). In white muscle, CPT-1 activity was also unaffected by dietary TTA. However, there was a significant increase in CPT-1 activity in liver and red muscle of trout fed the diet containing 0.5 % TTA. ACO activity mirrored CPT-1 activity in all tissues of trout fed TTA such that liver and red muscle ACO activity significantly increased whereas white muscle ACO activity was unaffected (Figure 6.3). There was a trend for dietary CLA to increase ACO in liver and red muscle, but this effect was only significant in red muscle in fish fed the higher level of CLA. The overall flux through the HUFA synthesis pathway, as measured by summing all the desaturated products of 18:3n-3, was unaffected by dietary CLA or TTA (Figure. 6.4). However, both 1 % CLA and 0.5 % TTA significantly increased the amount of radioactivity recovered in the hexaene fraction of the total radioactivity recovered from hepatic microsomes incubated with radiolabelled 18:3n-3. Conversely, the amount of radioactivity from labelled 18:3n-3 recovered in the pentaene fraction was significantly less in CLA-treated fish compared with controls.

Figure 6.1 Effects of CLA or TTA on peroxisomal β-oxidation capacity in tissue homogenates of liver, red and white muscle of rainbow trout.



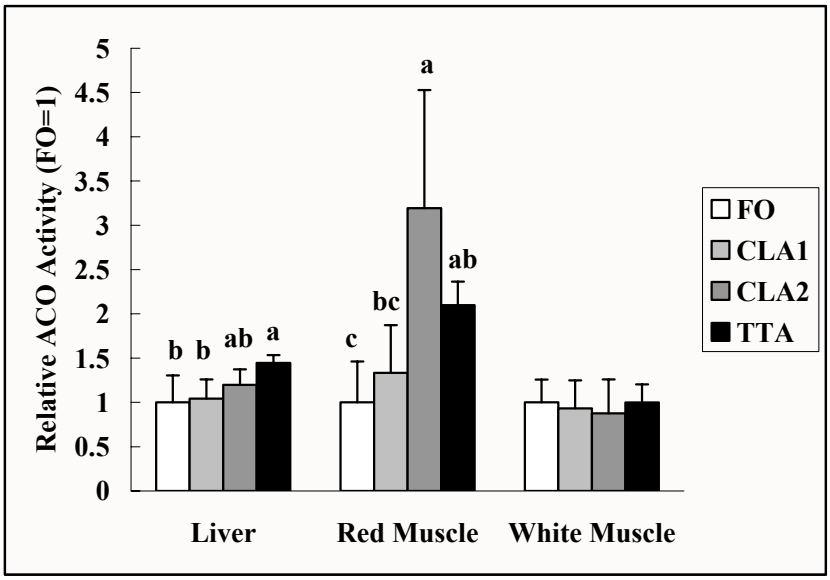
Results are presented relative to the activity in fish fed FO for each tissue and are means ± SD (n = 6). There were no statistically significant differences between dietary treatments within each tissue.

Figure 6.2 Effects of CLA or TTA on CPT-1 activity in tissue homogenates of liver, red and white muscle of rainbow trout.



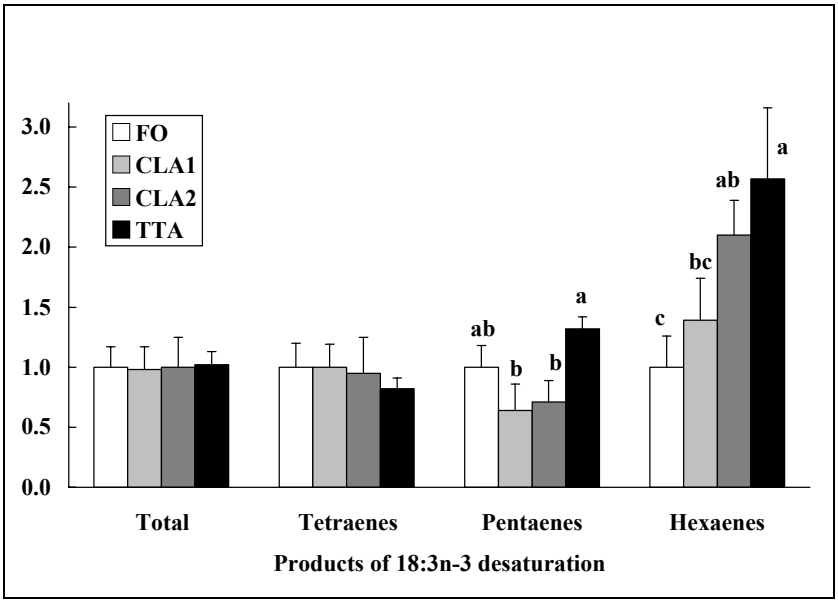
Results are presented relative to the activity in fish fed FO for each tissue and are means ± SD (n = 6). Different letters denote significant differences between dietary treatments within each tissue.

Figure 6.3 Effects of CLA or TTA on ACO activity in tissue homogenates of liver, red and white muscle of rainbow trout



Results are presented relative to the activity in fish fed FO for each tissue and are means ± SD (n = 6). Different letters denote significant differences between dietary treatments within each tissue.

Figure 6.4 Effects of dietary oil content and conjugated linoleic acid (CLA) and tetradecylthioacetic acid on highly unsaturated fatty acid (HUFA) synthesis in the liver microsomes

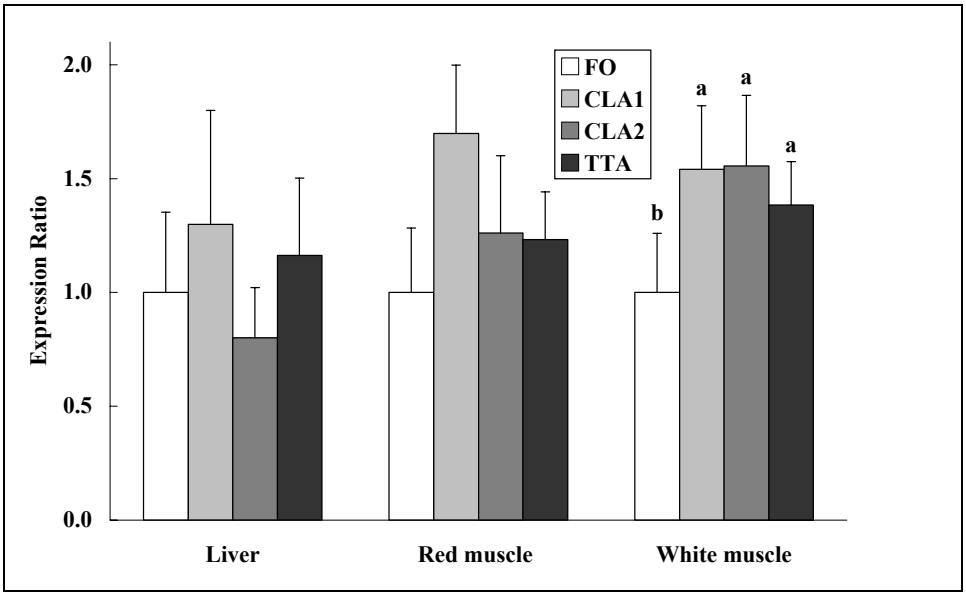


Results are presented relative to the activity in fish fed FO for each tissue and are means ± SD (n = 6). Different letters denote significant differences between dietary treatments within each tissue

6.3.6 Gene Expression of CPT-1in Liver, Red and White Muscles, and Fatty Acyl Δ6 Desaturase and PUFA Elongase in Liver

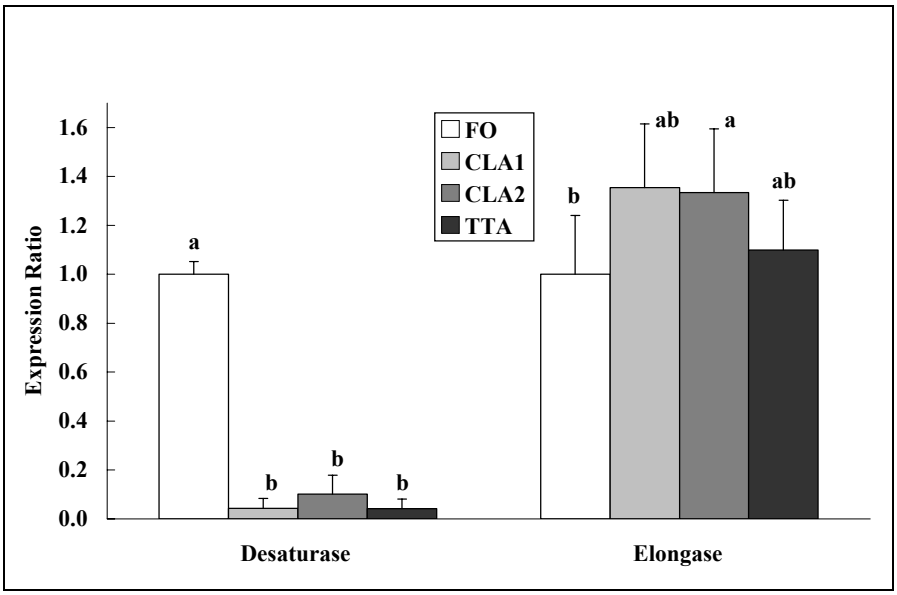
Neither dietary CLA nor TTA had any significant effect on the gene expression of CPT-1 in liver or red muscle compared to fish fed FO alone (Figure 6.5). However, CPT-1 expression was significantly increased in white muscle as a result of dietary CLA and TTA inclusion relative to fish fed FO alone. The expression of fatty acyl Δ6 desaturase was significantly lower in fish fed CLA and TTA compared to fish fed FO alone, whereas the expression of PUFA elongase was increased by dietary CLA (Figure 6.6).

Figure 6.5 Effects CLA or TTA on the expression of CPT-1 in the liver, red and white muscle of rainbow trout.



Results are presented relative to the activity in fish fed FO and are means ± SD (n = 6). Different letters denote significant differences between dietary treatments.

Figure 6.6 Effects of CLA or TTA on the expression of fatty acyl Δ6 desaturase and elongase genes in liver of rainbow trout.



Results are presented relative to the activity in fish fed FO and are means ± SD (n = 6). Different letter denote significant differences between dietary treatments.

6.3.7 Innate Immunological Response

There was no significant difference in the packed cell volume (haematocrit) of rainbow trout fed CLA or TTA relative to fish fed the control (FO). Likewise, there was no difference in red or white blood cell counts in rainbow trout fed either CLA or TTA compared with the control (Table 6.8). Furthermore, the serum lysozyme activity of fish was unaltered in response to either dietary CLA or TTA in rainbow trout.

Table 6.8 Effects of dietary oil content and conjugated linoleic acid (CLA) on basic haematology and selected immunological parameters of Atlantic salmon

Diet	RBC (FO =1)	WBC (FO =1)	PCV (%)	Lysozyme (U/min/ml)
FO	1.00 ± 0.09	1.00 ± 0.49	62.2 ± 6.5	430.0 ± 62.2
CLA1	1.12 ± 0.21	0.95 ± 0.25	65.9 ± 6.3	439.7 ± 45.2
CLA2	1.15 ± 0.20	0.90 ± 0.26	63.2 ± 3.6	444.5 ± 49.9
TTA	1.04 ± 0.23	0.93 ± 0.29	64.8 ± 7.0	456.0 ± 34.8

RBC, red blood cell count (relative to FO diet); WBC, white blood cell count (relative to FO diet); PCV, packed cell volume.

6.4 Discussion

Considering the myriad of beneficial effects TTA, and particularly CLA have elicited in many different animal models and cell culture systems, the main focus of this study was to test the hypothesis that these bioactive fatty acids can have similar beneficial effects in trout culture, namely through enhanced growth and/or altered lipid deposition. It was also proposed, as a result of feeding trout dietary CLA or TTA, that improved nutritional quality could be attained through incorporation of these bioactive fatty acids and additional n-3 HUFA levels in the flesh. Thus, it was also important to analyse the capacity for CLA and TTA to accumulate in rainbow trout flesh as this fish may provide a vehicle through which these fatty acids could be delivered to the consumer. An additional aim of this trial was, to determine the influence of both dietary TTA and CLA at a biochemical level by analysing the activity of key enzymes thought to be pivotal in

lipid metabolism, as well as give a preliminary insight into the immunological response of fish fed these bioactive fatty acids.

The results have shown that the hypotheses were only partly proved. These fatty acids can be easily incorporated into the diet; the flesh accumulates CLA and TTA to a relatively greater extent than liver that shows lower percentages of these fatty acids which, when combined with the relatively high muscle lipid content, means the flesh of trout can deliver a reasonable dose of the bioactive fatty acids. Specifically, this study indicates that both CLA and TTA were accumulated in rainbow trout flesh, with levels reaching 1.6 and 1.2 % of total fatty acids, respectively. Obviously, there is potential for an increase in market value of the fish fed CLA or TTA as a result of this finding, if indeed these bioactive fatty acids prove to be indubitably beneficial for human health and can be included in the diets at a cost-effective level. This is with particular reference to TTA, which has hitherto yet to achieve FDA approval. Indeed, TTA is still largely untested in humans (Berge *et al*., 2002), although two recent studies suggest it may have lipid-lowering and anti-inflammatory effects (Aukrust *et al*, 2003; Fredriksen *et* al., 2004). As mentioned previously, the effects of CLA and TTA on trout are not without precedent, however the number of papers in the peer-reviewed literature on fish are very few compared to studies conducted using mammalian models. Nevertheless, previous studies involving CLA supplementation in fish have indicated that this fatty acid can accumulate to a much higher level in fish tissues compared to the endogenous levels found in ruminant tissues. For instance, muscle (flesh) CLA concentrations in fish fed 1 % CLA have reached 8 % of total fatty acids in hybrid striped bass (Twibell *et al*., 2000), 3.4 % in European sea bass (Valente *et al*., 2007b), 2.9 % in perch (Twibell *et al.*, 2001) and 3.5 % in Atlantic salmon (section 3.3.5). Recent studies investigating the influence

of dietary CLA on smaller rainbow trout have also indicated that muscle CLA concentration can reach 4 – 5 % of the total lipid content (Bandarra *et al.*, 2006; Valente *et al.*, 2007a). Therefore, it is clear that the levels of CLA accumulated in the flesh in the present trial with large trout were lower than the levels obtained in previous studies involving smaller trout fed an equivalent CLA concentration. This is entirely consistent with the notion that the kinetics of fatty acid composition changes will be more rapid in smaller fish. Thus, it is likely that higher levels of dietary CLA and TTA could be accumulated in larger fish given a longer period of dietary supplementation. In addition, there appeared to be degree of selectivity between the individual CLA isomers, such that the c9t11 was more effectively incorporated into the liver and flesh of trout; a finding also acknowledged in previous studies involving trout (Bandarra *et al*., 2007; Valente *et al*., 2007a) and other animals (Ostrowska *et al*., 2003; Degrace *et al*., 2004). This may have implication on market quality considering the diverse physiological and biochemical effects attributed to each isomer. For instance, it is now accepted that t10c12 CLA is responsible for the observed attenuation of fat deposition in rodents in particular (Pariza *et al*., 2001).

Hitherto, the influence on dietary TTA on the fatty acid composition of rainbow trout had not been investigated. However, in Atlantic salmon TTA accumulated in muscle averaging 0.5 % of total fatty acids at dietary inclusion levels of 0.6 % (Kleveland *et al*., 2006). In the present study using fish that are more representative of market size, trout fed TTA at a slightly lower dietary concentration were able to accumulate more TTA in muscle compared to Atlantic salmon in the previous trial, that were of a significantly smaller size. Importantly, in trout, the accumulation of CLA and TTA did not significantly affect the levels of the n-3HUFA in flesh and therefore there was no effect

on flesh fatty acid compositions that could be considered detrimental or likely to compromise the established nutritional benefit of fish for the human consumer. This is in line with previous studies that showed no detrimental effect on 20:5n-3 and 22:6n-3 levels in muscle or whole body of salmon fed similar dietary inclusion levels of 0.6 % TTA and 1 % CLA, respectively (Berge *et al*., 2004; Kleveland *et al*., 2006). On the contrary, liver n-3 PUFA levels significantly increased in this study in response to dietary TTA and CLA in addition to a trend indicating a rise in the proportion of 22:6n-3 in fish fed these bioactive fatty acids. A related increase in 22:6n-3 and 20:5n-3 levels was also observed in the liver of hybrid striped bass fed CLA (Twibell *et al*., 2000). In the present study, the increase in hepatic n-3 PUFA and appreciable rise in 22:6n-3 level correlates with an increase in level of radioactivity recovered in the hexaene fraction of the HUFA synthesis assay. It is noteworthy that only 10 % of the total radioactivity recovered was represented by the hexaene fraction and that the majority of this fraction could only contain 24:6n-3 since the reaction was carried out in microsomes whereas peroxisomes are required for conversion of 24:6n-3 to 22:6n-3 in trout (Buzzi *et al*., 1997). However in the present study, total HUFA synthesis, as measured by the sum of all desaturated products recovered, was low in all dietary treatments, as expected in trout (Buzzi *et al*., 1996), and fish in general (Tocher, 2003), fed essentially FO diets, and there was no effect of CLA and TTA on overall HUFA synthesis. Thus, the effects of CLA and TTA were specifically to increase the proportion of the products of the pathway recovered as hexaenes rather than an increase in the overall activity of the HUFA synthesis pathway. Consistent with this, dietary CLA at inclusion levels up to 1 % had no effect on the HUFA synthesis pathway in salmon fed diets with similar oil content (section 4.3.1). It is noteworthy that the hepatic 18:3n-3/20:5n-3 ratio, regarded as an indicator of Δ6

desaturase activity in fish, was not significantly effected by either TTA or CLA. However, in the present trial, Δ6 desaturase expression was significantly less in fish fed CLA and TTA relative to fish fed FO diets alone; a result which was not replicated in salmon fed an equivalent dietary level of CLA. In contrast, as has been described previously, dietary CLA increased both Δ5 and Δ6 desaturase expression in salmon. Obviously, the discrepancy between HUFA synthesis and Δ6 desaturase expression in trout warrants further investigation, however, the general low level of HUFA synthesis, perhaps combined with an observed compensatory increase in PUFA elongase expression, may have mitigated the effect of decreased desaturase expression in this trial. Suppression of desaturase activity has been reported previously in studies investigating the effects of CLA in transformed yeast (Chuang *et al*., 2001a) and t10c12 CLA in HepG2 cells (Eder *et al*., 2002). In fact, a lack of a direct correlation between desaturase expression and activity has previously been observed in further studies involving yeast cell systems incubated with CLA (Chuang *et al*., 2004), and also between Δ6 desaturase fatty acid indices and expression in plasma from humans fed CLA (Thijssen *et al*., 2005).

In the present study the ratio of 18:0/18:1n-9, a surrogate marker for stearoyl CoA desaturase (SCD) activity, was increased in response to dietary CLA in liver and, to a lesser extent, flesh lipids. These data correlate well with studies previously in striped bass (Twibell *et al*., 2000), yellow perch (Twibell *et al*., 2001), salmon (Berge *et al*., 2004; section 4), cod (section 5) and sea bass (Valente *et al*., 2007b). In addition, dietary CLA increased the proportions of 18:0 and decreased percentages of 18:1 in liver, muscle and viscera of rainbow trout juveniles (Bandarra *et al*., 2006). Suppression of SCD expression and/or activity has also been observed in rats (Wendel and Belury, 2006; Purushotham *et al*., 2007), mice (Lee *et al*., 1998; Viswanadha *et al*., 2006), pigs (Smith

et al., 2002), poultry (Shang *et al*., 2005) and mammalian cell lines (Choi *et al*., 2001, 2002) in response to t10c12 CLA, c9t11 CLA or a combination thereof. Conversely, the data in the present trial indicate that TTA may have only a moderate effect on SCD activity in trout. The hepatic 18:0/18:1n-9 ratio or SCD index was only slightly altered and there was no effect on these indices in flesh of trout fed TTA. Previously, dietary TTA was shown to have no major effects on 18:0/18:1n-9 levels in salmon liver, gill and heart (Moya-Falcon *et al*., 2004) or salmon flesh lipids (Kleveland *et al*., 2006), or cod liver or flesh lipids (section 5.3.4). Similarly, TTA had no effect on 18:0/18:1n-9 levels in hepatocytes from salmon fed 0.6 % TTA or in hepatocytes incubated with 0.8 mM TTA (Moya-Falcon *et al*., 2006).

As aforementioned, another primary objective of this study was to determine whether CLA or TTA could reduce the deleterious consequences of feeding fish high fat diets, which include increased lipid deposition in liver and flesh in particular. It is widely accepted that dietary CLA can have a beneficial effect on body composition in mammals through decreased body fat mass and, in some instances, increased lean body mass, particularly in mice, rats and pigs (Wang and Jones, 2004). Similarly, TTA has been shown to prevent high fat diet-induced adiposity in mammals (Madsen *et al*., 2002). It is unlikely that any apparent change in SCD activity as speculated above, would lead to a reduction in body fat mass since studies carried out on SCD-null mice have shown that CLA does not effect body fat gain compared with wild type mice (Kang *et al.*, 2004). Certainly, evidence provided in the present study generally supports earlier work performed in fish, which shows that neither dietary TTA nor CLA have any effect on piscine muscle lipid content, despite the fact the SCD indices were altered. In the present trial, flesh lipid content was unaffected by dietary CLA or TTA. In two previous trials on

salmonids, total lipid content of flesh in salmon and trout was not reduced by dietary CLA, although these trials used smaller animals compared to the present trial, around 87 g for the salmon smolts and only 5 g for the trout (section 3.3.4; Bandarra *et al*., 2006). One caveat when studying juvenile fish is that they may not be at an optimum age to conclusively determine if CLA or TTA can have desirable effects on adiposity and lipid deposition. This is because fish of a smaller size do not actively deposit lipid to the same extent as larger fish. Hence, this investigation focused on trout which grew from over 400 to around 800 g, and the trial was performed in seawater.

Unfortunately, due to an on-site freezer malfunction while samples were awaiting transportation to Stirling, whole body proximate composition was not measured in the present trial. However, it may be reasoned from previous work detailing the influence of dietary CLA on whole fish proximate composition that it is unlikely that any significant effects would have been observed. Indeed, it has been consistently shown in previous trials that CLA had no significant effect on whole body proximate composition in fish (Twibell and Wilson, 2003; Berge *et al*., 2004; Figueirdo-Silva *et al*., 2005; Valente *et al*., 2007a). Similarly, 0.5 % TTA had no effect on cod proximate composition as observed in the similar study discussed in the previous chapter. In addition, there was no indication from any of the other biometric or tissue lipid analyses data that would suggest that reduction in overall body lipid content had occurred in trout fed CLA or TTA in the present study in trout. Therefore, the inability of CLA and TTA to display any commercially important effects on lipid deposition in studies using smaller fish is also reflected in the present study, despite the use of larger fish.

Despite the lack of alteration in flesh lipid levels, liver lipid was slightly lowered by dietary CLA in the present study, akin to results obtained previously in striped bass

(Twibell *et al*., 2000), yellow perch (Twibell *et al*., 2001) and 97 g trout (Valente *et al*., 2007a). Nevertheless, the decrease in liver lipid deposition as a consequence of CLA supplementation did not significantly influence the HSI and was therefore unlikely to represent a significant reduction in liver TAG content in the present study. This is in contrast to studies that have shown that dietary CLA can increase HSI in hybrid striped sea bass (Twibell *et al*., 2000) and increase hepatic steatosis in mice (Park *et al*., 1997; DeLaney *et al*., 1999; Clement *et al*., 2002; Takahashi *et al*., 2002). However, feeding CLA reduced liver TAG levels (Rahman *et al*., 2002) and decreased hepatic steatosis in Wistar rats (Purushotham *et al*., 2007). Thus, it is plausible that the effects of CLA on liver physiology are species specific.

In rodents, studies have suggested that CLA increased fatty acid oxidation via an increase in CPT-1 activity (Rahman *et al*., 2001; Degrace *et al*., 2004; Zabala *et al.*, 2006). However, in the present trial with trout, neither CPT-1 nor ACO activities were increased in liver by dietary CLA, and hepatic CPT-1 expression was also unaffected by either bioactive fatty acid. In concordance with this finding on ACO, peroxisomal β-oxidation capacity was also unaffected by dietary CLA. Collectively, these data suggest that the lipid lowering effect of CLA is unlikely to be due to an increase in hepatic fatty acid oxidation. However, it is important to note that the effects of CLA on hepatic CPT-1 activity are t10c12 isomer-dependent in hamsters (Macarulla *et al*., 2005) and mice (Degrace *et al*., 2004), and this may be the case in fish. It would be interesting to investigate whether the two main biologically active CLA isomers reveal antagonistic effects on CPT-1 activity. Certainly, it is clear from previous investigations that there is a lack of synergy between the c9t11 and t10c12 isomers in some cases (Brown *et al*., 2003a; Brandebourg and Hu, 2005). In contrast, TTA increased both CPT-I and ACO

activities in trout liver, but peroxisomal β-oxidation capacity remained unchanged compared with fish fed the control diet. The increase in ACO and CPT-1 activities was not reflected in lower liver lipid, similar results to those obtained previously in cod (section 5.3). The lack of association between tissue lipid levels and indicators of fatty acid oxidation was also observed in the trout muscle tissue since TTA increased CPT-1 and ACO activities in red muscle and 1 % CLA increased red muscle ACO activity with no measurable effect on muscle lipid deposition. However, it is known that red muscle comprises only a small proportion of total flesh, the remainder of which is composed mostly of white muscle. Thus, red muscle lipid and fatty acid metabolism may not influence overall flesh lipid composition to an appreciable extent. Although it is likely that mitochondrial β-oxidation is more prevalent than peroxisomal β-oxidation in salmonid muscle (Frøyland *et al.*, 2000), peroxisomal β-oxidation capacity was unaffected by dietary CLA or TTA in white and red muscle. This correlates with data which suggest that both CPT-1 and ACO activities were unaffected by CLA or TTA in white muscle. Considering the lack of effect of the bioactive fatty acids on CPT-I activity, the increased CPT-I expression in white muscle of fish fed both CLA and TTA was a contradiction, but could perhaps be attributed to posttranslational modification. In C57BL/6J mice, CPT-I activity was significantly more sensitive to malonyl-CoA inhibition in CLA-fed animals compared to controls (Degrace *et al.*, 2004). This has also been purported to be the case in hamsters supplemented with CLA (Bouthegourd *et al.*, 2002). A similar mechanism could account for the lack of association between CPT-1 expression and activity in the present trial, at least in fish fed CLA.

CLA has been studied to a far greater extent than TTA and so more potential effects are known. For instance, CLA may have effects on growth performance in

mammals as some studies have suggested that it can enhance growth and feed efficiency in young rodents (Pariza *et al*., 2001). However, these effects on growth have not been replicated in previous trials on a variety of fish species (Twibell *et al*., 2001; Twibell and Wilson, 2003; Berge *et al*., 2004; Yasmin *et al*., 2004; Manning *et al*., 2006), including smaller rainbow trout (Figuierdo-Silva *et al*., 2005; Valente *et al*., 2007a). The present trial with near market size fish also showed no effect of CLA or TTA on growth. Final weight, SGRs and FCRs were all unaffected by both CLA and TTA.

Previously, significantly depressed growth and increased mortality was reported in salmon smolts fed 0.6 % TTA (Moya-Falcon *et al*., 2004; Kleveland *et al*., 2006). It was particularly noteworthy that in the present study, there was no detrimental effect on growth or mortality in trout fed 0.5 % TTA. This was supported by the preliminary immunological analyses, which showed that TTA had no effect on blood cell number, packed cell volume or lysozyme activity. Of course, further investigations are required to determine whether the adverse effects associated with TTA in earlier studies were a result of using smaller fish, higher doses, or different species of fish. The ability of dietary CLA to modulate the innate immune response of juvenile rainbow trout has been documented previously (Clarke, 2003; Marshall, 2003). However, both studies concluded that growth was not affected by CLA in juvenile trout fed up to 2 % dietary CLA compared with control diets. Additionally, packed cell volume and blood cell count remained constant regardless of dietary treatment. Serum lysozyme activity was also shown to be unaltered in response to dietary CLA and a number of other immunological parameters, including respiratory burst by head kidney macrophages, phagocytic ability of head kidney macrophages and specific antibody titres were also unaffected at levels up to 1 % dietary CLA (Marshall, 2003). Thus, it may be surmised that CLA or TTA at

dietary concentrations equivalent to the present study, could be supplemented to rainbow trout at a commercial level without compromising the innate immune response of the fish. However, it is not possible to draw any conclusive result on the immunomodulatory response in rainbow trout fed either CLA or TTA, as only a select number of immunological parameters have been investigated.

In conclusion, the results of the present study only partly support the hypotheses tested. Thus CLA and TTA did not beneficially alter the lipid content of the flesh in commercial size rainbow trout grown in seawater. However, trout preferentially accumulated both CLA and TTA in the flesh compared to liver, with no detrimental effect on flesh n-3 HUFA levels. Both CLA and TTA could also be successfully incorporated into the diet with no evident detrimental effect on fish health. Therefore, if CLA or TTA are proved to be beneficial in the human diet, trout may be a convenient and consumer-friendly source of these fatty acids, thus enhancing the nutritional quality through provision of n-3 HUFA and bioactive fatty acids.

CHAPTER 7. DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

7.1 General Discussion

The overall objective of this research work was to determine the influence of dietary fatty acid nutraceuticals, namely conjugated linoleic acid (CLA) and tetradecylthioacetic acid (TTA) on growth performance, flesh quality, and a number of physiological and biochemical parameters in commercially important farmed finfish species. The scientific rationale for this work was based on previous studies in mammals, which provided strong evidence in favour of CLA and/or TTA eliciting beneficial effects including growth enhancement, augmentation of immune function, and decreased lipid deposition in particular. To this end, this project was designed to test the hypothesis that dietary CLA and TTA would elicit similar effects in finfish, the corollary of which could bring larger, leaner, healthier fish to market.

The first trial aimed to determine the effects of dietary CLA and oil level on growth, biometry, lipid and fatty acid metabolism of Atlantic salmon smolts. This trial also set out to elucidate a biochemical mechanism of action in salmon based on preceding cumulative evidence, which suggested that enhanced fatty acid oxidation was the primary mechanism for the reduction in body fat observed in rodents fed dietary CLA. The experiment was designed to include different dietary oil inclusion levels in order to determine the relationship between dietary CLA and fat levels and whether they interacted to moderate the effects of CLA supplementation. The results of this trial proved that dietary CLA, at the dose formulations used, did little to influence fish at a physiological level, irrespective of dietary oil level. Arguably the most important finding

at this stage in the research work was that the levels of CLA deposited in salmon flesh were much higher than levels that could be obtained naturally from meat and dairy produce alone. Ergo, it was ascertained that farmed Atlantic salmon could be used as a vehicle for the provision of this bioactive fatty acid, which in turn could lead to enhanced nutritional quality of the fish. Of course, the assumption that dietary CLA is indeed beneficial to human health is an ideal yet to be irrefutably proven.

The second part of this trial investigated mechanisms of action of CLA and established, for the first time in fish, the effects of dietary CLA on fatty acid β-oxidation, specifically peroxisomal β-oxidation capacity, CPT-1 gene expression and enzyme activity, and HUFA synthesis in tissues of Atlantic salmon. Despite the lack of significant effects at a whole body level and gross physiological evidence, data from this part of the study revealed that dietary CLA significantly increased both CPT-1 activity and expression in muscle, and also HUFA synthesis activity in liver in conjunction with increased expression of Δ5 and Δ6 fatty acyl desaturase genes. Thus, these data suggested that dietary CLA could enhance mitochondrial β-oxidation in muscle and may increase n-3 HUFA levels in liver. Indeed, levels of 22:6n-3 and 20:5n-3 in liver were significantly increased in response to dietary CLA although this was not reflected in flesh fatty acid compositions. Emerging evidence in studies involving mammals have implicated peroxisome proliferator-activated receptors (PPARs) in the regulation of lipid homeostasis (Desvergne *et al*., 2006) and suggested CLA can directly influence metabolism via interaction with these transcription factors (Moya-Camarena *et al*., 1999). Further to elucidating a possible biochemical *modus operandi*, the present research work aimed to investigate the effects of dietary CLA on PPAR transcript levels in muscle and liver of salmon. The results showed that PPAR transcript levels were significantly

increased in response to dietary CLA. However, recent evidence has proved that the effects on body fat distribution after CLA supplementation are not mediated by PPAR and that, unlike mammals, fish may have multiple forms of each PPAR isoform, some of which are not structurally homologous in comparison to their mammalian counterparts (Anderson *et al*., 2000: Leaver *et al*., 2005). The limited understanding of the role PPAR play in fish lipid metabolism made it difficult to interpret these results. However, based on recent evidence in mammalian studies (Desvergne *et al*., 2006), it is entirely reasonable to suggest that a rise in PPARα and PPARβ levels may have enhanced fatty acid oxidation as observed in this study.

The second major dietary trial aimed to test the hypothesis that dietary CLA or TTA could decrease fat deposition in Atlantic cod, specifically in liver, which is the major site of lipid deposition in this species. This entirely novel work concluded that neither dietary CLA nor TTA influenced growth or biometry or whole body proximate composition in Atlantic cod. At a biochemical level, the data obtained from the fatty acid oxidation studies provided evidence in favour of an increase in hepatic fatty acid oxidation specifically via increased ACO activity, which was concomitant with elevated peroxisomal β-oxidation capacity in cod fed both dietary CLA or TTA. However, unfortunately, lipid content of the liver remained unchanged in cod fed dietary CLA and TTA. The trial also showed that both CLA and TTA could be deposited in the flesh of cod, which could ultimately result in a consumer friendly route for provision of these bioactive fatty acids to the human diet.

At this stage of the project, it was theorised that juvenile fish of the size used thus far, may not be suitable candidates for analysis since smaller fish that are not actively depositing lipid may attenuate the many previously observed effects of feeding dietary

CLA or TTA found in other animal models. In consequence, a third major dietary trial was conducted for the first time with larger, closer to market-size rainbow trout in order to determine the effects of CLA and TTA on lipid and fatty acid metabolism, immunomodulation, and parameters of the fatty acid oxidation pathway. Unfortunately, the results again indicated that growth and biometry of these fish were not significantly altered in response to either dietary CLA or TTA. However, lipid content in liver was significantly decreased by CLA and there was a trend for decreased VSI in trout fed 2 % CLA, whereas TTA had no such effects. At a biochemical level, CLA increased CPT-I gene expression but not activity, whereas TTA increased the activity of both CPT-I and ACO, consistent with increased gene expression in the case of CPT-I, but increased ACO activity was not reflected in peroxisomal β-oxidation activity. Lack of correlation between CPT-I expression and activity as observed with CLA may be related to increased sensitivity of CPT-I to malonyl-CoA inhibition in CLA-fed trout; an incongruity previously observed in rodent studies (Degrace *et al*., 2004).

In line with previous studies (Chuang *et al*., 2001a; Eder *et al*., 2002), Δ6 desaturase transcript levels were significantly lower in trout fed up to 1 % CLA compared with the control. This is in contrast to the earlier trial with Atlantic salmon, which showed an increase in Δ6 desaturase gene expression in fish fed dietary CLA and was also consistent with previous work done in rodents (Takahashi *et al*., 2003). Whatever the cause for this difference in the effects of CLA on fatty acyl desaturase gene expression between the salmon and trout, it is apparent the mechanism of action of CLA (and TTA) is still unclear and warrants further investigation in both mammals and fish. Interestingly, one of the more consistent findings of this project was that dietary CLA increased 18:0 and decreased 18:1. Based on these data, it can be surmised therefore that

SCD was inhibited in fish fed dietary CLA. This particular finding correlated well with previous studies in fish fed dietary CLA (Twibell *et al*., 2000, 2001; Valente *et al*., 2007a).

Presently, the aquaculture industry relies heavily on vaccines and chemotherapeutants in the fight against disease. The use of antibiotics and chemicals to treat disease outbreaks are no longer favoured largely due to environmental issues, which in turn fuel consumer anxieties that farmed fish are not a safe alternative to wild fish. Compounding this issue is that while vaccination is a useful tool in combating disease, not all diseases affecting the aquaculture industry can be controlled via vaccination. Thus, a subsidiary aim of this research was to examine the potential for dietary CLA to enhance the immune function in farmed fish. Although this area was not extensively studied, the results indicated that CLA did not beneficially influence any of the parameters tested.

In summary, this research work has established that CLA and TTA do not confer any physiologically significant beneficial alterations in overall body composition or growth of a number of farmed finfish species. However, there are a number of methodological considerations, which may have had implication in the present work and that could be addressed in future work involving similar dietary trials in fish. Aside from the issue regarding the use of juvenile fish as abovementioned, it is possible that the type of CLA preparation used in these experiments, and all other dietary trials with fish, may have influenced the results obtained. This project, together with the majority of peer-reviewed literature, have used mixtures of c9t11 and t10c12 CLA, usually around 1:1, in dietary trials involving fish. In mammals, various *in vitro* and *in vivo* work has established that each of the two main CLA isomers confers different metabolic effects.

For instance, t10c12 CLA is now widely accepted as the isomer responsible for changes in body composition (Pariza *et al*., 2001). Moreover, there is some evidence to suggest that both CLA isomers act antagonistically towards one another. For example, *in vitro* studies using primary cultures of differentiating pre-adipocytes have shown that TAG content decreased when these cells were administered t10c12 CLA (Brown and McIntosh, 2003). In contrast, TAG levels rose subsequent to c9t11 CLA administration in the same cells. Similarly, c9t11 CLA tended to diminish the inhibitory effect of t10c12 CLA on glycerol-3-phosphate dehydrogenase activity in porcine adipocytes (Brandebourg and Hu, 2005). It is not unreasonable to suggest that similar antagonistic effects could have prejudiced the outcome of the results in terms of the hypotheses tested. Recent studies have ascertained that the moiety of both CLA and TTA can have a profound effect on the metabolic influence of these fatty acids. In COS-1 cells, the addition of a methyl group to the alpha position of CLA and TTA caused an increased activation of PPARα in comparison with non-methylated forms of these fatty acids (Larsen *et al.*, 2005). Additionally, methylated TTA increased peroxisomal, but not mitochondrial, β-oxidation in rats (Vaagenes *et al.,* 1999). Unfortunately, the majority of the papers detailing the effects of dietary CLA in fish have not specified whether CLA was supplied as either a methyl ester, or esterified in TAG and so it is impossible to compare these data in this regard. However, the research work described in this thesis incorporated CLA into the diets in the form of methyl ester. It is unclear whether structural modification of this type would influence the outcome of these data in the present research work involving fish. Nevertheless, this issue should be considered in future investigations involving dietary trials incorporating methyl ester derivatives of CLA and/or TTA.

There were some technical difficulties that arose during the course of this work. Most of these difficulties were imposed by logistical limitations intrinsic to the design of the experimental phase of the research. For example, it was impossible to perform assays involving radioactive isotopes on-site in Denmark and Ardtoe due to respective government legislation and licencing issues. Thus tissues were required to be frozen prior to analysis at the laboratories in Stirling. It was known that freeze/thawing of the samples disrupts the mitochondrial membranes and in doing so, prevented the analysis of total and mitochondrial β-oxidation capacity. However, peroxisomal integrity is reputed to be maintained after freeze-thawing and so measurement of peroxisomal β-oxidation was theoretically possible. To conclusively prove this, a pilot experiment was carried out at the laboratory in Stirling comparing β-oxidation capacity in fresh and frozen liver and muscle tissues in the presence or absence of potassium cyanide (KCN, data not shown). KCN inhibits mitochondrial β-oxidation by disrupting the electrochemical gradient between the membranes and so total β-oxidation is measured in absence of KCN and only peroxisomal β-oxidation activity is measured in the presence of KCN. This experiment elucidated that KCN-treated fresh tissue produced the same β-oxidation capacity as frozen tissue therefore it was concluded that peroxisomal β-oxidation could be accurately determined in these trials.

The second major constraint was the lack of immunological data that was obtained during this project. This was partly due to logistical constraints given that the necessary equipment to perform some of the assays was not available at the trial sites, and partly due to unforeseen experimental difficulties, which could not be rectified due to time constraints. Thus, it was difficult to draw any comprehensive conclusions on the influence of both dietary CLA and TTA on immunomodulation in the fish. However, as

aforementioned, overall the preliminary data obtained in this research did not suggest that there was any major or physiologically significant immunological benefit in feeding dietary CLA or TTA to farmed fish.

In discussion of the constraints in the present study, the cost of replicated nutritional trials must be acknowledged and appreciated. Costs are great and very often can be a limitation to dietary trials and compromises sometimes have to be considered. In the present study, all studies were fully replicated (triplicates) tank studies. However, providing sufficient funding was available there are several ways in which dietary trials on bioactive fatty acids such as CLA and TTA could be improved. Therefore, prospective work should encompass trials that are carried out during late summer and that utilise larger fish. Ideally, sea-caged Atlantic salmon of around 1 Kg, which are rapidly growing, increasing in weight and subsequently depositing more flesh lipid, should be used. Similarly, it may be possible to better gauge the influence of dietary CLA and TTA in cod that are a minimum of 500 g initial weight. In addition, hitherto, all dietary trials investigating the influence of CLA and TTA in fish have not exceeded 12 weeks (3 months) in duration. Thus, it would be interesting to carry out longer trials of a minimum of 6 months attaining finishing weights of around 2.5 - 3 Kg and 1 - 1.5 Kg for salmon and cod, respectively. In addition to testing the bioactive fatty acids in fish of a much more appropriate physiological stage, it would also be possible to ascertain the deposition levels of CLA and TTA in the flesh of fish ready for harvest and give a clearer indication of the levels that can be supplied to consumers via fish flesh and also the time required to reach maximum deposition.

Arguably another very important aim should be to determine the effects of feeding dietary CLA or TTA in combination with alternative, sustainable lipid sources,

i.e. diets containing VO. As aforementioned in Chapter 1, aquafeeds will increasingly contain plant products. The aim of the current EU FP6 integrated project, Aquamax, is to reduce FM to under 50 % of current use (replaced with plant meals etc.) and reduce FO down to possibly zero (100 % replacement with VO) in diets for Atlantic salmon. Obviously, this will affect fish fatty acid composition and also may affect lipid homeostasis and deposition patterns. Additionally, there are some perceived effects such as increased adiposity and free oil problems (oil leaking from flesh that can cause problems for processors such as smokers) associated with high marine product replacements. Thus, it would be interesting to determine whether dietary CLA or TTA could mitigate some of these potential problems.

The present research work has gone some way in bridging the gap between the extensive knowledge base with regards to the influence of dietary CLA in rodent and other mammalian models, and the relative lack of information from similar trials involving fish. Other than the studies described in this thesis, virtually no mechanistic studies have been performed with CLA in fish. Nonetheless, it is clear that further work is required in order to gain a more comprehensive understanding of the effects of dietary CLA and TTA at biochemical and molecular level, and thus of their mechanisms of action in farmed fish. For salmon the recently completed BBSRC project "TRAITS" has produced a large, trait-targeted 17K feature cDNA microarray for salmon. The traits targeted were lipid metabolism (alternative diets), protein metabolism (ration etc.), immune function and parr-smolt transformation. The TRAITS project has contributed to a relatively new field of research termed transcriptomics, which has provided for the first time, the possibility of determining changes in gene expression levels in salmon, at a global level, using so-called microarray technology. The potential implication of this

work would allow examination of the effects of CLA or TTA on thousands of genes within many different metabolic pathways; the application of which would lead to a much clearer understanding of the mechanism of action of these bioactive fatty acids. Two key examples of this would be the uncoupling proteins (UCP) and the sterol response element-binding protein (SREBP) transcription factors.

Recent studies involving CLA-fed mammals have elucidated a number of potential factors that are likely to contribute to the overall biochemical mode of action. For instance, UCPs (particularly UCP-2 and UCP-3) are key regulators of energy expenditure and diet-induced thermogenesis in mammals and are ubiquitously expressed throughout the tissues of the body. Both are known to be up-regulated in response to t10c12 CLA in *ob/ob* mice (Roche *et al*., 2002) whilst UCP-2 mRNA expression was increased in adipose and muscle tissue of rats fed c9t11 CLA (Ryder *et al*., 2001; Choi *et al*., 2004). Another study noted that UCP-2 expression in brown adipose tissue was increased in AKR/J mice as a result of 1 % dietary CLA (West *et al*., 2000). Moreover, UCP-2 expression in both brown adipose tissue and skeletal muscle tissue was significantly increased in ICR and C57BL/6J mice fed 2 % CLA (Takahashi *et al*., 2002). Feeding the CLA-producing bacteria *Lactobacillus rhamnosus* PL60 to C57BL/6J mice also resulted in increased UCP-2 mRNA expression in adipose tissue (Lee *et al.*, 2006). It is therefore reasonable to suppose that CLA supplementation causes an up-regulation of UCP-2 expression that could contribute to an increase in energy expenditure.

There is increasing evidence to suggest that a family of nuclear transcription factors, namely SREBPs, are also modulated by CLA both *in vitro* and *in vivo*. Collectively, SREBPs are involved in the transcriptional activation of more than 30 genes associated with cholesterol, fatty acid, TAG and PL synthesis. One isoform, SREBP-1a,

has the ability to activate all SREBP-responsive genes (Desvergne *et al*., 2006) SREBP-1c on the other hand, is responsible for mediating the expression of genes involved in fatty acid synthesis while SREBP-2 controls cholesterol synthesis. The majority of studies detailing the influence of dietary CLA on SREBP gene expression have shown that these transcription factors are significantly reduced as a result of supplementation. For instance, inhibition of proteolytic activation of SREBP-1 is thought to cause a suppression of milk fat synthesis in t10c12 CLA-fed dairy cows (Peterson *et al*., 2004). Recently, both SREBP-1a and SREBP-1c gene expression were down-regulated in adipose tissue of hamsters administered t10c12 CLA (Zabala *et al*., 2006). In pigs, pre-adipocyte proliferation and differentiation was inhibited in response to dietary t10c12 in a mechanism involving down-regulation of SREBP-1c gene expression (Brandebourg and Hu, 2005). Conversely, dietary c9t11 CLA has been known to increase SREBP-1c mRNA expression in adipose tissue of *ob/ob* mice (Roche *et al*., 2002), suggesting that metabolic effects of CLA may be isomer dependant and in this case, antagonistic. Importantly, SREBP-1c and PPARγ interact with each other to execute pre-adipocyte differentiation (Granlund *et al*., 2003). It is likely that concomitant decreases in SREBP-1c and PPARγ gene transcription observed in the abovementioned studies, are responsible for the overall reduction in transcriptional activation of lipogenic genes in animals administered t10c12 CLA. It may be that SREBP plays a more important role in fish in comparison to PPAR. However, the associated drop in transcript levels of SREBPs in other animals including pigs (Brandebourg and Hu, 2005) and hamsters (Zabala *et al*., 2006) was also associated with a drop in PPARγ levels, something not observed in the present study. Clearly, more studies will be required to determine whether dietary CLA exerts its effects via a SREBP-dependant mechanism in fish. In

addition, a more detailed evaluation of the effects of CLA and TTA on immune function is also required, particularly considering the potential for dietary TTA to cause increased mortality in Atlantic salmon (Kleveland *et al*., 2006).

In conclusion, the present research work has provided new insights to the effects of dietary CLA and TTA on the physiology and biochemistry of some key species of farmed fish. However, this novel research has also broached many ideas and hypotheses, which will require further elucidation in order to gain a more thorough understanding of the effects of both these bioactive fatty acids as dietary supplements in aquaculture.

7.1 Summary of Results and Conclusions

In summary, the results of this research work have indicated the following:-

- There were no effects on growth performance as evidenced through measurement of final body weight, SGRs or TGC
- There were no effects on feed efficiency as measured via FCR
- There were few physiologically significant effects on fat levels of fish as evidenced by no major effects on carcass proximate compositions or tissue (flesh or liver) lipid levels
- No evidence for any redistribution of body fat with no physiologically significant effects on HSI and VSI
- Both CLA and TTA could be incorporated into fish flesh thus providing a vehicle through which these bioactive fatty acids can be delivered to the consumer.
- There were a number of effects on fatty acid metabolism including CLA clearly inhibiting SCD (Δ9) desaturase leading to increased 18:0 and decreased 18:1 in

cod and trout in particular, and possibly increased Δ6 and Δ5 desaturase expression and activity (at least in salmon) leading to increased hepatic n-3HUFA

- There were some significant effects on gene expression of enzymes and transcription factors thought to be pivotal in modulating lipid and fatty acid metabolism however the data did not always correlate with activities. Morover, the effects on gene transcription and biochemistry had little impact at the whole body level
- Although not extensively studied, there were no indications that CLA (or TTA) had any major beneficial health effects as there were no effects on immune status, including basic haematology or some parameters of immune function
- No detrimental effects of the bioactive fatty acids (as had been previously reported in other trials), were observed in the present trials.

7.2 Future Perspectives

Considering the range of effects that CLA, and to a lesser extent other bioactive fatty acids like TTA, have been shown to have in mammals, both in dietary trials and cell model systems, many of which could be potentially beneficial in aquaculture, it is disappointing that in the trials to date, there have been no substantial advantages of including these functional fatty acids in diets for farmed fish. However, there are still some trials that could perhaps provide final definitive conclusions. Points 1-3 below summarise the applied research that is still required to conclusively establish if CLA, or TTA, could have significant beneficial effects in fish farming. Points 4 and 5 represent

the basic scientific research required to determine the mechanisms of action underpinning the actions of bioactive fatty acids like CLA.

1. Larger fish. It is important that larger fish at more appropriate time in their life/growth cycle be investigated. For salmon this would require a seawater pen trial on fish of around 1 Kg (probably around May-October), a stage when they will be growing rapidly, producing large increases in overall weight and actively depositing lipid in the flesh. Similarly, for cod a seawater pen trial using fish of around 0.5 Kg or more would be desirable – perhaps starting in pre-starved fish.
2. Increased length of trial. The length of trials should be longer and would, ideally, be at least double the duration of the trials in the present study, with probably around 6 months as a minimum. Combined with the larger initial size of the fish, this would result in fish at the end of the trials being much closer to market size and weights, around 2.5-3 Kg for salmon and at least 1-1.5 Kg for cod.
3. Alternative diets (i.e. VO replacement). Diets for farmed fish will increasingly contain plant products. This will have consequences for FA compositions, of course, but also may affect lipid homeostasis and deposition patterns etc. There are already reports of perceived effects such as increased adiposity and free oil problems associated with high marine product replacements. Clearly it is essential that a full investigation of the effects of bioactive fatty acids such as CLA and TTA should also include trials with these diets that although currently termed "alternative" will increasingly become the "standard". In terms of dietary formulation it is also worth considering the chemical form of the bioactive fatty

acid that is supplemented, and so a trial comparing, say, CLA added as methyl ester versus addition as TAG may be illuminating.

4. Additional analysis. a) Total and mitochondrial β-oxidation. The pathways of fatty acid oxidation are crucial in these trials with CLA and TTA that have known effects on these pathways. In the present trial, legal and logistic problems limited the β-oxidation studies, and so the assays performed were a compromise to obtain the maximum information possible with frozen tissue samples. Similar problems would arise with the trials described above as they all have to be performed at Feed Trial Units that are, essentially, simply fish farms. However, innovative ways should be sought for measuring total and mitochondrial β-oxidation. b) Transcriptomics. For salmon the trait-targeted 17K feature cDNA microarray will be extremely useful for looking at the effects of bioactive fatty acids on global gene expression, investigating many pathways and areas of metabolism simultaneously.
5. Individual isomers. In the case of CLA, it is already known in studies with mammalian systems that specific isomers are responsible for eliciting different individual effects. Investigation of individual isomers has not been attempted with fish and cannot be done with dietary trials due to then cost of purified isomers. However, several potentially very useful fish cell lines are available including those from Atlantic salmon (AS) and rainbow trout (RTH, hepatic and RTG, gonad). Although no cell line is available for cod, lines are available for other marine species including turbot (TF) and sea bream (SAF-1). As well as studying individual isomers, cell line studies could be very useful for more detailed

analysis, both biochemical (enzyme assays) and molecular (gene expression) in studies designed to elucidate the mechanisms of action of these interesting bioactive or functional fatty acids.

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APPENDIX I

10 X TBE electrophersis buffer, per litre

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

55g Boric Acid (BioRad labs, Hercules, CA, U.S.A)

8.3g EDTA (BDH)

LB Medium

25 g LB Broth (MERCK)

1 L Distilled H_2O

LB/Ampicillin/X-gal/IPTG plates

25g LB Broth (MERCK)

15 g Agar Bacteriological No. 1 (Oxoid, Hampshire, U.K.)

1 mg X-gal (5-bromo-4-chloro-3-indoyl-β-galactopyranoside)

0.8mg IPTG (isoproylthio-β-D-galactoside)

APPENDIX II

GFX™ Micro Plasmid Prep Kit (Amersham Biosciences)

Components of Kit.

Solution I: 100 mM Tris-HCl (pH 7.5), 10 mM EDTA, 400 µg/ml RNase I

Solution II: 1 M NaOH, 5.3% (w/v) SDS (5.3X concentrated solution)

Solution III Buffered solution containing acetate and chaotrope.

Wash Buffer Tris-EDTA buffer in 80% ethanol.

Cell Lysis

- 1.5 ml of an overnight *E. coli* culture was transferred to a 1.5 ml microcentrifuge tube then centrifuged at full speed for 30 sec to pellet the cells.
- As much supernatant as possible was removed by aspiration without disturbing the pellet.
- An additional aliquot of culture (to give 2-3 ml total volume of culture) was added to the microcentrifuge tube, centrifuged at full speed for 30 sec and the supernatant removed by aspiration as described above.
- The pellet was then resuspended in 300 µl solution I with vigorous vortexing
- Three hundred µl of solution II was added to the microcentrafuge tube then mixed by inverting the tube 10-15 times.

- Six hundred µl of solution III was added and mixed by inverting the tube until a flocculent precipitate appeared with inverting continuing until the precipitate was evenly dispersed.
- The microcentrifuge tube containing the flocculent precipitate was then centrifuged for 5 min at room temperature to pellet the cells.
- One GFX^{TM} column for each preparation was prepared by placing the column into a collection tube.

DNA purification

- Approximately half of the supernatant was transferred to the prepared GFX^{TM} column, incubated for 1 min and centrifuged at full speed for 30 s.
- The flow-through was discarded by emptying the collection tube and the remaining supernatant transferred to the same GFX^{TM} column.
- The GFX column was incubated for a further minute at room temperature before being centrifuged for 30 s at full speed.
- The flow-through was once again discarded as above.
- Four hundred µl of wash buffer was added to the column. The column was centrifuged at full speed for 30 s to remove the buffer and dry the matrix prior to elusion.
- The GFX column was transferred to a clean microcentrifuge tube and 50 to 75 µl of H_2O added to the top of the glass fibre matrix in the GFX column.
- The column was incubated at room temperature for 1 min before being centrifuged for 1 min to remove the purified plasmid DNA.

APPENDIX III – PUBLISHED PAPERS

Kennedy, S. R., P. J. Campbell, A. Porter and D. R. Tocher. 2005. Influence of dietary conjugated linoleic acid (CLA) on lipid and fatty acid composition in liver and flesh of Atlantic salmon (*Salmo salar*). *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.*, 141, 168-178.

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