THE EFFECT OF CONJUGATED LINOLEIC ACID ON LIPID METABOLISM IN THE HAMSTER AND THE SHEEP

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ABSTRACT

The term conjugated linoleic acid (CLA) refers to a range of geometric and positional isomers of linoleic acid. Recent research suggests a variety of potential health benefits with consumption of dietary CLA. These include a reduction in body fat deposition that has been demonstrated in a number of monogastric species including the mouse, rat, hamster and pig. This thesis describes the effects of CLA on lipid metabolism in sheep, where CLA may be useful in reducing carcass fat and improving fatty acid profile. The results are contrasted with those in the hamster, a model monogastric species, previously shown to respond to CLA.

Ovine adipose tissue metabolism was studied in explants maintained in culture and incubated with a mixture of CLA isomers and individual isomers. Total lipogenesis, and the formation of saturated and monounsaturated fatty acids were examined. Results show no effect of CLA (mixed or individual isomers) on total lipogenesis or desaturation of fatty acids. Furthermore, there was no effect on mRNA concentration for acetyl coenzyme A carboxylase (ACC) or stearoyl coenzyme A desaturase (SCD). The effect of feeding CLA (protected from rumen degradation) to sheep, on lipogenic gene expression was then investigated. While there was no evidence of a decrease in total fat deposition, there was a decrease in the proportion of monounsaturated fatty acids in the tissues. As there was no effect on SCD mRNA levels, it appears likely that CLA inhibits SCD activity rather than affecting gene expression.

A further feeding study was undertaken in Golden Syrian hamsters. As in the sheep, CLA feeding reduced the monounsaturated fatty acid content of the tissues but did not change the SCD mRNA concentration in adipose tissue or liver. This further supports the suggestion that CLA directly inhibits SCD activity. Unlike the sheep, there was an overall decrease, of approximately 10%, in total carcass fat. However, paradoxically, there was an increase in adipose tissue ACC and fatty acid synthase (FAS) mRNA concentrations. Thus, suppression of lipogenic enzyme expression does not appear to be the mechanism by which CLA reduces fat deposition.

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PUBLICATIONS

Some of the work in this thesis has already been published elsewhere:

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ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BMR	Basal metabolic rate
bp	Base pairs
BSA	Bovine serum albumin
BSE	Bovine Spongiform Encephalopathy
BW	Body weight
°C	Degrees centigrade
CHD	Coronary heart disease
CLA	Conjugated linoleic acid
CoA	Coenzyme A
CP	Crude protein
CPT-1	Carnitine palmitoyltransferase-1
CTP	Cytosine triphosphate
d	Day
DEFRA	Department for the Environment, Food and Rural Affairs
DM	Dry matter
DNA	Deoxyribonucleic acid
EDTA	Diaminoethanetetra-acetic acid
FA	Fatty acid
FAMEs	Fatty acid methyl esters
FAS	Fatty acid synthase
g	Gram

GTP	Guanosine triphosphate
h	Hour
HEPES	(N-[2-Hyrdrozyethyl]piperazine-N'-[2-ethanesulfonic acid])
HMG-CoA	Hydroxmethylglutaryl-CoA
IDDM	Insulin dependent diabetes mellitus
kDa	Kilodaltons
kg	Kilogram
l	Litre
LA	Linoleic acid
LPL	Lipoprotein lipase
M	Molar
MBq	Megabecquerels
ME	Metabolisable energy
mg	Milligram
MJ	Megajoule
ml	Millilitre
mM	Micromolar
MUFA	Monounsaturated fatty acid
n	Number of samples
NAC	No amplification control
NADH	Nicotinamide adenine dinucleotide dehydrogenase
NADPH	Nicotinamide adenine dinucleotide phosphate dehydrogenase
NEFA	Non-esterified fatty acid
ng	Nanograms
NIDDM	Non-insulin dependent diabetes mellitus
nM	Nanomolar
nm	Nanomoles

NTC	No template control
NTP	Nucleotide triphosphates
OA	Oleic acid
p	Probability
PCLA	CLA protected against rumen biohydrogenation
PCR	Polymerase chain reaction
pmol	Picomole
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator-response element
PUFA	Polyunsaturated fatty acid
R^2	Correlation co-efficient squared
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
s.e.d.	Standard error of the difference between the means
SCD	Stearoyl-CoA desaturase
SFA	Saturated fatty acid
SREBP	Sterol regulatory element-binding protein
TAE	Tris-acetate-EDTA
TAG	Triglyceride
TLC	Thin layer chromatography
µg	Micrograms
µl	Microlitre
µM	Micromolar
µm	Micromoles
UTP	Uridine triphosphate

1. INTRODUCTION

1.1. ANIMAL FAT AND HUMAN HEALTH

An increase in the number of people suffering from chronic diseases such as obesity, diabetes and cardiovascular disease is putting a huge financial strain on the National Health Service. In the western world, incidences of obesity and diabetes are on the increase, and cardiovascular disease is still the greatest cause of premature death. Obesity is caused when consumption of energy exceeds energy expenditure. Excess energy is stored as fat, primarily in adipose tissue depots and can accrue to comprise over 50% of body weight (Vernon, 1986). Obese people are generally less healthy and are at a higher risk of developing other diseases such as diabetes and cardiovascular diseases. Diabetes is a disease state where glucose is poorly handled by the body as a result of impaired insulin production or resistance to insulin. Insulin dependent diabetes mellitus (IDDM) results in high blood glucose concentrations due to the lack of insulin synthesis by the pancreas. Patients normally display symptoms in childhood and must be treated by daily insulin administration to correct the blood glucose concentration. Non-insulin dependent diabetes mellitus (NIDDM) is the most common form of diabetes, and is seen in older, usually obese patients who are producing high levels of insulin to overcome effects of insulin resistance induced by obesity. NIDDM occurs when the level of insulin production by the pancreas cannot be sustained, resulting in high blood glucose levels which cannot be corrected by insulin administration (Mangiapane & Salter, 1999). Cardiovascular disease is a chronic disease state, caused by arterial obstruction. Coronary heart disease (CHD) is caused by obstruction of the coronary arteries, and is usually preceded by the development of fatty deposits in coronary arteries which harden and increase in size. This results in a narrowing of blood vessels, increases the risk of blood clots and impairs blood flow to the heart, resulting in death of heart muscle and possible heart attack (Mangiapane & Salter, 1999). All of these diseases are associated with diet and the over-consumption of energy, leading to obesity, which can result in NIDDM and cardiovascular disease. The latest data on fat consumption indicates that in the UK, over 38% of total energy is supplied by fat (Defra, 2000). Fat is energy dense; 1g of fat contains 2.3 times the amount

of energy as 1g of glycogen, the form in which carbohydrate is stored in the body (McDonald *et al*, 1995). Therefore consumption of a small amount of fat supplies a large amount of energy, but no feeling of satiety, therefore there is a tendency to over-eat in terms of energy balance. It has been long established that the type of fat consumed has a major impact on the incidence of CHD. An increase in blood cholesterol has been shown to increase the risk of CHD, and saturated fatty acid intake increases blood cholesterol. In contrast, polyunsaturated fatty acids lower blood cholesterol and therefore the risk of developing CHD. In the UK an average of 15% of total energy is supplied from saturated fatty acids, with only 6.9% from polyunsaturated fatty acids (Defra, 2000). Consequently, government recommendations are to reduce consumption of saturated fats to no more than 10% and for the percentage of total energy from fat to be below 35% (Department of Health, 1994). 22% of total fat intake is accounted for by consumption of meat, poultry and meat products, while dairy products account for a further 15% (Department of Health, 1994). Thus there is a significant contribution of ruminant products to total fat intake and implementation of these recommendations has had an effect on meat consumption. This has implications for meat producers.

1.2. DIETARY FAT AND ANIMAL PRODUCTS

Animal products are a major source of dietary fat, with the majority of fatty acids coming from dairy products and meat. Consumption of meat has fallen in the U.K. over the last 30 years, and the last decade has seen a number of health and related scares involving meat products, including outbreaks of salmonella and Escherichia coli 0157, bovine spongiform encephalopathy (BSE) and foot and mouth disease. All these cases have weakened public confidence in the livestock industry and have resulted in reductions in sales of meat and meat products. In addition to these factors, government recommendations advise against the consumption of high levels of saturated fat, advising that only 10% of food energy from total fat should be derived from saturated fats (Department of Health, 1994). Dairy products and meat from ruminants are particularly high in saturated fats in comparison to meats from pigs or poultry (Table 1.1). This is due to the structure of the ruminant digestive tract and process by which their food is digested and absorbed, which will be discussed later. There is a perception that it is unhealthy due to the type and amount of fat it contains, so consumption of these meats has reduced (Figure 1.1). In addition red meat is more expensive than chicken or turkey, and therefore consumption of the latter is increasing at the expense of red meat. Clearly the continuation of such trends is worrying for the livestock industry, and the decline in meat consumption seen over previous years needs to be addressed. However the discovery of conjugated linoleic acid (CLA) in samples of ground beef (Pariza *et al*, 1987), and the fact that ruminant meat is particularly high in CLA suggests that ruminant meat is not all bad. A number of beneficial effects have been attributed to CLA, including its potential as an anti-carcinogen and reductions in fat deposition seen in animals fed diets supplemented with CLA. Thus the advantages of CLA in ruminant meat could be twofold; increased consumption of CLA contained in ruminant tissues could confer increased health benefits on consumers, and the feeding of CLA to animals may have favourable effects on the amount and type of fat associated with the meat. This is discussed in more detail in Sections 1.6 and 1.7. The Government has set up public bodies to monitor food standards and production in response to public concern regarding food safety, and organisations such as the Food Standards Agency have a role to play by advising consumers on aspects of food safety. Meat industry bodies

such as the Meat and Livestock Commission aim to promote meat and meat products, through campaigns, liaison with producers and advice on the production of a better product, more suited to consumers' needs. As previously mentioned, consumption of beef and lamb has fallen over the last 30 years, while poultry consumption has risen. If these trends continue they will have a devastating effect on the ruminant meat industry. A major problem is the fat content of ruminant tissues, and any attempt to meet consumers' requirements for beef and lamb needs to focus on changing the amount and type of fat present in the carcass. The rise in poultry consumption indicates that consumers prefer lean meat with little associated fat. Although poultry contains similar amounts of fat to ruminant meats, this fat is largely unsaturated and is usually removed from the meat prior to consumption. There are a number of ways that the amount and type of fat in ruminant meat could be changed, chief of which is to change the carcass composition, to raise the lean percentage and reduce associated fat. To do this effectively the process of fat deposition during the growth of ruminants and the effects on both the digestive and fat deposition processes in response to different dietary fats needs to be understood.

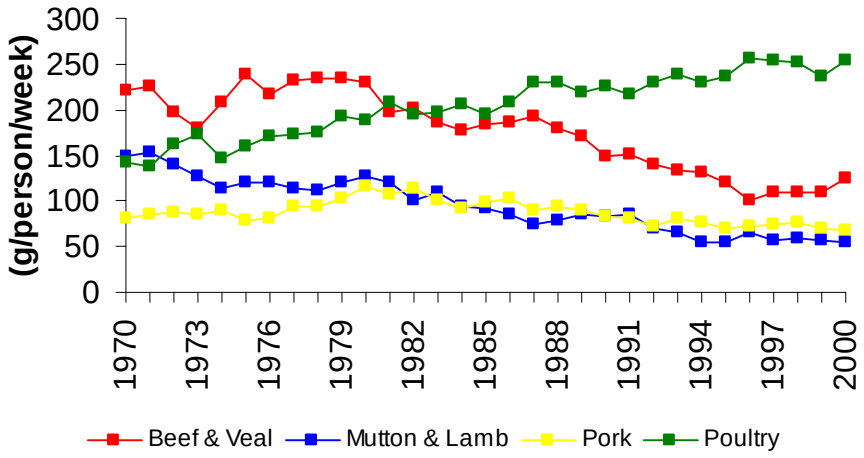
Table 1.1 Fatty acid composition of meat and dairy products

Fatty acid	Beef[a]	Lamb[a]	Pork[a]	Dairy products[b,c]
C4:0	NR	NR	NR	4.20
C6:0	NR	NR	NR	2.37
C8:0	NR	NR	NR	1.33
C10:0	NR	NR	NR	2.97
C12:0	0.08	0.31	0.12	3.40
C14:0	2.66	3.30	1.33	11.18
C16:0	25.0	22.2	23.2	26.56
C16:1	4.54	2.20	2.71	2.17
C18:0	13.4	18.1	12.2	11.13
C18:1 n-9	36.1	32.5	32.8	21.31
C18:2	2.42	2.70	14.2	2.19
C18:3 n-3	0.70	1.37	0.95	1.21

Data from [a]Enser *et al*, 1996; [b]Jensen, 1992 expressed as % weight of total fatty acids. [c]Dairy products are an average of fatty acid composition for milk and butter. NR = not reported.

Sources: Enser *et al*, 1996; Jensen, 1992

Figure 1.1 Meat consumption from 1970 - 2000



Source: National Food Survey 2000 (Defra)

1.3. LIPIDS

The term 'lipid' is a collective term for a number of compounds which are insoluble in water. This group of compounds includes fats, oils, phospholipids and sterols. They have many different functions, but a large number of fats and oils are the major forms of stored energy in many organisms, while phospholipids and sterols are the major constituents of biological membranes. Fats and oils are principally composed of fatty acids and their derivatives.

1.3.1. The structure and naming of fatty acids

Fatty acids are made up of a carboxyl (COOH) group bound to a hydrocarbon chain. This hydrocarbon chain can be of any length, but is usually even numbered in eukaryotes; short chain fatty acids are usually between 2 and 14 carbons long; and long chain fatty acids over 14 carbons in length. Fatty acids vary in their degrees of saturation, that is, how many double bonds and therefore hydrogen atoms are associated with them. A fatty acid is said to be saturated if it does not contain any double bonds and therefore has the maximum number of hydrogen atoms possible bound to each carbon atom. Unsaturated fatty acids contain one or more double bonds and therefore have less hydrogen atoms bound to them. The conversion of an unsaturated fatty acid to a saturated one requires the addition of hydrogen, and so saturated fatty acids are said to be hydrogenated. In fatty acids containing double bonds, there are two different configurations, *cis* and *trans*. These refer to the position of the remaining hydrogen atoms in relation to the fatty acid chain. The *cis* configuration has both remaining hydrogen atoms on the same side of the fatty acid chain, while the *trans* configuration has them on opposite sides (Figure 1.2). Changes in configuration produce geometric isomers of fatty acids, while changes in the position of double bonds produce positional isomers. The naming of fatty acids is done according to structural features, namely chain length and number of double bonds. The number of carbons in the chain is written first, followed by a colon, after which the number of double bonds contained within the carbon chain is written. For example, myristic acid, a saturated fatty acid with 14 carbons and no double bonds is written as 14:0, while palmitoleic acid, a monounsaturated fatty acid with 16 carbons and 1 double bond is written as 16:1. However, this system has its limitations when

dealing with a number of forms or isomers of the same molecule. Sometimes it is important to know not only the number of double bonds, but their configuration and location in the molecule. Numbering of the location of double bonds is usually done from the carboxyl end of the fatty acid, so palmitoleic acid is referred to as *cis-9* hexadecadienoic acid, indicating that the double bond is between the ninth and tenth carbons from the carboxyl end of the fatty acid chain, and it is in the *cis* configuration. Fatty acids also have systematic names (Table 1.2) by which they are more commonly referred to. The biosynthesis of fatty acids means that most naturally occurring fatty acids are even numbered.

Figure 1.2 Representation of *cis* and *trans* fatty acids

cis fatty acid *trans* fatty acid

R denotes the fatty acid chain

Source: Adapted from Gurr, Harwoood & Frayn (2002)

Table 1.2 Common saturated, monounsaturated and polyunsaturated fatty acids found in animal tissues and their systematic names

No. of carbon atoms and double bonds	Systematic name	Common name
2:0	*n*-Ethanoic	Acetic
3:0	*n*-Propanoic	Propionic
4:0	*n*-Butanoic	Butyric
6:0	*n*-Hexanoic	Caproic
8:0	*n*-Octanoic	Caprylic
10:0	*n*-Decanoic	Capric
12:0	*n*-Dodecanoic	Lauric
14:0	*n*-Tetradecanoic	Myristic
16:0	*n*-Hexadecanoic	Palmitic
16:1	*cis-9*-Hexadecanoic	Palmitoleic
18:0	*n*-Octadecanoic	Stearic
18:1	*trans*-9-Octadecenoic	Elaidic
18:1	*trans-11*-Octadecenoic	*trans*-Vaccenic
18:1	*cis-9*-Octadecenoic	Oleic
18:1	*cis*-11-Octadecenoic	Vaccenic
20:0	*n*-Eicosanoic	Arachidic
22:0	*n*-Docosanoic	Behenic

Source: Adapted from Gurr, Harwood & Frayn (2002)

1.3.2. Saturated fatty acids

Saturated fatty acids contain no double bonds, and the naturally occurring ones in animal tissues are up to 22 carbons in length. In these compounds, the atoms in the carbon-carbon bond are able to freely rotate, resulting in a very flexible fatty acid chain. The most stable conformation is a straight extended chain, due to the lack of interactions between atoms within a single bond.

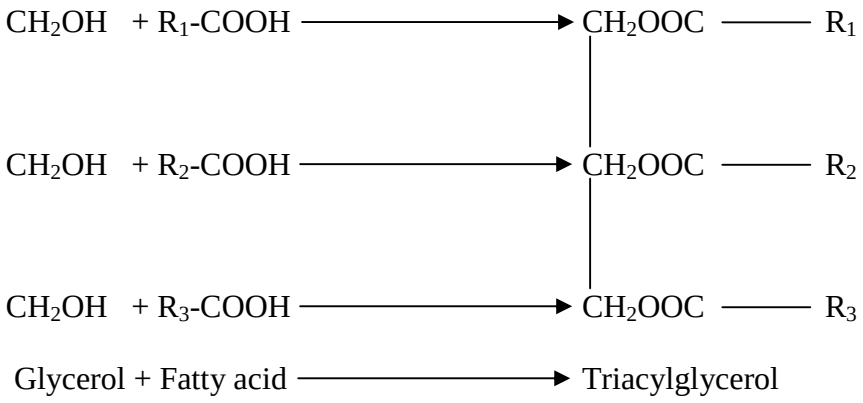
1.3.3. Unsaturated fatty acids

Unsaturated fatty acids have at least one double bond in their hydrocarbon chain. Monounsaturated fatty acids have a single double bond and are generally between 16 and 22 carbons in length. The degree of saturation has an effect on the alignment of the fatty acid chain, as the introduction of double bonds and the associated restriction of rotation in the acyl chain motion at that point, due to inter-atomic forces, causes the chain to kink or to be extended (Gurr, Harwood & Frayn, 2002). Polyunsaturated fatty acids originate from their monounsaturated counterparts; thus linoleic acid (18:2) is produced by desaturation of oleic acid (18:1), however this only happens in plants. Animals cannot synthesise linoleic acid and therefore it is an essential fatty acid which must be supplied in the diet.

1.3.4. Storage of lipids in animal tissues

Storage occurs when dietary energy intake exceeds that required by the body. Most lipids are stored as triacylglycerols, which are esters of glycerol and consist of three fatty acids in ester linkage with glycerol (Figure 1.3). Triacylglycerols are hydrophobic, like all lipids, and because of this do not carry associated water. Fat oxidation yields much more energy than that of carbohydrates (Lehninger, 2000). Triacylglycerols are stored primarily in adipose tissue in animals, which has the advantage of being able to expand greatly to accommodate vast stores of energy.

Figure 1.3 Triacylglycerol structure

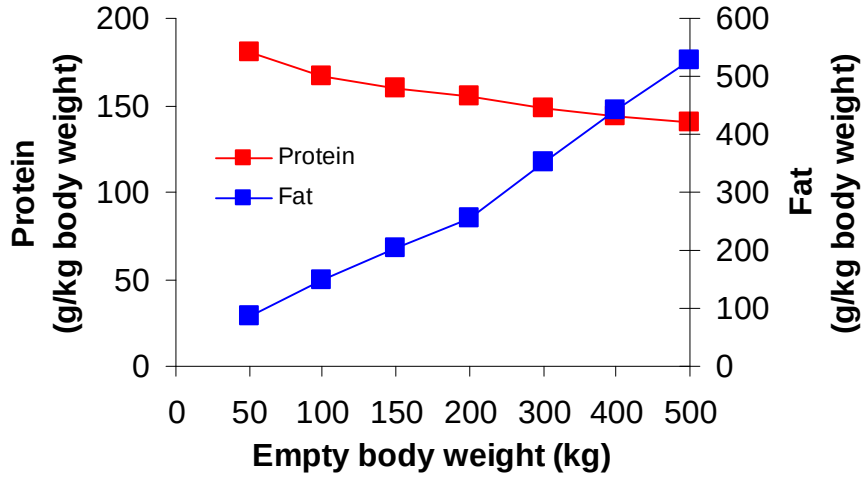


R represents the fatty acid chain

1.3.5. Growth and fattening

Animals consist of four major types of tissue; nervous, bone, adipose and lean. These tissues are in turn made up of different cells. For the purpose of this thesis, adipose and lean tissues are discussed. Adipose tissue is mainly made up of adipocytes, whose primary function is to esterify and store fat. Lean tissue is made up of a number of different cell types, forming muscle proteins organised into muscle fibres; and water. In the immature animal, the rate of growth is very rapid. Initially protein or lean tissue deposition rate is greater than that of fat, but as the animal reaches its mature weight, the rate of fat deposition increases to exceed that of protein (Figure 1.4). If fat growth is not restricted, fat depots can expand massively to comprise over 50% of total body weight. Lean tissue is still being produced but the amount present in the body remains approximately constant due to the turnover of constituent proteins, where those which are old or damaged are degraded and new ones are synthesised.

Figure 1.4 Composition of gains in empty body weight for castrate cattle of a medium-sized breed



Source: Agricultural Research Council (1980)

1.4. LIPID METABOLISM

Lipid metabolism in ruminants and monogastrics is different, in that ruminants have four stomachs instead of one. The largest of them is the rumen, which is colonised by micro-organisms which help to digest the plant matter which forms a large part of ruminant diets. Ruminants also have a true stomach, or abomasum, which contains stomach acid and enzymes as in the monogastric. In the non-lactating ruminant, the major site of fatty acid synthesis is adipose tissue and little occurs in liver. In humans and birds the liver is the major site of fatty acid synthesis (Vernon, 1980), but mice, rats and rabbits synthesise fatty acids in both tissues (Jansen *et al*, 1966; Leveille, 1966; Leung & Bauman, 1976). The differences in diet and digestion strategy in these two types of animals results in differing pathways of lipid metabolism, and therefore are considered separately in this section.

1.4.1. Lipid metabolism in the monogastric

The monogastric animal has a simpler approach to digestion and absorption of fats relative to the ruminant. It has only one stomach, the equivalent of the abomasum in ruminants, which secretes acid and pepsinogen to hydrolyse proteins. The stomach then gives way to the small intestine, where digesta entering is mixed with bile salts, secretions from the pancreas and alkaline fluid from the duodenum. Pancreatic lipase acts on fats to produce non-esterified fatty acids and glycerol. The small intestine of the monogastric processes much more triglyceride than the ruminant, as fat passes through the stomach and enters the small intestine as large globules, which are difficult to hydrolyse quickly. Bile salts emulsify fats, allowing hydrolysis to occur more effectively. Fats are then solubilized into micelles, which are absorbed due to the action of bile salts. After absorption, as in the ruminant, triacylglycerols are resynthesised and packaged into lipid droplets called chylomicrons, which then enter the circulation for distribution to the rest of the body.

1.4.2. Dietary fat

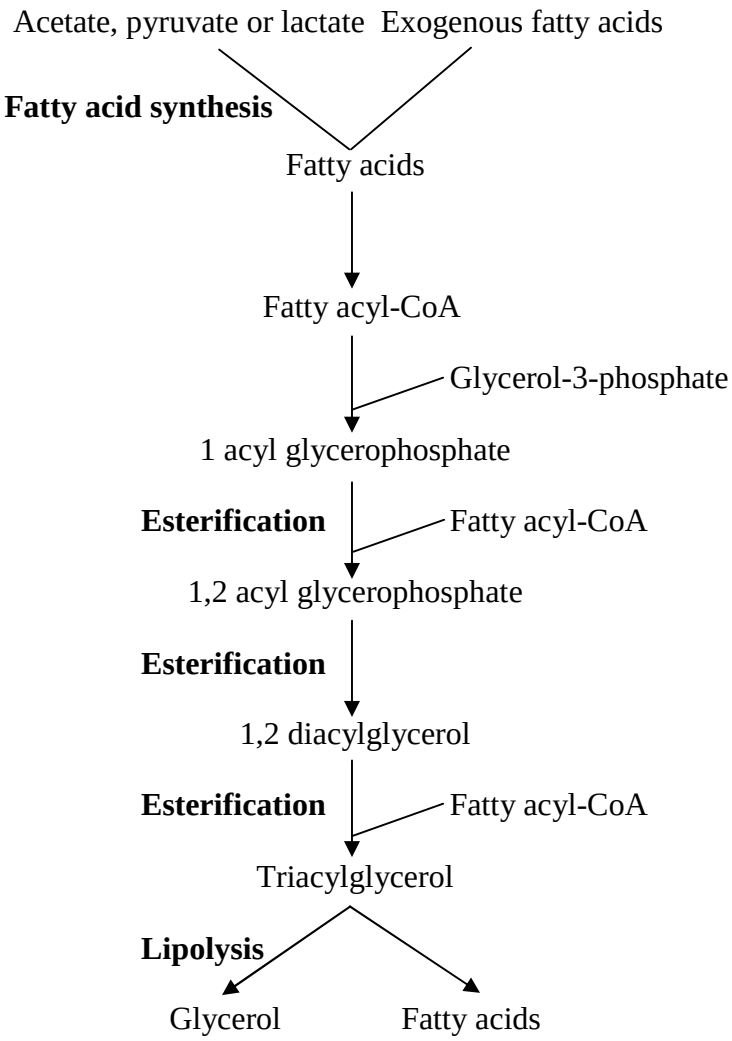
Fat present in the body comes from two sources; dietary fat or endogenously synthesised fat. Dietary fat is hydrolysed during digestion then re-esterified or oxidised on reaching the tissues depending on the energy state of the animal; endogenously synthesised fat is made by lipogenesis. Adipose tissue contains stored or esterified fat. This is an energy store which can be mobilised when bodily energy requirements exceed energy intake. Fat is hydrolysed to form glycerol and non-esterified fatty acids, which can then be used to meet the energy requirements of the animal. Dietary fats affect lipogenesis. In humans consuming a high fat diet with 40% energy as fat, *de novo* lipogenesis (fatty acid and triacylglycerol (TAG) synthesis from glucose and amino acids) is not a major contributor to fat storage. In animals consuming a high carbohydrate diet, lipogenesis is much more important and contributes significantly more to fat deposition. Lipogenesis is particularly important in the ruminant, which consumes a low fat diet and must synthesise fatty acids and TAG from the products of digestion which are discussed in Section 1.4.5.

1.4.3. Lipogenesis

Fatty acid synthesis occurs via a process known as lipogenesis, which is the formation of acetyl-coenzyme A from acetate, glucose or lactate. Acetate is the major precursor in adult ruminants, with glucose and lactate contributing more to lipogenesis in foetal, pre-ruminant animals and monogastrics (Vernon, 1986). Glucose is largely produced by gluconeogenesis in the ruminant (Ballard *et al*, 1969), due to the lack of glucose in the diet. Little is available for fatty acid synthesis, as much of it is diverted to tissues that cannot use fatty acids, such as the brain. The process of lipogenesis is initiated by the conversion of acetate to acetyl-coenzyme A by acetyl-CoA synthetase (Figure 1.6). This occurs in the cytosol of the cell (Ballard, 1972; Knowles *et al*, 1974). Acetyl-CoA carboxylase (ACC), another enzyme present in the cytosol, converts acetyl-coenzyme A to malonyl-coenzyme A. This enzyme is hormonally regulated, and exists in active and inactive states depending on phosphorylation of serine residues. This is the first committed step in fatty acid synthesis and a key regulatory point of the process (Vernon, 1992). Malonyl-coenzyme A goes through a series of reactions which elongate the fatty acid

chain by 2 carbons. These reactions are catalysed by fatty acid synthase (FAS) which consists of seven polypeptides that are associated in a complex. Each of these acts on the fatty acid chain, and extends it by 2 carbons. This is repeated seven times until palmitic acid is formed and leaves the cycle. Palmitic acid is the precursor for a number of long chain fatty acids; the chain may be lengthened by addition of acetyl groups by elongases present in the endoplasmic reticulum and mitochondria (Figure 1.6); or a double bond introduced to form monounsaturated fatty acids by the action of stearoyl-CoA desaturase (SCD). Palmitoleic and oleic acids are the most common monounsaturated fatty acids present in animal tissues. Further desaturation to linoleic and linolenic acids is not possible in animals, so these fatty acids are essential in the diet of animals for synthesis of long chain polyunsaturated fatty acids (Lehninger, 2000).

Figure 1.5 Fatty acid metabolism



Adapted from Vernon (1981)

1.4.3.1. Acetyl-CoA carboxylase (ACC)

This enzyme is the first enzyme in the catalysis of fatty acid and ultimately lipid synthesis. ACC is a heterotetrameric protein, with a molecular weight of approximately 265kDa (Salati & Goodridge, 1996) present in the cytosol. Although its actions are usually described as a single step, in reality there are two steps. ACC contains a biotin prosthetic group, which acts as a carrier of CO_2, which is then transferred to acetyl-CoA, forming malonyl-CoA (Lehninger, 2000). The biotin prosthetic group must be able to interact with the biotin carboxylase and the carboxytransferase sites for the enzyme to be active (Gurr, Harwood & Frayn, 2002). Regulation is by phosphorylation in response to hormones.

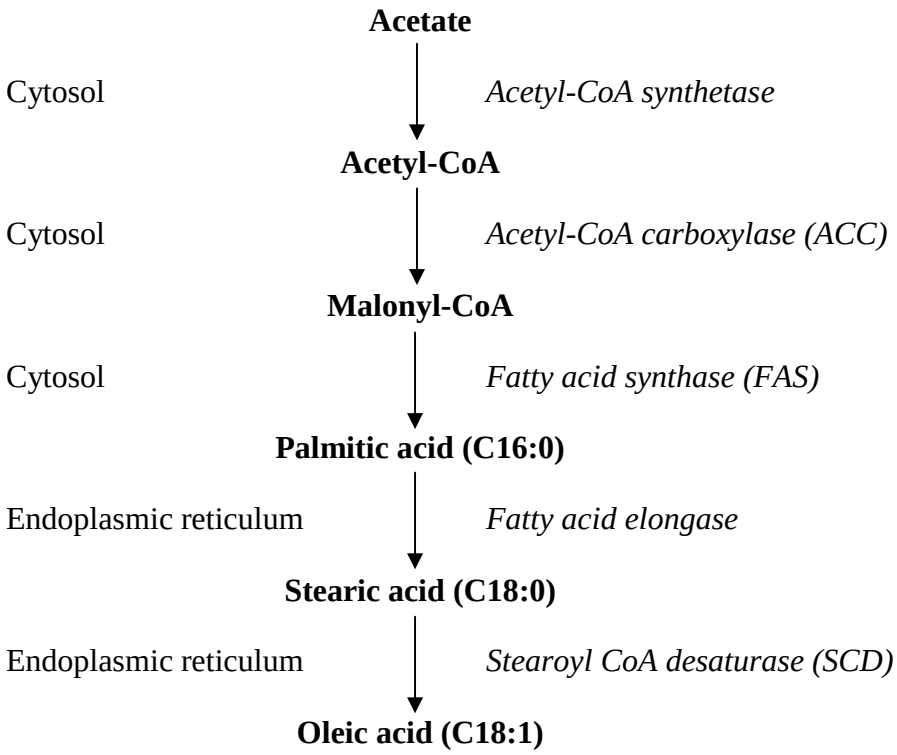
1.4.3.2. Fatty acid synthase (FAS)

Animal fatty acid synthase is a dimeric enzyme, which contains seven catalytic domains, each of which needs to be active for the enzyme to be completely active. FAS in mammalian and avian liver has a molecular weight of approximately 270kDa (Gurr, Harwood & Frayn, 2002). The major product is palmitic acid, although in mammary gland C4:0-C16:0 can be formed. FAS is regulated by dietary fat and level of nutrition (Salati & Goodridge, 1996).

1.4.3.3. Stearoyl-CoA desaturase (SCD)

This enzyme complex consists of three proteins: NADH-cytochrome b_5 reductase, cytochrome b_5 and a desaturase component, all of which are cyanide sensitive. The complex has a molecular weight of approximately 112kDa (Cook, 1996). The enzyme catalyses the removal of hydrogen from saturated fatty acyl chains. SCD always introduces a double bond between carbons 9 and 10, hence its other name, delta-9 desaturase.

Figure 1.6 Fatty acid synthesis



From Lehninger (2000)

1.4.4. Lipolysis

Lipolysis is the hydrolysis of triacylglycerol to form three free fatty acids and glycerol. Studies in non-ruminants show that hormone sensitive lipase acts on the triacylglycerol to form a diacylglycerol which is further hydrolysed to form free fatty acids and glycerol (Shapiro, 1977; Vaugham & Steinberg 1965). Lipolysis in the ruminant is postulated to occur in the same way (Vernon, 1981).

1.4.4.1. Lipoprotein lipase (LPL)

Lipoprotein lipase is a key enzyme in lipolysis of triacylglycerols and acts at the capillary endothelium of muscle and adipose tissues, on triacylglycerol-rich lipoproteins. LPL is constituted of 448 amino acids (Fielding & Fielding, 1996) and is similar to pancreatic lipase. LPL action produces unesterified fatty acids and glycerol, as a result of the serine hydrolase action of the enzyme. This results in the uptake of fatty acids into tissues for oxidation (eg. muscle) or re-esterification (eg. adipose tissue).

1.4.5. Lipid metabolism in the ruminant

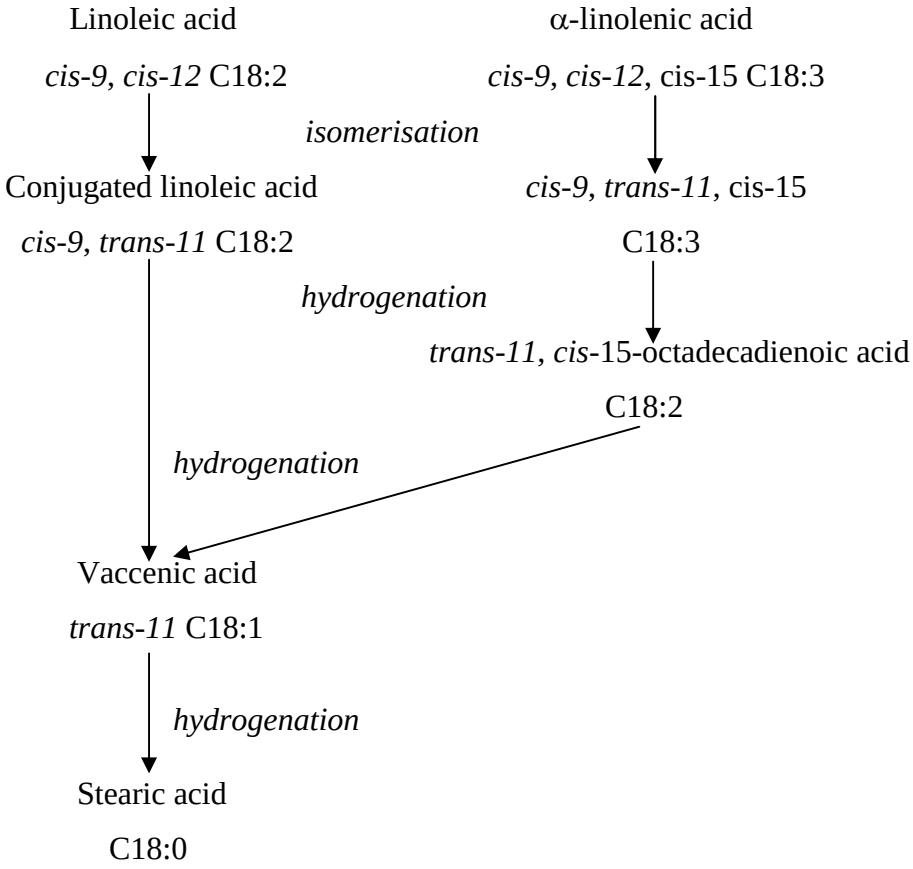
Ruminants consume an inherently low-fat diet (1-2% fat), in contrast to monogastrics, with lipids being provided in forage crops and supplements or concentrates made up of crushed cereal grains with oil seeds or their extracts added (Harfoot and Hazlewood 1988). Ruminant lipid metabolism is different to that in animals with single stomachs, due to the colonization of the rumen by micro-organisms. Ruminant diets cannot contain high amounts of fat, especially unsaturated fatty acids, as these are potent inhibitors of microbial fatty acid metabolism in the rumen (Jenkins, 1993). Consequently *de novo* lipogenesis is very important in the ruminant animal. The action of rumen micro-organisms on dietary fat is an important part of lipid metabolism. In an animal consuming pasture, the major fatty acid being supplied to the rumen is linolenic acid, whereas in an animal consuming a dietary supplement, or a concentrate diet, quantities of linoleic acid as triglyceride enter the rumen (Harfoot, 1981). Therefore there are two different pathways by which linoleic and linolenic acids are hydrogenated.

1.4.5.1. Lipid metabolism in the rumen

Lipids entering the rumen are acted on initially by microbial lipases, which hydrolyse ester linkages to form free or non-esterified fatty acids. Unsaturated fatty acids may then be acted on by other rumen bacteria in a process known as biohydrogenation (Figure 1.7), the product of which is ultimately stearic acid (C18:0). The initial lipolysis step is essential for isomerisation to occur as a free carboxyl (COOH) group is required for the isomerase to be active (Kepler *et al*, 1970). The final hydrogenation step is rate limiting, and conversion of *trans-11* vaccenic acid to stearic acid can be affected by conditions within the rumen; excessive amounts of linoleic acid irreversibly inhibit this step (Harfoot *et al* 1973), while feed particles and cell-free ruminal fluid stimulate conversion to stearic acid (Kellens *et al*, 1986). This final step can be slow compared to the preceding ones, leading to a build up of 18:1 *trans-11* in the rumen, some of which can pass out into the tissues through the rumen wall, incompletely biohydrogenated. This has implications for further lipid metabolism in the tissues and is a route by which unsaturated fat is stored by the ruminant. In addition to biohydrogenation, the microbes are also able to carry out *de novo* fatty acid synthesis, usually forming stearic and palmitic

acids (Knight *et al*, 1978). When microbes pass out of the rumen, into the abomasum or true stomach, the fatty acids contained within them are available for absorption by the animal. Fermentation processes occurring in the rumen act on carbohydrates and proteins, and produce short chain or volatile fatty acids, with fatty acid chains between two and five carbons in length. The three major ones are butyric, propionic and acetic acids. These can pass through the rumen wall and supply the ruminant animal with a considerable proportion of the total energy available. Of these, acetic acid is the most abundant, although the production of individual acids can be changed by feeding different diets and altering fermentation patterns in the rumen. The ruminant therefore uses acetic acid as acetate for the precursor for *de novo* lipogenesis, not glucose. Much of the glucose produced from breakdown of dietary carbohydrate is used by the rumen micro-organisms and is not available to the animal, hence the low contribution of glucose to lipogenesis. The hydrogenation of most of the fatty acids fed and the limitations in the amount of fat that can be fed means that changing carcass fat is extremely difficult in the ruminant animal.

Figure 1.7 Biohydrogenation of lipids in the rumen



Source: Adapted from Christie (1981)

1.4.5.2. Post-ruminal lipid metabolism

Passage of digesta through the omasum and abomasum in the ruminant is swift due to its uniform consistency after ruminal digestion. The composition of digesta does not change appreciably, but micro-organisms passing through are subjected to acid digestion and are broken down to release their fatty acids for absorption by the small intestine. Although most of the fatty acids in the small intestine of the ruminant are present in the non-esterified form, they are still associated with particles of feed and must be separated before absorption can take place. This is done by pancreatic and biliary secretions which form micelles in a similar way to that in monogastric animals. Studies have shown that in animals where bile production is limited, fatty acid absorption from the small intestine fell to 17% of the amount seen in the normal animal (Heath & Hill, 1969). Bile addition helps to transfer unesterified fatty acids into solution (Polonovski & Bourrillon, 1952) to be absorbed and therefore it is hardly surprising that in the absence of bile there is reduced fatty acid absorption. Fatty acids entering the duodenum as unhydrolysed triglycerides, such as those protected against rumen degradation are acted upon by pancreatic lipases and broken down to form non-esterified fatty acids and glycerol and then absorbed through mucosal cells. The main fatty acid available for absorption is stearic acid, much of which is converted to oleic acid by desaturases prior to resynthesis of triglycerides for storage or use in other parts of the body.

1.4.5.3. Triacylglycerol synthesis

95% of the fatty acids synthesised by the pathway described above are esterified; however this does not mean that they are incorporated into triacylglycerol stores, because there is a certain amount of lipolysis occurring, liberating glycerol and free fatty acids. The glycerol-3-phosphate pathway is assumed to be the route by which triacylglycerol is synthesised in ruminant adipose tissue (Vernon, 1981), but this requires glucose from the blood, as adipose tissue has virtually no glycerol kinase activity, ruling out the use of glycerol liberated by lipolysis (Vernon, 1980). Triacylglycerols are supplied to adipose tissue via the blood, contained in the centre of large lipoproteins called chylomicrons. On arrival at the adipose tissue, the enzyme lipoprotein lipase (LPL) is secreted by the adipocytes and attached to the cell membrane of endothelial capillary cells where it catalyses the hydrolysis of triacyglcerols

subsequent to their re-esterification within adipocytes (Vernon, 1992, Lehninger, 2000). Studies suggest that dietary fatty acids are more readily incorporated into adipose tissue (Vernon, 1992), and this is supported by a reduction in the contribution of fatty acids synthesised *de novo* to esterification and storage in the adipose tissue of cows when exogenous fatty acids were added (Baldwin *et al*, 1973).

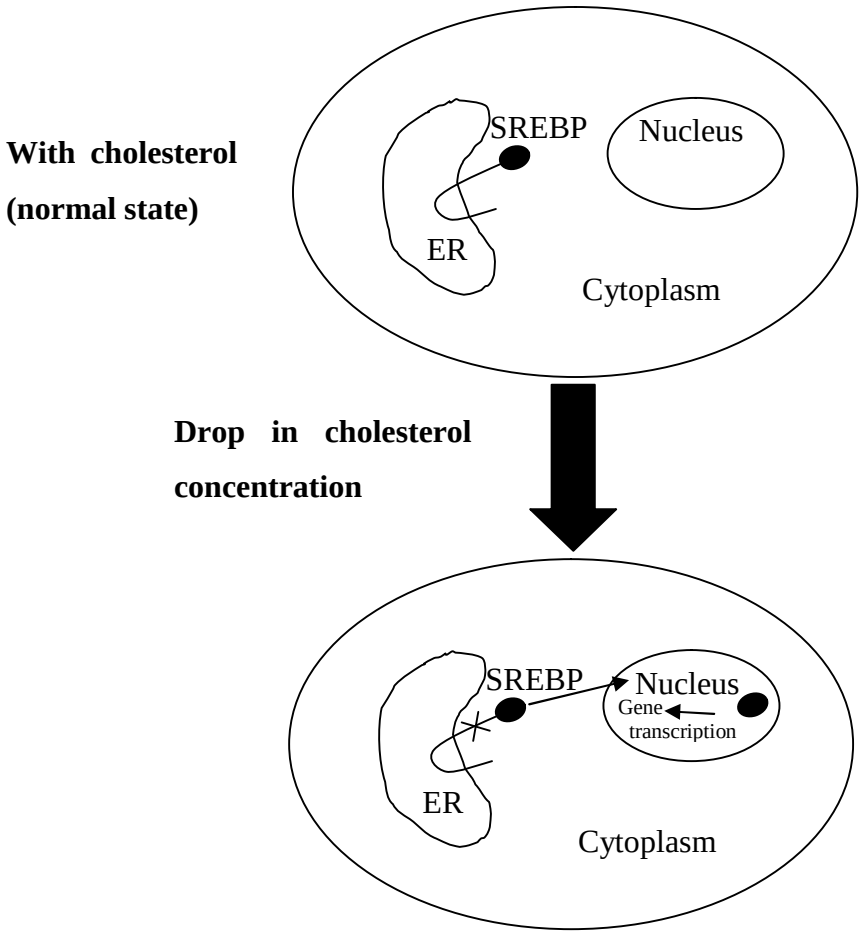
1.4.6. Transcription factors and receptors involved in lipid metabolism

In recent years a number of transcription factors have been identified and studied. Transcription factors are regulatory proteins which bind to DNA (Lehninger, 2000), and activate transcription, the production of RNA from DNA. To synthesise the proteins which make up the various enzymes involved in lipid metabolism, an RNA template is needed, therefore the process of transcription is the first response to a signal for increased fatty acid synthesis or lipolysis for example. In addition to transcription factors, specific receptors are also capable of activating gene expression and work in conjunction with certain transcription factors. Two such systems which play an important role in lipid metabolism are described here.

1.4.6.1.1. Sterol regulatory element-binding proteins (SREBPs)

Sterol regulatory element-binding proteins (SREBPs) are synthesised as precursors bound to the membrane of the endoplasmic reticulum. The protein forms a loop, with both amino and carboxyl ends protruding into the cytoplasm (Osborne, 2000). There are 3 forms of SREBP: 1a, 1c and 2. The latter is activated in response to a drop in cellular cholesterol (Latchman, 1998) and controls cholesterol biosynthesis, but SREBP 1a and 1c regulate fatty acid metabolism (Gurr, Harwood & Frayn, 2002). The precursor is cleaved and the active form of the protein enters the nucleus and binds to the sterol regulatory element. This is present in all of the genes which SREBP controls and activates the expression of cholesterol biosynthetic genes (Figure 1.8). SREBP-1c is activated in response to high insulin levels (Gurr, Harwood & Frayn, 2002), which are known to increase fatty acid synthesis. Increased SREBP-1c activation then increases gene transcription in the nucleus of enzymes involved in fatty acid synthesis. Table 1.3 shows some of the genes which SREBPs affect. Clearly SREBPs are key regulators of lipid metabolism, and substances that have been postulated to have a direct effect on certain genes (eg. insulin on ACC) may be acting through SREBP. The SREBPs are a major control point in the system and manipulation of their activation status has the potential to change many different factors in the lipid metabolism pathway, with far reaching effects on the biological system.

Figure 1.8 Activation of SREBP



Source: Latchman (1998)

When cholesterol is present in the cell, the SREBP transcription factor remains anchored in the endoplasmic reticulum membrane and is prevented from passing into the nucleus. When levels of cellular cholesterol drop, the SREBP precursor is cleaved. This releases the active form of the protein which enters the nucleus and activates transcription of genes involved in cholesterol biosynthesis such as ACC and FAS.

Table 1.3 Genes affected by activation of SREBPs

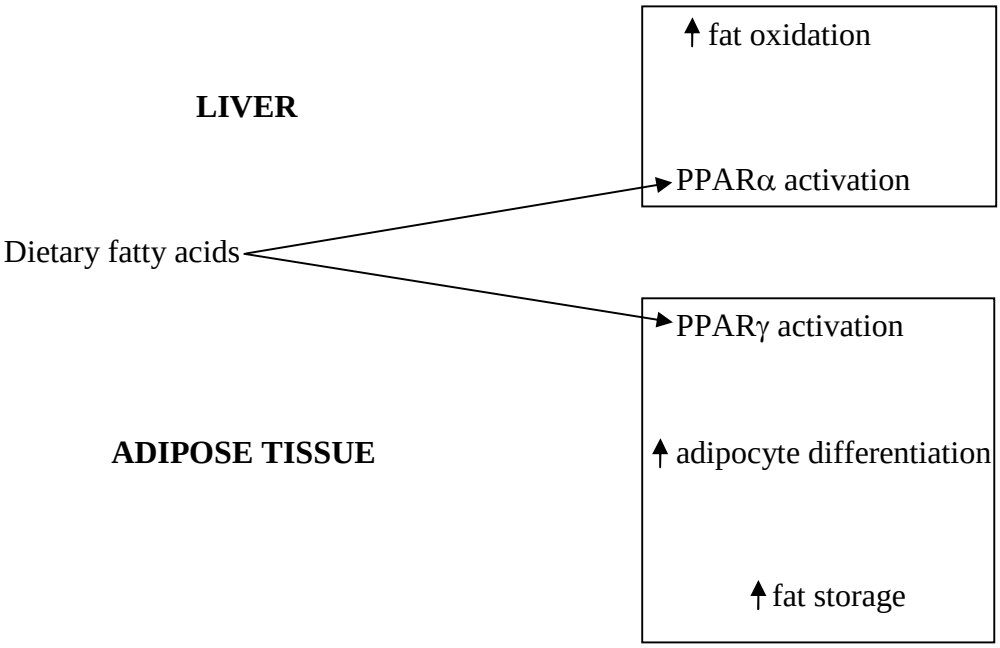
Gene	Controlling isoform	Function	Reference
Acetyl-CoA carboxylase	1c	*De novo* fatty acid synthesis	Kim & Spiegelman, 1996; Lopez *et*
Fatty acid synthase	1c	*De novo* fatty acid synthesis	*al*, 1996; Magana & Osborne, 1996;
Lipoprotein lipase	1c	Import of lipoprotein-triacylglycerol fatty acids	Shimano *et al*, 1996; Tontonoz *et al*,
Stearoyl-CoA desaturase	1c	Synthesis of oleate	1993
Glycerol 3-phosphate acyltransferase	1c	Triacylglycerol and phospholipid synthesis	Sul & Wang, 1998
HMG-CoA synthase	2	*De novo* cholesterol synthesis	Goldstein & Brown, 1990
PPAR-γ	1c	Induction of adipocyte differentiation	Kliewer *et al*, 1995
Squalene synthase	2	*De novo* cholesterol synthesis	Guan *et al*, 1996
LDL receptor	2	Import of LDL particles	Hussain *et al*, 1999
HMG-CoA reductase	2	Inhibition of cholesterol biosynthesis	Brown & Goldstein, 1986; Sabine, 1983

Adapted from Gurr *et al*, 2002

1.4.6.1.2. Peroxisome proliferator-activated receptors (PPARs)

PPARs are nuclear hormone receptors responsible for the regulation of fatty acid metabolism (Kersten, 2001). There are three different isoforms: α, β (δ) and γ. PPARα is mainly expressed in the liver, while PPARγ is primarily present in adipose tissue (Gurr, Harwood & Frayn, 2002). PPARγ has an important role in adipogenesis, which is the differentiation of pre-adipocytes into mature adipocytes, and the sensitivity of tissues to insulin (Auwerx, 1999). Expression of PPARγ is increased by high levels of fat and reduced by either fasting or low levels of fat (Vidal-Puig *et al*, 1996). Insulin also causes increased gene expression of PPARγ (Rieusset *et al*, 1999). Fatty acid derivatives are ligands for PPARs, and PPARs interact with peroxisome proliferator response elements, which are present in the sequences of genes involved in lipid metabolism (Gurr, Harwood & Frayn, 2002). This activation of PPARγ results in increased adipogenesis and fatty acid storage. PPARα increases high density lipoprotein production and fatty acid oxidation in the liver, which provides glycerol 3-phosphate for triacylglycerol synthesis, so the two isoforms work co-ordinately (Figure 1.9). There is evidence that SREBP-1c, expressed in adipocytes causes increased fatty acid synthesis, increasing concentrations of ligands for PPARγ (Auwerx, 1999). The most potent ligands for PPARγ are eicosanoids and unsaturated fatty acids (Kliewer *et al*, 1997; Krey *et al*, 1997); fatty acids that have been shown to bind are linolenic (18:3), eicosapentaenoic (20:5) and docosahexaenoic (22:6). Any factor which has an effect on fatty acid availability will affect the PPARs and therefore the actions that they have.

Figure 1.9 Actions of PPARs α and γ in liver and adipose tissue



Source: Gurr, Harwood & Frayn (2002)

1.4.7. Regulation of lipid metabolism

There are a number of factors regulating lipid metabolism in animals, and as such these can all be manipulated to have different effects on metabolism. The substrates for lipogenesis, namely glucose and acetate, have stimulatory effects on fatty acid synthesis and esterification, while growth hormone has inhibitory effects on fatty acid synthesis and stimulatory effects on lipolysis (Vernon, 1982). Insulin has stimulatory effects on fatty acid synthesis but inhibits lipolysis, and on the other hand glucagon has the opposite effects to insulin. Dexamethasone, a synthetic glucocorticoid has an inhibitory effect on fatty acid synthesis (Faulconnier *et al*, 1994; Vernon *et al*, 1991). Fatty acids, whose effects on lipid metabolism are the subject of this thesis, have inhibitory effects on both fatty acid synthesis and lipolysis, but stimulate esterification (Vernon, 1981).

1.4.7.1. Hormonal regulation - Insulin and glucocorticoids

A body system can be in a state of anabolism or catabolism. In the former, the effects of the hormone insulin are manifested, while in the latter the glucocorticoids (eg. glucagon) and the catacholamines (eg. adrenaline) are in control. It has been demonstrated by a number of researchers that insulin increases lipogenesis in ruminant and non-ruminant animals. Vernon (1979)

showed that ovine adipose tissue was metabolically active for 3 days in culture and that including insulin in the culture medium resulted in an increase in the rate of fatty acid synthesis as measured by [1-^{14}C] acetate incorporation. [U-^{14}C] glucose incorporation was also measured, but rates of fatty acid synthesis from this were much lower than from acetate and representative of lipogenesis in the ruminant, where 95% of the carbon for fatty acid synthesis is derived from acetate (Smith & Prior, 1986, Vernon, 1979). Faulconnier *et al* (1997) investigated the effects of insulin and dexamethasone (a synthetic glucocorticoid) on lactate production by bovine and ovine adipose tissue. The major products of glucose metabolism are carbon dioxide and lactate, and although acetate is the predominant source of carbon for fatty acid synthesis, ruminant adipose tissue uses glucose to produce NADPH via the pentose phosphate pathway, which then is involved in the production of ribose-5-phosphate for nucleic acid synthesis. Faulconnier *et al* (1997) showed that insulin increased the ratio of lactate production to glucose utilization, dexamethasone reduced it and that dexamethasone when added to insulin supplemented media could not reduce this ratio. Chilliard & Faulconnier (1995) studied the effects on acetate and glucose utilization of insulin and/or dexamethasone treatment on ovine and bovine adipose tissue explants cultured for seven days. This study showed differences between ovine and bovine tissue, but the stimulatory effects of insulin, both with and without dexamethasone were still present in both species, and the inhibitory effects of dexamethasone were evident. Again, the acetate utilization was greater than that of glucose, consistent with the former being the major carbon source for ruminant lipogenesis (Smith & Prior, 1986, Vernon, 1979). Vernon *et al* (1991) cultured ovine adipose tissue for 48 hours with insulin and dexamethasone and showed that insulin increased the rate of fatty acid synthesis and dexamethasone decreased it. Additionally, and as shown in the current results, insulin and dexamethasone acted together to increase fatty acid synthetic rate. Miller *et al* (1991) showed that insulin significantly increased lipogenesis in bovine adipose tissue explants treated with insulin both short term (2h) and long term (48h). Tissue was still capable of responding to insulin treatment after 48h, but levels of acetate relative to total lipid were reduced compared to explants cultured for 2h. This has been demonstrated previously (Miller *et al*, 1989; Etherton & Evock, 1986). The effect of insulin on lipogenesis is not

confined to ruminant animals; Walton and Etherton (1986) demonstrated an increase in glucose incorporated into porcine adipose tissue maintained in culture with insulin, compared to controls. Glucagon and the catacholamines, namely adrenaline, reduce lipogenesis and stimulate lipolysis, while insulin reduces lipolysis and stimulates lipogenesis. Insulin is produced by the pancreas in response to an increase in blood glucose levels and stimulates lipogenesis and inhibits lipolysis. Glucagon is produced by the pancreas in response to a decrease in blood glucose levels and stimulates lipolysis and inhibits lipogenesis. Adrenaline, produced by the adrenal gland, also acts on the liver to promote glycogen breakdown and increase blood glucose levels (Frayn, 2003).

1.4.7.2. Dietary fat

Fatty acids have been shown to have effects on lipid metabolism. High fat diets inhibit lipogenesis in both liver and adipose tissue (Herzberg, 1983) and long term feeding of polyunsaturated fatty acids (PUFAs) suppresses hepatic lipogenic enzyme synthesis (Shillabeer *et al*, 1990). Lipogenesis is the synthesis of fatty acids from precursors, so any increase in these precursors will translate into an increase in lipogenesis. When high carbohydrate diets are fed, animals show increased lipogenesis. When levels of fat in the diet are reduced, as seen in ruminants and other animals consuming a low fat diet, TAG must be synthesised from other dietary components. Treatment of cultured rat hepatocytes with PUFAs in response to treatment with insulin, dexamethasone and triiodothyronine (T_3) caused a reduction in ACC and FAS mRNA levels (Fukuda *et al*, 1992; Armstrong *et al*, 1991). It has since been shown that the inhibitory effect on lipogenic enzyme synthesis seems to be specific to liver (Girard *et al*, 1994), with no changes in FAS mRNA levels in adipose tissue of rats fed high levels of polyunsaturated fat (Shillabeer *et al* 1990) or in cultured rat adipocytes treated with linoleate and stimulated with insulin and dexamethasone (Girard *et al*, 1994). There is no effect of saturated or monounsaturated fatty acids on lipogenic enzymes in liver or adipose tissue (Ntambi, 2001; Shillabeer, 1990). The length of time that diets are fed for can have marked effects. Short term feeding or treatment of cells (48h to 2 weeks) results in small changes in lipogenic enzyme activity, but over time could have significant influence on the type of fatty acids and composition of fat stored in

adipose tissue (Shillabeer *et al*, 1990). Feeding saturated fatty acids over an extended period of time (12-18 months) does not reduce lipogenesis as effectively as a PUFA diet (Dupont *et al*, 1978), indicating that PUFAs are able to interact with the processes of lipid metabolism. PUFAs reduce hepatic lipogenesis by suppressing lipogenic genes expressed in liver; ACC, FAS and SCD (Xu *et al*, 1999; Jump *et al*, 1994), and stimulating fatty acid oxidation (Clarke, 2001). However the latter pathway is not as sensitive to PUFA regulation, so PUFAs seem to have their effects largely on lipid synthesis (Clarke, 2001). The key features that fatty acids seem to need to possess are a chain containing at least 18 carbons, and at least two double bonds in the 9 and 12 positions (Clarke & Jump, 1993). PUFA directly increase the amount of peroxisome proliferator-activated receptor α (PPARα), by acting as ligands (Clarke, 2001). Up-regulation of this receptor induces transcription of genes involved in fatty acid oxidation (Xu *et al*, 1999), one of which is responsible for the production of malonyl-CoA. The drop in malonyl-CoA concentration results in increased fatty acid entry into the mitochondria and peroxisomes which leads to increased fatty acid oxidation (Zammit, 1999). n-3 PUFAs are more potent than n-6, but metabolites of PUFAs are the most potent (Clarke, 2001). As previously mentioned, high levels of PUFAs are the most effective at suppressing fatty acid synthesis and stimulating fatty acid oxidation, but studies in porcine stromal-vascular adipocytes indicated that high levels of fatty acids (>100μM) may have a cytotoxic effect, with many cells treated with long chain PUFAs being lost from the plates (Ding & Mersmann, 2001). The researchers put forward several possible explanations for this: 1) PUFA may be more effective detergents and cause a loss of cell integrity; 2) Large amounts of certain PUFAs may be incorporated into cell membranes, changing function and attachment to the plate; 3) Oxidation products of PUFA may have adverse effects on the cells; 4) A metabolite of the PUFA; possibly an eicosanoid may be the trigger for cell loss; 5) Apoptosis may be stimulated (Ding & Mersmann, 2001). In support of this it has been noted that PUFA metabolites, eicosanoids and oxidised fatty acids are more potent transcriptional activators than PUFAs (Clarke, 2001). Clearly the feeding of PUFAs will result in greater absorption from the digestive tract, resulting in higher plasma non-esterified fatty acid (NEFA) levels. This results in an increase in the activity of LPL (Peterson *et al*, 1990), which is able to bind the fatty acids, but in doing so, its affinity for lipid

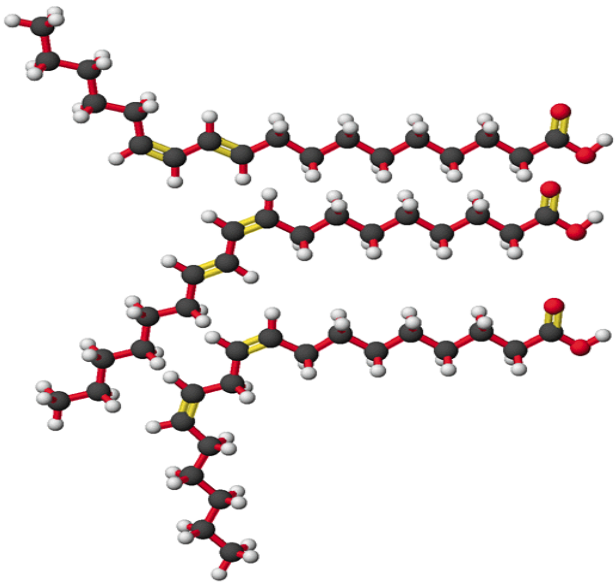
droplets is reduced (Posner & DeSanctis 1987; Bengtsson & Olivecrona, 1980). Albumin can reverse this, by binding to the fatty acids (Bengtsson-Olivecrona & Olivecrona, 1987; Posner & DeSanctis 1987; Bengtsson & Olivecrona, 1980; Scow & Olivecrona, 1977). In contrast to the PUFAs, oleic acid has been shown to have a stimulatory effect on LPL during the differentiation of porcine adipocytes in culture. Expression levels of LPL are indicative of the lipogenic condition of the cell (Ding & Mersmann, 2001). As concentrations of oleic acid increased, LPL mRNA levels rose, as did differentiation of the cells. There is a peroxisome proliferator-response element (PPRE) in the promoter area of the LPL gene, and this in conjunction with the concomitant rise in PPARγ mRNA suggests that the effects of oleic acid on LPL mRNA concentrations are mediated through PPARγ (Ding & Mersmann, 2001). The effect of oleic acid was acute (mRNA level increases seen at 1-5d and no change thereafter), suggesting that the fatty acid affects transcription, rather than acting through membrane compositional change. SCD mRNA levels also increase during the differentiation of 3T3-L1 preadipoctyes (Ntambi, 1995), and it is likely that PPARγ is involved. Inhibition of hepatic SCD1 by 50, 55 and 95% respectively was seen in rats fed trilinolein, trilinolenin and triarachidonin, compared with fat free controls (Ntambi, 1992). PUFAs with longer chain lengths seem to be most potent. There was no difference in SCD1 mRNA levels between rats fed saturated and monounsaturated fat. This was demonstrated in adipose tissue of rats fed linoleic acid, where a 75% decrease in SCD1 mRNA levels was seen (Jones *et al*, 1996). SCD1 mRNA levels decreased dose-dependently, and were accompanied by reduced levels of palmitoleic acid, the product of SCD1 activity. A reduction in SCD1 activity was also seen in the liver of rats fed linoleic acid (Jeffcoat & James, 1978), providing a further mechanism for fatty acid effects. Ntambi (1992) observed that PUFAs act at the level of gene transcription to reduce induction of hepatic SCD1. It remains to be seen whether this is true for adipose tissue as well, or if there are effects on enzyme activity rather than transcription. Studies in 3T3-L1 pre-adipocytes treated with arachidonic acid showed a dose dependent inhibitory effect on both SCD enzyme activity and mRNA levels (Sessler *et al*, 1996) Treatment with linoleic and linolenic acid also reduced SCD1 mRNA levels but stearic and oleic acids had no effect. By comparing enzyme activity with mRNA levels, Sessler *et al*

(1996) suggested that the fatty acids were affecting mRNA stability as the reductions in the activity and mRNA were not equal. Transcriptional run-on assays confirmed that the mRNA half-life was reduced. The discovery of SREBPs and their effects on many of the genes involved in lipid metabolism allowed the effects of fatty acids to be seen from a different perspective. A number of studies have shown that suppression of the SREBP-1 gene is accompanied by low expression of lipogenic genes (Xu *et al*, 1999; Yahagi *et al*, 1999), and that SREBP mRNA levels are suppressed by PUFAs, but not saturated fatty acids (SFAs) or monounsaturated fatty acids (MUFAs) (Xu *et al*, 1999; Yahagi *et al*, 1999). Evidence for the action of SREBP as a "master switch" for lipogenic genes came from the study of FAS, which was shown to contain DNA binding sites for SREBP-1 (Magana & Osborne, 1996). It has been suggested that the effects of PUFAs on SREBP and subsequently lipogenic genes is mediated by an impairment of the proteolytic release of SREBP (see Section 1.4.6.1.1.) by inhibition of transcription factors involved in insulinemic and carbohydrate control of lipogenesis, thereby reducing the nuclear content of mature SREBP (Xu *et al*, 1999). This would result in an increased rate of SREBP mRNA degradation as seen in SCD (Sessler *et al*, 1996) and ACC (Katsurada *et al*, 1990) which would result in a lowering of precursor SREBP anchored in the endoplasmic reticulum. However, there are links between PPARs and SREBP. Activation of SREBP-1c results in increased FAS mRNA in adipose tissue, leading to increased fatty acid synthesis and generation of ligands for PPARγ (Gurr, Harwood & Frayn, 2002). However, it is clear that the existence of PPARs and SREBPs cannot explain all the effects of fatty acids on lipid metabolism, as PPAR$\alpha^{-/-}$ mice fed PUFAs still showed reduced mRNA levels for lipogenic enzymes, so clearly PUFAs can have their effects either through a different transcription factor or directly on the genes themselves.

1.5. CONJUGATED LINOLEIC ACID

Conjugated linoleic acid is a collective term for a number of positional and geometric isomers of linoleic acid, an 18 carbon fatty acid with two double bonds. They are referred to as conjugated because the double bonds are separated by 2 carbon atoms between which is a single bond. The two most abundant isomers are *cis-9, trans-11* and *trans-10, cis-12* CLA (Figure 1.10). These are positional isomers, because the double bonds are between different carbon atoms in each isomer. Double bonds are also possible between different carbons in the fatty acid chain; *cis-cis*, *trans-trans*, *cis-trans* and *trans-cis* combinations for double bonds at C7, 8, 9, 10, 11, 12, 13 have been reported (Lavillonniere *et al*, 1998; Sehat *et al*, 1998; Yurawecz *et al*, 1998; Parodi, 1977). The conjugated double bond structure is thought to be responsible for the many biological effects of CLA that have been reported.

Figure 1.10 Structures of the two major isomers of CLA and linoleic acid



trans-10, cis-12 CLA

cis-9, trans-11 CLA

cis-9, cis-12 LA

Source: Steinhart (1996)

1.6. PRODUCTION OF CLA

1.6.1. Ruminal CLA production

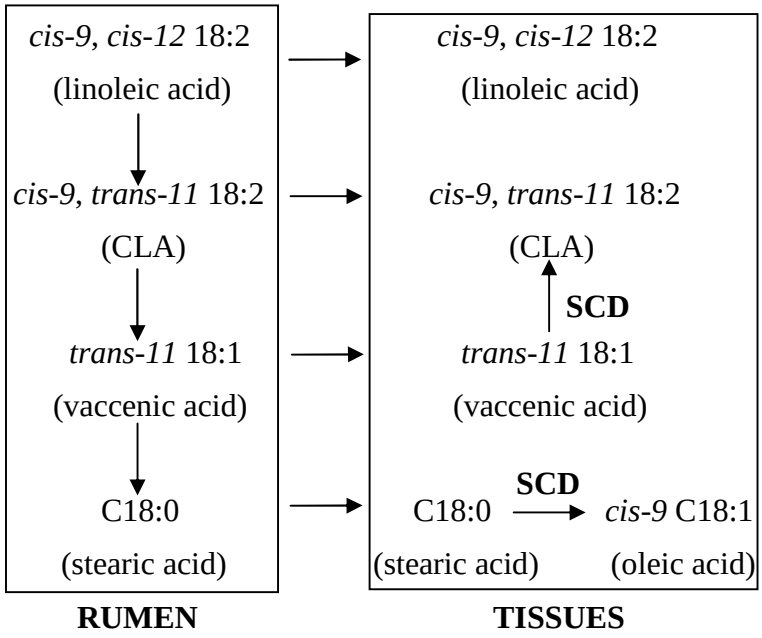
In ruminants, there are two methods by which CLA can be synthesised. The first is by biohydrogenation of dietary linoleic acid by rumen bacteria. The initial step is isomerisation (Section 1.4.5.1) by linoleate isomerase to form *cis-9, trans-11* CLA. This is then converted to *trans-11* 18:1. The latter happens very quickly, so consequently concentrations of *cis-9, trans-11* CLA do not build up in the rumen (Kellens *et al*, 1986). A study comparing levels of CLA in meat from ruminants and non-ruminants (Chin *et al*, 1992) found that levels of CLA were higher in the former, and that the major isomer present was *cis-9, trans-11* Chin *et al* (1992) concluded that since linoleate isomerase produces *cis-9, trans-11* CLA, rumen microbial activity must be the source of this isomer. The accumulation of CLA in the tissues due to incomplete biohydrogenation of CLA was also reported by Kelly *et al* (1998). But it became clear that the presence of CLA in the tissues could not be explained by this mechanism of CLA production alone. There was a shortfall between amounts of CLA produced in the rumen and measured in the tissue, and it was clear that there was something else producing the CLA in the tissues (Griinari & Bauman, 1999). Although *trans-11* 18:1 can pass out of the rumen and be absorbed in the gastrointestinal tract, because CLA is so quickly hydrogenated, little is available for absorption in the gastrointestinal tract and therefore CLA produced in this way is not the only contributor to tissue concentrations of CLA.

1.6.2. Endogenous CLA production

The discrepancy between amounts of ruminally produced CLA and tissue concentrations led to a number of suggestions for the presence of CLA in ruminant tissues. It was known that cultured rat hepatocytes were capable of desaturating *trans-11* 18:1 to *cis-9, trans-11* CLA (Mahfouz *et al*, 1980) and this led to hypotheses that this was happening in ruminant tissue (Griinari *et al*, 1997). This seemed more likely, after the discovery that the desaturase enzyme SCD was present in mammary gland (Kinsella, 1972) combined with the fact that desaturation occurs readily in adipose tissue, with preference given to fatty acids synthesised *de novo* (Pothoven *et al*, 1974). As mentioned in Section

1.4.3, SCD acts on palmitic and stearic acids to form C16:1 and *trans-11* C18:1 respectively, but Griinari *et al* (2000) demonstrated that it was also able to form *cis-9, trans-11* CLA from *trans-11* 18:1 (Figure 1.11). An experiment inhibiting SCD in tissues by abomasal infusion of sterculic oil demonstrated that as levels of *trans-11* 18:1 reduced, there was a concomitant reduction in tissue CLA concentrations (Griinari, 2000). A number of other studies have also demonstrated a close relationship between CLA and *trans-11* 18:1, and that this is applicable over a large range of *trans-11* 18:1 concentrations (Griinari & Bauman, 1999).

Figure 1.11 CLA biosynthesis in ruminants



Adapted from Griinari & Bauman (1999)

However, there is another major isomer of CLA to consider, which is also found in the milk and tissues of ruminants and other animals. The *trans-10, cis-12* isomer has been identified in cow milk (Griinari & Bauman, 1999), and feeding a low fibre diet resulted in the *trans-10* isomer being the most abundant in milk fat (Griinari, 1998). There is no evidence to suggest that mammals possess a delta-12 desaturase (Pariza *et al*, 2001), to act on *trans-10* 18:1 and convert it to *trans-10, cis-12* by the insertion of a double bond at position 12 in the fatty acid chain. This would suggest that its origins are in the rumen, and formation by biohydrogenation, followed by absorption from the gastrointestinal tract, however this merits further research to fully elucidate the mechanism by which *trans-10, cis-12* CLA is incorporated into the tissues.

1.6.3. Desaturase indices

Fatty acid composition of tissues provides an indication of enzyme activity. The Δ9 desaturase index provides an estimate of SCD enzyme activity (Corl *et al*, 2001), and examines the ratio of the substrate for SCD and product of SCD action. The ratio can be calculated as: C18:1 *cis-9* / C18:0 or C16:1 *cis-9* / C16:0. Although these fatty acids are found in the tissues normally, changes in the indices are a sign of changes in SCD enzyme activity.

1.7. EFFECTS OF CLA

1.7.1. CLA as an anti-carcinogen

Ha *et al* (1987) first isolated CLA from grilled minced beef. It was of particular interest at that time because it was found to inhibit carcinogenesis. Ip *et al* (1991) showed that dietary CLA fed at 0.5, 1 and 1.5% could prevent mammary carcinogenesis in rats exposed to 7,12-dimethylbenz(a)anthracene at 50d of age. This anti-carcinogenic effect was dose dependent, but feeding greater than 1% CLA did not further reduce tumour incidence appreciably. Subsequent studies have determined that the anti-carcinogenic effects of CLA are seen at 0.1%, indicating high potency. The majority of studies examining carcinogenesis in response to CLA treatment have been done using mammary tumour models, but effects have been seen in 5 different tissues and in several strains of both mice and rats (Scimeca, 1999). A further dose-response study showed conclusively that CLA is effective at low concentrations in the diet, with chronic feeding of CLA at levels of 0.05-0.5% in the diet dose dependently inhibiting rat mammary tumours induced by 7,12 dimethylbenz(a)anthracene (Ip *et al*, 1994) Chronic feeding is more effective than acute feeding, as rats fed for 4 weeks subsequent to carcinogen treatment with 1% CLA showed no reduction in tumour incidence. CLA feeding for 8 and 20 weeks delayed appearance of tumours by 2-3 weeks and significantly reduced tumour appearance respectively (Ip *et al*, 1997). It is possible that there may be a concentration which must be attained before CLA is effective in preventing development of cancerous cells, supported by the emergence of evidence that for CLA to be effective in blocking malignant tumours it must be present in the organ affected. CLA is primarily incorporated into triacylglycerols, which are neutral lipids (Banni *et al*, 2001) and a major

constituent of the mammary gland. This explains the high level of incorporation of CLA seen in mammary gland in response to CLA feeding (Corl *et al*, 2003; Ip *et al*, 1997). Clearly the conjugated diene structure of CLA is important in the mechanism of effect in carcinogenesis, as linoleic acid-supplemented diets have been shown to increase tumours when compared to those supplemented with CLA (Cesano *et al*, 1998) and when high levels of linoleic acid were fed to female Sprague-Dawley rats during growth of mammary tissue they developed significantly more tumours than rats fed low levels (Lu *et al*, 1995). The differentiation and maturation period for the mammary gland appears to be a critical point in the process of carcinogenesis. Ip *et al* (1994) suggested that changes in gland development and morphogenesis could be a mechanism by which CLA exerts its effects. This is supported by work done on rat mammary epithelial cells in primary culture where cells were treated with a mixture of CLA isomers (Ip *et al*, 1999). Cells treated with CLA at increasing concentrations showed a dose-dependent reduced growth of mammary epithelial cell organoids (MEO), with 32μM inhibiting growth by 50%. An equi-molar concentration of linoleic acid had no effect, indicating that the conjugated diene structure of CLA has distinct biological effects. There is evidence of maintenance of this structure through *in vivo* elongation and desaturation of CLA; Banni *et al* (1995) detected conjugated dienes (CD) C18:3 and C20:3 in the livers of rats fed 0.04% CLA for 1 week. CD 20:4 may compete with arachidonic acid for cyclooxygenase (COX) and lipooxygenase (LOX), which control the synthesis of eicosanoids, including prostaglandins, thromboxanes and leukotrienes. In adipose tissue, it is conceivable that CLA or a metabolite of CLA may be released from the triacylglycerol pool contained in the adipocyte (Ip *et al*, 1997) and change eicosanoid synthesis in a similar way. It is possible that CLA may through the actions of its metabolites, indirectly modulate this process. It is clear that CLA feeding during maturation of mammary gland is most effective in reducing carcinogenesis. CLA was shown to inhibit DNA synthesis in cultured mammary epithelial cells when culture proliferation rate was high (Ip *et al*, 1999). There was also evidence that CLA induced apoptosis in differentiated mammary cell organoid cell cultures. It appears that CLA has a direct effect prior to initiation on development by limiting growth and proliferation of normal mammary epithelium, and that it does this by reducing DNA synthesis

and triggering apoptosis of mammary epithelial organoids (Ip *et al*, 1999; Ip *et al*, 1994). Another suggestion is that CLA could activate peroxisome proliferator activated receptors (PPARs) which are capable of altering epithelial growth either directly or indirectly (Houseknecht *et al*, 1998). Dietary CLA has been shown to be incorporated into mammary gland neutral lipids, with levels increasing while CLA feeding is occurring. Upon withdrawal of CLA a rapid reduction (return to basal levels in 4 weeks) in tissue CLA was seen and this correlated extremely well with tumour incidence (Ip *et al*, 1997), suggesting that the presence of CLA is needed to prevent tumour appearance. CLA incorporation into the phospholipids of mammary gland was noted to be much lower (0.5% of total fatty acids) than that of neutral lipids (3% of total fatty acids) and take much longer for withdrawal to have an effect. This reflects the resistance to compositional change in phospholipids, which are a major constituent of biological membranes (Ip *et al*, 1997). The observation that CLA is incorporated into phospholipids provides another possible mechanism for action of CLA. Any change in fatty acid composition will affect membrane fluidity and as such, will affect receptor activation, and affinities of factors for their respective receptors (Ip *et al*, 1999). As explained previously, CLA is a mixture of many different isomers, and there is evidence that they may have different effects due to their different shapes (Figure 1.10). Until recently, synthesis and isolation of pure isomers was extremely difficult, so effects seen with the mixtures of CLA used in experiments could not be attributed to one isomer, however this is now not the case and it has been possible to show that the *cis-9, trans-11* isomer is responsible for the anti-carcinogenic effects. This is demonstrated in rats with chemically induced mammary tumours fed a sunflower oil (LA rich) diet, a CLA mix or purified *cis-9, trans-11* CLA. The two CLA treatments reduced tumour incidence by 44 and 45% respectively, when compared to sunflower oil (Lavilloniere *et al*, 2003), but it is of note that other studies have shown that linoleic acid promotes tumour development (Ip *et al*, 1997), so inhibitory effects of CLA on tumour development may have been exaggerated when expressed relative to linoleic acid. The formation of *cis-9, trans-11* CLA from C18:1 *trans-11* by SCD in the tissues is well documented (Section 1.6), and as such, a number of studies have examined the effects of feeding other fatty acids on tissue CLA concentration and tumour incidence. Rats fed 1, 2 and 3% vaccenic acid showed proportionately

increased concentrations of CLA and its metabolites in liver and mammary gland up to 2%, after which levels remained constant (Banni *et al*, 2001). Levels of CLA in mammary gland with 2% vaccenic acid feeding were equivalent to those which had been previously shown to inhibit tumour incidence. A second linked study fed 2% vaccenic acid to rats treated with carcinogens, and demonstrated that there was a correlation between vaccenic acid feeding and the number of pre-malignant mammary lesions. This strongly suggests that vaccenic acid feeding could be used as an anticancer drug, and has its effects by being the precursor for *cis-9, trans-11* CLA synthesis endogenously by SCD. A similar study examined the additive effects of feeding both vaccenic acid and *cis-9, trans-11* CLA in rats with chemically induced mammary carcinomas. There was an additive effect of CLA and vaccenic acid on reduction of tumours, as well as a dose response effect of both fatty acids. The effect of CLA was not as significant as that of vaccenic acid, due to concentrations of the former being at the low end of the dose response curve (Corl *et al*, 2003). Feeding vaccenic acid and CLA gives a further inhibitory effect on tumour incidence due to the action of SCD. Clearly there are a number of ways by which CLA and its associated metabolites and precursors could be utilised to treat cancer. In conclusion, CLA is a potent anti-carcinogen, efficacious at dietary concentrations close to those consumed by humans (Ip *et al*, 1994). In addition, although it seems that *trans-10, cis-12* CLA may cause hyperinsulinemia and insulin resistance (Roche *et al*, 2002; Clement *et al*, 2002), there have been no such serious effects seen in animals fed *cis-9, trans-11* CLA. Dietary manipulation seems to be the most economic way to deliver the favourable effects of CLA through vaccenic acid/CLA *cis-9, trans-11* and there is no doubt that endogenous production of *cis-9, trans-11* CLA by SCD is an important route for treatment and prevention of cancer.

1.7.2. Effects on fat and lean repartitioning

The first evidence for an effect of CLA on body composition was shown in mice (Park *et al*, 1997), where body fat mass in male and female mice fed 0.5% mixed isomer CLA was reduced by 57 and 60% respectively. In addition to this, lean mass was significantly elevated, by 5 and 14% in male and female mice respectively. Further studies in mice (West *et al*, 1998) showed a reduction in fat pad weights and body weight in mice fed high and low fat diets, both with and without CLA. This effect was also shown in other rodents; in rats fed 3% CLA and subjected to a diet that increased fat store mobilization, body fat reduced and body protein increased compared to rats fed sunflower oil (Stangl, 2000), and in sheep fed CLA prior to weaning body lipid was reduced (Mir *et al*, 2000). The increase in mRNA for uncoupling proteins (UCP) 2 and 3 during starvation, which parallels increased fat breakdown, may be the mode of action for CLA repartitioning in this case. Similarly, rats fed 2% CLA showed reduced TAG content in white adipose tissue (Yamasaki *et al*, 1999) suggesting that CLA may inhibit lipid filling of adipocytes. It is of note that the reduction in fat pad weight occurred despite an increase in food intake in rats fed CLA compared to controls. This is indicative of a specific effect on lipid metabolism, and may be due to an increase in energy expenditure. Increased levels of UCP-2 mRNA were seen in adipose tissue from mice fed CLA (Tsuboyama-Kasaoka *et al*, 2000), and this may have resulted in apoptosis of adipocytes due to decreased efficiency of ATP synthesis due to metabolic uncoupling. From the rodent studies that have been done, it is evident that mice respond much more dramatically to CLA feeding than rats in terms of fat reduction; in rats fed 0.5% CLA the reduction in fat pad weight was 15-25%, in mice it was 50-80% (Yamasaki *et al*, 1999). Some studies have shown a reduction in food intake (West *et al*, 1998), which seems consistent with reduced body fat mass; however this cannot be entirely responsible, because an increase in energy expenditure was also seen (West *et al*, 1998; Park *et al*, 1997) measured by changes in metabolic rate and an increase in muscle carnitine palmitoyl transferase-1 (CPT-1) activity respectively. Park *et al* (1997) suggested that the effects of CLA were manifested in a reduction of fat deposition, combined with increased lipolysis in adipocytes and potentially an increase in fatty acid oxidation in muscle and adipocytes. The same group have

shown that the *trans-10, cis-12* isomer is responsible for the repartitioning effect seen in CLA feeding studies (Park *et al*, 1999). There was no effect of the *cis-9, trans-11* isomer, but a mixture of isomers and *trans-10, cis-12* CLA reduced body fat. Possible mechanisms for this were suggested as a result of work in cultured adipocytes (fat storage) and skeletal muscle cells (major site of fat oxidation). To support these mechanisms, reductions in intracellular TAG and glycerol were seen, together with enhanced glycerol release into the culture media and reduced LPL activity indicating less fatty acid uptake and a reduction in TAG synthesis. In rats fed 1% *trans-10, cis-12* CLA and a CLA mix, an increase in adipose tissue CPT-1 enzyme activity was seen (Martin *et al*, 2000). This indicated that beta oxidation was increased, but there was no evidence that peroxisome proliferation was happening. This is in agreement with recent research findings (Moya-Camarena *et al*, 1999), and contradicts earlier findings which indicated that peroxisome proliferation was seen with increased beta oxidation (Belury *et al*, 1997). No evidence for a reduction in TAG synthesis was seen when phosphatidate phosphohydrolase (the rate limiting enzyme in *de novo* TAG synthesis in adipocytes) enzyme activity was measured (Martin *et al*, 2000). The increase in beta oxidation may result in lower levels of *trans-10, cis-12* in tissue lipids, as seen in hamsters fed CLA (de Deckere *et al*, 1999), due to the geometric and positional differences between the major two conjugated isomers which favour oxidation of *trans-10, cis-12* CLA over *cis-9, trans-11* CLA (Martin *et al*, 2000). Lower amounts of *trans-10, cis-12* CLA, linoleic and linolenic acids in the liver of hamsters fed *trans-10, cis-12* CLA and a CLA mix, indicate that the *trans-10, cis-12* isomer is responsible for an increase in oxidation of 18 carbon PUFAs (de Deckere *et al*, 1999). Another characteristic seen in animals fed CLA is the increase in liver weight (Tsuboyama-Kasaoka *et al*, 2000; de Deckere *et al*, 1999; West *et al*, 1998), often combined with a reduction in adipose tissue weight. Several researchers have suggested that this is due to lipid filling (Tsuboyama-Kasaoka *et al*, 2000; de Deckere *et al*, 1999; West *et al*, 1998). Reports that *trans-10, cis-12* CLA and a mixture of isomers induces peroxisome proliferation in mice and therefore liver hypertrophy (Belury *et al*, 1997) have been contradicted by a study which found no effect of CLA on palmitoyl CoA peroxidase and carnitine acetyl transferase, markers of peroxisome proliferation (de Deckere *et al*, 1999). It seems that the increase in liver weight is due primarily to

hypertrophy. There have also been a number of studies done in the 3T3-L1 preadipocyte cell line to study the effects of CLA on cell differentiation during adipogenesis, and after confluence on mature adipocytes. Post confluent 3T3-L1 adipocytes treated with 100μM CLA for up to 96 hours had more apoptotic cells than BSA and linoleic acid treated cultures (Evans *et al*, 2000). This supports the study reported previously (Tsuboyama-Kasaoka *et al* 2000), which suggested that CLA stimulated apoptosis of adipocytes. Preadipocyte proliferation was inhibited by CLA, indicating a mechanism by which adipogenesis was reduced, which could be active in reducing fat pad weights. Adipogenic differentiation factor PPARγ2 mRNA was reduced with CLA treatment (Brodie *et al*, 1999), indicating a clear effect on differentiation. In addition, CLA may have effects on mature cells; post-confluent cultures treated with 50-200μM CLA had less TAG and smaller cell sizes compared to cultures treated with linoleic acid. Treatment of cells with *trans-10, cis-12* CLA reduced TAG content significantly when compared to a mixture of CLA isomers (Evans *et al*, 2000); this is further evidence that the *trans-10, cis-12* CLA isomer is responsible for effects on lipid metabolism. The effects of CLA on differentiation are unprecedented, as fatty acids stimulate adipogenesis (Amri *et al*, 1991), so CLA has potential for altering lipid metabolism in terms of reducing fat synthesis. CLA has been shown to have marked effects on fatty acid composition of tissues; sheep fed CLA had reduced levels of oleic acid in adipose tissue (Mir *et al*, 2000), and liver microsomes from mice fed CLA supplemented diets had reduced levels of MUFAs than those from mice fed 5% corn oil (Lee *et al*, 1998). There was also a decrease in the desaturase index (see Section 1.6.3), likely to reflect a decrease in the enzyme activity; this has also been seen in pigs where CLA feeding increased SFAs and reduced MUFAs in TAG (Dugan *et al*, 2000). 100μM *trans-10, cis-12* CLA reduced mouse liver microsomal SCD activity, in contrast to *cis-9, trans-11* CLA where activity was not different to control levels (Park *et al*, 2000). *Trans-10, cis-12* CLA reduced hepatic SCD activity in rat liver microsomes dose dependently (Bretillon *et al*, 1999), but there was no effect of *cis-9, trans-11* CLA. Taken together, these studies suggest that *trans-10, cis-12* CLA is responsible for the reduction in MUFAs and increase in SFAs seen in animals fed a mixture of CLA isomers. In mice, CLA feeding reduced levels of hepatic SCD mRNA relative to beta actin, levels of which were unchanged with CLA (Lee *et al*,

1998), increased levels of SFAs and reduced those of MUFAs. This was a clear link between enzyme activity and gene expression and indicates that down regulation of the SCD gene is accompanied by a reduction in protein levels and enzyme activity, resulting in a drop in the levels of palmitoleic and oleic acids. Choi *et al* (2000) demonstrated a reduction in SCD1 gene expression in 3T3-L1 adipocytes treated with *trans-10, cis-12* CLA. Based on the observation that CLA had been shown to reduce MUFAs (Lee *et al*, 1995), they hypothesised that *trans-10, cis-12* CLA reduced fat (Evans *et al*, 2000) by lowering levels of MUFA in TAG, which leads to small lipid droplets and smaller cells, as oleic and palmitoleic acids make up 58% of the fatty acids in the lipid droplets of these cells (Lee *et al*, 1995). Effects of *trans-10, cis-12* CLA on 3T3-L1 preadipocyte differentiation are likely to be through inhibition of the SCD gene, as no effects of CLA on PPARγ2 were seen (Choi *et al*, 2000). However, in light of previous studies where inhibitory effects of CLA on PPARγ2 were seen (Brodie *et al*, 1999; Satory & Smith, 1999), this is still unclear. The identification of a PUFA response element in the SCD gene, along with other lipogenic genes (Clarke & Jump, 1993) suggests that CLA may act by reducing SCD1 mRNA stability and/or gene transcription. Notably, CLA treatment has been shown to consistently reduce MUFAs and increase SFAs by an as yet undiscovered mechanism. CLA has also been implicated in the reduction of milk fat observed in dairy cows fed certain diets. Abomasal infusion of CLA decreased milk fat (Chouinard *et al*, 1999; Bauman *et al*, 1998; Loor & Herbein, 1998), and this effect was most pronounced on *de novo* fatty acid synthesis and desaturation. The former was manifested in a reduction in short and medium chain fatty acids that are synthesised by the mammary epithelial cells (Bauman *et al*, 1998). Ratios of the SFA:MUFA increased dose dependently, indicating inhibition of SCD (Chouinard *et al*, 1999), and there is evidence that FAS and SCD activity are inversely related to CLA uptake (Jayan *et al*, 1998). Abomasal infusion of individual CLA isomers demonstrated that only *trans-10, cis-12* CLA was able to reduce milk fat (Baumgard *et al*, 2000), and that this isomer was responsible for increasing the ratio of 18:0:18:1, and therefore reducing the activity of SCD. There was a greater reduction in fatty acids from *de novo* synthesis than pre-formed fatty acids, in line with Bauman *et al* (1998), indicating an effect on fatty acid synthesis. Indications that CLA may work by reducing the activities and

expression of key lipogenic genes came from work done in cows fed milk-fat depressing diets. Loor & Herbein (1998) had already suggested that CLA may reduce ACC, and in cows fed milk fat depressing diets, ACC and FAS activity decreased by 61 and 44% respectively, and ACC mRNA levels were comparatively reduced (Piperova *et al*, 2000). This is consistent with a reduction in mammary *de novo* fatty acid synthesis mediated through down-regulation of ACC, leading to reduced ACC and FAS enzyme activity. *Trans-10, cis-12* CLA has been shown to depress milk fat, and the mechanism by which ACC and FAS was affected in response to a non-CLA-containing but milk fat depressing diet is likely to be the mode of action for this CLA isomer.

1.8. PROJECT HYPOTHESES

1) The fat reducing effect of CLA is due to decreased lipogenesis in adipose tissue

2) The fat reducing effects seen in rodents and other monogastric animals will occur in ruminants provided that sufficient CLA reaches the tissues

1.8.1. Experimental objectives

The first series of experiments examined lipogenesis, fatty acid formation and expression of key lipogenic enzymes in sheep adipose tissue explants treated with *cis-9*, *trans-11* and *trans-10 cis-12* CLA, both together and as individual isomers over a range of different concentrations. The effect of CLA on mRNA levels for ACC and SCD in sheep was investigated further in an *in vivo* trial, together with fatty acid composition of tissues. The final experiment examined potential mechanisms of CLA action on lipid metabolism in the hamster, a small monogastric animal model that has been shown to respond to CLA treatment, by examining carcass and fatty acid composition and mRNA levels of lipogenic genes in response to different supplementation levels of CLA.

It is important to determine how CLA has its fat reducing effects to ascertain any possible adverse effects on lipid metabolism such as an increase in the saturated fatty acid profile or hepatosteatosis as a result of supplementing diets with CLA. The effects of the individual isomers need to be determined, as some of the effects attributed to CLA may be as a result of feeding different purities of CLA isomers, containing many different forms of CLA, which may have differing effects on lipid metabolism. This thesis describes the effects of CLA on adipose tissue lipid metabolism in the sheep, through direct treatment of tissue with CLA and dietary supplementation. The effects of dietary CLA supplementation on lipid metabolism in the hamster are also described. This thesis provides new evidence that CLA follows different routes of action in ruminant and monogastric animals.

2. MATERIALS AND METHODS

2.1. REAGENT SOURCES

Applied Biosystems

Amplitaq Gold with 10X PCR Gold buffer and $MgCl_2$ solution.

BDH Laboratory Supplies, Merck Ltd., Lutterworth, Leicestershire

Mercaptoethanol.

Fisher Scientific, Loughborough, Leicestershire

Chloroform, EDTA, Ethanol, Glacial acetic acid, Hexane, Isopropanol, Methanol, Phenol, Sodium acetate, Sodium chloride, Sodium hydroxide, Sodium methoxide, Toluene, Whatman filter paper.

Natural Lipids, Norway

Conjugated linoleic acid isomers *cis-9*, *trans-11* and *trans-10*, *cis-12*, Tonalin™.

Promega UK Ltd., Chilworth Research Centre, Southampton, Hampshire

Blue/orange 6X loading dye, NTPs (ATP, CTP, GTP and UTP), Random hexamers, Reverse transcriptase, RNAse, DNAse with 10x buffer, 100 and 25 base pair DNA ladders.

Rhone Merieux, Harlow, Essex

Sodium pentabarbitone – 60mg/ml Sagatal.

Special Diet Services, Witham, Essex

RM 1: 2% fat maintenance ration rodent chow .

Sigma Chemical Company, Poole, Dorset

Bovine serum albumin, Boron tri-fluoride, Chloroform-iso-amyl alcohol, Conjugated linoleic acid (1:1 *cis-9, trans-11*:*trans-10, cis-12*), Dexamethasone, Diethyl pyrocarbonate, Gentamycin, Guanidinium thiocyanate, HEPES, Insulin from bovine pancreas, Linoleic acid, Medium 199, Oleic acid, Penicillin G, Phenol:chloroform:isoamyl alcohol, Phosphate buffered saline, Potassium chloride, Sodium hydrogen carbonate, streptomycin sulphate, Water (Ribonuclease free).

2.2. ANIMALS AND TRIALS

2.2.1. Explant culture of ovine subcutaneous adipose tissue

The aim of this project was to examine the effects of fatty acids, particularly the isomers of conjugated linoleic acid on fatty acid metabolism in animals, and specifically their effects on lipogenesis in adipose tissue and liver.

2.2.1.1. Animals

Sheep approximately 12 months old were used for the explant experiments, and were housed in individual pens in the metabolism unit on the University farm. The sheep were Mule x Charolais ewes and were fed a pelleted diet containing 225g barley, 450g oats, 225g grass meal and 100g Nutramol 30 (molassed feed meal) per kg. Body weight was recorded immediately prior to slaughter and animals were killed by stunning and exsanguination. Samples of adipose tissue were removed from the subcutaneous depot (above the base of the tail) for culture.

2.2.2. Effect of protected CLA feeding in sheep on lipid metabolism

The aim of this trial was to examine the effects of feeding CLA to sheep on ACC and SCD RNA levels in adipose tissue depots and liver in order to investigate whether CLA affects lipogenic gene expression *in vivo*. The cooperation of Dr. Richard Wynn in conducting the animal feeding trial is gratefully acknowledged. Full details of this trial and the results obtained are described in Wynn (2003), particularly the effects of protected CLA on the rates of fat deposition in the sheep.

2.2.2.1. Animals

36 weaned ewe lambs were used for this study with an initial weight of approximately 28kg. Lambs were individually penned with free access to water and mineral licks. Prior to feeding trial diets, lambs were given basal diet (Tables 2.1 and 2.2) for 2 weeks to allow them to acclimatise to diet and accommodation. Animals were also wormed during this time (Nilverm Gold, Schering Plough Animal Health).

Table 2.1 Composition of basal lamb fattener diet

Diet component	Amount in diet (g/kg fed weight)
Barley	550
Oats	350
Molassed feed meal	50
Extracted soybean meal	25
Sheep vitamins & minerals (Frank Wright Ltd.)	25

Table 2.2 Analysis of basal lamb fattener diet

Crude Protein (g/kg dry matter)	Gross energy (MJ/kg dry matter)	Estimated metabolisable energy (MJ/kg dry matter)	Lipid (g/kg)	Dry matter (g/kg)
109	18.42	12.54	29	874.2

2.2.2.2. Experimental diets

Lambs were fed a lamb fattener diet as ground meal, the composition of which is displayed in Table 2.1, to grow at approximately 180g per day. Supplementation of ruminally protected CLA-80 (80% CLA; Natural ASA, Hovdebygda, Norway), or Megalac™ (Volac International Ltd., Royston, Hertfordshire) was added by hand to diets 1-6 in differing amounts (Table 2.3). Although exact details of the protection method are not in the public domain, protection of the CLA-80 from rumen biohydrogenation was understood to be achieved by coating it with specific hydrogenated fatty acids, which pass virtually unchanged through the rumen (Trouw Nutrition UK, Wincham, Northwich, Cheshire). Megalac is a commercially available lipid source containing fatty acids bound as calcium salts which are protected against rumen breakdown but dissociate in the acidic conditions of the abomasum, releasing the fatty acids for absorption. The protected CLA (PCLA) contained 67.7% (w/w) lipid. Of the lipid component (as free fatty acid equivalents) 12.5% was the *cis-9, trans-11* isomer and 12.5% was the *trans-10, cis-12*

isomer of conjugated linoleic acid, 58% was C18:0 and 10% was C16:0. The protection against rumen degradation was assessed by Dr. Richard Wynn (see Wynn, 2003) by feeding the material to sheep fitted with rumen and proximal duodenal cannula and assessing the flow of CLA to the proximal duodenum. The dual phase marker technique of Faichney (1975) was used to assess the flow of digesta to the duodenum. The material was found to be 65.8% protected against rumen biohydrogenation while the unprotected CLA fed in the same experiment was only 8.5% protected.

2.2.2.3. Animals and treatments

Thirty-six female Mule x Charollais lambs (age 72 ± 3d, live weight 27.9 ± 1.4kg), which had been with their mothers at grass, were individually housed in an environmentally controlled metabolism unit with continuous access to water and a mineral block. During a three week adaptation period all animals were fed a concentrate diet (Tables 2.1, 2.2) as a meal to achieve a growth rate of approximately 180g/d. After the adaptation period animals were randomly assigned to one of seven treatment groups and fed the same concentrate diet to grow at 180g/d plus one of the following: 25g PCLA/kg DM (Low PCLA), 50g PCLA/kg DM (Medium PCLA), 100g PCLA/kg DM (High PCLA), 21.7g Megalac/kg DM (Low Megalac), 43.3g Megalac/kg DM (Medium Megalac), 86.6g Megalac/kg DM (High Megalac) (all n = 5) or no added supplement (Control; n = 6). Megalac (Volac Ltd., Royston, Herts, U.K.) was used to control for the total lipid content of the PCLA and both supplements were balanced for energy content at each intake level and provided extra energy above that of the basal ration (Table 2.3). The gross energy content of the PCLA was 28.73MJ/kg fed weight and that of the Megalac 33.17MJ/kg fed weight. The chemical composition of the PCLA has been described above. The Megalac however contained 812mg lipid/g and the major fatty acids were palmitic (C16:0; 48.8%) and oleic acid (C18:1; 34.4%). Diets were fed for 10 weeks and all the animals were weighed twice weekly to monitor growth rate and adjust feed intakes accordingly. Levels of feed were calculated according to the Ministry of Agriculture, Fisheries and Food (1975) and were adjusted once a week according to the mean weight of the treatment group. Throughout the trial all animals were fed once daily at 08.00h and feed refusals were noted.

Table 2.3 Supplement inclusion with crude protein (CP) and gross energy (GE) in treatment diets

Diet	Supplement addition (g/kg diet)	CLA (c9,t11 & t10,c12) (g in dry diet as fed/kg basal diet fed)	Estimated GE (MJ/kg dry diet fed/day)	Crude protein (g in dry diet as fed/kg basal diet fed)
1. Low PCLA	25.0	4.23	19.14	109
2. Medium PCLA	50.0	8.46	19.86	109
3. High PCLA	100.0	16.9	21.30	109
4. Low Megalac	21.7	-	19.14	109
5. Medium Megalac	43.3	-	19.86	109
6. High Megalac	86.6	-	21.30	109
7. Control	Nil	-	19.14	109

Lambs were fed for 8 weeks, reaching a final body weight of approximately 45kg.

2.2.2.4. Slaughter and collection of samples

Animals were randomly slaughtered within 36h by stunning and exsanguination, and samples collected. Small amounts of subcutaneous, perirenal and omental adipose tissue were taken and snap frozen in liquid nitrogen and stored at -80°C for RNA extraction and fatty acid composition analysis as described in Sections 2.3.9 and 2.3.10. A sample of liver was also taken and snap frozen for the same purpose. RNA was extracted from tissue and used to make complementary strand DNA, amounts of which were quantified by Taqman RT-PCR as described in Sections 2.3.11 and 2.3.14.

2.2.3. CLA feeding trial in hamsters

The aim of this experiment was to examine the effects of a 90% pure CLA mixture, consisting of a 50:50 mix of *cis-9, trans-11* and *trans-10, cis-12* CLA on body weight and composition, plasma cholesterol and triglyceride levels, adipose tissue depot weights, liver weight, fatty acid composition of these tissues and mRNA levels for key lipogenic genes.

2.2.3.1. Animals

40 male 6 week old weanling Golden Syrian hamsters were purchased from Harlan and individually housed in shoebox cages with free access to water and powdered diet, supplied in powder hoppers to minimise spillage. Hamsters were weighed before being assigned to their treatment groups, to ensure that the initial average weight was the same across groups. Animals were fed control diet for 1 week before their assignation to a treatment group and introduction to the treatment diets.

2.2.3.2. Experimental diets

A 2% standard rat chow (RM 1, SDS, Witham, Essex) with 2% added fat as rapeseed oil (food grade, J Sainsbury plc, London) was fed. The 2% added fat was replaced with CLA in the treatment diets, which were fed for 11 weeks. The treatment diets consisted of the following:

Control diet (2% fat standard rodent chow + 2% added fat as rapeseed oil)
0.5% CLA diet (2% standard rodent chow + 1.5% rapeseed oil and 0.5% CLA)
1.0% CLA diet (2% standard rodent chow + 1.0% rapeseed oil and 1.0% CLA)
2.0% CLA diet (2% standard rodent chow + 0% rapeseed oil and 2.0% CLA)

Hamsters were weighed weekly to monitor growth rate and well-being and any hamster losing more than 10% of total body weight during this period was withdrawn from the trial. Feed refusals were weighed three times weekly and fresh diet supplied.

2.2.3.3. Sacrifice and collection of samples

Hamsters were terminally anaesthetised with 15mg Sagatal, administered intraperitoneally. Body weight was measured prior to blood sampling by cardiac puncture. Blood was collected into EDTA blood tubes and placed on ice for plasma recovery. The entire liver was removed and weighed, and snap samples were taken and rapidly frozen in liquid nitrogen for RNA extraction and fatty acid composition analysis. Epididymal, perirenal and intrascapular fat pads were dissected out in their entirety, weighed and frozen in liquid nitrogen for analysis as described for the liver. The carcasses were retained at -20ºC for total body composition analysis.

2.2.3.4. Plasma recovery from blood samples

Blood was spun at 3000rpm for 20 minutes at 4ºC and the plasma layer removed using a Pasteur pipette into a clean LP4 tube. 200µl of plasma was stored in microtubes at -20ºC for triglyceride and cholesterol measurement.

2.3. ROUTINE MATERIALS AND METHODS

2.3.1. Preparation of adipose tissue explants

Adipose tissue was aseptically removed from a sheep immediately following slaughter, placed in warm buffer and rapidly transferred to the laboratory. Blood vessels and connective tissue were dissected out and the tissue was chopped into small (10-15mg) pieces. The pieces were then washed with culture media before incubation for 24 hours at 5% CO_2, 39°C in media with no treatment. This was to allow the tissue to recover and re-synthesise any damaged hormone receptors.

2.3.2. Culture media

Medium 199 containing Earle's salts and L-glutamine was made up with the following additions: HEPES: 5.2g/l (cell culture grade), $NaHCO_3$: 2.2g/l and anhydrous sodium acetate: 0.3602g/l, gassed with 95% O_2 / 5% CO_2 (Air Products) and the pH was adjusted to 7.4 using 5M NaOH. The media was then sterile filtered by means of a 0.22μm bottle top filter (Fahrenheit) and stored at 4°C until use. On the day of use, the following were also added to the media: Penicillin G: 0.044g/l, streptomycin sulphate: 0.05g/l, gentamycin sulphate: 0.044g/l and 0.5g/l of 50mg/ml BSA solution.

2.3.3. Explant culture

Following the 24 hour pre-incubation period, treatment culture media was made up as before, but treatments of oleic acid and conjugated linoleic acid *cis-9, trans-11* and *trans-10, cis-12* isomers, complexed to BSA (as described in Section 2.3.4) were added to the plates at 10μM concentrations for a further 24 hours. The media was refreshed after 24 hours, and left for a further 24 hours, prior to lipogenesis measurements being made.

2.3.4. Reagent preparation

2.3.4.1. 24% bovine serum albumin solution

12g of essentially fatty acid free BSA was added to 35ml 150mM NaCl in small amounts over a 5 hour period, using a magnetic stirrer to ensure that it was completely dissolved. The pH of the solution was then adjusted to 7.4 using 5M NaOH and the final volume made up to 50ml using 150mM NaCl. Aliquots were stored at -20°C until required.

2.3.4.2. Bovine serum albumin – linoleic acid / CLA / oleic acid complex

The complex was prepared according to Bruce (1996). 84mg of CLA isomers *cis-9, trans-11* or *trans-10, cis-12* was mixed well with 2ml ethanol and 100µl 5M sodium hydroxide. The ethanol was removed under a stream of nitrogen and 10ml of 150mM sodium chloride was added to the dried sodium salt of the fatty acid. The solution was heated for 3-5 minutes on a Dri-Block, and then transferred to a magnetic stirrer at room temperature. While the solution was still warm 12.5ml of ice cold 24% bovine serum albumin was added and the solution stirred for 10 minutes. The final volume was adjusted to 25ml with 150mM sodium chloride to give a concentration of 12mM and aliquots were frozen at -40°C until required. For oleic acid and linoleic acid, 97mg and 96mg of their sodium salts respectively were heated in 10ml of 150mM sodium chloride, and then prepared in the same way as conjugated linoleic acid to give concentrations of 12.7 and 14.3mM respectively.

2.3.5. Measurement of lipogenesis using radio-labelled acetic acid

Culture media was prepared as before, with treatments of oleic acid and CLA isomers *cis-9*, *trans-11* and *trans-10*, *cis-12* complexed to BSA added to individual flasks. Lipogenesis was measured by looking at the incorporation of radio-labelled acetate into the adipose tissue explants as previously done in this laboratory (Daniel *et al*, 2004; Dawson *et al*, 1999). [1-^{14}C] acetate (Amersham) was added to the culture media at 0.27µl/ml media, giving a specific activity of 0.1MBq/ml. Each flask contained 5ml of culture media. The tissue was transferred to the flasks, which were gassed with 95% O_2 / 5% CO_2

(Air Products), stoppered, and incubated in a shaking water bath at 39°C for 2 hours. T_0 flasks, which measured the incorporation of acetate in the instant that tissue was added to the media, were placed on ice before tissue addition, and then placed back in the ice bath immediately after tissue was added. After 4 hours, the flasks were removed from the water bath and placed in an ice bath to kill the tissue. The total weight of the explants was recorded and these were stored in plastic scintillation vial inserts at -40°C for extraction of lipid. The media in each flask was then collected for glycerol assay and stored at -40°C in plastic scintillation vial inserts.

2.3.6. Folch lipid extraction

Lipid was extracted from tissue using the method of Folch *et al* (1957). Approximately 200mg of the frozen tissue from the lipogenesis step was weighed out into a 50ml conical Sarstedt tube to which 5ml of methanol was added. The sample was then homogenized using a Polytron™ homogenizer for 30 seconds. 10ml of chloroform was added to the homogenate and the sample was homogenized further for 30 seconds. The tubes were vortexed and left to stand for 10 minutes. A Folch wash was then performed by adding 3.75ml 0.88% potassium chloride solution to the tubes and mixing thoroughly. The tubes were centrifuged at 3000rpm and 25°C for 10 minutes to enable the phases to separate. The lower organic phase (the chloroform) was collected using a short form Pasteur pipette into an oven dried, desiccated and weighed scintillation vial and dried under a stream of nitrogen to complete dryness. The vials were then placed in an oven at 80°C for 3-4 hours to dry off further and then desiccated and weighed to determine the weight of lipid extracted.

2.3.6.1. Measurement of acetate incorporated into lipid

8ml of Emulsifier-Safe scintillant (Packard) was added to each vial, and the vials warmed for 2 minutes in the oven at 80°C to dissolve the lipid. The vials were then vortexed until a clear solution was obtained and placed in a scintillation counter for 12 hours prior to counting for ^{14}C. Each vial was counted for a minute to give disintegrations per minute (dpm) of the carbon-14 present in the [1-^{14}C] acetate.

2.3.6.2. Measurement of fatty acid formation

In the measurement of the above it was necessary to distinguish between the different types of fatty acid present and to preserve them in their different forms they were stored under nitrogen and not dried in an oven after extraction of lipid. This helped to prevent oxidation of the lipid so that the different types of fatty acids could be separated out. To get a measure for percentage lipid recovered from the tissue, 3 samples of tissue were extracted then dried in an oven as described above. The average percentage from these samples was then used to determine the weight of lipid extracted from the rest of the samples.

2.3.7. Acid-base methylation of lipid

Lipid extracted from adipose tissue and PCLA and Megalac supplements was methylated according to Kramer *et al* (1997) and Christie (1999). Briefly, a 25mg sample of lipid was dissolved in 1ml toluene in a 15ml methylating tube to which 2ml of 0.5M sodium methoxide in methanol was added. The tube was capped and the contents mixed prior to methylation at 60°C for 20 minutes, shaking halfway through. 2ml of 14% boron trifluoride was added and mixed and the tubes were returned to the oven for a further 20 minutes at 60°C. After cooling, 5ml of deionised water was added and the tubes were vortexed before addition of 2 x 2ml hexane to extract the fatty acid methyl esters. The hexane was drawn off, transferred to a clean tube and dried under nitrogen to leave the fatty acid methyl esters, which were then redissolved in 1ml hexane.

2.3.8. Argentiation thin layer chromatography

Plastic backed, silica coated TLC plates (Merck, 20cm x 20cm, silica gel 60) were soaked in a 4% silver nitrate solution made up in 9:1 water:methanol for 30 minutes to allow the silver ions to impregnate the plate. The plate was dried in a fan oven for 20 minutes at 80°C and cooled before loading the fatty acid methyl esters in hexane using a hairdryer. 2-5mg of lipid was loaded onto each column of the plate. The plate was run to separate the fatty acid methyl esters using an equilibrated TLC tank of 90:10 petroleum ether:diethyl ether to within 1cm of the top of the plate. The fatty acid bands were visualised by spraying the dried plate with 0.1% 2,7 dichlorofluorescein in methanol, and marked immediately. The highlighted bands on the TLC plate were cut out and placed

in glass scintillation vials to which 15ml Emulsifier Safe scintillation fluid was added. The vials were transferred to a scintillation counter where they were left for 2 hours prior to counting the ^{14}C present for 10 minutes.

2.3.9. Analysis of fatty acid methyl esters by gas chromatography

Gas chromatographical analysis was performed by Dr. R. Wynn, using a Perkin Elmer Autosystem fitted with an autosampler and flame ionization detector in conjunction with Turbochrom 4 software (Perkin Elmer, Beaconsfield, Buckinghamshire) and using a 100m column. Samples were placed in 2ml vials or 100µl inserts, which were sealed using a crimped aluminium cap. Sample fatty acids were identified by reference to standards and were expressed as a percentage of total fatty acid methyl esters present. The calibration standard used contained 19 fatty acid methyl esters (Sigma 189-20) from C8:0 to C22:1. A methylated sample of Tonalin CLA-90 (Natural Lipids, Hovdebygda, Norway) was added as well as more C18:1 *trans* isomers: C18:1 trans-6, C18:1 *trans*-9, C18:1, *trans-11*, C18:1, *trans*-12 (Sigma Aldrich Chemical Company Ltd., Poole, Dorset).

2.3.10. Ribonucleic acid extraction

2.3.10.1.Acid-guanidinium-thiocyanate-phenol-chloroform extraction

Tissue previously snap frozen in liquid nitrogen was prepared by wrapping in foil, and smashing into smaller pieces using a hammer, and then grinding to a powder using a pestle and mortar filled with liquid nitrogen. This had to be done very rapidly to prevent the tissue thawing and the RNA degrading. The ground tissue was transferred into 50ml polypropylene screw-topped tubes (Sarstedt) and stored at -80°C. Approximately 2g of the frozen ground adipose tissue was placed into 10ml of denaturing solution in a 50ml polypropylene conical tube (Fahrenheit). The sample was homogenised using a Polytron™ homogeniser, rinsing the probe between samples. The samples were then frozen in the tubes and allowed to thaw in order to shear the DNA. 10ml of chloroform was added to the tube and mixed well to extract the fat from the samples. The phases were separated out by centrifugation at 3000rpm for 10 minutes at 4°C. The chloroform layer was pipetted off and the extraction

repeated. 1ml of 2M sodium acetate at pH4 was added, followed by 10ml of water saturated phenol and 2ml of chloroform-isoamyl alcohol 49:1. The samples were vortexed between additions, transferred into plastic 30ml tubes and centrifuged at 10000rpm for 30 minutes at 4°C. This left the RNA in the top aqueous phase, DNA and proteins in the interphase and phenol and chloroform in the bottom phase. 30ml glass tubes with 10ml isopropanol in were prepared and the top phase from the previous spin was transferred to these. The tops were covered with foil and left at -20°C overnight to precipitate the RNA. The tubes were spun at 10000rpm for 30 minutes at 4°C and the isopropanol poured off, leaving the precipitated RNA at the bottom of the tube. This was dissolved in 3ml of denaturing solution and vortexed until it dissolved completely. 3ml of isopropanol was added to the denaturing solution and the tubes were covered and left at -20°C overnight. The tubes were then spun at 10000rpm under the same conditions as before. The liquid was then poured off, the pellet washed with 4ml of 75% ethanol and centrifuged again. The ethanol was poured off and the tubes left to dry. When dry, the RNA was redissolved in 2ml of ribonuclease-free water for quantification. For liver an additional step to remove glycogen was carried out where the RNA pellet was dissolved in 2.4ml ribonuclease free water and vortexed before addition of 7.5ml of 4M sodium acetate at pH 7. The tubes were then left at 0°C overnight to aid precipitation. Following precipitation, tubes were centrifuged at 10000 rpm for 30 minutes at 4°C. The resulting pellet was washed with 4ml of 75% ethanol and dissolved in 2ml of ribonuclease free water, pending measurement of the RNA.

2.3.10.2.Quantification of RNA

1ml aliquots of RNA were transferred into a quartz cuvette and their absorbance measured at 260 and 280nm on a spectrophotometer (Pharmacia LKB Ultrospex III). One absorbance unit at 260nm is equivalent to 40μg of RNA per μl and from this information the RNA content of the samples could be calculated. The absorbance at 280nm measures protein present in the sample. To check that the RNA was of good quality and not contaminated with protein the ratio of the 260:280 readings was calculated. Ideally this ratio

should be between 1.7 and 2.0, and samples below 1.5 should not be used. The RNA yield per gram of starting tissue was then calculated. Aliquots of 10µg or 20µg of RNA were stored as a precipitate in 1.5ml microtubes with 8 volumes of ethanol and 0.1 volumes of 3M sodium acetate at pH 5.5. The samples were stored at -80°C for further analysis. To check that the RNA was intact, a small amount of each sample was run on a 1% agarose gel, and the presence of the 18S and 28S bands verified.

2.3.10.3.Trizol™ extraction

This method extracts RNA from small amounts of tissue so is suitable for smaller samples such as those obtained from rodents. Tissue was prepared as in section 2.3.10.1 and the resulting powder stored in 1.5ml microtubes at -80ºC. Approximately 100mg of tissue was weighed into autoclaved 15ml glass tubes and 1ml Trizol™ reagent was added. Tissue was homogenized using a Polytron™ homogeniser. The homogenate was poured into autoclaved 1.5ml microtubes and freeze thawed at -80ºC to shear DNA. Tubes were thawed and centrifuged at 12000g for 10 minutes at 4ºC. Excess fat was removed from the top and the supernatant was transferred to a clean tube. Tubes were left at room temperature for 10 minutes, and then 200µl of choloroform was added. Samples were shaken for 15 seconds to mix the tube contents and left at room temperature for 2-3 minutes, then centrifuged at 12000g for 15 minutes at 4ºC. The clear aqueous top phase contains the RNA, with DNA and protein in the interphase, and phenol and chloroform in the lower organic phase. The top phase was transferred into a clean tube and the RNA was precipitated. For adipose tissue samples 500µl of isopropanol was added to precipitate the RNA, but in the case of liver, which contains glycogen, a glycogen removal step is necessary; therefore to precipitate the RNA from liver samples 250µl of 0.8M sodium citrate and 1.2M sodium chloride solution and 250µl of isopropanol were added. For both adipose tissue and liver samples, tubes were left at room temperature for 10 minutes and centrifuged at 12000g for 10 minutes at 4ºC. The resulting supernatant was poured off and 1ml 75% ethanol was added to the RNA pellet, vortexed and centrifuged at 7500g for 5 minutes at 4ºC. The resulting supernatant was poured off and the pellet air dried for 5-10 minutes, before being redissolved in RNAse-free water for quantification.

2.3.10.4.Quantification of RNA extracted using Trizol™

A sample (usually 10µl) of the total volume was dissolved in 45µl of RNAse-free water for quantification using a Gene-Quant spectrophotometer, which uses a 50µl volume and measures at 230nm, 260nm, 280nm and 320nm to ensure clean, pure RNA as previously described in section 2.3.10.2. The concentration of the stock solution was calculated and diluted to 0.75µg/µl for DNAse treatment.

2.3.10.4.1. DNAse treatment

RNA was treated with DNAse to ensure that any remaining DNA contamination which could affect results during Taqman analysis was removed. 40µl of sample RNA at a concentration of 0.75µg/µl was pipetted into a 1.5ml microtube. 5µl of 10x DNAse buffer and 5µl of DNAse were pipetted into the tube and the contents mixed. The tubes were incubated in a 37ºC waterbath for 30 minutes, then 200µl of RNAse-free water and 150µl of phenol:chloroform:isoamyl alcohol were added. Samples were vortexed and spun at 12000g for 5 minutes to allow phase separation. 150µl of the aqueous top phase which contained the RNA were pipetted into 1.5ml microtubes containing 375µl 100% ethanol and 15µl 3M pH 5.5 sodium acetate and stored at -80ºC. RNA was precipitated and quantified as described previously in Section 2.3.10 and diluted to 0.1µg/µl ready for synthesis of complementary strand DNA (cDNA) as described in Section 2.3.11.

2.3.11. Reverse transcriptase polymerase chain reaction (RT-PCR)

This technique was used as an alternative to the ribonuclease protection assay to detect the presence of stearoyl CoA desaturase (SCD) and acetyl CoA carboxylase (ACC) messenger RNA in RNA samples extracted from adipose tissue and liver of sheep used in the CLA feeding trial, and levels of ACC, FAS, LPL and SCD mRNA in adipose tissue and liver of hamsters fed CLA. This was done as a two step method so that both the reverse transcriptase and the polymerase chain reaction could be optimised independently.

2.3.11.1. Reverse transcriptase reaction

The reverse transcriptase (RT) reaction synthesises complementary strand DNA. Clean and precipitated RNA was used for the RT reaction at a concentration of 0.1μg/μl. 5μl of RNA was mixed with 1μl of random hexamers and 9μl of RNAse free water. This was incubated for 5 minutes at 70°C to denature the secondary structure, and then put on ice while the following reagents were added, in order (Table 2.4).

Table 2.4 Contents of reverse transcriptase reaction

Reagent	Volume (μl)	Final concentration
MMLV Reverse transcriptase buffer x5	5	--
Nucleotides at 10mM (dNTPs)	1.25	0.5mM
RNAse inhibitor	0.5	25U
MMLV Reverse transcriptase	1	200U
Water (RNAse/DNAse free)	2.25	--

The tubes were incubated for 10 minutes at room temperature and then 1 hour at 42°C. The total volume of 25μl containing the cDNA made by the reverse transcriptase reaction was made up to 100μl with DNAse and RNAse free water and stored at -20°C prior to amplification by polymerase chain reaction (PCR).

2.3.11.2. Polymerase chain reaction

This reaction uses the cDNA made during the reverse transcriptase step (Section 2.3.11.1) in a process of amplification using the ABI 7700 'Taqman' machine which takes 96 x 0.2ml well plates. This is a real time PCR system which detects and quantifies fluorescence produced by a reporter present in the well. The fluorescence of this reporter increases in proportion to the amount of product formed during the course of a reaction. Two different reporters were used, one of which was Sybr Green, which binds any double stranded DNA present in the well. This can be a disadvantage, as apart from true PCR product, the Sybr Green may bind primer dimers, or DNA present in the RNA (especially likely if the sample has not been DNAsed) and the concentration of

PCR product may be exaggerated. An alternative, more specific method involves using a Taqman probe containing a fluorescent dye attached to the 3' base and a quenching dye on the 5' base. The probe sits on either of the two DNA strands between the two primers. When it is irradiated the dye transfers energy to the quenching molecule, so producing a non-fluorescent substrate. PCR involves replication of the DNA to which the probe is bound and as the polymerase enzyme moves along the strand, digesting bases, the 5' end is lost into solution. The reaction was set up on a 96 well 0.2ml plate, with samples being run in triplicate. Table 2.5 shows the reagents in each well.

Table 2.5 Reagents and cDNA added to each well of the reaction plate

Reagent	Volume + probe	Volume - probe	Concentration
2x Taqman Universal PCR MM*	12.5µl	-	-
2x SybrGreen PCR MM*	-	12.5µl	-
Forward primer (10pmol/µl)	0.75µl	0.75µl	0.3µM
Reverse primer (10pmol/µl)	0.75µl	0.75µl	0.3µM
Probe (10pmol/µl)	0.5µl	-	0.2µM
cDNA (≡ 5ng total RNA/µl)	2µl	2µl	10ng RNA
Water (molecular grade)	8.5µl	9.0µl	-

*MM = mastermix

A mastermix was made up (Table 2.6), sufficient to run each sample in triplicate. To ensure there was enough reagent, 2.2 times the reagent was made up, leaving some spare.

Table 2.6 Mastermix to be added to cDNA

Reagent	Volume + probe	Volume - probe
2x Taqman Universal PCR MM*	40µl	-
2x SYBR Green PCR MM*	-	40µl
Forward primer (10pmol/µl)	2.4µl	2.4µl
Reverse primer (10pmol/µl)	2.4µl	2.4µl
Probe (10pmol/µl)	1.6µl	-
Water (molecular grade)	27.2µl	28.8µl

***MM = mastermix**

24.5µl of Mastermix (Table 2.6) was added to a labelled tube, followed by 2.1µl of cDNA. The tube contents were mixed and briefly spun down to the bottom of the tube. 25µl was pipetted into a well on the 96 well reaction plate in duplicate or triplicate for each sample run. The wells were capped with an optically clear cap strip and the plate was placed into the machine for PCR.

2.3.12. Design of primers and probes for sheep and hamster genes

Primers and probes were designed for ovine ACC, SCD and beta actin and hamster ACC, FAS, LPL, SCD and beta actin (see Section 2.4.6 and 2.4.7) by entering the sequences into the Primer Express™ software. Primers were then selected from the output. When primer-probe sets had been designed, the primers were tested on sheep and hamster cDNA to verify that the correct product was being formed. This product consisted of the region from which the primers were taken and the sequence between the primers. In most cases the product length was approximately 70-80 base pairs. Samples from the PCR and a DNA ladder to identify band sizes were run on a 4% metaphor agarose gel at 100V for 1-2 hours, stained using ethidium bromide for 20 minutes and visualised under UV light. The sheep primers and probes were designed with Dr. Z. Daniel of the University of Nottingham and the sequences are displayed in Table 2.7. However, in the case of the hamster, after several attempts to design primers and probes for ACC, FAS, LPL and SCD (Sections 2.4.7 and 2.4.8) a paper which had used Taqman RT-PCR on hamster tissue reported

primer and probe sequences for these genes (Guo *et al*, 2001). The exact sequences for ACC, FAS, LPL and SCD together with the original genetic sequences used to design the primers and probes were kindly supplied (Table 2.15; Qiu Guo, personal communication). These primers and probes were synthesised and used in the subsequent RT-PCR analysis on the hamster tissue.

Table 2.7 Primers and probes used in measurement of ovine SCD, ACC and beta actin mRNA levels

	Sequence	T_m (°C)
SCD		
Forward primer	CGA ACC TAC AAA GCT GGG CT	65.4
Reverse primer	TGG AAC GCC ATG GTG TTG	67.0
Probe	CCC CTA CGG GTC TTC CTG ACA TCG	74.4
ACC		
Forward primer	CAT GTC TGG TTT GCA CCT AGT CA	66.0
Reverse primer	TCA CTT TAT TCC CAC CAA AAC GA	66.1
Probe	CGA GAC CGA AAG AAA ATA GAC TCA CAG CGA	75.0
Beta actin		
Forward primer	TGT GCG TG CAT CAAGGA GAA	66.8
Reverse primer	CGC AGT GGC CT CTC CTG	68.3
Probe	CTG CTA CGT GGC CCT GGA CTT CGA	75.6

2.3.12.1.1. Making and testing of PCR product

Complementary strand DNA was made from hamster RNA extracted from adipose tissue and liver using a reverse transcriptase reaction as described in Section 2.3.11.1. A PCR reaction was then done on the cDNA from the reverse transcriptase reaction. For multiple reactions a mastermix was made up which contained all the components except the template and the enzyme. Table 2.8 shows the components of the mastermix.

Table 2.8 Mastermix for single PCR reaction

Reagent	Volume (μl)
Nucleotides at 10mM (dNTPs)	1.0μl
Forward oligo at 10pmol/μl	1.25μl
Reverse oligo at 10pmol/μl	1.25μl
PCR buffer	5.0μl
$MgCl_2$	3.0μl
RNAse/DNAse free water	28μl

The mastermix was made up as in table 2.6, and 39.5μl was added to 0.5ml autoclaved microtubes containing 10μl of cDNA and 0.5μl of Amplitaq Gold enzyme. Tubes were spun briefly to mix contents and concentrate at the bottom of the tube, before adding to the PCR block for the run. The PCR block was pre-programmed as shown in Table 2.9.

Table 2.9 PCR block programme

Step 1	95°C for 10 minutes
Step 2	94°C for 30 seconds
Step 3	Melting temperature of primer - 5°C
Step 4	72°C for 1 minute
	Repeat steps 2-4 34 times
Step 5	72°C for 5 minutes
Step 6	Hold at 4°C

After PCR, products were stored at -20°C, prior to running on a gel.

2.3.13. Casting and running of 4% metaphor agarose gel

4g of metaphor agarose was sprinkled into 100ml TAE buffer whilst rapidly stirring to prevent clumps forming. The stirring bar was removed and the agarose soaked in the buffer for 15 minutes before heating. The solution was weighed and heated for 1 minute on medium in a microwave and left on a bench for 15 minutes. The gel was heated for a further 2 minutes, removed and gently swirled to resuspend settled powder and gel pieces. The gel was then heated on high for 1-2 minutes or until the solution boiled, before being held at boiling point for 1 minute, ensuring all particles had dissolved. Sufficient hot distilled water was added to make the gel solution up to its initial weight. The gel was allowed to cool to 50-60°C before casting. A gel former was prepared by sealing at both ends using tape or a clamp, and set up with a comb to accommodate all the samples to be run. The cooled gel was carefully poured in to avoid bubbles forming and left to set at 4°C for an hour. Once set, the tape or sealer was removed from the gel, and the gel former containing the gel was set in an elecrophoresis tank. This was filled with enough TAE to cover the surface of the gel. The comb was removed to allow the buffer to fill the wells before sample loading. 10µl of PCR product was run on the gel with 3µl DNA loading dye. The gel was run until the markers were approximately 60% of the way down the gel and stained using ethidium bromide solution for 20 minutes.

2.3.14. Measurement of mRNA levels for hamster and sheep genes

Complementary strand DNA was synthesised as described in section 2.3.11.1 from RNA extracted from hamster liver, epididymal, perirenal and intrascapular adipose tissue (section 2.3.10.3) using Trizol™ reagent. RNA was extracted from sheep liver, subcutaneous, perirenal and omental adipose tissue (section 2.3.10.1), using an acid-guanidinium-thiocyanate-phenol-chloroform extraction. Samples from individual animals were run in duplicate on the plate so that a single tissue and gene could be run on the same plate to reduce variability. A 7 point standard curve was also run, using a pooled sample of 5µl of cDNA from each animal. The dilutions for the standard curve are expressed in terms of RNA, because the cDNA is not quantified after synthesis. The dilutions are detailed below (Table 2.10).

Table 2.10 Standard curve dilutions for Taqman RT-PCR

Dilution factor	Volume of stock (µl)	Volume of water (µl)
1 in 5: 0.2x stock	40	160
1 in 10: 0.1x stock	40	360
1 in 20: 0.05x stock	100 of 1 in 10 dilution	100
1 in 50: 0.02x stock	20 of 1 in 5 dilution	180
1 in 100: 0.01x stock	20 of 1 in 10 dilution	180

Two other solutions completed the standard curve – a stock solution, given the name 1x stock and a 5x stock solution, where 5 times the normal volume of stock was added to the mastermix. In addition to the standard curve, a no template control (NTC) and a no amplification control (NAC) were also run in duplicate. These consisted of water and RNA added in place of cDNA template respectively. The NTC checked that there was no contamination on equipment or in solutions and the NAC checked that there was no DNA present in the RNA used to make the cDNA template, which could be being amplified in the sample templates, and over-estimating the gene expression.

2.3.15. Fatty acid analysis of hamster adipose tissue, liver and treatment diet lipids

Lipid was extracted from adipose tissue as previously described in Section 2.3.6, according to Folch *et al* (1957).

2.3.15.1. Extraction of hepatic lipids

A hepatic homogenate was prepared from 250mg of frozen liver to which 5ml of cold Tris-NaCl buffer (50mM Tris/150mM NaCl) was added and homogenised using a Polytron™ homogeniser. 1ml of this homogenate was then taken and transferred into a 15ml graduated conical polypropylene tube on ice. 3.75ml of cold freshly made chloroform:methanol (1:2v/v) was added to the tube and the contents mixed well. At room temperature 1.25ml chloroform was added to the tube and the contents vortexed, then 1.25ml distilled water was added and the tube vortexed again. This gave a final extraction solvent ratio of chloroform:methanol:water of 10:10:9 v/v. To complete phase

separation, tubes were centrifuged at 1100rpm at 25°C for 5 minutes. The lower organic chloroform phase, which contained the lipid, was collected into a solvent resistant LP4 tube using a Pasteur pipette. The lipid was dried down under a stream of nitrogen gas, rinsing the tubes down with choloroform to ensure all lipids were extracted.

2.3.15.2.Extraction of diet lipids

Approximately 1g of each diet was weighed out in triplicate into a 50ml graduated conical polypropylene tube. 5ml of chloroform:methanol (1:2 v/v) was added and the tube contents vortexed thoroughly. To pellet the diet particles, samples were centrifuged at 1100rpm and 25°C for 10 minutes. The upper organic phase, which contained the extracted lipids, was collected using a Pasteur pipette into a graduated conical polypropylene tube. This procedure was repeated on the diet samples twice. The three extracted portions were mixed and centrifuged at 1100rpm and 25°C for 10 minutes to pellet any remaining diet particles. The organic phase was collected into a pre-weighed 15ml graduated conical polypropylene tube using a glass Pasteur pipette. The lipid was dried down under nitrogen gas to complete dryness and the weight of the capped tubes was recorded to determine lipid yield from the diet samples.

2.3.16. Methylation of lipid samples

Lipid samples were methylated and analysed by Dr. A.L. Lock, Cornell University, Ithaca, N.Y.

2.3.17. Body composition analysis on hamsters

Hamster carcasses were frozen at -20°C immediately after removal of adipose tissue and liver samples as described in Section 2.2.3.3. Once completely frozen, carcass weights were recorded prior to commencement of freeze drying. Carcasses were freeze dried to remove all water for 4-5 days and weights recorded again on the 4^{th} and 5^{th} days to check that weight was remaining constant. Carcasses were broken up and homogenised using a blender to ensure that a representative sample could be taken for percentage fat analysis. 1g of the homogenised carcass was taken and analysed using a rapid Soxhlet extraction at a running temperature of 150°C, boiling time of 35

minutes and extraction time of 50 minutes. The percentage fat in the carcass was then calculated from the extract weight and starting sample weight.

2.3.18. Cholesterol and triglyceride assays

Cholesterol and triglyceride standards were made up from the solutions supplied with the kit (Table 2.11).

Table 2.11 Preparation of standards for cholesterol and triglyceride assays

Cholesterol conc mM	Triglyceride conc mM	µl standard	µl water
0	0	0	1000
0.517	0.339	100	900
1.030	0.678	200	800
2.070	1.356	400	600
3.100	2.034	600	400
4.140	2.712	800	200
5.170	3.390	1000	0

10µl of blank (water), standard or sample was pipetted into wells on a 96 well plate. 250µl of Trace Cholesterol or Triacylglycerol assay reagent was added, and then the plate was incubated at 37ºC for 15 minutes to allow the assay reaction to proceed. The plate was read at 550nm on a Dynatech MR5000 micro-plate reader.

2.4. METHOD DEVELOPMENT

2.4.1. Development of explant culture conditions

The aim of these experiments was to investigate the effect of conjugated linoleic acid upon lipogenesis in adipose tissue explants. A culture period of 24 hours was used, followed by a 2-hour incubation using 1-^{14}C acetic acid as a label. The starting point for the development of the explant culture technique used in this thesis was provided by work done by Dawson *et al* (1993). Krebs Ringer bicarbonate buffer solution at pH 7.4 was used as the culture medium initially but it became clear that it was unable to support the tissue for a 24 hour period prior to lipogenesis measurement. No ^{14}C above that of background levels was detected, so it was concluded that the tissue was dead. Dawson *et al* (1993) had been using the culture medium only for the 2 hour incubation immediately after slaughter, so this cast doubt on its suitability as a long term culture medium. A different culture medium was tried – Medium 199 (Sigma); one which previous work in this laboratory had shown to be successful in culturing explants for up to 48 hours post-slaughter. (Daniel 2002, Richards, 1997) There was discussion as to whether a pre-incubation period in untreated media was required immediately post slaughter. It was decided to include this and incubate the explants in media without treatment substances in order to allow the tissue to recover after chopping. This improves the quality of the explants because receptors that may have been damaged in the preparation process can be re-synthesized. For the purposes of technique development, a positive control was used to check that the experiment was working using this procedure. Insulin is known to increase the rate of lipogenesis of adipose tissue by activating the enzyme acetyl CoA carboxylase (Vernon *et al*, 1999, Vernon, 1993), so the efficacy of the technique could be established.

2.4.1.1. Supply of fatty acids to adipocytes

Fatty acids are solubilized for transport in the circulatory system due to binding to albumin. This blood protein has a high affinity for free fatty acids, although it does carry other components, but their binding is weakened when concentrations of free fatty acids are high. This is the route of supply of non-esterified fatty acids to adipocytes, and therefore it is essential to bind fatty acids to be supplied to the adipocytes to albumin to facilitate their uptake (Noble, 1981). Work suggests that a molar ratio of 4:1 fatty acid:bovine serum albumin is used (Peck *et al*, 1996). The ratio that was used in the explant experiments was 7:1 but the amounts added were still below the toxicity level of 100μM for BSA-bound fatty acids as reported by (Peck *et al*, 1992).

2.4.1.2. Measurement of lipogenesis using radio-labelled acetic acid

Lipogenesis is calculated by the measurement of radiolabelled ^{14}C taken up by the explants from [1-^{14}C] acetate supplied over an incubation period. From the specific activity of the acetate, and its concentration, the amount of fatty acid formed can be calculated. Previous work done in this laboratory had used [1-^{14}C] acetate with a specific activity of 0.02MBq per ml of media, corresponding to 135μl of acetate per 50ml of media (Daniel *et al*, 2004). This produced some high counts and consequently it was decided to work with a tenth of the amount, which brought the counts down significantly and gave a specific activity of 27dpm/nmol acetate. Instead of performing a lipid extraction on the tissue, a solubiliser was used instead to dissolve the adipose tissue before addition to the scintillation fluid. This did not consistently produce the results expected for the positive control, and it was considered possible that acetate that was not incorporated into the lipid but adherent to the associated connective tissue was being counted. The experiment was to look at acetate incorporated into the lipid as a measure of lipogenesis, so a Folch lipid extraction (Folch *et al*, 1957) was used. This removed the non-lipid fraction and associated acetate so only the acetate bound to the lipid was counted for ^{14}C. The changes being investigated were so small that it was thought better to use a lipid extraction to ensure that only the bound lipid was being analysed.

2.4.2. Measurement of stearoyl CoA desaturase (SCD) activity

The focus of the project shifted from investigating the effects of CLA isomers and other fatty acids on lipogenesis and lipolysis to looking at their effects on the stearoyl CoA desaturase enzyme. Its activity can be measured by looking at the type of fatty acids formed in adipose tissue incubated for a time period in culture media containing ^{14}C acetate. The incubation with radio-labelled acetate was carried out as previously described in section 2.3.5 and the lipid was extracted from the explants, but instead of counting the total lipid formed immediately, the individual fatty acids as fatty acid methyl esters were separated according to their degree of saturation using a silver – impregnated thin layer chromatography plate (sections 2.3.7 and 2.3.8). This was based on a technique previously done in this laboratory using silver-impregnated florisil columns (Daniel *et al*, 2004). At the start of using this technique very low counts were being seen, possibly due to the low specific activity of the acetate present so it was decided to raise this to 1360dpm/nmol acetate (0.32MBq per flask)

2.4.2.1. Preparation of fatty acid methyl esters

Initially, 5% sulphuric acid in methanol was used to methylate the lipid at 90°C for 2.5 hours. The fatty acid methyl esters (FAMEs) were extracted using 2x 2ml petroleum ether. During the methylation the lipid turned brown, and after extraction of the FAMEs, the lipid would not dissolve in the petroleum ether. The methylation being used is a strong one, especially with heating at 90°C for 2.5 hours, and it was suspected that the fatty acids were being oxidized. Christie (1982) recommends using a further solvent to dissolve lipid in prior to methylating because methanol, or reagents composed primarily of methanol will not allow non-polar lipids such as triacylglycerols to dissolve in them and will not react in a reasonable time. A milder base methylation (Christie, 1999, 2001) was used, which was much quicker and used 0.5M sodium methoxide in methanol at 50°C for 10 minutes. The esters were extracted with hexane. This was much more successful so this method was adopted.

2.4.2.2. Separation of fatty acid methyl esters using silver ion thin layer chromatography

Silver ion thin layer chromatography is effective at separating saturated fatty acids from unsaturated ones due to the interaction of the silver with the double bond of the unsaturated fatty acids. The monounsaturated fatty acid band is approximately 1cm below the saturated fatty acid band due to the retardation of the double bond by the silver. Previously, silica coated glass TLC plates have been used, but with care it is possible to use plastic backed plates, impregnating them with silver by immersion in a 4% silver nitrate solution in 9:1 water:methanol (Wood & Snyder, 1966). One of the limiting factors was the amount of lipid that could be loaded onto the plate. It is not possible to load all the lipid that is contained in the average flask of explants (approximately 100mg) because of the likelihood of smearing the sample up the plate and not achieving a clean separation. The best separations are achieved by using between 2 and 20mg of lipid – if too little is used, it is impossible to detect in the bands, but if too much is used, a clean separation is not achieved. The loading limit posed a problem, because the radiolabel being detected using 100mg of lipid was sufficient for the scintillation counter to detect and accurate enough to calculate from, but if a twentieth of that amount was used it would not be possible to distinguish between background counts and sample counts. Due to the safety risk of using increased levels of radiation, TLC plates were run using different amounts of lipid, to determine quality of separation and detection of sample. Three amounts of lipid were investigated; 2.7mg, 13mg and 27mg. The clearest separation was given with 2.7mg of lipid, while the bands tended to run into each other using 13mg. The 27mg of lipid did not separate at all and just smeared up the plate. Gas chromatography revealed that no separation had taken place within the band. The 2.7mg of lipid had separated into bands, with the unsaturated fats below the saturated fats, but amounts were very low, so would be unlikely to contain sufficient ^{14}C to be detected by the scintillation counter. The 13mg lipid band had separated, but was less clear in that some of the bands had run into each other. It was obvious that amounts of lipid in excess of 13mg could not be run successfully on the TLC plate, and that 13mg of lipid gave a smeared separation, so it was decided

to run amounts of 2mg and 5mg to check clarity of separation. It was possible to get a clear separation using 5mg of lipid, so this was used for all future TLC plates.

2.4.3. Retrieval of fatty acid methyl ester bands

Once the TLC plates had been run and the bands visualised, the next step was to extract the fatty acid methyl esters from the plates. Initially this was done using two hexane washes for 30 minutes each before centrifuging the solvent to pellet any silica and drying it off. The analysis from the gas chromatograph showed that there was very little unsaturated fatty acid present, even in the monounsaturated band. There was very little oleic acid present which was unusual for ovine adipose tissue. It was suspected that not all the fatty acids were being removed from the silica, especially the monounsaturated ones. This may be because hexane is not a polar enough solvent to lift the unsaturated fatty acids off the silica. The fact that the silver retards the double bond of the monounsaturated fatty acids is an indication that they are more firmly bound to the adsorbent than saturated ones. Previous work in this laboratory (Richards, 1997) has shown that oleic acid may be eluted with 1:1 diethyl ether: petroleum ether, and that 3% diethyl ether in petroleum ether elutes all saturated fatty acid methyl esters. Thus it was decided to use different solvent mixtures for each band to improve detection. This produced levels of monounsaturates in excess of saturates in the correct band. These solvent systems were used for all future extraction work.

2.4.4. Elution of the fatty acid methyl esters from the TLC plate

The individual bands on the TLC plate were cut up and placed in methylation tubes to which 2ml of solvent was added. The tubes were vortexed and left for 30 minutes to allow the lipid to dissolve in the solvent. The solvent was then transferred to an LP4 tube using a Pasteur pipette. This solvent wash was repeated and the liquid transferred to the LP4 tube which was then centrifuged at 3000rpm for 10 minutes in order to pellet the remaining silica at the bottom of the tube. The solvent was transferred into another LP4 tube, leaving the silica behind, and dried off to leave lipid. This was resuspended in 180µl of hexane prior to gas chromatograph analysis.

2.4.5. Verification of fatty acid bands

Following the initial experiment, it was found that although the plates showed a saturated fatty acid band and a monounsaturated fatty acid band, there was another band close to the loading area. Upon exposure to film, it became clear that there was no or little radioactivity in the monounsaturated band, but some in the saturated or top band and the lower band. The lower band would be expected to be polyunsaturated fatty acids, and it was unclear why there should be no oleic acid being formed, when polyunsaturated fatty acids were present. Standards of palmitic and oleic fatty acid methyl esters were run on a silver TLC plate to verify that the oleic acid band did run to 1cm below the palmitic acid band. This was found to be correct, so the next step was to add some radiolabelled oleic and palmitic fatty acids to the standards and methylate them before running on a silver TLC plate. This was done to check that the acetate was not preventing the oleic acid from running up the plate to its usual position. The plate was imaged to detect radiolabelled fatty acid methyl esters. The oleic acid was 1cm below the palmitic acid band, so the radiolabel was clearly not preventing upward travel of the oleic acid. Attention then turned to the methylation being used. The base methylation is suitable for lipids present as triacylglycerol, but does not work well for lipids present as free fatty acids (FFAs). An acid-base methylation would methylate all lipids, whether present as TAG or FFAs. It may be possible that the oleic acid formed during the incubation is present as FFAs, due to the time taken for it to be formed. Palmitic acid is formed relatively quickly, but lipid destined for oleic acid must be elongated in the endoplasmic reticulum, desaturated and then re-esterified. It may be that the incubation was not long enough for the process to be completed. An experiment was done using an acid-base methylation in addition to a base methylation and plates compared for oleic acid bands. Clear MUFA bands were seen, containing radioactive acetate, and the lower band that was previously visible, just above the loading point was absent. It was concluded that this band seen under base methylation conditions had been composed of unmethylated free fatty acids, and therefore base–acid methylation was used thereafter.

2.4.6. Design of primers and probes for hamster genes

Primer and probe sets were required for hamster ACC, beta actin, FAS, LPL and SCD. Relatively little sequencing work has been done on the hamster genome, so initially mouse and rat sequences were used which were likely to be highly conserved across other rodent species and therefore suitable for use with hamster DNA. Sequences for the above genes in rat and mouse were found under the following accession numbers in the GenBank database: Rat ACC: NM 053922; Rat beta actin: NM 031144; Rat FAS: NM 017332; Rat LPL: NM 012598; Rat SCD1: NM 139192; Rat SCD2: NM 031841; Mouse ACC: BC022940; Mouse beta actin: NM 007393; Mouse FAS: NM 007988; Mouse LPL: NM 008509; Mouse SCD: NM 009127; Mouse SCD2: NM 009128. A BLAST™ search was done to check that the gene sequences for rat and mouse were a good match and suitable to design the primers from. Primer-probe sets for the genes of interest are shown in Table 2.12. It was not possible to design a suitable primer-probe set for the individual SCD genes due to the presence of 2 isoforms in rat and 4 in mouse. Finding a region of the sequence that was not conserved across isoforms proved difficult and such regions did not yield any primer-probe sets.

Table 2.12 Primers designed from mouse and rat sequences

Gene	Forward primer	Reverse primer
ACC	CAGATCCGGAAATATCATGTTTCA	TGATCAATACGCCCTATGTCACC
Beta actin	CGTGAAAAGATGACCCAGATCA	ACGTAGCCATCCAGGCTGTG
FAS	TCCTGGAACGAGAACACGATCT	CAAGTCCAGGAGTGACACGTCTC
LPL	CAAGATTCACTTTTCTGGGACTGA	TCTCTGTACGGCACAGTGGC

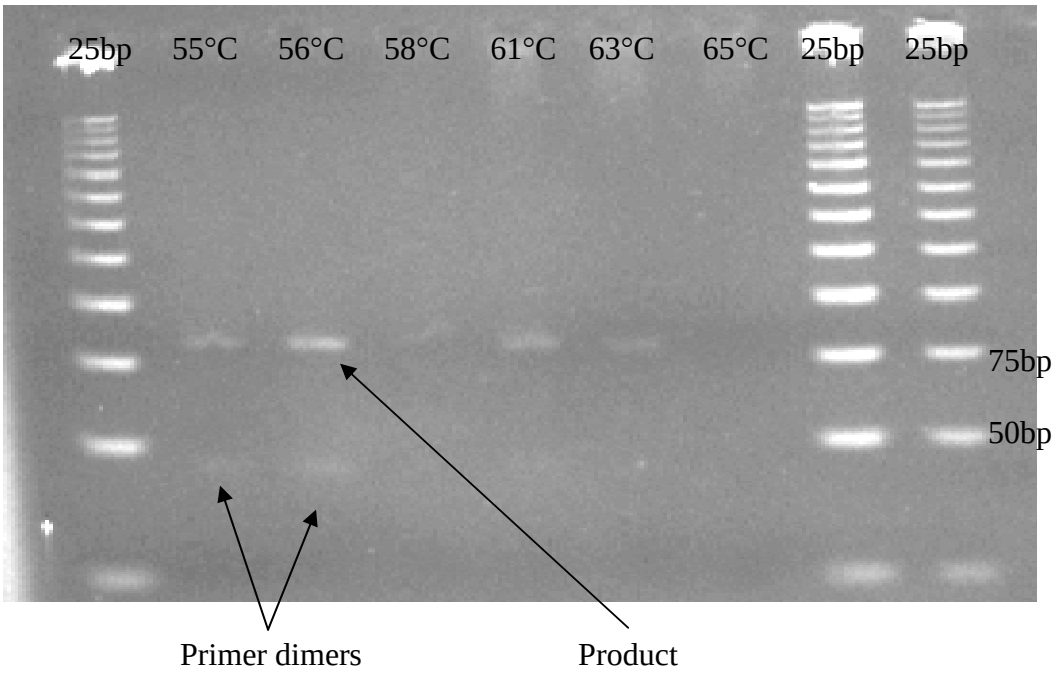
2.4.7. PCR optimization

It was necessary to verify that the PCR products being formed with the primers were the correct size to ensure that the desired region of sequence was being amplified.

2.4.7.1. 1st design primers

RNA was extracted from hamster liver as described in Section 2.3.10.4 and complementary DNA was synthesized from this template. A PCR reaction was set up as previously explained in section 2.3.11 across a temperature gradient to check at which temperature the primers worked best. Ideally the annealing temperature should be 5 degrees below the melting temperature of the primers. The Primer Express program is set up to design primers and probes with T_m values of 60°C and 70°C respectively. With this in mind, I set up a temperature gradient from 55°C to 65°C for all primers and amplified up cDNA from hamster liver. The resulting PCR products were then run on a gel against a DNA ladder to confirm size and check that the correct product was being formed (Figure 2.1).

Figure 2.1 PCR using hamster LPL primers over a temperature gradient



The LPL primers worked well across the temperature gradient, with the clearest band being seen at 56°C. The product size calculated from the

sequence is 78 base pairs, which indicated that the product band seen just above the 75 base pair marker was likely to be the correct one. The bands seen at 55 and 56°C just below the 50 base pair marker are probably primer dimers, and disappear as the annealing temperature increases.

2.4.7.2. Verifying PCR product

To confirm that the PCR reaction is working successfully with the primers, one clear sharp band needs to be seen on the gel. At temperature extremes, several bands may be seen, with the main one not clear. As the ideal temperature is approached, the main band will sharpen up and the others will become less evident, eventually not being seen. This is the temperature at which the primers will work best. Ideally the primers needed to work best at 60°C, because the probe is designed to work with a differential of 10°C from the primers. If this differential changes, the efficiency of the reaction is altered, so the annealing temperatures for the probe and primers should be 70°C and 60°C respectively. The temperature gradient for the PCR reaction is a way of checking if the primers will successfully amplify up cDNA. If only one temperature setting is used, it cannot be confirmed whether the primers really do not amplify or whether they do not amplify at that particular temperature. If all of the primers were to work and product bands to be seen and conditions had to be changed for each gene, using a probe would not have been possible because primer-probe annealing temperature differentials must be 10°C. In this case, using Sybr Green, a dye that binds to double stranded DNA would be better to measure amplification and therefore gene expression. In the case of LPL, where the best annealing temperature is 56°C, using Sybr Green would be the best approach unless it was possible to find another primer pair that would work best at 60°C.

2.4.7.3. 2nd design primers

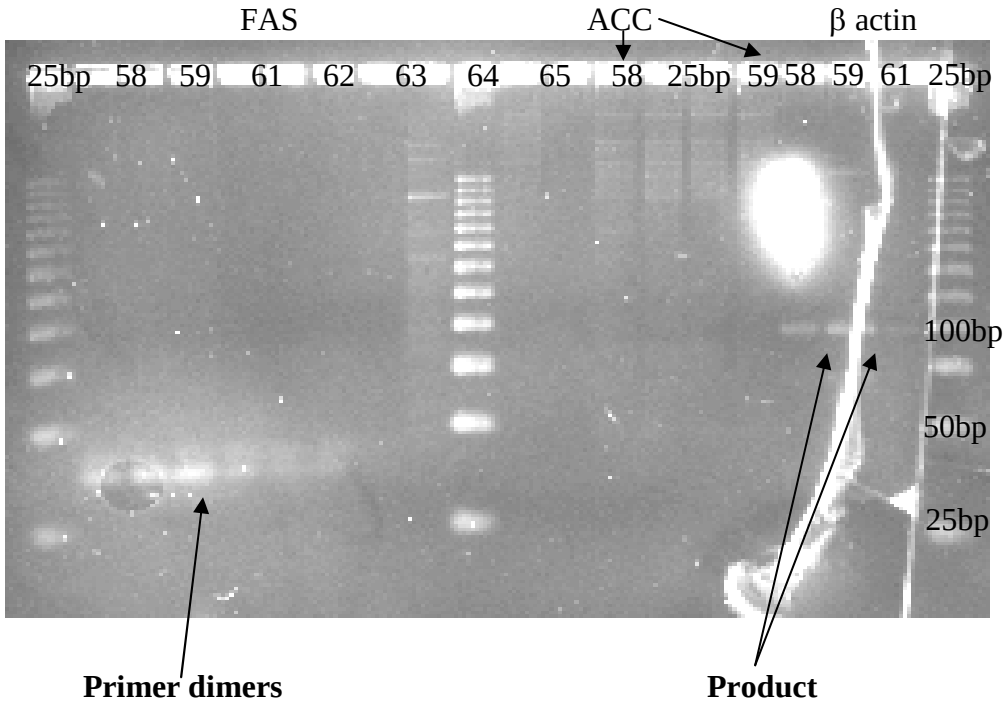
ACC, beta actin and FAS showed no bands on the gel, so these primers did not produce products under the conditions of the PCR reaction. New primers were designed and checked in the same way (Table 2.13).

Table 2.13 Sequences for 2nd design primers

Gene	Forward primer	Reverse primer
ACC	TGGATCCGCTTACAGAGACTTTT	GCCGGAGCATCTCATTCG
Beta actin	GGCGCTTTTGACTCAGGATT	GGGATGTTTGCTCCAACCAA
FAS	TCGTGATGAACGTGTACCGG	CTTTGTAAACGTCCTCACCCG

cDNA was synthesized and a PCR reaction set up as in Section 2.3.11. A gel was run to look at products and check sizes (Figure 2.2).

Figure 2.2 PCR products using FAS, ACC and beta actin primers



This gel shows bands at 58 and 59°C for beta actin, approximately 100 base pairs in size. The expected product was 70 base pairs, but it is possible to see a second band at 59°C, which is likely to be the predicted fragment. The 2 bands indicate that the primers are clearly not producing a single clean product so are unlikely to be suitable for use in Taqman analysis. No product bands were detected using FAS or ACC primer pairs. The FAS primers formed primer dimers which can also be seen on the gel.

2.4.8. Final primer and probe sequences

2.4.8.1. ACC, FAS and LPL primers

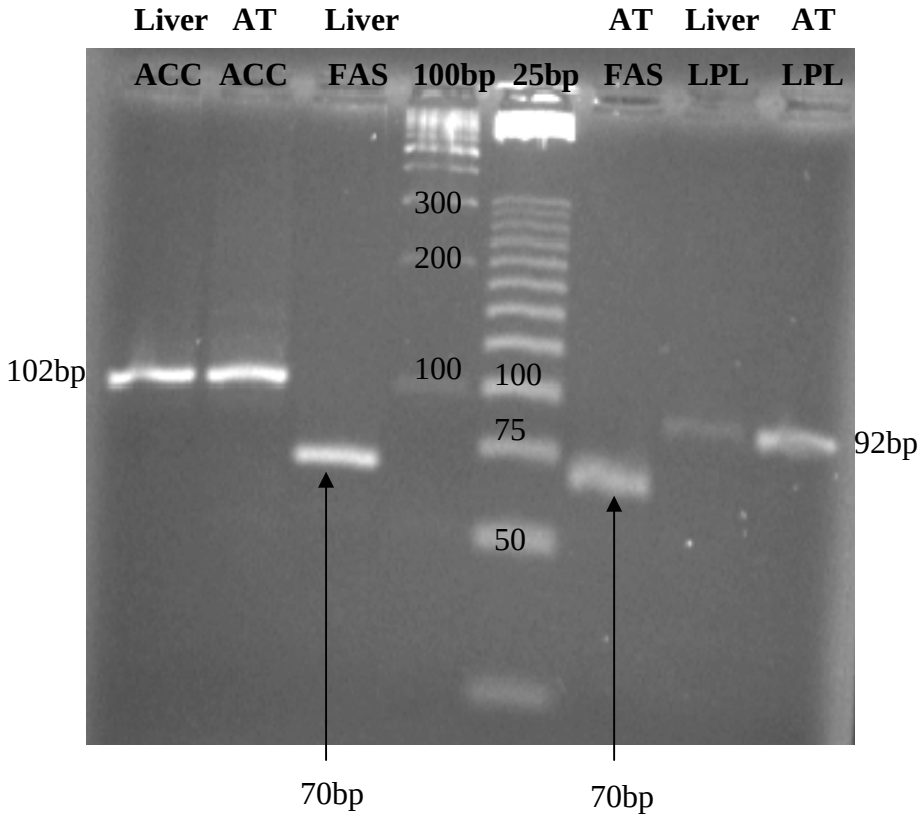
Similar work (Guo *et al*, 2001) has looked at gene expression using Taqman analysis for a number of genes in Chinese hamsters, including ACC, FAS and LPL. Sequences for these have been published on GenBank under the following accession numbers: ACC: AF 356089; FAS: AF 356086 and LPL: AF 356087. From this work I was able to obtain probe and primer sequences for ACC, FAS, LPL and SCD (Guo, personal communication). Primer and probe sequences were checked against the gene sequences from the database and located in FAS and LPL. Primers for ACC were found, but the probe did not match any of the sequence. Initially, only primers were ordered, to check that they were able to amplify up hamster cDNA. Table 2.14 shows the primers that were tested.

Table 2.14 Primers from Qiu Guo

Gene primers	Primer Sequence	T_m (°C)
ACC Forward primer	CTGCTGGAGACCGAAAGCTT	65.7
ACC Reverse primer	CAACATGGTGTCAGGACGTTCT	66.1
FAS Forward primer	AAGGACCAGGTGGAGGATGC	67.5
FAS Reverse primer	CCTGGACAAGGACTTTGCCA	67.4
LPL Forward primer	TTTAACTACCCCCTGGACAATGTC	65.6
LPL Reverse primer	ACCTTCTTGTTGGTCAGACTTCCT	65.1
SCD Forward primer	CCGAGAGACTTCAGGGAACTTG	66.1
SCD Reverse primer	GGTAGGCAGGTATGCTCCGA	66.2

Primers were tested to check that they amplified hamster cDNA and produced the correct size products. These primers had been designed to work on the Taqman, which uses an annealing temperature 10°C less than the probe at 70°C so I expected these to work well with an annealing temperature of 60°C, so a single temperature setting was used. The product sizes for ACC, FAS and LPL were calculated to be 102bp, 70bp and 92bp respectively. Figure 2.3 shows the product bands from the PCR using these primers.

Figure 2.3 PCR products using primer pairs for ACC, FAS and LPL

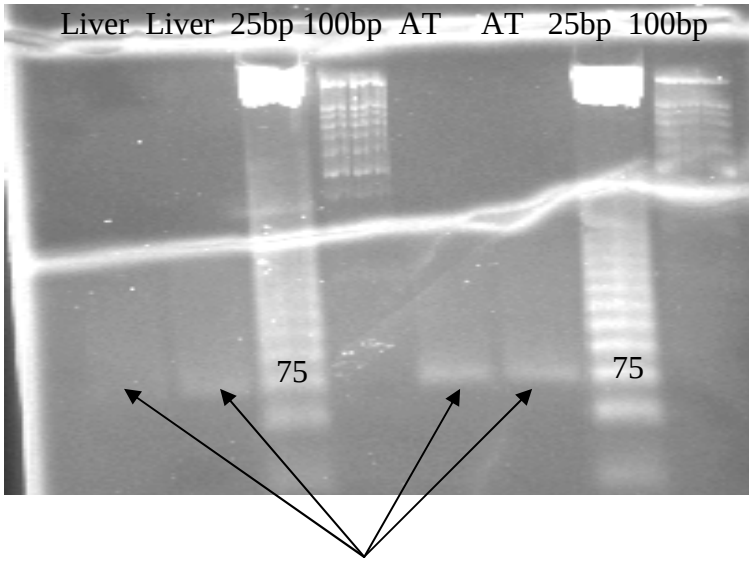


Clear single bands were formed for each gene and for both liver and adipose tissue cDNA. The markers indicated that the band sizes agreed with the expected product. It is interesting to see that while the ACC bands from liver and adipose tissue are of equal intensity, those from FAS and LPL in adipose tissue and liver vary. The FAS product formed from liver cDNA is more intense than that formed from adipose tissue cDNA. This is likely to reflect relative proportions of the different genes in the 2 tissues. Similarly, the LPL product from adipose tissue is more intense than that from liver. It is known that LPL is more evident in adipose tissue than in liver, and the gel seems to confirm this well.

2.4.8.2. SCD primers

The primers for SCD were tested in the same way, and a gel of PCR products run. Figure 2.4 shows the product bands that were seen.

Figure 2.4 PCR products using primer pairs for SCD



The expected product size was approximately 70 base pairs, and the bands are level with the 75 base pair marker, so this is likely to be the correct product.

Taqman optimization

2.4.9. Testing of primers and probes for ACC, FAS, LPL and SCD

Probes were synthesised for ACC, FAS, LPL and SCD by Sigma Genosys. The sequences of the probes are shown in Table 2.15.

Table 2.15 Probes for ACC, FAS, LPL and SCD

Gene	Probe sequence	T_m (°C)
ACC	TCTGTCCAGCCAGCCAGTGTCGA	75.5
FAS	TCCGCTACATGGCTCAGGGAAAACA	74.5
LPL	AGCCTTGGAGCCCACGCTGCT	75.2
SCD	CAGTTGCTCCTGGCTGTGGTGAAGTCT	74.6

A stock solution of hamster cDNA was made as described in section 2.3.14, and from this standard dilutions were made up to form a standard curve. Standard curves for ACC, FAS, LPL and SCD were run, to check for linearity. Figures 2.5-2.8 show the resulting standard curves.

Figure 2.5 Standard curve using ACC primers and probe

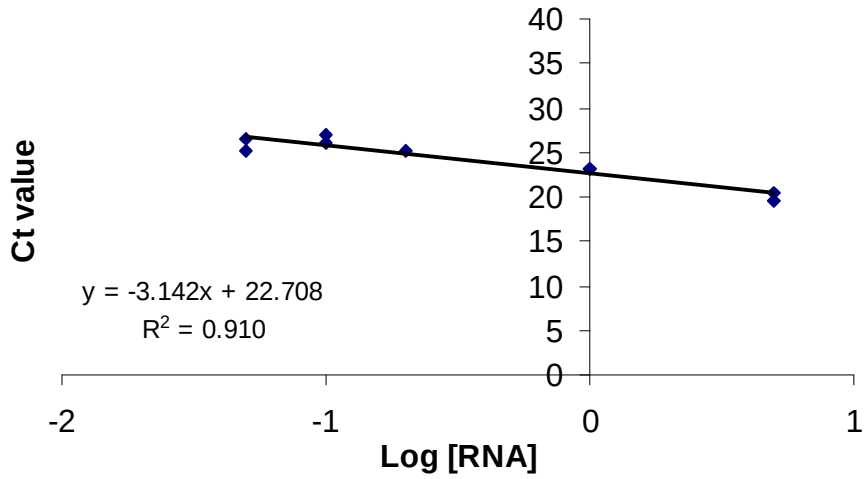


Figure 2.6 Standard curve using FAS primers and probe

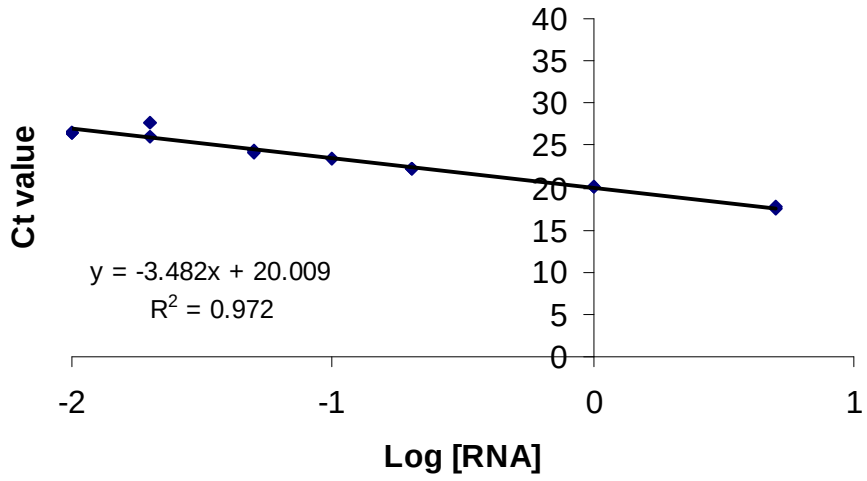


Figure 2.7 Standard curve using LPL primers and probe

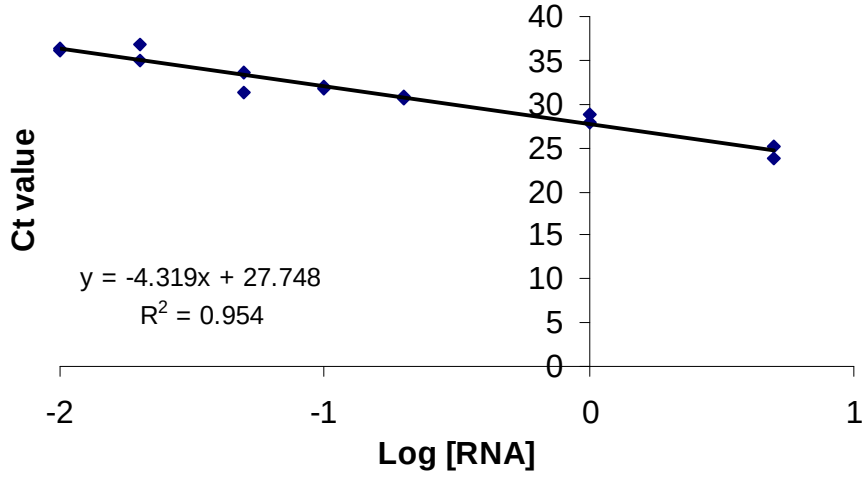
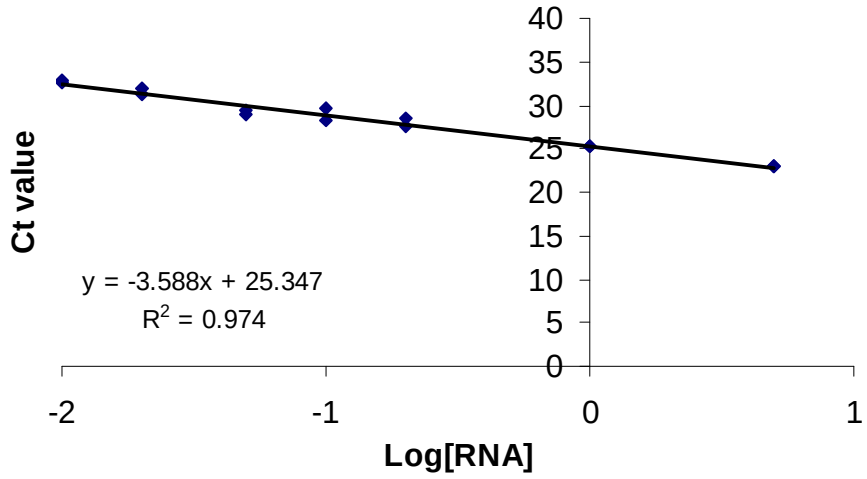


Figure 2.8 Standard curve using SCD primers and probe



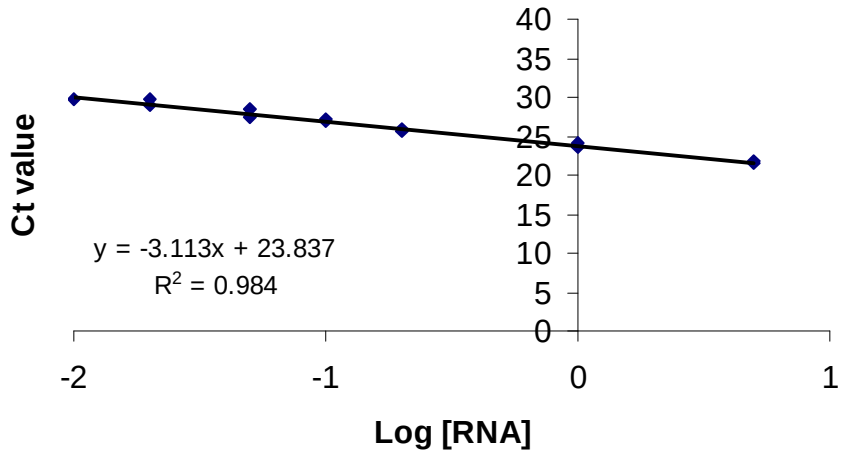
2.4.10. Testing of primers and probe for beta actin

On testing the probe with the primers the standard curve was not linear, so primers alone and Sybr Green mastermix were used. The standard curve was still not linear, so primers designed from rat and sheep gene sequences were tested. These primers worked well and gave a linear standard curve so could be used for sample analysis, especially using the probes which had been designed to work with them. Primer and probe matches to the hamster sequence were examined. The rat primers were conserved between sequences, but the probe was not so only the primers could be used in any analysis. The sheep primers and probe were not conserved so these were discarded as an option. The beta actin primer and probe sequence designed for use with hamster tissue was from a mouse beta actin sequence so this was aligned with the hamster sequence to check how conserved the primers were. The reverse primer was not conserved between species so the hamster sequence was entered into Primer Express. Due to the constraints imposed by the Taqman method for design of primers and probes no suitable amplicon could be found, so primers were manually designed around the existing beta actin probe, keeping within these constraints. The resultant primers used in the final analysis (Table 2.16) were tested by running a standard curve (Figure 2.9).

Table 2.16 Primers designed manually around existing hamster sequence probe

	Sequence	T_m (°C)
Forward primer	GGCGCTTTTGACTCAGGATTTA	66.1
Probe	AACTGGAACGGTGAAGGCGACAGC	74.7
Reverse primer	GGGATGTTTGCTCCAACCGA	69.3

Figure 2.9 Standard curve using beta actin primers and probe



2.5. STATISTICAL ANALYSIS

Data from the *in vitro* sheep adipose tissue culture experiments (Chapter 3) using one variable was subjected to one-way analysis of variance (ANOVA), blocking for animal (Genstat 6th edition, Lawes Agricultural Trust, Rothamsted). Data from experiments where two different variables were applied was analysed by two-way ANOVA. Experiments using two different fatty acids compared to a single control group were analysed by analysis of variance, with treatments partitioned into the control group versus the rest of the treatments (control vs rest), type of fat and amount of fat. Results were deemed significant at $p \leq 0.05$.

Data from the *in vivo* protected CLA sheep feeding trial (Chapter 4) was analysed by analysis of variance using Genstat, with variation in treatment partitioned into the control group versus the rest of the fat treatments (control vs rest), type of fat and amount of fat. Results were deemed significant at $p \leq 0.05$.

Data from the *in vivo* hamster CLA feeding trial (Chapter 5) was analysed by one-way ANOVA using Genstat, with the exception of the fatty acid composition data, which was analysed by fitting a regression model to the fatty acid composition data within an ANOVA analysis. Results were deemed significant at $p \leq 0.05$.

3. EFFECT OF CONJUGATED LINOLEIC ACID ON LIPID METABOLISM IN OVINE ADIPOSE TISSUE EXPLANTS

3.1. CONTROL OF LIPOGENESIS IN OVINE ADIPOSE TISSUE

The initial experiments concentrated on verifying that the explant culture technique was able to detect changes in lipogenesis caused by hormonal stimulation. Insulin is known to increase the rate of lipogenesis (Vernon, 1979) in ovine adipose tissue explants, so this was used as a treatment.

Tissue from 10 ±2 month old Mule x Charollais non-lactating ewes fed on a basal concentrate diet as described in Section 2.2.1.1 was used for all explant experiments.

3.1.1. Examination of control of lipogenesis by insulin and dexamethasone

To study the effect of insulin on lipogenesis, adipose tissue explants were cultured with insulin for 24 hours before being transferred to culture media containing acetate with the 1st carbon labelled with ^{14}C at a specific activity of 0.02MBq/ml and incubated in a shaking water bath for a further 2 hours. Tissue was then solubilised and counted using a scintillation counter for ^{14}C. A time course experiment measuring acetate incorporation into adipose tissue was carried out initially to check that incorporation remained linear over the time period being studied. This was repeated once using a different sheep. The experiment treating explants with insulin was carried out on subcutaneous adipose tissue from 4 different sheep and the results shown are data combined from the four sheep and blocked for individual animal in the analysis of variance. Experiments were also carried out using insulin and the hormone dexamethasone (a synthetic glucocorticoid) on 2 different sheep.

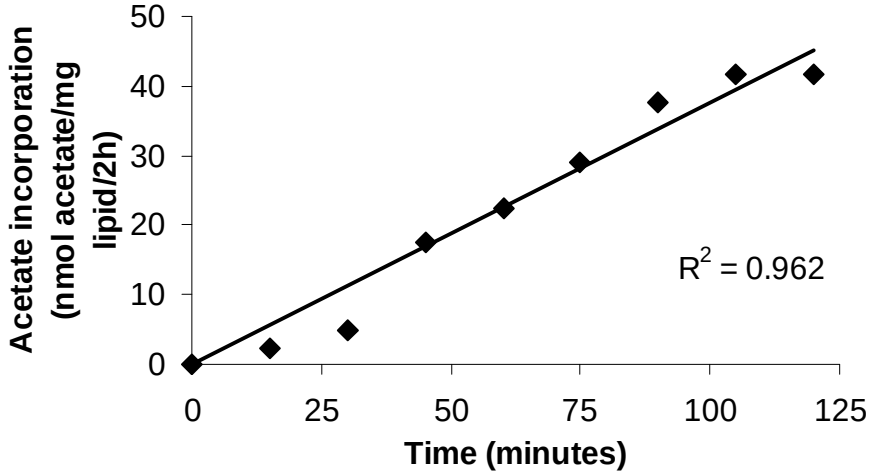
3.1.1.1. Effect of time on lipogenesis

Preliminary studies (Figure 3.1) confirmed that acetate incorporation is linear over time and can be used as a measure of lipogenesis in explant culture studies. The pattern of acetate incorporation was best explained when fitted to a linear regression, with an R^2 value of 0.962 (analysed by ANOVA), therefore acetate incorporation was concluded to be a suitable marker for the measurement of lipogenesis in adipose tissue explants. A repeat of the experiment confirmed these observations.

3.1.1.2. Effect of insulin and dexamethasone on lipogenesis

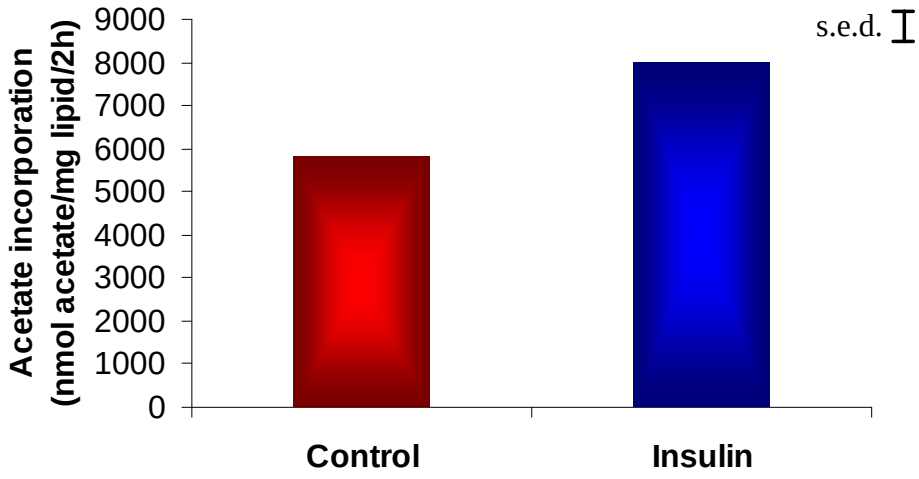
Insulin at a concentration of 20nM significantly increased acetate incorporation (Figure 3.2) and therefore lipogenic rate over a 24 hour period in culture. This is consistent with previous work in this laboratory (Richards, 1997). Treatment of ovine adipose tissue explants independently with insulin and dexamethasone (Figure 3.3) resulted in a significant increase and decrease respectively in lipogenesis ($p<0.001$). However, when the two hormones were supplied together levels of lipogenesis increased but not significantly above those of control levels.

Figure 3.1 A time course experiment measuring acetate incorporation into subcutaneous ovine adipose tissue explants incubated in the absence of insulin in Medium 199



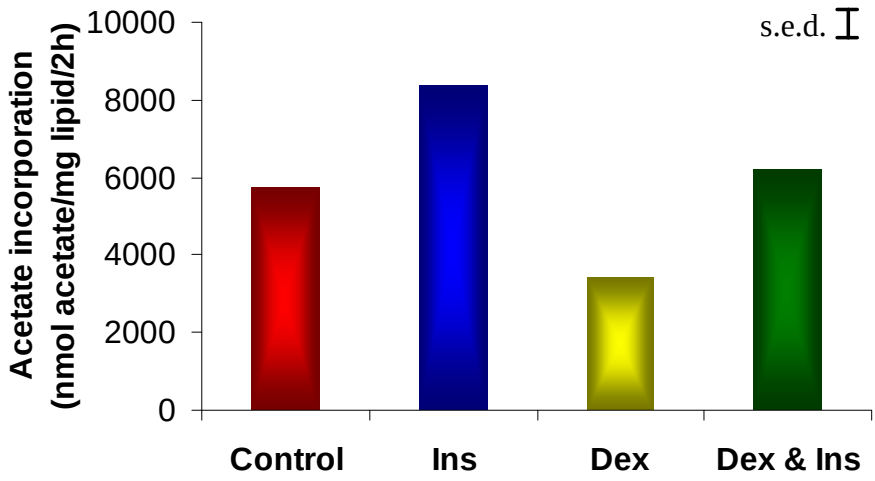
Data presented are group mean values and is from one experiment. Data was subjected to an analysis of variance test, and fitted to a linear regression model (R^2 value presented). Error bars are standard errors of the differences of means.

Figure 3.2 1-[^{14}C] acetate incorporation as a measure of lipogenesis in ovine subcutaneous adipose tissue explants subjected to 24h pre-incubation, followed by 24h treatment with insulin (20nM), then 2h incubation with treatments and radio-labelled acetate.



Data is from 4 independent experiments using 4 different sheep and was subjected to analysis of variance, blocking for experiment using Genstat. Error bars are standard errors of the differences of means of treatment groups. Results were considered significant at p = 0.05.

Figure 3.3 1-[^{14}C] acetate incorporation as a measure of lipogenesis in ovine subcutaneous adipose tissue explants subjected to 24h pre-incubation, followed by 24h incubation with no insulin (control), 20nM insulin (Ins), 20nM insulin and 10nM dexamethasone (Ins & Dex) or 10nM dexamethasone (Dex) alone then 2h incubation with treatments and radio-labelled acetate.



Data is from 2 independent experiments using 2 different sheep and was subjected to one-way analysis of variance, blocking for experiment using Genstat. Error bars are standard errors of the differences of means of treatment groups. Results were considered significant at p = 0.05.

3.1.2. Examination of control of lipogenesis by fatty acids

Having successfully demonstrated that ovine adipose tissue explants in culture are sensitive to both lipogenic and lipolytic hormones, the effects of fatty acids on lipogenesis were examined.

3.1.2.1. Effect of different concentrations of fatty acids on lipogenesis

Tissue was treated with an isomeric mixture of 90% pure conjugated linoleic acid, consisting of approximately equal amounts of the two major isomers *cis-9, trans-11* and *trans-10, cis-12* at four different concentrations and compared to oleic acid at the same concentrations (Table 3.1). There was an interaction between concentration and type of fatty acid ($p<0.001$), with explants treated with 1µM CLA incorporating significantly less acetate than those treated with oleic acid. Conversely, acetate incorporation in explants treated with 100µM CLA was significantly higher than those treated with 100µM oleic acid.

Table 3.1 1-[^{14}C] acetate incorporation as a measure of lipogenesis in ovine subcutaneous adipose tissue explants subjected to 24h pre-incubation, followed by 24h incubation in insulin-containing media, with either oleic or conjugated linoleic acid at different concentrations then 2h incubation with treatments and radio-labelled acetate.

	Acetate incorporation
Control	3.94
1µM oleic acid	8.18
10µM oleic acid	6.58
100µM oleic acid	5.59
1000µM oleic acid	3.79
1µM CLA	5.34
10µM CLA	7.52
100µM CLA	8.47
1000µM CLA	4.24
s.e.d.	
Control vs rest	0.577
Type of fatty acid	0.385
Concentration of fatty acid	0.544
Interaction	0.770
P-value	
Control vs rest	<0.001
Type of fatty acid (F)	0.354
Concentration of fatty acid (C)	<0.001
Interaction (F x C)	<0.001

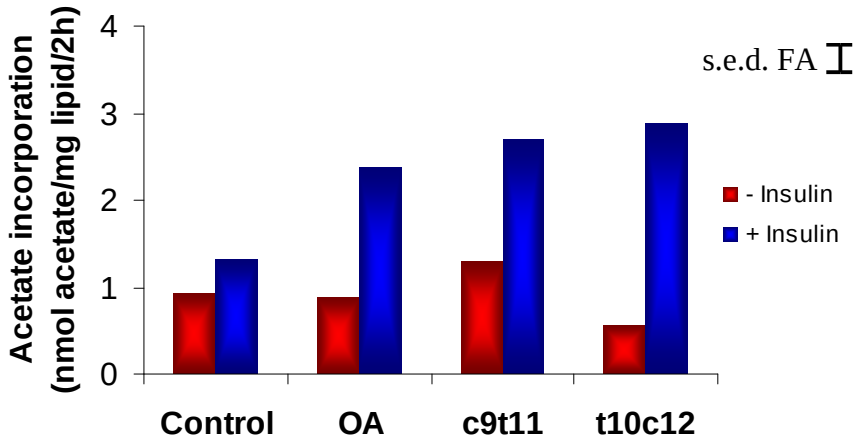
Data is expressed as nmol acetate/mg tissue/2h and is from 3 independent experiments using 3 different sheep with 4 plates for each and was subjected to a two-way ANOVA analysis blocking for experiment using Genstat and comparing control with the rest of the treatments (Control vs rest). F x C = fatty acid-concentration interaction; F = fatty acid; C = concentration. Results were considered significant at p = 0.05.

3.1.2.2. Effect of individual isomers of CLA on lipogenesis

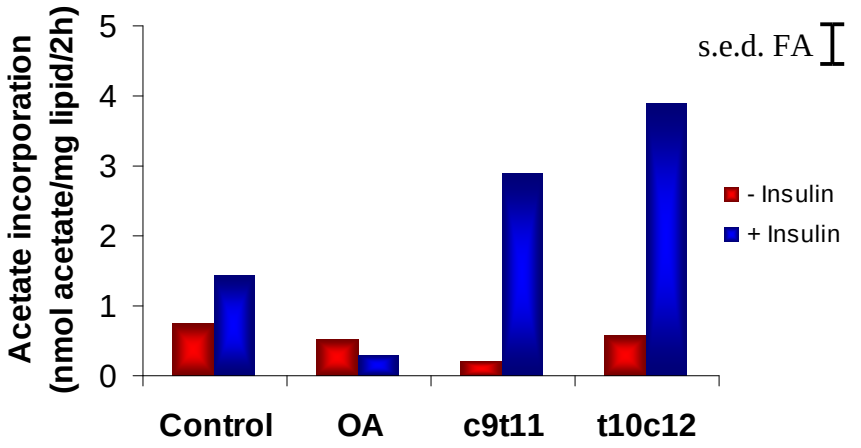
Having established a concentration effect of fatty acids on lipogenesis (Table 3.1), fatty acids were added to cultured explants at a concentration of 10µM. It was thought that any effect of the different fatty acids would be detected at this concentration. To further increase rates of lipogenesis, incubation with treatment fatty acids was increased to 48h, as a result of a time course experiment showing better responses to insulin with time (data not shown). There is evidence that different CLA isomers have different effects, so the effects of the two major isomers (*cis-9, trans-11* and *trans-10, cis-12* CLA) on lipogenesis were studied in the presence and absence of insulin, using oleic acid as a comparative fatty acid and BSA as a control, to take into account any lipogenic effects of the BSA which the fatty acids were complexed to. There was much variation in the tissue response between different preparations (Figure 3.4), with no consistent effect of treatments being seen. When the results were pooled together and analysed, there was a significant ($p<0.001$) interaction between fatty acid and insulin (Table 3.2). When insulin was present, *cis-9, trans-11* and *trans-10, cis-12* CLA significantly increased acetate incorporation, but in the absence of insulin, *trans-10, cis-12* CLA reduced acetate incorporation relative to control incubations, so the inhibitory effects of *trans-10, cis-12* CLA seem to be overcome by the stimulatory effect of insulin on lipogenesis. Oleic acid reduced acetate incorporation relative to control values both with and without insulin. However, in light of the variation between individual experiments and the lack of consistent results it is not possible to reach a conclusion regarding these results.

Figure 3.4 Acetate incorporation into subcutaneous ovine adipose tissue explants subjected to 24h pre-incubation, followed by 48h incubation with 10µM of treatment fatty acids followed by 2 hours further incubation with radio labelled acetate by individual experiment

a) Experiment 1

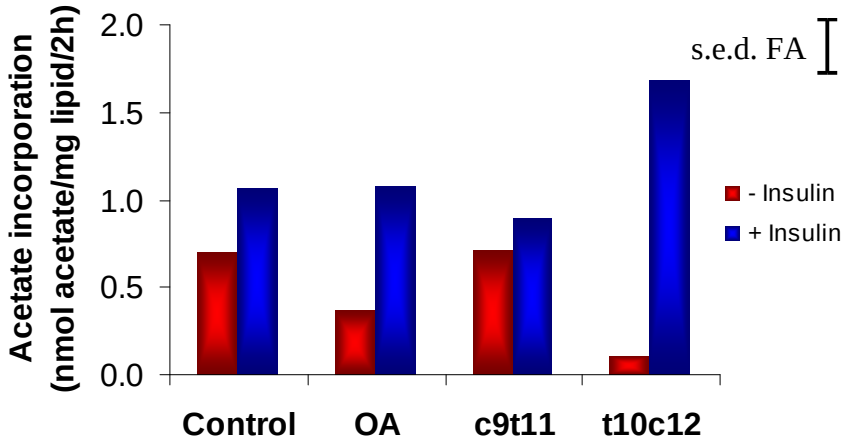


b) Experiment 2



Fatty acids were oleic acid (OA), *cis-9, trans-11* CLA (c9t11) and *trans-10, cis-12* CLA (t10c12) at a concentration of 10µM or with BSA control (Control) at the same concentrations present in the fatty acid-containing plates. s.e.d. is the standard error of the difference between the means for fatty acid (FA) treatment. A one-way ANOVA was performed on the data (Genstat) which is expressed as nmol acetate/mg lipid/2h and is presented as the mean of 3 plates for each treatment.

c) Experiment 3



Fatty acids were oleic acid (OA), *cis-9, trans-11* CLA (c9t11) and *trans-10, cis-12* CLA (t10c12) at a concentration of 10µM or with BSA control (Control) at the same concentrations present in the fatty acid-containing plates. s.e.d. is the standard error of the difference between the means for fatty acid (FA) treatment. A one-way ANOVA was performed on the data (Genstat) which is expressed as nmol acetate/mg lipid/2h and is presented as the mean of 3 plates for each treatment**.**

Table 3.2 Acetate incorporation into subcutaneous ovine adipose tissue explants subjected to 24h pre-incubation, followed by 48h incubation with 10µM of treatment fatty acids followed by 2 hours further incubation with radio labelled acetate.

	Fatty acid[a]						
	Con	**OA**	**c9t11**	**t10c12**	**Average**	**s.e.d.[b]**	**p-value[c]**
-Ins	0.65	0.39	0.61	0.19	0.46	f = 0.27	0.002
+Ins	1.10	0.73	2.16	2.82	1.70	i = 0.19	<0.001
Average	0.87	0.56	1.39	1.50		f.i = 0.38	<0.001

[a]Fatty acids were oleic acid (OA), *cis-9, trans-11* CLA (c9t11) and *trans-10, cis-12* CLA (t10c12) at a concentration of 10µM or with BSA control (Con) at the same concentrations present in the fatty acid-containing plates.

[b]s.e.d. is the standard error of difference of the means of treatment groups.

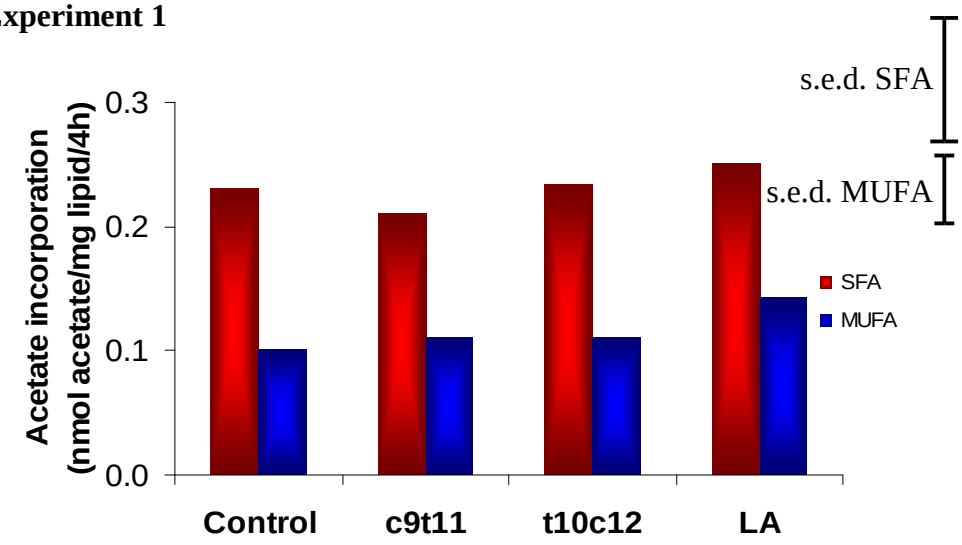
[c]Statistical analyses were performed using a two-way ANOVA (Genstat) to examine interactions between fatty acid and insulin; f denotes fatty acid effect, i denotes insulin effect. Data is expressed as nmol acetate/mg lipid/2h and is from 3 independent experiments using 3 different sheep and statistical analysis was carried out blocking for experiment. Results were considered significant at p = 0.05.

3.1.3. Effect of fatty acids on acetate incorporation into saturated and monounsaturated fatty acids

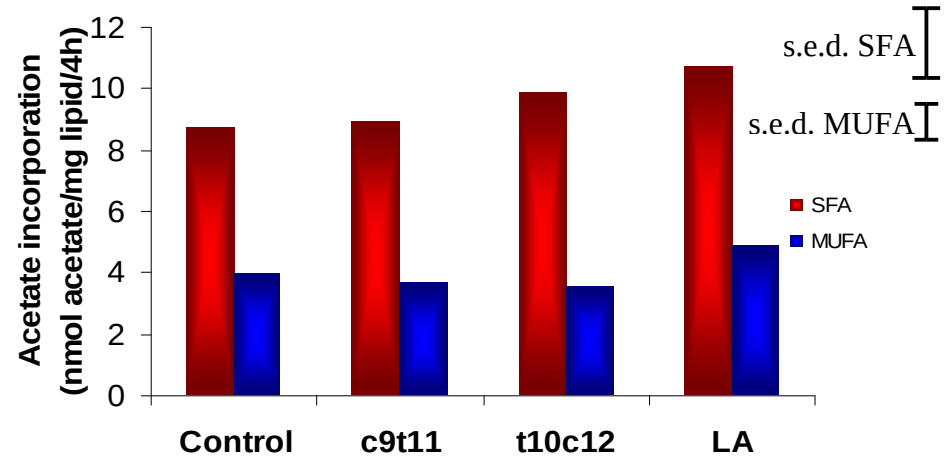
Having examined the effect of CLA and other fatty acids on total lipogenesis, effects on acetate incorporation into saturated and monounsaturated fatty acids were studied (Sections 2.3.3, 2.3.6, 2.3.7 & 2.3.8). CLA has been shown to affect SCD activity, and an indication of enzyme activity can be gained by studying the formation of monounsaturated fatty acids. The following experiments were designed to identify if there are any effects of CLA on SCD activity as measured by the relative synthesis of saturated and monounsaturated fatty acids. Individual isomers were used as in Section 3.1.2.1 and 3.1.2.2, but linoleic acid was used as a control fatty acid as it is atomically the same as the CLAs but lacks the conjugated double bond structure which is characteristic of these fatty acids. There was no effect of fatty acid on acetate incorporation into either saturated or monounsaturated fatty acids (Table 3.3), however there was an indication that *trans-10, cis-12* CLA reduced monounsaturated fatty acid formation, but this was not significant. There was no significant effect of fatty acid on the ratio of saturated to monounsaturated fatty acids, so it can be concluded that treatment of explants with any of the fatty acids used here does not reduce the ratio of formation of saturated and monounsaturated fatty acids. There was a large amount of variation between tissue preparations' responses to fatty acids (Figure 3.5) and inconsistent responses between individual experiments indicate that there is no detectable effect of fatty acids on saturated or monounsaturated fatty acid synthesis in ovine adipose tissue.

Figure 3.5 Acetate incorporation into saturated and monounsaturated fatty acids of subcutaneous ovine adipose tissue explants subjected to 24h pre-incubation, followed by 48h incubation in insulin-containing media with no fatty acid (BSA), and 10µM of either oleic acid, *cis-9*, *trans-11* or *trans-10,cis-12* CLA and 4 hours further incubation with radio labelled acetate by individual experiment

a) Experiment 1

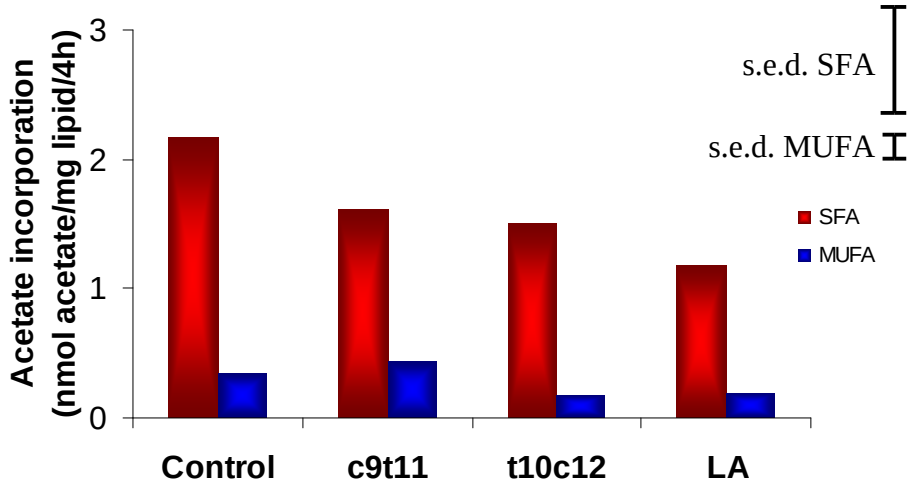


b) Experiment 2



Fatty acids were linoleic acid (LA), *cis-9, trans-11* CLA (c9t11) and *trans-10, cis-12* CLA (t10c12) at a concentration of 10µM or no fatty acid (Control), both with and without insulin. Each graph is from one experiment. s.e.d. is the standard error of the difference between the means of treatment groups. A one-way ANOVA was performed on the data (Genstat) which is expressed as nmol acetate/mg lipid/4h and is presented as the mean of 3 plates for each treatment.

c) Experiment 3



Fatty acids were linoleic acid (LA), *cis-9, trans-11* CLA (c9t11) and *trans-10, cis-12* CLA (t10c12) at a concentration of 10µM or no fatty acid (Control), both with and without insulin. Each graph is from one experiment. s.e.d. is the standard error of the difference between the means of treatment groups. A one-way ANOVA was performed on the data (Genstat) which is expressed as nmol acetate/mg lipid/4h and is presented as the mean of 3 plates for each treatment.

Table 3.3 Acetate incorporation into saturated and monounsaturated fatty acids of subcutaneous ovine adipose tissue explants subjected to 24h pre-incubation, followed by 48h incubation in insulin-containing media with no fatty acid (BSA), and 10µM of either oleic acid, *cis-9, trans-11* or *trans-10,cis-12* CLA and 4 hours further incubation with radio labelled acetate.

	Fatty acid[a]					
	Con	**LA**	**c9t11**	**T10c12**	**s.e.d.[b]**	**p-value[c]**
SFA	3.70	4.04	3.58	3.85	f = 0.71	0.925
MUFA	1.47	1.75	1.39	1.27	f = 0.42	0.701
SFA:MUFA ratio	3.57	3.66	2.84	4.47	f = 0.66	0.130

[a]Fatty acids were linoleic acid (LA), *cis-9, trans-11* CLA (c9t11) and *trans-10, cis-12* CLA (t10c12) at a concentration of 10µM or no fatty acid (Con), all with insulin.

[b]s.e.d. is the standard error of difference of the means of treatment groups.

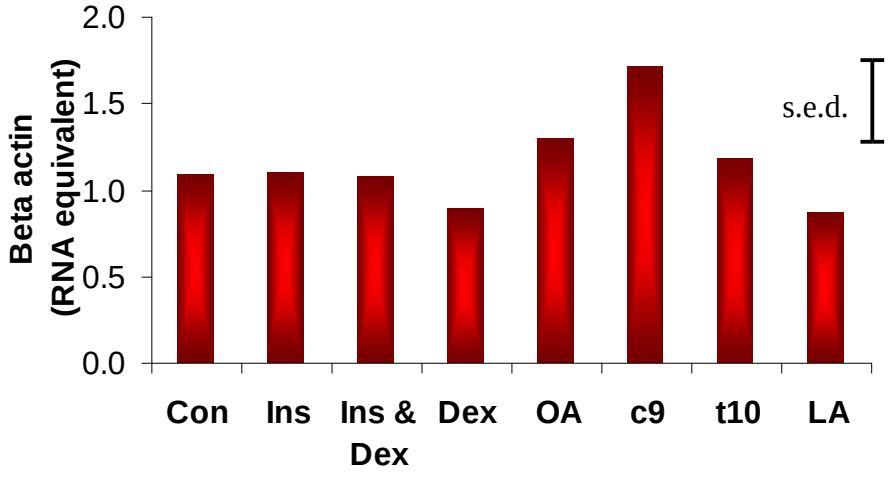
[c]Statistical analyses were performed using a one-way ANOVA (Genstat) to examine effects of fatty acids on acetate incorporation; f denotes fatty acid effect.

Data is expressed as nmol acetate/mg lipid/4h and is from 3 independent experiments using 3 different sheep and statistical analysis was carried out blocking for experiment. Results were considered significant at p = 0.05.

3.1.4. Effect of fatty acids on steady-state RNA levels in ovine adipose tissue

Having concluded from the work studying acetate incorporation into monounsaturated fatty acids that there was no reproducible effect of CLA on lipogenic activity in ovine adipose tissue explants cultured in vitro, the key genes controlling lipogenesis and desaturation in sheep were examined to determine if CLA had effects at the gene transcription level. Beta actin was used as a housekeeping gene and did not change significantly with treatment across all experiments (Figure 3.6). Acetyl-CoA carboxylase is the first enzyme in the lipogenesis pathway, and is also rate limiting, so this was used as a marker of lipogenic rate. SCD controls desaturation as previously mentioned (Section 1.4.3) and was also studied. Preliminary experiments using the hormones insulin and dexamethasone as in the total lipogenesis experiments were carried out before using the fatty acids.

Figure 3.6 Beta actin steady-state mRNA levels in subcutaneous ovine adipose tissue subjected to 24h pre-incubation, followed by 48h with no insulin (Con), 20nM insulin (Ins), 20nM insulin and 10nM dexamethasone (Ins & Dex), 10nM dexamethasone (Dex), 10µM of either oleic acid (OA), *cis-9, trans-11* CLA (c9), *trans-10, cis-12* CLA (t10) or linoleic acid (LA)



Data is from one experiment and was subjected to one way ANOVA using Genstat. The experiment was repeated twice and the same results obtained. s.e.d. is the standard error of the difference between the treatment group means which are presented as the mean of 3 plates for each treatment.

There was a significant effect ($p<0.001$) of treating explants with insulin and dexamethasone on ACC RNA levels (Figure 3.7). This was the same as seen in the total lipogenesis studies and indicates that, in the case of ACC, gene expression is a good indicator of enzyme activity. Treatment with insulin alone significantly increased ACC mRNA levels, but treatment with dexamethasone failed to significantly reduce those levels, possibly due to the low levels of ACC mRNA measured in both control and dexamethasone treated explants. There was a significant effect ($p = <0.001$) of both hormones on SCD mRNA levels (Figure 3.8). Insulin and dexamethasone significantly increased SCD mRNA levels, as did insulin alone. Dexamethasone alone significantly reduced SCD mRNA levels. Effects seen on SCD mRNA levels were 4 fold greater than controls, but those seen on ACC mRNA levels were only 2 fold greater than controls. This and the effects latterly described were consistent with Daniel *et al*, 2004, using ribo-nuclease protection assays. Figures 3.7 and 3.8 demonstrate that steady-state RNA levels as quantified by Taqman real-time PCR are as reliable as quantification by ^{32}P ribo-nuclease protection assay.

Figure 3.7 ACC steady-state mRNA levels in ovine subcutaneous adipose tissue subjected to 24h pre-incubation, followed by 48h incubation with no insulin (control), 20nM insulin (Ins), 20nM insulin and 10nM dexamethasone (Ins & Dex) or 10nM dexamethasone (Dex) alone

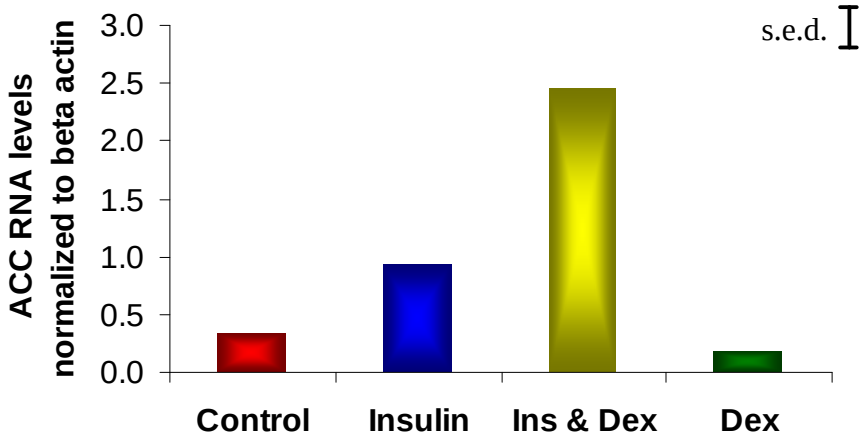
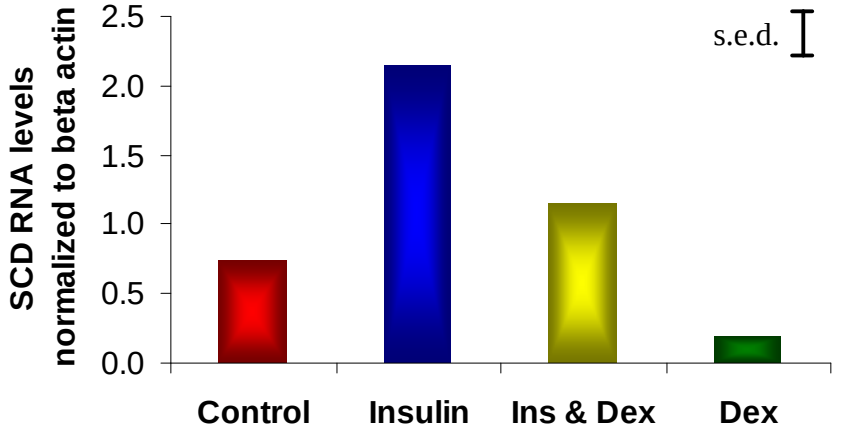


Figure 3.8 SCD steady-state mRNA levels in ovine subcutaneous adipose tissue subjected to 24h pre-incubation, followed by 48h incubation with no insulin (control), 20nM insulin (Ins), 20nM insulin and 10nM dexamethasone (Ins & Dex) or 10nM dexamethasone (Dex) alone

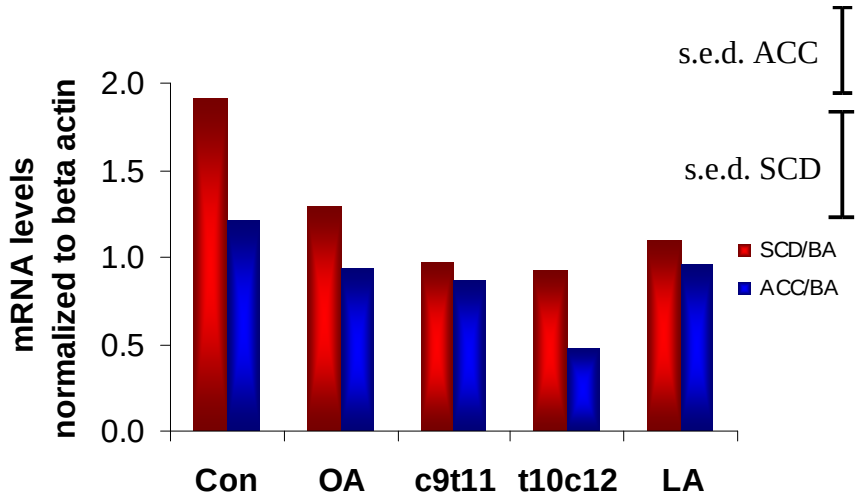


Data is expressed relative to beta actin and is from 3 independent experiments using 3 different sheep and was subjected to one-way analysis of variance, blocking for experiment using Genstat. Error bars are standard errors of the difference between treatment group means. Results were considered significant at $p = 0.05$.

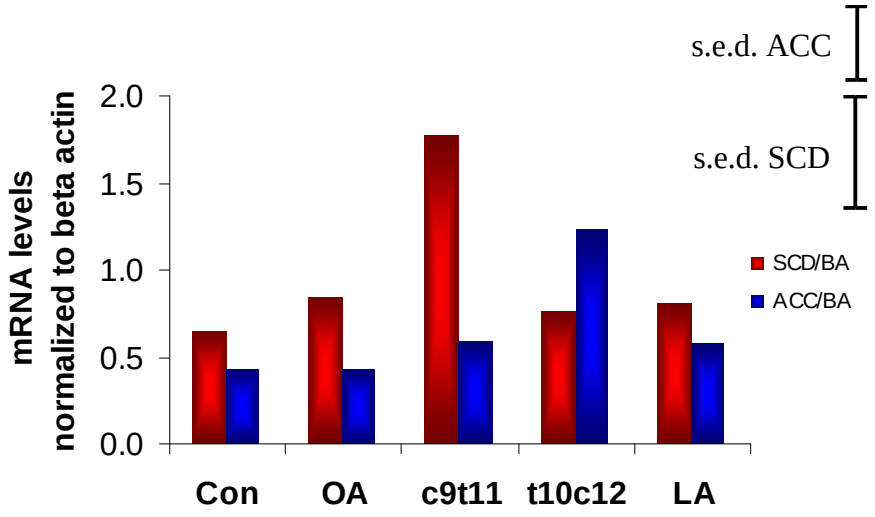
There was no significant effect of fatty acid (p=0.768 and 0.212 respectively) on either SCD or ACC RNA levels (Figure 3.10). Linoleic acid increased ACC RNA levels but the difference relative to control values was not significant. This is consistent with the total lipogenesis results (Table 3.3). There was variation between experiments (Figure 3.9) making it difficult to ascertain whether there was an effect of fatty acid on mRNA levels.

Figure 3.9 **SCD and ACC steady-state RNA levels in subcutaneous ovine adipose tissue explants subjected to 24h pre-incubation, 48h incubation in insulin-containing media with no fatty acid (control), and 10µM of either oleic acid, linoleic acid, *cis-9, trans-11* or *trans-10, cis-12* CLA displayed as individual experiments**

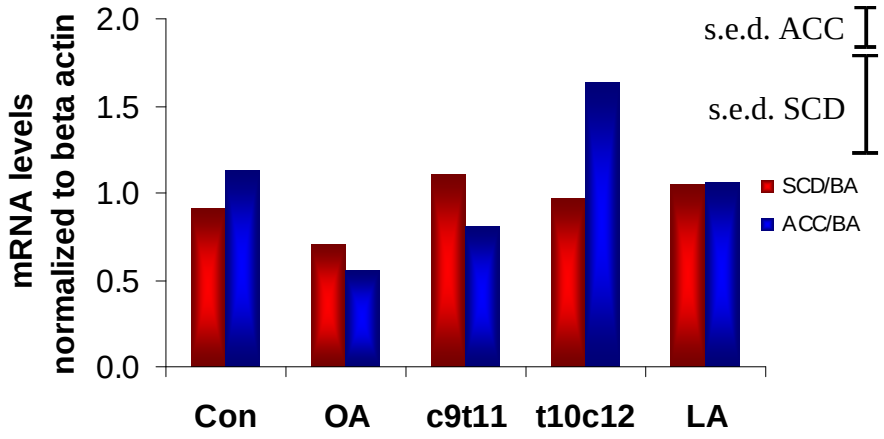
a) Experiment 1



b) Experiment 2

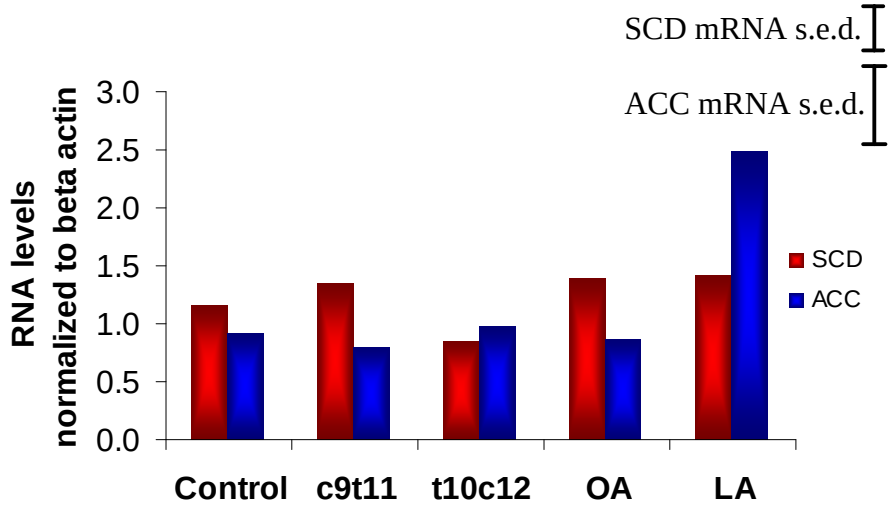


c) Experiment 3



Fatty acids are linoleic acid (LA), oleic acid (OA), *cis-9, trans-11* CLA (c9t11) and *trans-10, cis-12* CLA (t10c12) at a concentration of 10µM or no fatty acid (control). Each graph is from one experiment. s.e.d. is the standard error of the difference between the means of treatment groups. A one-way ANOVA was performed on the data (Genstat) which is expressed as relative to the housekeeping gene beta actin and is presented as the mean of 3 plates for each treatment.

Figure 3.10 SCD and ACC steady-state RNA levels in subcutaneous ovine adipose tissue explants subjected to 24h pre-incubation, 48h incubation in insulin-containing media with no fatty acid (control), and 10µM of oleic acid, linoleic acid, *cis-9, trans-11* or *trans-10, cis-12* CLA



Fatty acids are linoleic acid (LA), oleic acid (OA), *cis-9, trans-11* CLA (c9t11) and *trans-10, cis-12* CLA (t10c12) at a concentration of 10µM or no fatty acid (Control).

Data is expressed relative to beta actin and is from 3 independent experiments using 3 different sheep and was subjected to one-way analysis of variance, blocking for experiment using Genstat. Error bars are standard errors of the differences between the treatment group means. Results were considered significant at p = 0.05.

3.2. DISCUSSION

The aim of these experiments was to determine whether CLA had direct effects on lipogenesis in sheep adipose tissue and whether these effects were a result of modulation of expression of mRNA coding for lipogenic genes. Experiments were performed to specifically look at total lipogenesis and the synthesis of saturated and monounsaturated fatty acids separately.

3.2.1. Hormonal regulation of lipogenesis

Ovine adipose tissue explant culture is a well established technique for the study of lipogenesis in response to hormones and the effects of insulin, growth hormone and the glucocorticoids have been extensively studied in a number of species. In ruminant animals acetate is the major substrate for lipogenesis (Smith & Prior, 1986; Vernon, 1979) and therefore acetate and not glucose was used for the tissue culture work presented here. It has been demonstrated by a number of researchers that insulin increased lipogenesis in ruminant animals (Miller *et al*, 1991; Vernon *et al*, 1991; Etherton & Evock, 1986; Vernon, 1979), therefore insulin was used as a positive control in all of the adipose tissue culture experiments to test that the tissue was capable of responding to hormonal stimuli. Dexamethasone, a synthetic glucocorticoid, was also used, as this has been shown to reduce lipogenesis (Daniel *et al*, 2004) in ovine adipose tissue explants. Initial experiments confirmed that the ovine explants were responsive to hormones with lipogenesis increasing and decreasing in the presence of insulin and dexamethasone respectively. However it is of note that different preparations of adipose tissue explants varied significantly in their response to insulin with the level of stimulation varying from 61 to over 200% of controls.

3.2.2. Effect of exogenous fatty acids on lipogenesis

Having established that the explant culture technique was capable of responding to hormonal stimuli, the effects of fatty acids on lipogenesis were examined. In initial experiments, in the presence of insulin, the response to oleic acid was concentration dependent. At low concentrations (1µM) oleic acid appeared to stimulate lipogenesis. At high concentrations of oleic acid

lipogenesis appeared to fall and at 1000µM were similar to control levels. It is possible that the relative reduction at a concentration of 1000µM of fatty acid may be related to the toxic effect of this high concentration. There was a similar pattern with the CLA mix with an initial stimulation of lipogenesis on feeding a CLA mix, up to 100µM and then an apparent decrease at 1000µM. Contrary to these findings, Dawson & Herbein (1996) reported that bovine mammary cells cultured with CLA had reduced *de novo* synthesis of palmitic acid and that this effect increased as cellular uptake of CLA increased. 3T3-L1 preadipocytes treated with 50 and 200µM CLA presented with significantly lower triglyceride levels than cells treated with the same concentrations of linoleic acid (Evans *et al*, 2000). There was no dose dependent reduction of triglyceride accumulation with CLA treatment, but linoleic acid increased triglyceride accumulation in a dose dependent manner. Since there is a large amount of evidence to suggest that the two major isomers of CLA have different effects, and it is primarily the *trans-10, cis-12* isomer that is responsible for effects on fat metabolism (Park *et al*, 1999; Choi *et al*, 2000), explants were cultured with individual isomers at a concentration of 10µM with and without insulin (Table 3.2). Results from these experiments were somewhat variable but there was little evidence to suggest any major effect of either isomer on lipogenesis. Brown *et al* (2001b) cultured 3T3-L1 preadipocytes with individual isomers of CLA in the presence of insulin, and found that the *trans-10, cis-12* isomer reduced the triglyceride content of the cells, but that treatment with the *cis-9, trans-11* isomer increased triglyceride levels above those seen with linoleic acid. The present results do not confirm these findings in ovine adipose tissue explants. This may be the result of species differences in responsiveness to CLA. However it is of note that there was much variation in basal and insulin stimulated lipogenesis between different explant preparations. The increase in lipogenesis in response to treatment with insulin is well documented (Miller *et al*, 1991; Etherton & Evock, 1986; Vernon, 1979), and yet in some of the experiments there is a very small increase in lipogenesis with insulin, while others show larger increases. Fatty acids seem to stimulate lipogenesis, but effects are highly variable, and clearly there are differences between tissue preparations evident by these

variable insulin responses. It is likely that genetic and physiological differences between animals together with differences in the viability of the final explants may all contribute to this variation in responsiveness. The availability of fatty acids to all of the cells within the tissue may also represent a specific problem when looking for a response. While relatively small, water soluble hormones may readily diffuse through the tissue to reach cells in the centre of the explants, this may not be the case for hydrophobic fatty acids, even when complexed to albumin. It must be concluded that any direct effects of fatty acids on lipogenesis are not of a magnitude that can be detected using the adipose tissue explant system.

3.2.3. Effect of fatty acids on saturated and monounsaturated fat

Previous studies in other species have suggested that CLA can markedly affect the fatty acid composition of tissue by reducing the relative rate of desaturation of saturated fatty acids to monounsaturated fatty acids through modulating the activity of SCD. Having failed to demonstrate a clear effect of CLA on total lipogenesis, effects of CLA treatment on acetate incorporation into different classes of fatty acids were studied. Previous work from our laboratory showed that this technique could be used to demonstrate specific effects of insulin and dexamethasone on the relative synthesis of these fatty acid classes in ovine adipose tissue explants (Daniel *et al*, 2004). However in the present experiments, no consistent effect of this isomer, or any other fatty acid, on the relative synthesis of saturated fatty acids or MUFA could be demonstrated. Studies in HepG2 cells treated with *trans-10, cis-12* CLA showed an increase in saturated fatty acids and a concomitant decrease in monounsaturated fatty acids compared to BSA fatty acid free controls (Choi *et al*, 2001). There was no indication that formation of monounsaturated fatty acids was inhibited by CLA in sheep as has been previously reported in pigs (Demaree *et al*, 2002; Smith *et al*, 2002; Ramsay *et al*, 2001), rats (Alasnier *et al*, 2002; Poulos *et al*, 2001), sheep (Mir *et al*, 2000). Much of the adipose tissue lipid is comprised of TAG, and changes in the proportions of saturated and monounsaturated fatty acids can be used as an indication of SCD activity (Demaree *et al*, 2002; Smith *et al*, 2002). Although fatty acid composition was not measured in these experiments, it is *de novo* fatty acid synthesis which is responsible for tissue

fatty acid composition and therefore measurement of radiolabel incorporated into fat, or the amount of saturated or monounsaturated fatty acid synthesis occurring is another method of estimating SCD enzyme activity. Obviously the lack of any response in the explants may represent a tissue specific difference in response to CLA isomers. Alternatively, this may reflect a difference in responsiveness of different species. However, the specific problems associated with explants, as detailed in Section 3.2.2 may be responsible for the lack of responsiveness.

3.2.4. Effect of fatty acids on enzyme RNA levels in adipose tissue

Previous work from our laboratory has indicated that ovine adipose tissue explants can be used to demonstrate the effect of hormones on the mRNA concentrations for lipogenic enzymes (Daniel et al, 2004; Richards, 1997). Insulin was shown to increase the levels of both ACC and SCD mRNA while dexamethasone had the opposite effect (Daniel *et al*, 2004). Furthermore the same technique was used to demonstrate the effects of retinoic acid on ACC and SCD gene expression (Daniel, 2002). It was therefore decided to look at the effects of different fatty acids, including the two major CLA isomers on mRNA concentrations for these two enzymes. Initial experiments confirmed the effects of insulin and dexamethasone and produced similar results to those previously published (Daniel *et al*, 2004). However, there was no detectable change in these mRNA levels with CLA, or any other fatty acid.

3.2.5. Summary

The present data fails to demonstrate any consistent effect of fatty acid on lipogenesis, or expression of lipogenic enzymes in ovine adipose tissue explants. There are many reports of the effects of dietary fat on adipose tissue lipogenesis in the literature, many of which are conflicting. Variously *de novo* lipogenesis was not suppressed (Nelson *et al*, 1987; Clark *et al*, 1977) was suppressed to a greater extent (Waterman *et al*, 1975; Vernon *et al*, 1975) or was less suppressed (Demeyer *et al*, 1974; Du & Kruger, 1972) by a saturated fat than a polyunsaturated fat diet. Compounding these findings is the time scale of experiments which can vary from 48h to 2 weeks. Therefore very small, insignificant changes in the type and amount of lipid may be seen in this time which would be significant if observations were made over a period of months. Effects of CLA on whole body systems may have their effects on a number of other hormones, transcription factors and metabolites which are not present in the tissue culture systems. It may be that CLAs have their effects by acting on factors external to the system, which then have their effects on lipogenic genes and/or enzymes. In addition, at present there is more evidence for inhibition of hepatic lipogenesis by fatty acids than adipose tissue, although an inhibition of lipogenesis with increasing concentrations of fatty acids has been demonstrated in ovine adipose tissue (Vernon, 1975). It is of interest to note that no difference in lipogenesis between the 2 treatment fatty acids, linoleic acid and palmitic acid was seen. The results presented here with respect to CLA could reflect a lack of responsiveness of mature sheep to the fat-reducing properties of CLA. However, careful interpretation of *in vitro* studies is needed and ideally these should be confirmed during *in vivo* trials. For this reason it was decided to undertake the CLA feeding trial described in the next chapter.

4. EFFECT OF CONJUGATED LINOLEIC ACID ON LIPID METABOLISM IN SHEEP

There have been few studies undertaken to look specifically at the effects of CLA on ruminant carcass composition and tissue fatty acid composition in the context of lipogenic enzyme gene expression. The objective of this trial was to examine the effects of feeding CLA to sheep on carcass composition, tissue fatty acid composition and ACC and SCD mRNA levels in adipose tissue depots and liver in order to elucidate how CLA affects fat metabolism at a transcriptional level. It was predicted that increasing the amount of CLA available for absorption into tissues would decrease carcass fat by reducing the mRNA levels of lipogenic enzymes ACC and SCD. This trial was carried out in conjunction with R. Wynn of the University of Nottingham (see Materials and Methods). The fatty acid composition data presented here are from the analyses of Dr. R. Wynn and was presented as part of a PhD thesis (Wynn, 2003).

4.1.1. Experimental design

36 weaned ewe lambs, initial weight 28kg were fed a lamb fattener diet (Table 2.1) as ground meal, supplemented with CLA-80 or Megalac™ at three levels for 10 weeks as described in Section 2.2.2. The two fat supplements were protected against rumen degradation and were broken down in the acidic conditions of the abomasum. The protected CLA inclusion rates were 25, 50 and 100g/kg diet, equivalent to 4.23, 8.46, 16.92g CLA (with equal amounts of *cis-9, trans-11* and *trans-10, cis-12* isomers). Previous studies in this laboratory (Wynn, 2003) had established that the CLA-80 was 66% protected against rumen degradation (see Section 2.2.2.2). Megalac™ is a commercially available ruminally protected lipid supplement which contains fatty acids bound to calcium. The calcium salts are protected from degradation in the rumen, but release the fatty acids for absorption in the duodenum. Megalac was fed to control for the energy value of the PCLA supplement, which is why the amounts added to the diet are different from those of CLA. The CLA and Megalac™ diets were isoenergetic with each other at each supplementation

level. A basal lamb fattener diet containing no Megalac or CLA was fed as a control treatment. Sheep were individually penned with access to water and mineral licks. Sheep were slaughtered and samples collected as described in Section 2.2.2.3.

4.1.2. Effect of PCLA on carcass composition

There was no effect of feeding protected CLA or Megalac on the growth rate, final carcass weight or percentage carcass fat of lambs (Table 4.1). The weights of perirenal and omental adipose tissue were also unchanged by supplementation of diets with PCLA or Megalac.

Table 4.1 Animal growth rates, carcass weights, body fat content, fat depot weights and liver weights for lambs fed a basal lamb fattener diet supplemented with Megalac or protected CLA (PCLA)

	Growth rate (g/d)	Carcass cold weight (kg)	Body fat content (% DM)	Perirenal fat weight (g)	Omental fat weight (g)	Liver weight (g)
Control	197.0	20.2	54.8	472.8	643.1	663.5
Low Megalac	204.3	20.7	57.3	534.0	662.5	677.8
Medium Megalac	175.5	19.7	58.6	555.4	663.4	674.3
High Megalac	178.0	19.9	55.8	488.1	594.6	629.3
Low PCLA	186.8	20.3	57.3	491.7	645.4	617.4
Medium PCLA	182.2	20.5	57.1	564.5	662.6	616.3
High PCLA	198.4	20.8	55.9	523.0	705.6	652.1
s.e.d						
Control vs rest	10.27	0.55	1.58	69.10	88.70	32.80
Type of fat	11.10	0.59	1.71	74.60	95.80	35.40
Amount of fat	11.86	0.63	1.82	79.70	102.40	37.90
Interaction	13.91	0.77	2.23	97.70	125.50	46.40
P-value						
Control vs rest	0.362	0.907	0.185	0.447	0.888	0.569
Type of fat	0.708	0.350	0.697	0.992	0.672	0.244
Amount of fat	0.280	0.840	0.440	0.697	0.989	0.977
Interaction	0.193	0.437	0.856	0.852	0.737	0.366

4.2. FATTY ACID COMPOSITION OF SHEEP DIETS

Figure 4.1 Fatty acid composition of Megalac supplement

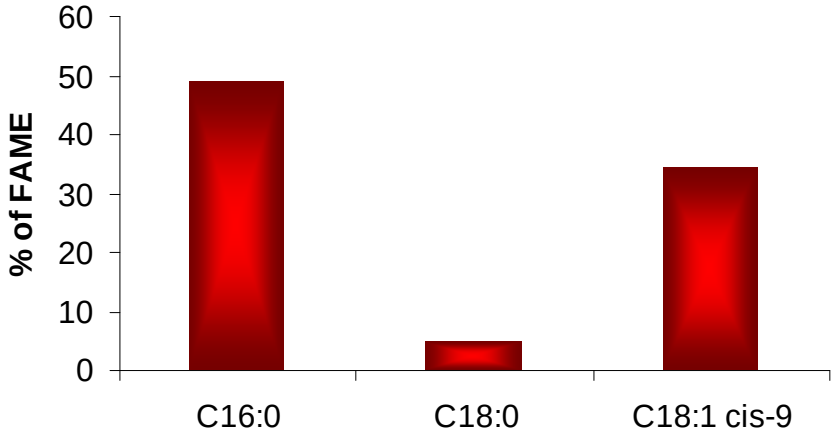
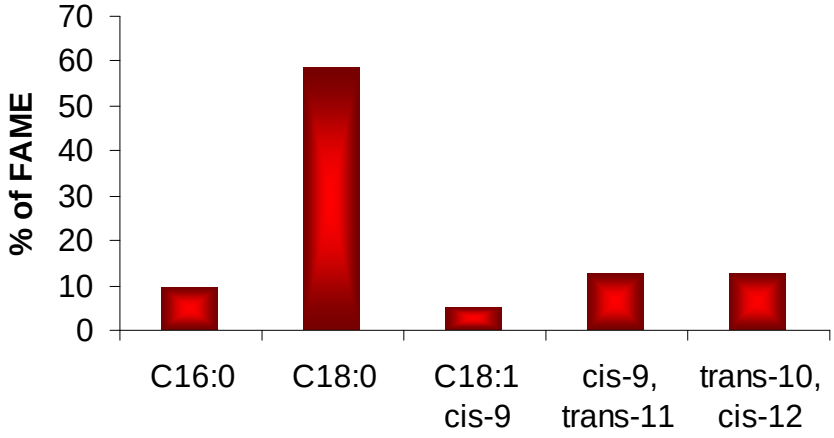


Figure 4.2 Fatty acid composition of PCLA supplement



FAME were prepared using 0.5M sodium methoxide/methanol followed by 14% BF_3/methanol as in Section 2.3.7 and analaysed by GC using a BPX 70 60m column under the conditions described in Section 2.3.9.

4.3. FATTY ACID COMPOSITION OF SHEEP TISSUES

4.3.1. CLA content of adipose tissue depots

Lipid was extracted from subcutaneous, perirenal and omental adipose tissues, methylated and analysed by gas chromatography to examine incorporation of *cis-9, trans-11* and *trans-10, cis-12* CLA as described in Sections 2.3.6, 2.3.7 & 2.3.9. There was a significant effect ($p<0.001$) of amount and type of fat on incorporation of both CLA isomers into all adipose tissue depots (Table 4.2 and there was a significant interaction between the two. When the basal lamb fattener diet was compared against all other treatments there was a highly significant ($p = 0.001$) effect of fat treatment on the incorporation of both isomers into all depots, with the exception of *cis-9, trans-11* CLA in the subcutaneous adipose tissue depot ($p = 0.080$). The amount of CLA present in the adipose tissue increased with increasing supplementation. This indicates that CLA was present in the tissues to potentially affect transcription of the genes under investigation. The *cis-9, trans-11* isomer was present as a greater proportion of total FAME than *trans-10, cis-12* CLA.

4.3.2. CLA content in liver

Lipid was extracted from liver tissue, methylated and analysed by gas chromatography to examine incorporation of *cis-9, trans-11* and *trans-10, cis-12* CLA as described in Sections 2.3.6, 2.3.7 & 2.3.9. There was a significant ($p<0.001$) effect of the amount and type of fat fed on incorporation into lamb liver of both CLA isomers (Table 4.3). The proportion of *cis-9, trans-11* is greater than that of *trans-10, cis-12*, as seen in the adipose tissue depots (Table 4.2) As supplementation of CLA increases the amount seen in the tissues also increases, indicating that CLA is incorporated into liver in a dose dependent manner.

4.3.3. Fatty acid composition of adipose tissue

There was a significant decrease in C16:1 and C18:1 *cis-9* with PCLA feeding (Table 4.4) in subcutaneous adipose tissue ($p<0.001$). There was a significant decrease ($p<0.001$) in C16:1 in perirenal and omental adipose tissue. There was a significant interaction between type and amount of fat for C18:1 *cis-9* ($p = 0.002$ and 0.015 respectively).

4.3.4. Fatty acid composition of liver

There was a significant decrease in C16:1 with PCLA feeding (Table 4.3; $p=0.046$) and a significant interaction between type and amount of fat for C18:1 *cis-9*.

4.3.5. Desaturase activity in adipose tissue depots and liver

The lipid extracted from adipose tissue and liver was methylated and subjected to gas chromatography as described in Section 2.3.9 to identify the individual fatty acids present in tissues. The ratios of two pairs of fatty acids were calculated; Palmitoleic acid (C16:1) and palmitic acid (C16:0); and oleic acid (C18:1 *cis-9*) and stearic acid (C18:0). This was used as a measure of stearoyl-CoA desaturase activity, which acts on stearic and palmitic acids to form oleic and palmitoleic acids (Ntambi, 1999). By comparison of fatty acid composition in animals fed CLA, specifically of these fatty acids, and RNA levels in tissues, it is possible to identify whether CLA acts directly on the enzyme complex or at a transcriptional level. The palmitoleic:palmitic acid desaturase ratio was

significantly reduced with CLA supplementation in subcutaneous, perirenal and omental adipose tissue ($p = 0.003$, <0.001 and 0.002 respectively), indicating that CLA was reducing the activity of SCD (Table 4.5). The stearic:oleic acid desaturase ratio was significantly reduced by CLA feeding in subcutaneous, perirenal and omental adipose tissue depots ($p = 0.027$, <0.001, 0.008). The results seem to indicate that the subcutaneous adipose tissue depot is less responsive than the internal depots. There was no significant effect of increasing dose on either of the desaturase ratios, but there was an interaction between fat type and amount in perirenal tissue for C16:1/C16:0 and C18:1/C18:0 ($p = 0.084$ and 0.042 respectively). Desaturase ratios were also calculated for liver in the same way as for adipose tissue. There was a significant effect of amount of fat fed on the palmitoleic:palmitic ratio (Table 4.3; $p = 0.028$), but no significant effect on the stearic:oleic ratio ($p = 0.388$). There was no effect of type of fat on either desaturase ratio, but there were significant ($p = 0.072$ and 0.026) interactions between type and amount of fat fed for both palmitoleic:palmitic and stearic:oleic ratios respectively.

Table 4.2 Proportions (% of FAME) of *cis-9, trans-11* and *trans-10, cis-12* CLA found in subcutaneous, perirenal and omental adipose tissue of lambs fed a basal lamb fattener diet supplemented with Megalac or protected CLA (PCLA)

	Subcutaneous		**Perirenal**		**Omental**	
	Cis-9, trans-11	*trans-10, cis-12*	*cis-9, trans-11*	*Trans-10, cis-12*	*Cis-9, trans-11*	*trans-10, cis-12*
Control	0.91	0.04	0.46	0.04	0.68	0.05
Low Megalac	0.80	0.02	0.47	0.07	0.73	0.07
Medium Megalac	0.95	0.10	0.50	0.13	0.82	0.08
High Megalac	0.86	0.08	0.52	0.24	0.75	0.10
Low PCLA	1.12	0.19	0.70	0.21	0.98	0.19
Medium PCLA	1.38	0.39	1.03	0.38	1.27	0.37
High PCLA	2.01	0.78	1.66	0.78	1.82	0.77
s.e.d.						
Control vs rest	0.150	0.033	0.074	0.048	0.098	0.033
Type of fat	0.162	0.035	0.079	0.052	0.105	0.036
Amount of fat	0.174	0.038	0.085	0.055	0.113	0.038
Interaction	0.213	0.046	0.104	0.068	0.138	0.047
P-value						
Control vs rest	0.080	<0.001	<0.001	<0.001	<0.001	<0.001
Type of fat	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Amount of fat	0.015	<0.001	<0.001	<0.001	<0.001	<0.001
Interaction	0.021	<0.001	<0.001	0.001	<0.001	<0.001

FAME were prepared as described in Section 2.3.7 and analysis was carried out by gas chromatography on a 100m CP-Sil 88 column. Data was analysed by ANOVA, comparing control with the other treatment groups and testing for effects of fat, fat type, fat amount and a type-amount interaction.

Table 4.3 Proportions (% of FAME) of *cis-9, trans-11* and *trans-10, cis-12* CLA, C16:0, C16:1, C18:0 and C18:1 *cis-9* found in the liver of lambs fed a basal lamb fattener diet supplemented with Megalac or protected CLA (PCLA) and desaturase ratios

	Liver	**Liver**	**Liver**	**Liver**	**Liver**	**Liver**	**Liver**	**Liver**
	Cis-9, trans-11	*trans-10, cis-12*	C16:0	C16:1	C18:0	C18:1 *cis-9*	C16:1/C16:0	C18:1/C18:0
Control	0.39	0.05	15.60	1.43	21.54	16.19	0.091	0.760
Low Megalac	0.40	0.10	14.79	1.20	22.83	16.08	0.080	0.715
Medium Megalac	0.42	0.11	15.67	1.22	22.46	16.97	0.077	0.758
High Megalac	0.54	0.14	15.43	1.67	21.35	20.07	0.106	0.943
Low PCLA	0.76	0.20	15.55	1.21	22.69	17.17	0.078	0.763
Medium PCLA	1.20	0.34	15.87	1.09	22.17	17.86	0.070	0.812
High PCLA	2.32	0.65	14.51	0.89	22.59	16.15	0.061	0.726
s.e.d.								
Control vs rest	0.074	0.036	0.446	0.174	0.894	0.776	0.0098	0.0536
Type of fat	0.080	0.039	0.482	0.188	0.966	0.838	0.0106	0.0579
Amount of fat	0.086	0.042	0.515	0.201	1.032	0.896	0.0113	0.0619
Interaction	0.105	0.051	0.631	0.247	1.264	1.097	0.0138	0.0758
P-value								
Control vs rest	<0.001	<0.001	0.514	0.225	0.373	0.135	0.208	0.628
Type of fat	<0.001	<0.001	0.971	0.046	0.714	0.316	0.028	0.388
Amount of fat	<0.001	<0.001	0.192	0.777	0.679	0.178	0.583	0.222
Interaction	<0.001	<0.001	0.175	0.071	0.648	0.004	0.072	0.026

FAME were prepared as described in Section 2.3.7 and analysis was carried out by gas chromatography on a 100m CP-Sil 88 column. Data was analysed by ANOVA, comparing control with the other treatment groups and testing for effects of fat, fat type, fat amount and a type-amount interaction.

Table 4.4 Proportions (% of FAME) of fatty acids in subcutaneous, perirenal and omental adipose tissue depots of lambs fed a basal lamb fattener diet supplemented with Megalac or protected CLA (PCLA)

	Subcutaneous				Perirenal				Omental			
	C16:0	C16:1	C18:0	C18:1 *cis-9*	C16:0	C16:1	C18:0	C18:1 *cis-9*	C16:0	C16:1	C18:0	C18:1 *cis-9*
Control	24.41	1.48	18.61	31.11	23.72	1.10	27.75	27.72	25.25	1.14	23.91	26.38
Low Megalac	25.05	1.44	19.10	31.22	25.33	1.05	27.63	26.41	26.19	1.12	23.65	26.27
Medium Megalac	25.72	1.47	18.91	30.97	25.79	1.09	27.24	27.24	26.51	1.14	23.23	27.52
High Megalac	24.96	1.40	17.77	31.40	24.64	1.17	25.55	28.61	26.61	1.13	22.82	27.35
Low PCLA	24.27	1.09	22.17	28.49	23.34	0.82	29.73	25.84	25.17	0.95	24.36	26.03
Medium PCLA	24.98	1.08	18.70	28.34	24.27	0.78	28.08	24.08	25.49	0.86	24.68	24.16
High PCLA	24.24	1.00	19.89	25.29	24.11	0.72	28.84	21.81	25.15	0.88	23.58	21.84
s.e.d.												
Control vs rest	0.614	0.125	1.476	1.046	0.680	0.064	0.887	0.787	0.552	0.070	1.044	0.843
Type of fat	0.664	0.135	1.594	1.130	0.735	0.069	0.958	0.850	0.597	0.076	1.128	0.910
Amount of fat	0.709	0.144	1.704	1.208	0.785	0.074	1.024	0.908	0.638	0.081	1.206	0.973
Interaction	0.869	0.177	2.087	1.480	0.962	0.091	1.255	1.113	0.781	0.099	1.477	1.192
P-value												
Control vs rest	0.456	0.074	0.588	0.092	0.214	0.018	0.992	0.014	0.283	0.078	0.854	0.321
Type of fat	0.149	<0.001	0.180	<0.001	0.022	<0.001	0.015	<0.001	0.015	<0.001	0.265	<0.001
Amount of fat	0.410	0.823	0.378	0.308	0.525	0.969	0.148	0.512	0.844	0.855	0.689	0.167
Interaction	0.999	0.983	0.528	0.186	0.556	0.256	0.574	0.002	0.898	0.725	0.925	0.015

FAME were prepared as described in Section 2.3.7 and analysis was carried out by gas chromatography on a 100m CP-Sil 88 column. Data was analysed by ANOVA, comparing control with the other treatment groups and testing for effects of fat, fat type, fat amount and a type-amount interaction.

Table 4.5 Desaturase ratios (calculated as the proportions of C16:1/C16:0 and C18:1 *cis*-9/C18:0) in subcutaneous, perirenal and omental adipose tissue depots of lambs fed a basal lamb fattener diet supplemented with Megalac or protected CLA (PCLA)

	Subcutaneous		**Perirenal**		**Omental**	
	C16:1/C16:0	C18:1 *cis*-9/C18:0	C16:1/C16:0	C18:1 *cis*-9/C18:0	C16:1/C16:0	C18:1 *cis*-9/C18:0
Control	0.061	1.72	0.046	1.00	0.045	1.12
Low Megalac	0.058	1.71	0.041	0.97	0.043	1.14
Medium Megalac	0.057	1.64	0.042	1.00	0.043	1.18
High Megalac	0.056	1.77	0.048	1.14	0.043	1.20
Low PCLA	0.045	1.38	0.035	0.88	0.038	1.09
Medium PCLA	0.043	1.53	0.032	0.86	0.034	0.98
High PCLA	0.041	1.33	0.030	0.78	0.035	0.94
s.e.d.						
Control vs rest	0.0051	0.1546	0.0025	0.0536	0.0026	0.0736
Type of fat	0.0056	0.1669	0.0027	0.0579	0.0028	0.0795
Amount of fat	0.0059	0.1785	0.0029	0.0619	0.0030	0.0850
Interaction	0.0073	0.2186	0.0036	0.0758	0.0037	0.1041
P-value						
Control vs rest	0.055	0.319	0.004	0.236	0.028	0.730
Type of fat	0.003	0.027	<0.001	<0.001	0.002	0.008
Amount of fat	0.875	0.960	0.782	0.798	0.771	0.814
Interaction	0.994	0.581	0.084	0.042	0.698	0.346

FAME were prepared as described in Section 2.3.7 and analysis was carried out by gas chromatography on a 100m CP-Sil 88 column. Data was analysed by ANOVA, comparing control with the other treatment groups and testing for effects of fat, fat type, fat amount and a type-amount interaction.

The data displayed previously (Tables 4.3, 4.4 and 4.5) suggested that CLA was having an effect on SCD and that there was a change in the amount of fatty acid desaturation occurring. The aim of quantifying the mRNA for SCD and ACC was to examine whether the effects of CLA on desaturation were due to reduced transcription or direct inhibition or activation of the enzyme, and if there was any effect on lipogenesis in terms of gene transcription.

4.4. ACC AND SCD MRNA LEVELS IN SHEEP TISSUES

RNA was extracted from liver tissue and the subcutaneous, perirenal and omental adipose tissue depots of sheep as described in Section 2.3.10. Section 2.3.11 describes the measurement of tissue mRNA levels in detail. Briefly, complementary strand DNA was synthesized and sequences were amplified using Taqman™ primers and probes. Amplification was measured using the ABI 7700 'Taqman' machine, which quantifies the amount of RNA present. Data was expressed relative to the gene beta actin, which was shown not to change significantly across treatment groups; $p = 0.340$; 0.358; 0.249; 0.549 for subcutaneous, perirenal, omental adipose tissue and liver respectively (Figures 4.3-4.6).

Figure 4.3 Subcutaneous adipose tissue depot beta actin mRNA levels

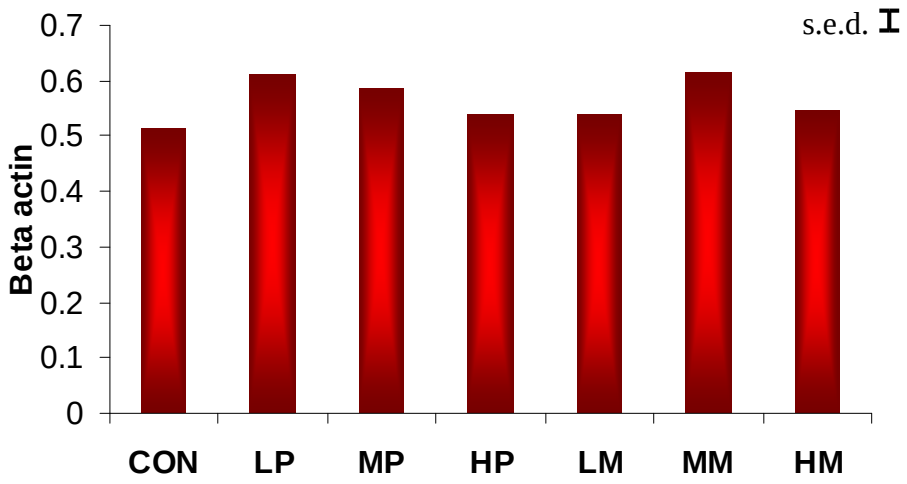
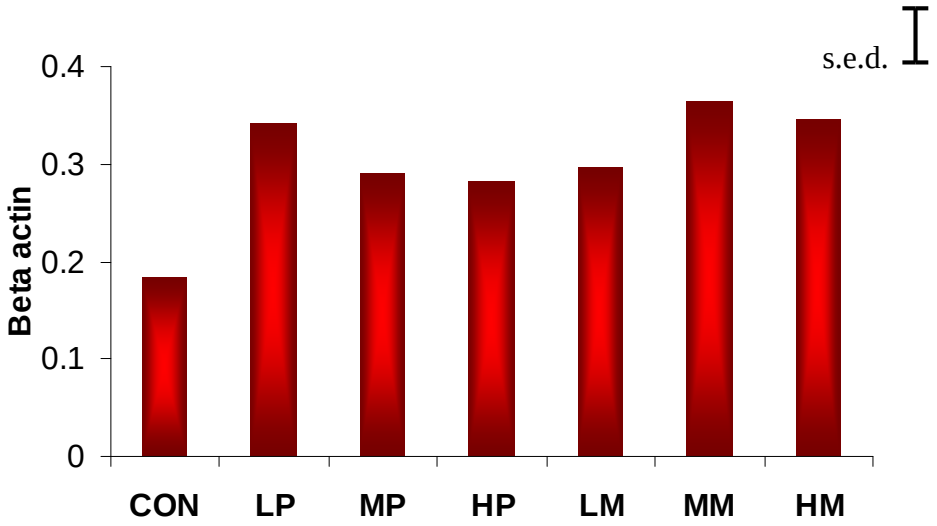


Figure 4.4 Perirenal adipose tissue depot beta actin mRNA levels



Data is presented as the average of treatment groups: CON (control), LP (Low PCLA), MP (Medium PCLA), HP (High PCLA), LM (Low megalac), MM (Medium megalac), HP (High megalac). Data was analysed by ANOVA; s.e.d. is he standard error of the differences between the group means.

Figure 4.5 Omental adipose tissue depot beta actin mRNA levels

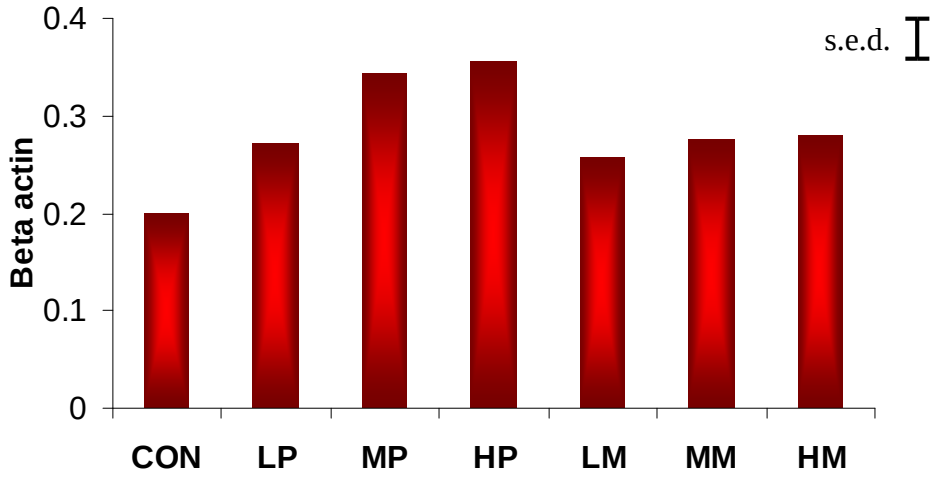
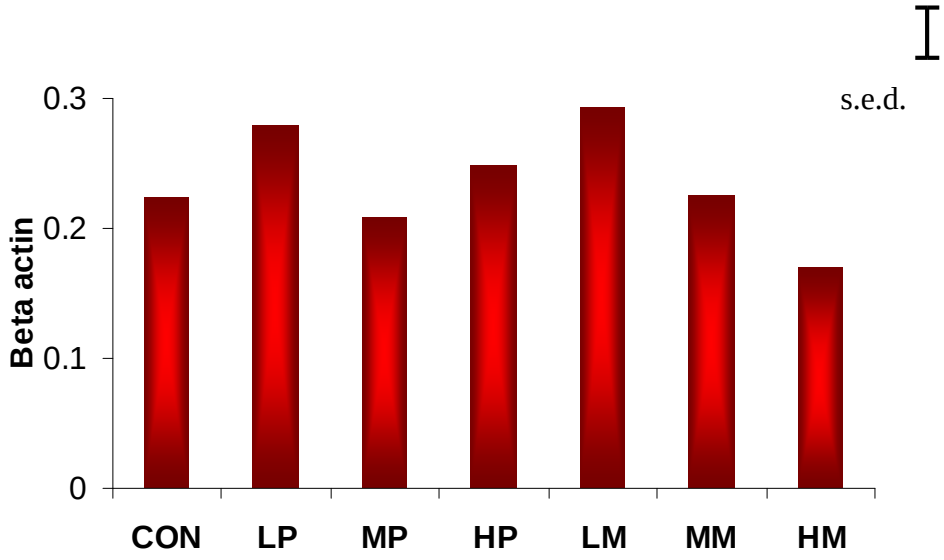


Figure 4.6 Liver beta actin mRNA levels



Data is presented as the average of treatment groups: CON (control), LP (Low PCLA), MP (Medium PCLA), HP (High PCLA), LM (Low megalac), MM (Medium megalac), HP (High megalac). Data was analysed by ANOVA; s.e.d. is he standard error of the differences between the group means.

4.4.1. ACC mRNA levels in adipose tissue depots and liver

There was no significant effect of feeding protected CLA on ACC mRNA in subcutaneous and perirenal adipose tissue depots (Table 4.6) , however, there was a significant interaction between the type and amount of fat in omental adipose tissue and liver ($p = 0.051$ and 0.013 respectively). The effect of amount of fat feeding is different for the two types of fat. Increasing Megalac supplementation results in an increase in ACC mRNA levels in omental adipose tissue, while increasing CLA levels reduces ACC mRNA. There is no pattern to ACC mRNA levels in liver in response to increasing amounts of fat.

4.4.2. SCD mRNA levels in adipose tissue depots and liver

There was a significant effect of fat feeding on SCD mRNA (Table 4.6) in perirenal adipose tissue and liver ($p = 0.026$ and 0.024 respectively) and this resulted in an effect of fat feeding on the ratio of SCD:ACC in the same tissues ($p = 0.026$ and 0.005 respectively). Increasing CLA supplementation tended to reduce SCD mRNA in perirenal adipose tissue, particularly at the highest level of supplementation. There was no pattern to SCD mRNA levels in liver with increased fat supplementation, consistent with hepatic ACC.

4.4.3. Ratio of SCD:ACC mRNA levels in adipose tissue and liver

Feeding both Megalac and protected CLA significantly increased ($p = 0.005$) the ratio of SCD:ACC in liver (Table 4.6). Conversely, feeding the same amounts resulted in significant reduction of this ratio in perirenal adipose tissue ($p = 0.026$). There was no significant change in ACC mRNA in either of these tissues. In light of the SCD data, CLA seems to have an effect on SCD only.

Table 4.6 Stearoyl-CoA desaturase and acetyl-CoA carboxylase mRNA levels in liver, subcutaneous, perirenal and omental adipose tissue depots expressed relative to beta actin mRNA levels

	Subcutaneous			Perirenal			Omental			Liver		
	SCD	ACC	SCD/ACC	SCD	ACC	SCD/ACC	SCD	ACC	SCD/ACC	SCD	ACC	SCD/ACC
Control	0.651	0.545	1.258	1.263	1.293	0.971	1.361	1.198	1.039	0.609	0.606	0.969
Low Megalac	0.676	0.564	1.239	1.161	1.310	0.890	1.055	0.978	1.085	0.744	0.668	1.127
Medium Megalac	0.724	0.596	1.232	1.038	1.127	0.890	1.110	1.032	0.991	0.733	0.660	1.126
High Megalac	0.613	0.571	1.076	1.072	0.975	0.851	1.274	1.210	1.055	0.710	0.552	1.300
Low PCLA	0.675	0.656	1.037	1.085	1.170	0.859	1.282	1.316	0.972	0.667	0.635	1.055
Medium PCLA	0.637	0.656	1.161	1.063	1.187	0.904	1.188	1.123	1.058	0.624	0.524	1.190
High PCLA	0.608	0.603	1.020	0.997	1.258	0.792	1.117	1.044	1.092	0.738	0.674	1.105
s.e.d.												
Control vs rest	0.034	0.0588	0.074	0.082	0.097	0.045	0.110	0.097	0.063	0.039	0.041	0.060
Type of fat	0.028	0.0455	0.060	0.067	0.079	0.037	0.119	0.079	0.051	0.032	0.033	0.049
Amount of fat	0.034	0.0588	0.074	0.082	0.097	0.045	0.127	0.097	0.063	0.039	0.041	0.060
Interaction	0.048	0.0789	0.104	0.116	0.137	0.064	0.156	0.137	0.088	0.055	0.057	0.085
P-value												
Control vs rest	0.897	0.271	0.087	0.026	0.221	0.026	0.096	0.413	0.967	0.024	0.758	0.005
Type of fat	0.274	0.193	0.078	0.538	0.397	0.498	0.589	0.278	0.949	0.109	0.644	0.176
Amount of fat	0.089	0.784	0.145	0.529	0.438	0.244	0.914	0.760	0.689	0.511	0.342	0.192
Interaction	0.380	0.863	0.556	0.785	0.112	0.719	0.231	0.051	0.318	0.201	0.013	0.113

FAME were prepared as described in Section 2.3.7 and analysis was carried out by gas chromatography on a 100m CP-Sil 88 column. Data was analysed by ANOVA, comparing control with the other treatment groups and testing for effects of fat, fat type, fat amount and a type-amount interaction.

4.5. DISCUSSION

4.5.1. Incorporation of CLA into sheep tissues

Supplementation of lamb diets with protected CLA increased adipose tissue and liver *cis-9, trans-11* and *trans-10, cis-12* CLA (Tables 4.2 & 4.3). This is consistent with incorporation of dietary CLA into adipose and liver tissue and has been shown in pigs fed CLA (Smith *et al*, 2002; Ramsay *et al*, 2001; Bee, 2000) and demonstrates that the CLA is protected against rumen degradation. The dose dependent increase in CLA incorporation suggests that the presence of both CLA isomers is due to incorporation of dietary CLA rather than *de novo* synthesis in the adipose tissue. CLA content of adipose tissue in pigs fed different amounts of CLA has been shown to increase with supplementation level (Ramsay *et al*, 2001). Previous work has shown no effect of CLA supplementation on incorporation of isomers into ovine adipose tissue (Mir *et al*, 2000), however studies were done with pre-ruminant lambs, before weaning to a barley-based pelleted diet, and the period of CLA supplementation was only 3 weeks, so effects may not have been evident within such a short feeding period. CLA incorporation into liver tissue was slightly lower than that in adipose tissue. This is probably due to the different proportions of lipid classes present in liver and adipose tissue. Adipose tissue is made up of 90% TAG, whereas liver has a much higher proportion of phospholipids. CLA is incorporated preferentially in TAG (Banni *et al*, 2001b), and the role of phospholipids in cell membrane function resists major fatty acid compositional changes as this affects fluidity and membrane function. The reduction in liver CLA incorporation is consistent with the lower proportion of TAG present compared to adipose tissue. There was more *cis-9, trans-11* CLA than *trans-10, cis-12* CLA present in tissues (Tables 4.2 & 4.3) of sheep fed CLA. The diet contained equal amounts of the two major isomers, so the imbalance is likely to be due to *in vivo* synthesis, and the action of SCD on vaccenic acid (Griinari & Bauman, 1999), or preferential metabolism of the *trans-10, cis-12* isomer (Alasnier *et al*, 2002; Martin *et al*, 2000). These results indicated that CLA was present within animal tissues and potentially able to affect gene transcription.

4.5.2. Effect of CLA on fatty acid composition of sheep tissues

Fatty acid composition of adipose tissue depots and liver was examined, with particular focus on the amounts of the major saturated and monounsaturated fatty acids, namely palmitic and stearic (C16:0 and C18:0) and palmitoleic and oleic (C16:1 and C18:1). The ratio of these pairs (referred to as the desaturase index) has been shown to be a good indicator of SCD enzyme activity (Choi *et al*, 2001), therefore fatty acid composition and ratios of these key monounsaturated and saturated fatty acids have been used in a number of species, cell and tissue culture systems to determine whether treatments affect SCD activity (Choi *et al*, 2002; Demaree *et al*, 2002; Smith *et al*, 2002; Choi *et al*, 2001; Corl *et al*, 2001; Griinari *et al*, 2000; Park *et al*, 2000; Bretillon *et al*, 1999). These ratios and the effects of different fats upon them are discussed in Section 4.5.3. The fatty acid composition of tissues is first considered in relation to the fatty acid composition of the treatment diets. There was a decrease in levels of palmitoleic and oleic acids in adipose tissue and liver (Tables 4.3 and 4.4) of sheep fed CLA. It is possible that the drop in oleic acid seen in sheep fed CLA relative to Megalac could be due to the amounts of oleic acid present in the diets (Figures 4.1 & 4.2). However, there was no difference in the levels of palmitic acid present in sheep fed PCLA and Megalac, despite levels of 10% and 50% in PCLA and Megalac preparations respectively. Additionally, if the fatty acid composition of sheep tissues was due to diet, a greater amount of stearic acid would have been expected in sheep fed PCLA than those fed Megalac, due to the amounts of stearic acid in PCLA and Megalac (Figures 4.1 & 4.2). This was not evident, indicating that there was no effect of dietary fatty acids on fatty acid composition. Palmitoleic acid was not present in the diet, and yet levels fell significantly with PCLA feeding compared to control and Megalac fed animals. There was no significant difference in levels of palmitoleic acid between control animals and those fed Megalac (Tables 4.3 and 4.4). Palmitoleic acid can only be present in the tissues as a result of endogenous synthesis, via the action of SCD on palmitic acid. These observations indicate that there was inhibition of SCD enzyme activity with PCLA feeding. The palmitic and palmitoleic acid data suggest that the drop in tissue levels of oleic acid was due to an inhibition of SCD

enzyme activity, rather than changes in the relative abundance of this particular fatty acid in the two diets.

4.5.3. Effect of CLA on SCD enzyme activity

CLA supplementation in growing lambs reduced the desaturase indices for C16 and C18 fatty acids in all adipose tissue depots (Table 4.5); however there was no further reduction with increased supplementation. This is consistent with studies in pigs fed CLA where levels of palmitic and stearic acids increased and oleic acid decreased relative to controls (Smith *et al*, 2002; Ramsay *et al*, 2001). CLA also reduced desaturase ratio in pigs fed CLA (Demaree *et al*, 2002). Abomasal infusion of CLA reduced desaturase activity in the mammary gland of dairy cows (Chouinard *et al*, 1999). Increasing amounts of CLA were infused and this resulted in dose dependent reductions in C16:1 and C18:1 desaturase ratios. However, this group infused 0, 50, 100 and 150g/day of CLA isomers as CLA-60, and this was directly available for absorption at the level of the small intestine. Levels of CLA available for absorption at the level of the small intestine in sheep in this study were between 0 and 5.4g/kg diet, whereas those in cows were between 0 and 2.3g/kg diet (Chouinard *et al*, 1999). This does not explain why there was no dose response effect seen in sheep, but the method of fat supply may have had an effect. The CLA present for absorption at the level of the small intestine in sheep is calculated on the basis of the degree of protection from ruminal degradation, and the actual amount of CLA absorbed may have been lower than the amount calculated. Therefore the range of supplementation may have been too limited to show an effect. CLA feeding significantly reduced the C16 desaturase ratio in liver but had no effect on the C18 ratio. This is consistent with the reduced incorporation of CLAs into liver lipids and reflects the lower proportion of TAG and increased long chain fatty acids present within liver. Liver has a greater proportion of phospholipids, which are resistant to changes in fatty acid composition, so this could explain why there was a lesser effect of CLA on desaturase ratios in liver. A number of other researchers have studied the effects of individual isomers on fatty acid composition and SCD enzyme activity. Abomasal infusion of *trans-10, cis-12* CLA reduced oleic acid and increased stearic acid in mammary tissue from dairy cows (Baumgard *et al*, 2002), indicating an effect on SCD enzyme

activity with this CLA isomer. SCD activity in mouse liver was inhibited with 0.5% dietary CLA (Lee *et al*, 1998), while in rat liver microsomes treated with *trans-10, cis-12* CLA SCD activity was inhibited (Bretillon *et al*, 1999), but this was not the case with *cis-9, trans-11* CLA. The degree of inhibition with *trans-10, cis-12* CLA was increased dose dependently (Bretillon *et al*, 1999). This is consistent with the changes in desaturase ratios observed in sheep fed PCLA (Tables 4.3 & 4.5) where the there was a trend towards ratios reducing with increased supplementation levels, although this was not statistically significant. Desaturase ratios were reduced in murine 3T3-L1 adipocytes treated with *trans-10, cis-12* CLA and a mixture of CLA isomers (Choi *et al*, 2000) and in HepG2 cells, where the level of palmitoleic and oleic acids were reduced and stearic acid was increased (Choi *et al*, 2001). The results of enzyme activity studies with both pure and mixed isomers of CLA indicate that *trans-10, cis-12* CLA is responsible for the reduction in SCD activity and that fatty acid composition can be used to infer changes in enzyme activity. However, in light of the fatty acid composition of the treatment diets (Section 4.5.2), it suggests that careful consideration of this data, particularly the oleic:stearic ratio is needed. The palmitoleic:palmitic ratio is more reliable, because palmitoleic acid can only be made; it cannot come from the diet, and this did reduce with PCLA feeding, therefore it does indicate that SCD activity may have been inhibited by PCLA. A better measure to use would have been the ratio of myristoleic:myristic acid (14:1:14:0) as this can only be synthesised by the tissues. However, despite these comments, in view of the fatty acid composition data discussed in Section 4.5.2, it appears that SCD enzyme activity was directly inhibited in sheep adipose tissue and liver as a result of PCLA feeding.

4.5.4. Effect of CLA on SCD and ACC mRNA levels

Having determined that there was an effect of CLA on SCD enzyme activity in terms of effects on fatty acid composition, the effects of CLA on mRNA levels of SCD and the lipogenic rate limiting enzyme ACC were examined. Changes in enzyme activity are often correlated to changes in the amount of mRNA coding for the enzyme, so the next step was to determine if the changes in enzyme activity were due to an effect of CLA on transcription. There was a

significant interaction between type and amount of fat for omental adipose tissue and liver. This is due to different responses to the amount of fat between Megalac and PCLA. ACC mRNA increases with increasing Megalac feeding and reduces with increasing PCLA feeding. This is consistent with inhibition of transcription of the ACC gene as shown in mice fed a mixture of CLA isomers where ACC mRNA levels were lower than controls (Tsuboyama *et al*, 2000) and in dairy cows abomasally infused with both a mixture of CLA isomers and *trans-10, cis-12* CLA alone (Loor & Herbein, 1998; Baumgard *et al*, 2002). *Trans-10, cis-12* CLA has been shown to be the isomer responsible for the reduction in milk fat of dairy cows fed CLA (Baumgard *et al*, 2000) with *cis-9, trans-11* CLA showing no effect. Lipid metabolism in mammary tissue has been extensively studied, and effects of CLA have formed a large part of this research. Mammary tissue is extremely metabolically active, and this may be the reason for the significant reductions in fat synthesis in this tissue. There was a significant interaction between fat type and amount in liver, but there was no clear trend to the changes in ACC mRNA with amount or type of fat. There was no effect of PCLA on levels of ACC mRNA in subcutaneous or perirenal adipose tissue. Taking the data presented here together with the body of research in this area, there is no evidence to suggest that lipogenesis as measured by ACC mRNA levels in adipose tissue or liver in sheep is affected by dietary CLA. There was a significant effect ($p = 0.026$ and 0.024) of fat feeding on SCD mRNA levels in perirenal adipose tissue and liver respectively. SCD mRNA abundance reduced with fat feeding in perirenal adipose tissue, but increased slightly in liver. As previously mentioned, there was no effect of fat feeding on ACC mRNA in perirenal adipose tissue and liver, and as a result of the changes in SCD mRNA, there was a significant reduction in the ratio of SCD mRNA/ACC mRNA in perirenal adipose tissue, and an increase in liver. These different results seem to be due to the relative changes in ACC mRNA within the tissue (Table 4.6). There was no effect of CLA feeding specifically on SCD mRNA, but dietary fat had a significant effect. High fat diets have been shown to have an effect on SCD mRNA levels (Jones *et al*, 1996; Sessler *et al*, 1996). Polyunsaturated fatty acids have been shown to affect SCD mRNA levels in murine liver (Ntambi *et al*, 1992), adipose tissue of Zucker rats and 3T3-L1 adipocytes (Jones *et al*, 1996; Sessler

et al, 1996). SCD mRNA is positively regulated by low fat, high carbohydrate diets (Ntambi, 1992) and negatively regulated by PUFAs. Longer chain PUFA are more effective in reducing RNA levels; linoleic, linolenic and arachidonic acids fed as triacylglycerides to mice reduced hepatic SCD mRNA, but there was no effect of palmitic, stearic or oleic acids (Ntambi, 1992). The effect of dietary fat on SCD mRNA offers a reason for the significant effect seen in sheep fed diets supplemented with fat in the current study. There is evidence that PUFA suppress SCD mRNA dose dependently; this has been demonstrated in 3T3-L1 adipocytes, together with reductions in palmitoleic acid, which suggests there was also inhibition of SCD activity (Jones *et al*, 1996). A mixture of CLA isomers has been shown to reduce SCD mRNA levels in murine liver cells, while there was no effect of culturing with purified *cis-9, trans-11* CLA (Lee *et al*, 1998). The CLA mix of isomers also reduced C16 and C18 desaturase ratios relative to control cultures. There was no significant effect on ratios with *cis-9, trans-11* CLA treatment. Treatment of 3T3-L1 cells with purified isomers of CLA revealed that there was no effect of *cis-9, trans-11* CLA on SCD mRNA levels, but that *trans-10, cis-12* CLA significantly reduced mRNA levels (Choi *et al*, 2000). SCD enzyme activity was also measured and this changed in the same way as the mRNA results, indicating that *trans-10, cis-12* CLA was acting on transcription and not directly on enzyme activity, as has been suggested by the results presented in the current study. There was no effect of *cis-9, trans-11* CLA on SCD enzyme activity. Changes in fatty acid composition were studied in response to treatment of cell cultures with *cis-9, trans-11* and *trans-10, cis-12* CLA. *Trans-10, cis-12* CLA significantly reduced levels of palmitoleic and oleic acids relative to controls, while *cis-9, trans-11* CLA had no effect (Choi *et al*, 2000). This is in agreement with the findings in sheep where the proportions of palmitoleic and oleic acids reduced and palmitic and stearic increased with CLA feeding. Taken together with the lack of change in SCD mRNA levels with CLA feeding, it may be that the effects of CLA are acting directly on the enzyme complex, possibly by CLA competing with the stearic and palmitic acids for the active site of the enzyme and reducing the amount of desaturation occurring in this way. Evidence to support this theory of post-translational regulation of SCD by direct inhibition of the enzyme activity has come from studies in

HepG2 cells where *trans-10, cis-12* treatment suppressed desaturase activities without affecting SCD mRNA levels. Clearly *trans-10, cis-12* CLA has a greater inhibitory effect on SCD action than *cis-9, trans-11* CLA. Infusion of purified *trans-10, cis-12* into the abomasum of lactating dairy cows resulted in a suppression of SCD mRNA in mammary tissue (Baumgard *et al*, 2002). In conjunction with this, a reduction in the products of SCD activity (palmitoleic and oleic acids) was also evident, together with an increase in the saturated fatty acids which are substrates for SCD (Baumgard *et al*, 2002). The method of action of CLA in bovine mammary tissue seems to be via transcriptional changes; however there is no evidence to suggest that this is the case in the current study in sheep. Previous studies examining the effects of *cis-9, trans-11* CLA on milk fatty acid composition confirmed that this isomer had no effect on desaturation (Baumgard *et al*, 2000), in agreement with work done in 3T3-L1 cells (Choi *et al*, 2000). Taken together with the effects of *cis-9, trans-11* CLA on enzyme SCD mRNA levels and activity in 3T3-L1 cells (Choi *et al*, 2000) and those of *trans-10, cis-12* and a mixture of CLA isomers (Baumgard *et al*, 2002; Loor & Herbein, 1998) on SCD mRNA levels and enzyme activity inferred from changes in fatty acid composition, these effects seem to be due to the *trans-10, cis-12* isomer. Only one study has shown an increase in SCD mRNA with CLA feeding (Peters *et al*, 2001); it is generally accepted that CLA treatment reduces SCD mRNA, and indeed the authors suggested that their results could be due to the different genotype of the mouse strain used in the study. The mechanism by which some polyunsaturated fatty acids, including CLA reduce SCD mRNA is at present unknown. Studies in 3T3-L1 adipocytes have suggested a role for a reduction in mRNA stability, via increased turnover of SCD mRNA (Sessler *et al*, 1996). This has been seen in cells treated with linoleic, linolenic and arachidonic acids. It is possible that CLA has its effects by the same mechanism, and that positional differences in the two major isomers are responsible for their different effects. The current study provides no evidence to suggest that CLA is having effects on transcription of ACC or SCD; however feeding fat per se did affect SCD mRNA levels.

4.6. SUMMARY OF RESULTS

Feeding ruminally protected CLA resulted in increased incorporation of both isomers into adipose tissue depots and liver in a dose dependent manner. The desaturase ratios for 16:1:16:0 and 18:1 *cis-9*:18:0 reduced with CLA feeding in all adipose tissue depots. These changes were not dose-dependent. The desaturase ratio in the liver was less resistant to change, with only the 16:1:16:0 ratio reducing with increase in CLA supplementation. ACC mRNA was reduced with CLA feeding, but only significantly in omental adipose tissue. SCD mRNA was reduced with CLA feeding in perirenal adipose tissue. There was no clear effect of CLA feeding on SCD or ACC mRNA levels in liver. In view of these results, there seems to be very little effect of CLA on messenger RNA levels in either adipose tissue depots or liver in sheep. The fatty acid composition data provides evidence to suggest a direct inhibitory effect on SCD activity, resulting in changes in fatty acid composition in ovine adipose tissue and liver. There is no evidence to suggest that the effects of CLA on fatty acid composition were mediated through transcriptional regulation.

5. EFFECT OF CONJUGATED LINOLEIC ACID ON LIPID METABOLISM IN HAMSTERS

5.1. EXPERIMENTAL AIM

The objective of this experiment was to examine the effects of a 90% pure CLA mixture, consisting of a 50:50 mix of *cis-9, trans-11* and *trans-10, cis-12* CLA on body weight and composition, plasma cholesterol and triglyceride levels, adipose tissue depot weights, liver weight, fatty acid composition of these tissues and mRNA levels for the key lipogenic genes ACC, FAS, LPL and SCD.

5.1.1. Experimental design

6 week old male Golden Syrian hamsters were fed a 2% fat chow diet as detailed in Sections 2.1 and 2.2.3.2 supplemented with a further 2% fat as rapeseed oil and/or CLA at 0.5, 1 and 2% for 11 weeks. CLA was added at the expense of rapeseed oil, which contains high levels of oleic acid.

5.1.2. Carcass measurements

A number of measurements were taken at sacrifice as described in Section 2.3.3, including body weight, fat pad and liver weights. Hamster carcasses were also retained for body composition analysis. There was no effect of CLA supplementation on feed efficiency, and final bodyweight and feed intake was not significantly different between groups (Table 5.1). There were no significant differences in body weights of animals fed differing levels of CLA, and this was reflected in the fat pad weights from epididymal and perirenal adipose tissue which also did not change with CLA supplementation (Table 5.2). However, there was a tendency for perirenal fat pad weight to be lower at 0.5%, but this failed to reach significance (Tables 5.2 & 5.3). There was a significant reduction in intrascapular fat pad with 0.5% and 1% CLA compared to control, but this was not sustained at 2% CLA supplementation. When the data was expressed relative to body weight (Table 5.3) there were still no significant differences between treatments in epididymal and perirenal adipose tissue. There was a significant difference ($p<0.001$) between treatments in intrascapular adipose tissue, with a reduction in the percentage of bodyweight accounted for by the intrascapular fat pad at 0.5 and 1% CLA. However, this was not evident at 2% CLA, where there was no difference from control values. Liver weight significantly increased in animals fed 2% CLA comparative to controls (Table 5.2), and this was more significant when expressed as a percentage of final body weight (Table 5.3). There was a trend towards increased liver weights with increased CLA supplementation, but this failed to reach significance at 0.5 or 1% CLA. There was a reduction in hamster carcass fat of 10% when 0.5% CLA was fed (Figure 5.1). This significant reduction ($p = 0.002$) was not dose dependent, and increasing dosage did not cause further reduction in body fat.

Table 5.1 Body weight and feed consumption in hamsters fed 0, 0.5, 1 and 2% CLA

% CLA	0% CLA	0.5% CLA	1% CLA	2% CLA	s.e.d.	p-value
Initial weight (g)	58.6	58.6	59.5	59.3	1.42	0.901
Final weight (g)	118.2	114.3	113.7	115.2	5.12	0.823
Feed intake (g)	456.6	458.6	443.7	431.7	16.72	0.356
Feed efficiency*	0.1294	0.1198	0.1198	0.1293	0.007	0.288

*Feed efficiency is expressed as body weight gain relative to weight of feed consumed. 6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.).

Table 5.2 Fat pad and liver weights expressed as absolute weights

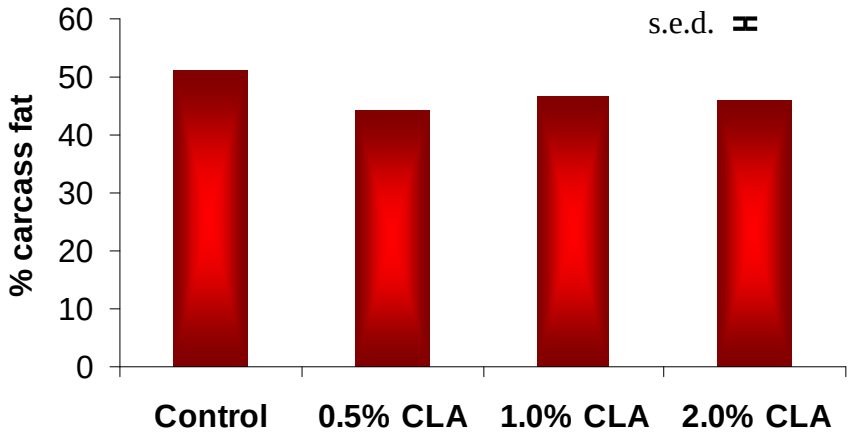
Body and depot weights (g)	0% CLA	0.5% CLA	1% CLA	2% CLA	s.e.d.	p-value
EP depot weight	2.40	2.51	2.52	2.69	0.213	0.609
PR depot weight	1.91	1.59	1.86	1.86	0.177	0.263
IS depot weight	0.48	0.37	0.44	0.52	0.034	<0.001
Liver weight	4.16	4.61	4.53	4.96	0.2549	0.030

Table 5.3 Fat pad and liver weights expressed relative to body weight

% of BW	0% CLA	0.5% CLA	1% CLA	2% CLA	s.e.d.	p-value
EP depot	2.03	2.20	2.16	2.32	0.1359	0.23
PR depot	1.62	1.39	1.60	1.59	0.1147	0.20
IS depot	0.42	0.32	0.38	0.45	0.0283	<0.001
Liver	3.52	4.00	3.98	4.30	0.1300	<0.001

*EP = Epididymal fat depot, PR = Perirenal fat depot, IS = Intrascapular fat depot
6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat) as absolute values (Table 5.1) and relative to final bodyweight (Table 5.2). Errors are presented as the standard error of the difference between the means (s.e.d.).

Figure 5.1 Hamster carcass fat percentages



6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). Level of significance p = 0.002.

5.1.3. Blood cholesterol and triglyceride concentrations

Blood was collected from animals at sacrifice and plasma separated. The plasma was then analysed for cholesterol and triglyceride as described in Section 2.3.18.

There was no significant effect ($p = 0.241$) of CLA on blood cholesterol levels (Figure 5.2), but there was a trend towards levels increasing with CLA supplementation. CLA had no significant ($p = 0.989$) effect on blood triglyceride levels (Figure 5.3).

Figure 5.2 Blood cholesterol concentrations

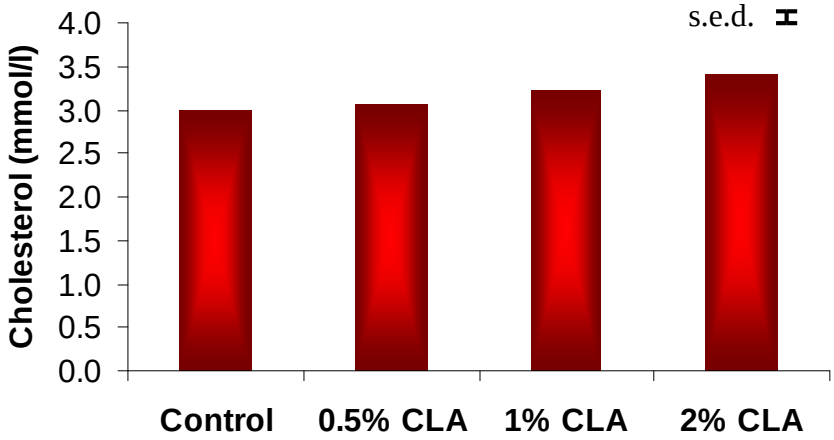
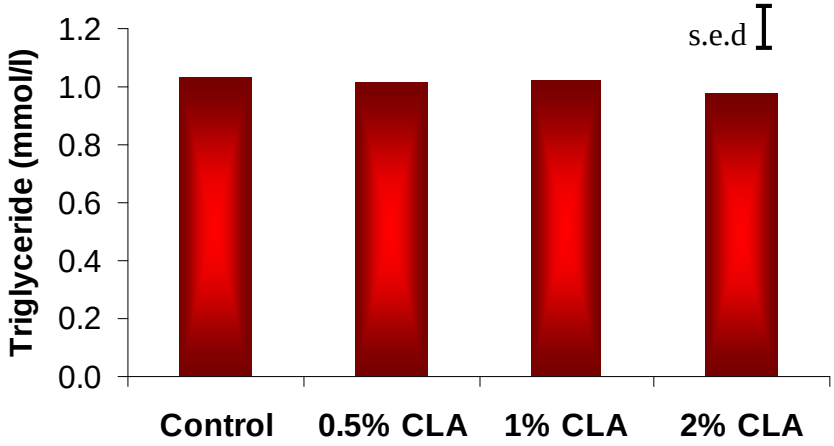


Figure 5.3 Blood triglyceride concentrations



6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Blood samples were taken at sacrifice and plasma prepared for cholesterol and triglyceride assay. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). Level of significance p = 0.241 and 0.989 for cholesterol and triglyceride respectively.

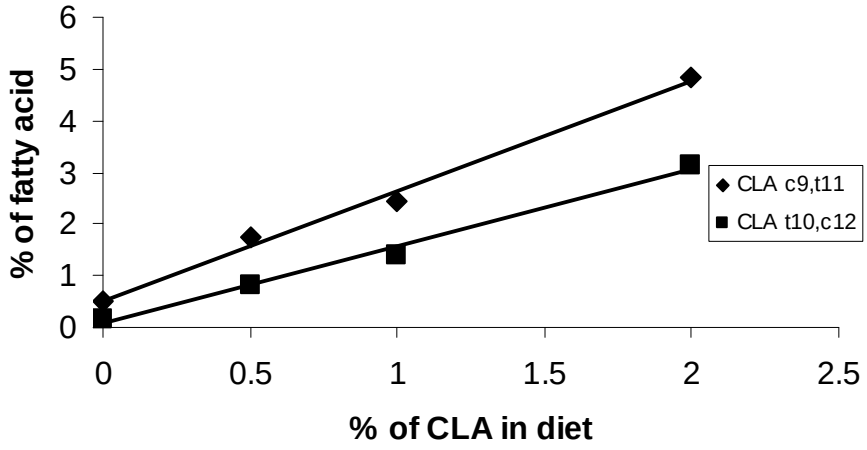
5.1.4. Fatty acid composition of hamster adipose tissue

Fat pads and liver were removed from the hamsters as described in Section 2.3.3, and prepared for gas chromatography analysis as described in Section 3.18 to see if fatty acid composition changed with increasing levels of dietary CLA. A general analysis of variance test incorporating regression analysis was performed using the Genstat™ statistical package to determine the significance of the results.

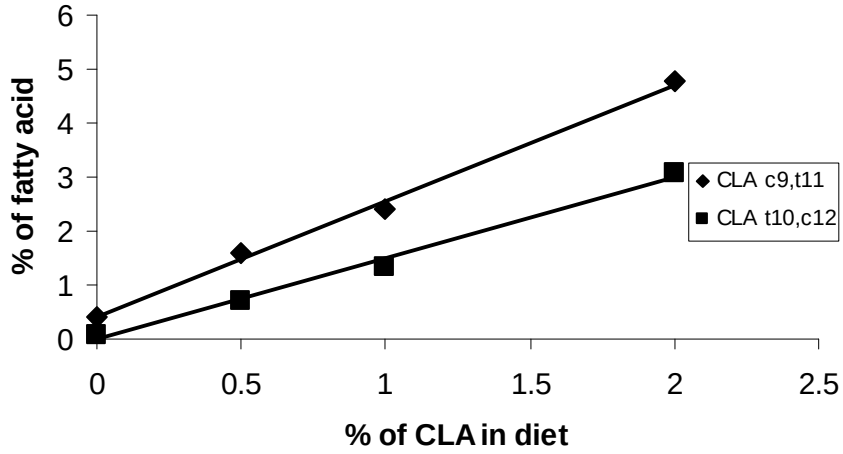
Both isomers of CLA were incorporated into the fat in a dose dependent manner (Figure 5.4). There was more of the *cis-9, trans-11* isomer present compared to the *trans-10, cis-12* isomer, as indicated by the steeper slope of the line for the *cis-9, trans-11* CLA isomer. There was a significant difference ($p<0.001$) in CLA incorporated into both fat pads between treatment diets, with levels of 5% and 3% of the *cis-9* and the *trans-10* isomer respectively present in animals fed 2% CLA.

Figure 5.4 Proportions of CLA *cis-9, trans-11* and CLA *trans-10, cis-12* in hamster fat pad

a) Epididymal fat pad



b) Perirenal fat pad

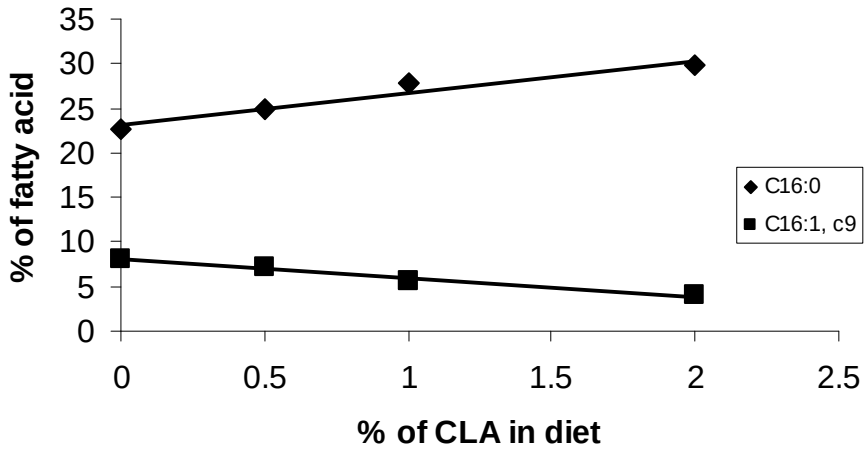


6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Samples of adipose tissue were taken at sacrifice for lipid extraction and methylation of fatty acids, which were then analysed by gas chromatography. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). EP CLA c9,t11 s.e.d. 0.251; EP CLA t10,c12 s.e.d. 0.165; Level of significance for EP CLA c9t11 and EP CLA t10c12 $p<0.001$. PR CLA c9,t11 s.e.d. 0.180; PR CLA t10,c12 s.e.d. 0.124; Level of significance for PR CLA c9t11 and PR CLA t10c12 $p<0.001$.

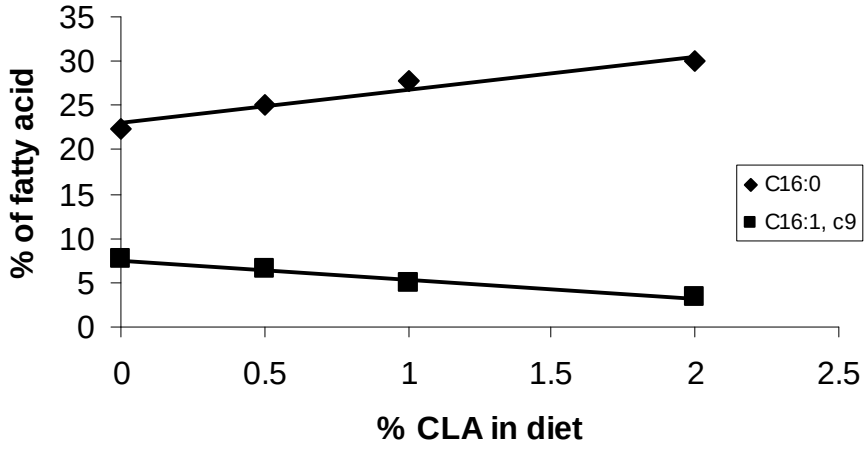
The proportion of fatty acids present as palmitic acid increased with an increase in the level of dietary CLA and that of palmitoleic acid decreased in both adipose tissue depots (Figure 5.5).

Figure 5.5 Proportions of palmitic and palmitoleic acid in hamster fat pad

a) Epididymal fat pad



b) Perirenal fat pad

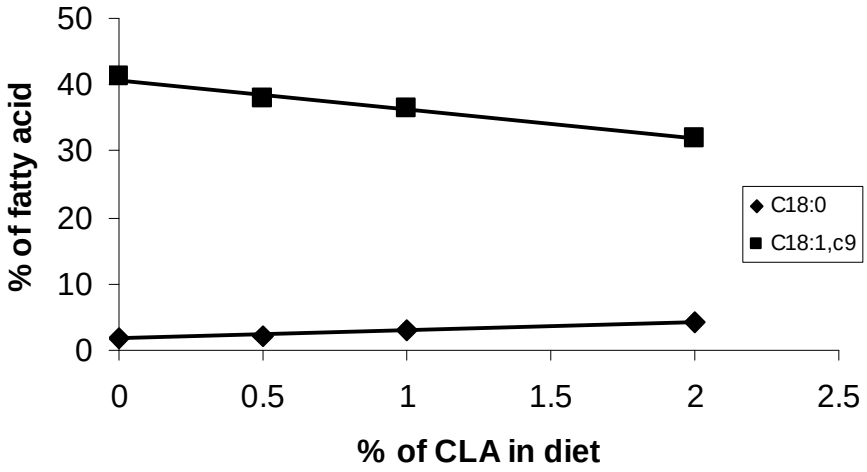


6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Samples of adipose tissue were taken at sacrifice for lipid extraction and methylation of fatty acids, which were then analysed by gas chromatography. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). EP C16:0 s.e.d. 0.500; EP C16:1 *cis-9* s.e.d. 0.280. Level of significance for EP C16:0 and EP C16:1 *cis-9* $p<0.001$. PR C16:0 s.e.d. 0.400; PR C16:1 *cis-9* s.e.d. 0.260. Level of significance for PR C16:0 and PR C16:1 *cis-9* $p<0.001$.

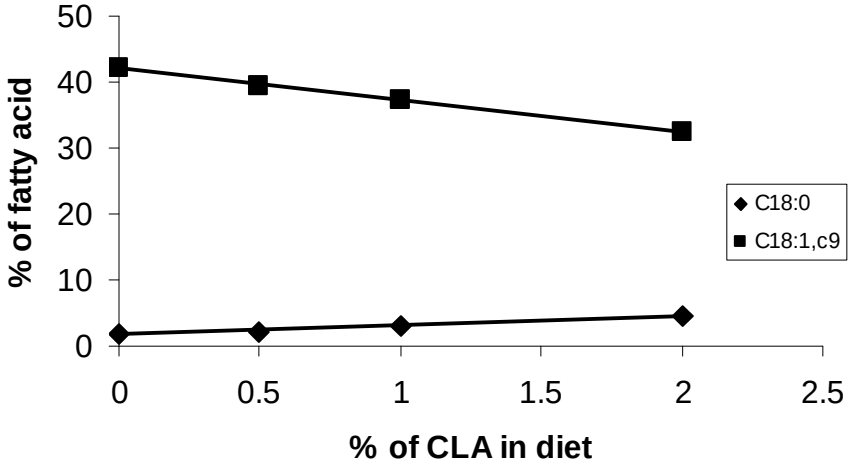
A much smaller percentage of fatty acids were found as stearic and oleic acids. Stearic acid shows a significant increase ($p<0.001$) with increasing CLA dose in both adipose tissue depots (Figure 5.6). Conversely, oleic acid levels decreased ($p<0.001$) as the dietary CLA dose increases. There is a 10% reduction in animals fed the 2% CLA diet ($p<0.001$). Levels of unsaturated fatty acids in hamster fat pads significantly reduced ($p<0.001$) with increasing CLA supplementation (Figure 5.7).

Figure 5.6 Proportion of stearic and oleic acids in hamster fat pad

a) Epididymal fat pad



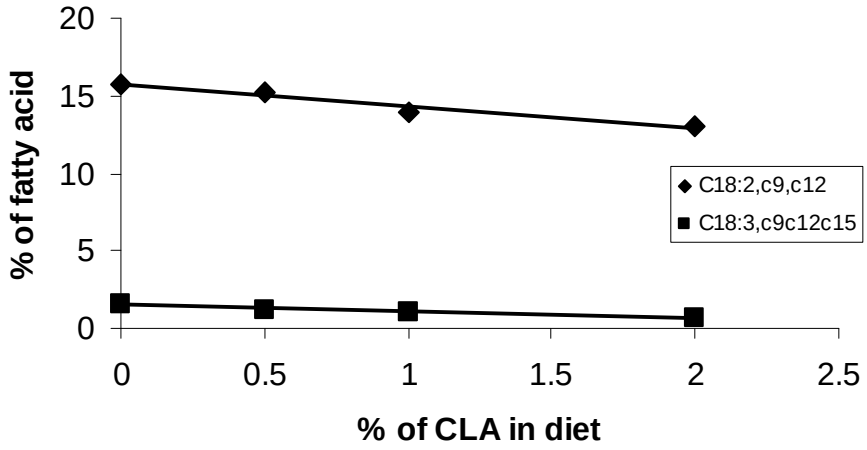
b) Perirenal fat pad



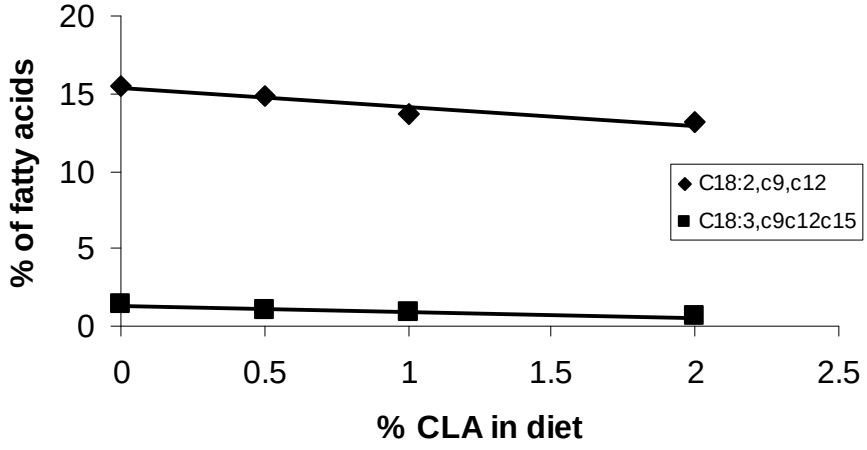
6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Samples of adipose tissue were taken at sacrifice for lipid extraction and methylation of fatty acids, which were then analysed by gas chromatography. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). EP C18:0 s.e.d. 0.100; EP C18:1 *cis-9* s.e.d. 0.750. Level of significance for EP C18:0 and EP C18:1 *cis-9* $p<0.001$. PR C18:0 s.e.d. 0.100; PR C18:1 *cis-9* s.e.d. 0.530. Level of significance for PR C18:0 and PR C18:1 *cis-9* $p<0.001$.

Figure 5.7 Proportions of linoleic and linolenic acids in hamster fat pads

a) Epididymal fat pad



b) Perirenal fat pad

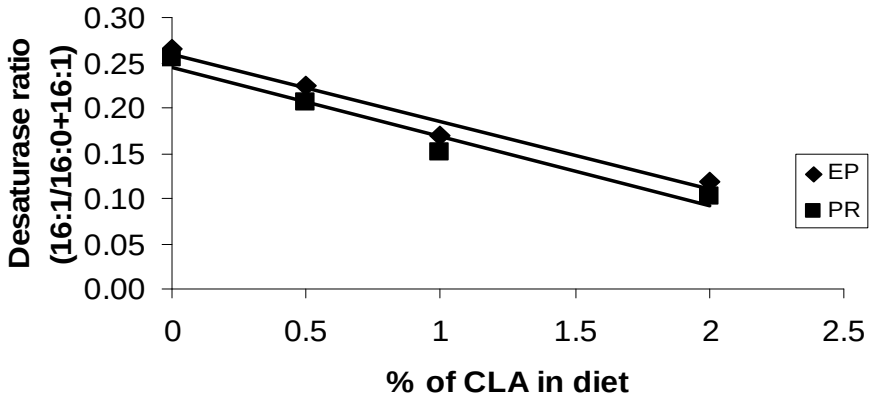


6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Samples of adipose tissue were taken at sacrifice for lipid extraction and methylation of fatty acids, which were then analysed by gas chromatography. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). EP C18:2 *cis-9, cis-12* s.e.d. 0.559; EP C18:3 *cis-9,cis-12*,cis-15 s.e.d. 0.096. Level of significance EP C18:2 *cis-9, cis-12* and EP C18:3 *cis-9,cis-12*,cis-15 $p<0.001$. PR C18:2 *cis-9, cis-12* s.e.d. 0.555; PR C18:3 *cis-9,cis-12*,cis-15 s.e.d. 0.074. Level of significance PR C18:2 *cis-9, cis-12* and PR C18:3 *cis-9,cis-12*,cis-15 $p<0.001$.

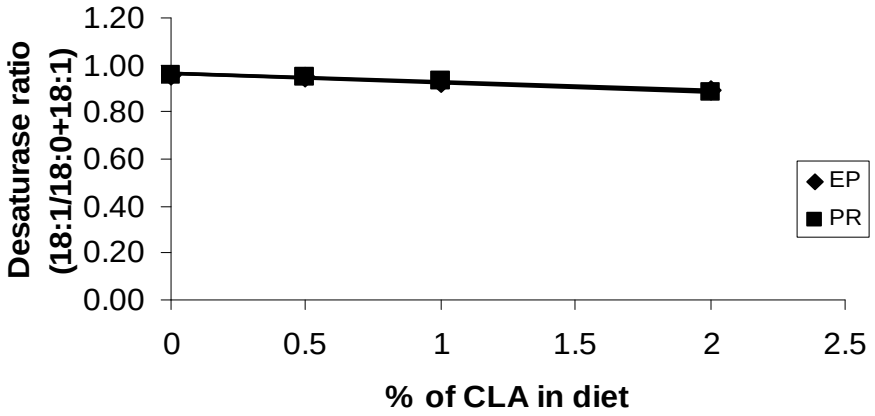
The ratio of saturated fatty acids to unsaturated fatty acids significantly reduces with CLA feeding. (Figure 5.8)

Figure 5.8 16:1 and 18:1 desaturase ratios in hamster adipose tissue

a) 16:1 desaturase ratio in epididymal (EP) and perirenal (PR) adipose tissue



b) 18:1 desaturase ratio in epididymal (EP) and perirenal (PR) adipose tissue

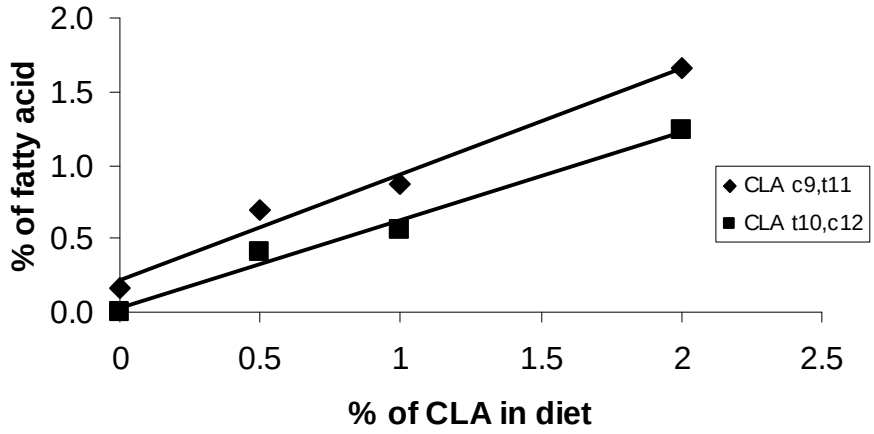


16:1 desaturase ratio = 16:1 / (16:0 + 16:1); 18:1 desaturase ratio = 18:1 / (18:0 + 18:1). 6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Samples of adipose tissue were taken at sacrifice for lipid extraction and methylation of fatty acids, which were then analysed by gas chromatography. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). 16:1 EP s.e.d. 0.010; 16:1 PR s.e.d. 0.009. Level of significance for 16:1 EP and 16:1 PR $p<0.001$. 18:1 EP s.e.d. 0.004; 18:1 PR s.e.d. 0.005. Level of significance for 18:1 EP and 18:1 PR $p<0.001$.

5.1.5. Fatty acid composition in hamster liver

There was less CLA incorporation in liver than in adipose tissue, and the isomers were incorporated in a similar way, with less *trans-10, cis-12* CLA than *cis-9, trans-11* (Figure 5.9). The slopes of the lines are also different (Figure 5.9, footnote), indicating that *cis-9, trans-11* is preferentially incorporated into the tissue over *trans-10, cis-12* CLA.

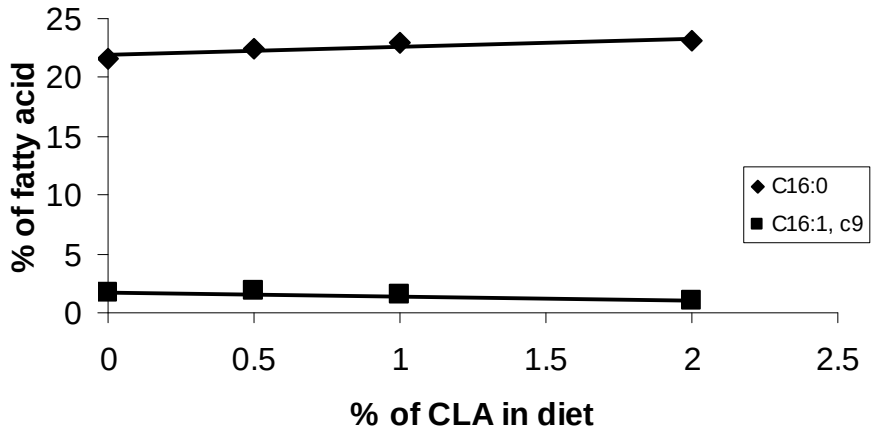
Figure 5.9 Proportions of *trans-10, cis-12* and *cis-9, trans-11* CLA in hamster liver



6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Samples of liver were taken at sacrifice for lipid extraction and methylation of fatty acids, which were then analysed by gas chromatography. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). CLA *cis-9, trans-11* s.e.d. 0.056; CLA *trans-10, cis-12* s.e.d. 0.040. Level of significance for CLA c9, t11 and CLA 10, c12 $p<0.001$. Equations of the lines fitted to the data are: CLA c9,t11 $y = 0.7212x + 0.2172$ $R^2 = 0.9827$; CLA *trans-10, cis-12* $y = 0.5942x + 0.0308$ $R^2 = 0.9855$

There was a small but significant decrease in palmitoleic acid levels in hamster liver and a concomitant increase in palmitic acid (Figure 5.10). These changes are not as pronounced as in adipose tissue.

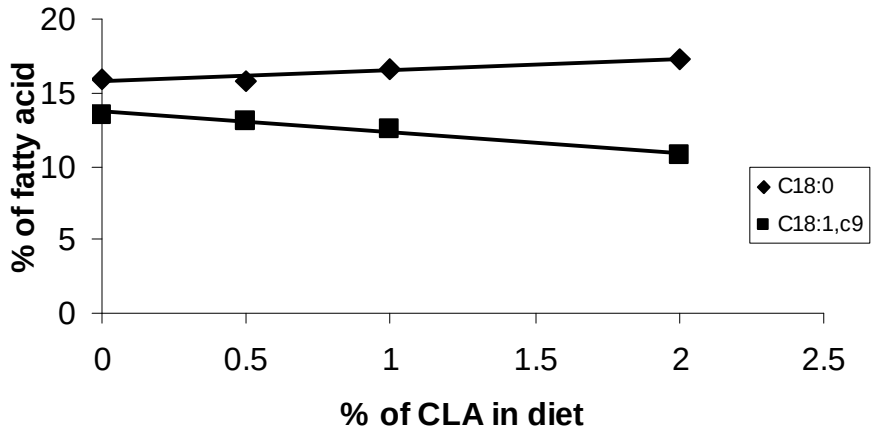
Figure 5.10 Proportions of palmitic and palmitoleic acid in hamster liver



6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Samples of liver were taken at sacrifice for lipid extraction and methylation of fatty acids, which were then analysed by gas chromatography. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). C16:0 s.e.d. 0.526; C16:1 *cis-9* s.e.d. 0.164. Levels of significance for C16:0 and C16:1 *cis-9* $p = 0.024$ and <0.001 respectively.

There was a significant increase in stearic acid with CLA feeding (Figure 5.11), although the change with levels of dietary CLA was not equivalent to that seen in adipose tissue. The decrease in levels of oleic acid with CLA feeding was also significant, but this was not as great as the change seen in adipose tissue.

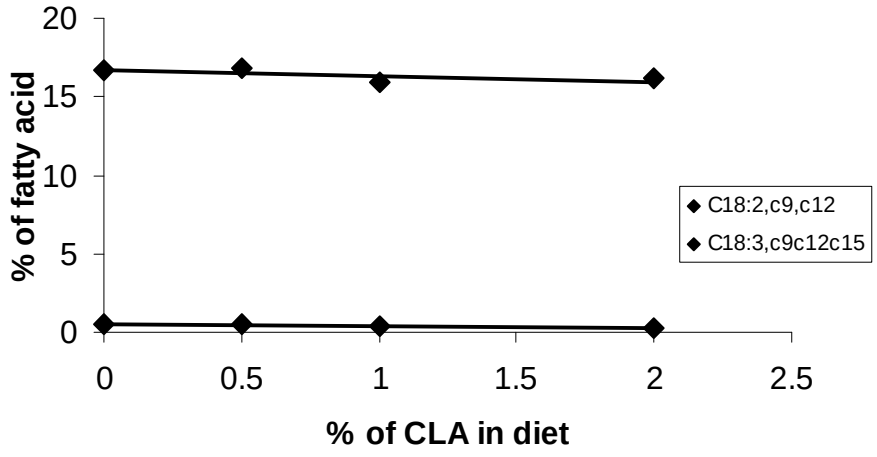
Figure 5.11 Proportion of stearic and oleic acids in hamster liver



6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Samples of liver were taken at sacrifice for lipid extraction and methylation of fatty acids, which were then analysed by gas chromatography. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). C18:0 s.e.d. 0.563; C18:1 *cis-9* s.e.d. 0.895. Levels of significance for C18:0 and C18:1 *cis-9* p = 0.045 and 0.026 respectively.

Linoleic acid levels remained relatively constant with increasing dietary CLA (Figure 5.12), while levels of linolenic acid reduced with increasing dietary CLA. These changes are likely to be a reflection of the amount present in the diet as animals cannot make this particular fatty acid. The level and changes in linolenic acid concentrations was very small, and probably reflected the low amounts present in the diet and therefore the small changes which were seen.

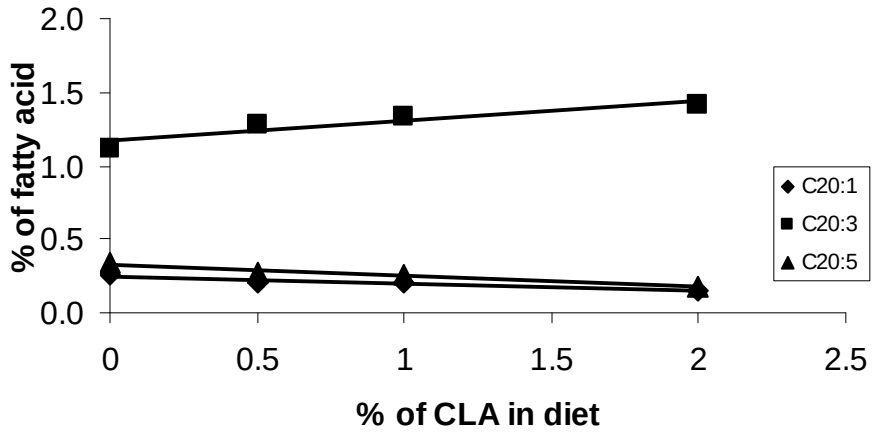
Figure 5.12 Proportion of linoleic and linolenic acids in hamster liver



6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Samples of liver were taken at sacrifice for lipid extraction and methylation of fatty acids, which were then analysed by gas chromatography. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). C18:2, *cis-9, cis-12* s.e.d. 0.296; C18:3, *cis-9,cis-12,*cis-15 s.e.d. 0.031. Levels of significance for C18:2 *cis-9, cis-12* and C18:3 *cis-9, cis-12,* cis-15 $p = 0.010$ and <0.001 respectively.

There was a significant decrease in levels of both 20:1 and 20:5 with CLA feeding (Figure 5.13). Levels of 20:3 increased with CLA feeding.

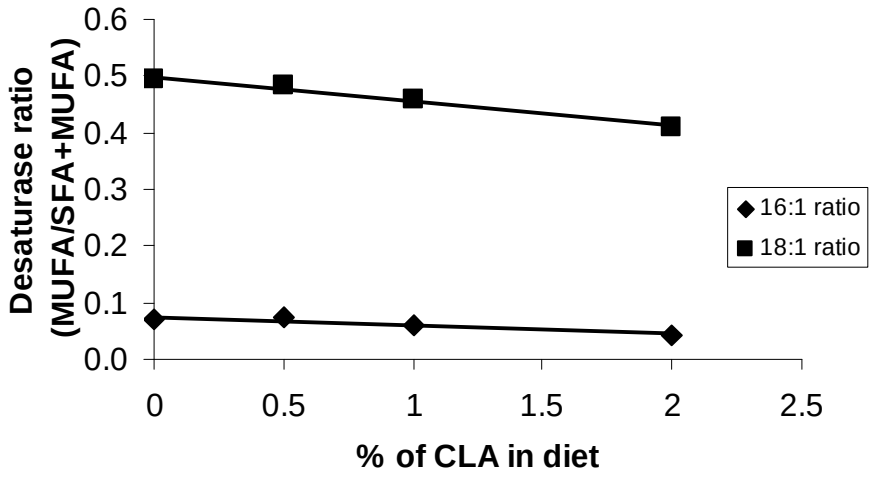
Figure 5.13 Proportions of long chain fatty acids 20:1, 20:3 and 20:5 in hamster liver



6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Samples of liver were taken at sacrifice for lipid extraction and methylation of fatty acids, which were then analysed by gas chromatography. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). 20:1 s.e.d. 0.012; 20:3 s.e.d. 0.066; 20:5 s.e.d. 0.026. Levels of significance for C20:1, C20:3 and C20:5 $p<0.001$, 0.001 and <0.001 respectively.

The desaturase ratio was also reduced in hamster liver (Figure 5.14), but was lower than that in adipose tissue.

Figure 5.14 Desaturase ratios in hamster liver

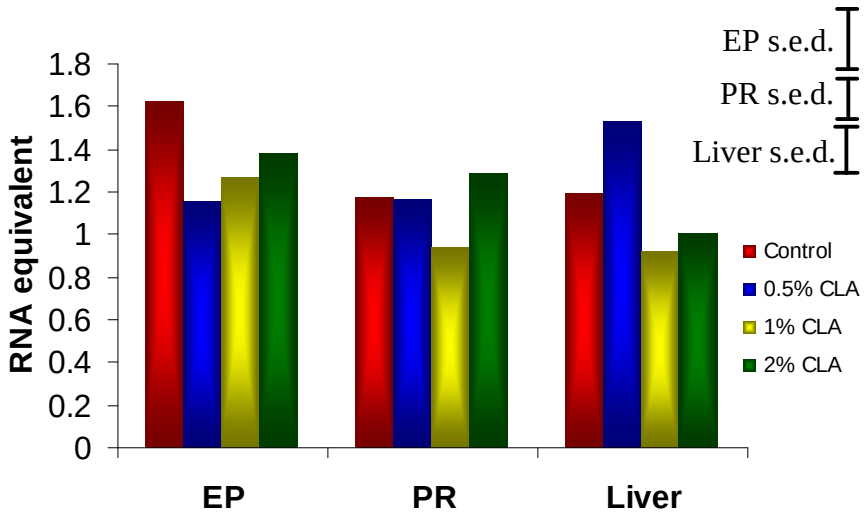


6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Samples of liver were taken at sacrifice for lipid extraction and methylation of fatty acids, which were then analysed by gas chromatography. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). 16:1 desaturase ratio s.e.d. 0.008; 18:1 desaturase ratio s.e.d. 0.036. Levels of significance for 16:1 and 18:1 are $p<0.001$ and 0.001 respectively.

5.1.6. Steady-state mRNA levels in hamster tissues

Much work has focused on the effects of fatty acids at a transcriptional level. Gene expression was measured using real time PCR and expressed relative to a constant housekeeping gene, beta actin. There was no change in beta actin mRNA levels in epididymal, perirenal adipose tissues or liver with CLA feeding (Figure 5.15). Beta actin was therefore suitable for use as a housekeeping gene and all other genes of interest were expressed relative to beta actin values. Expression of data relative to beta actin did not change the pattern of the bars, but reduced the errors associated with the data (Figure 5.16).

Figure 5.15 Beta actin steady-state mRNA levels in hamster tissues



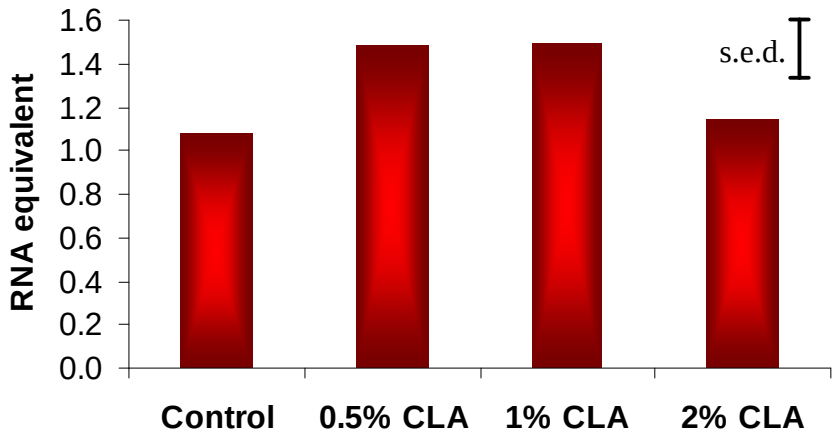
EP = epididymal adipose tissue; PR = perirenal adipose tissue
6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Samples of adipose tissue and liver were taken at sacrifice for RNA extraction and cDNA synthesis, which was then quantified by real-time PCR. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). Levels of significance for EP $p = 0.720$; PR $p = 0.750$; Liver $p = 0.234$.

5.1.6.1. Adipose tissue depots

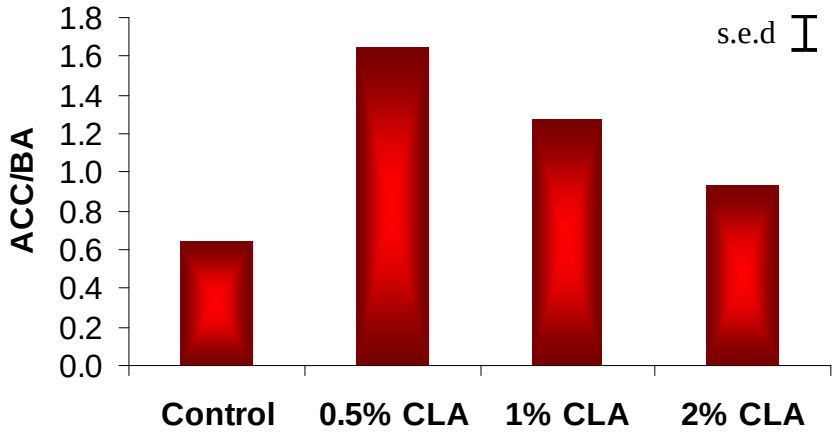
Levels of ACC, FAS, LPL and SCD mRNA were quantified in adipose tissue depots and corrected for beta actin, which did not change with treatment (Table 5.16).

Figure 5.16 ACC steady-state mRNA levels in hamster perirenal adipose tissue expressed as absolute values and relative to beta actin

a) Absolute values



b) Relative to beta actin

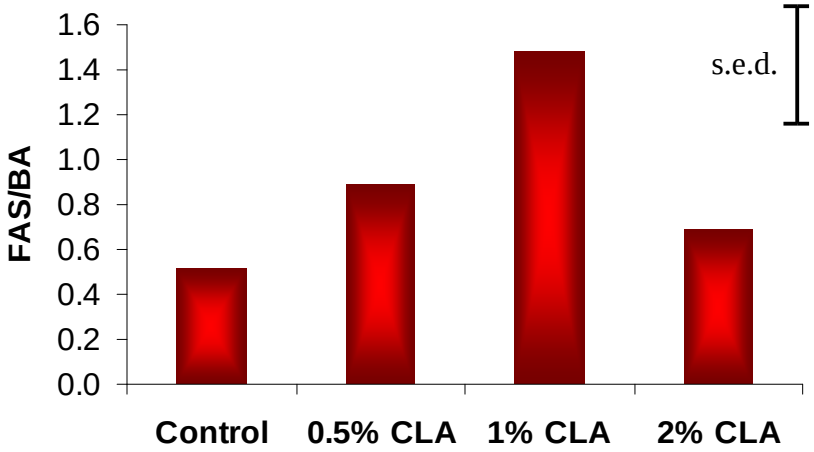


6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Samples of adipose tissue were taken at sacrifice for RNA extraction and cDNA synthesis, which was then quantified by real-time PCR. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). Level of significance ACC uncorrected for beta actin $p = 0.529$. Level of significance ACC corrected for beta actin $p<0.001$.

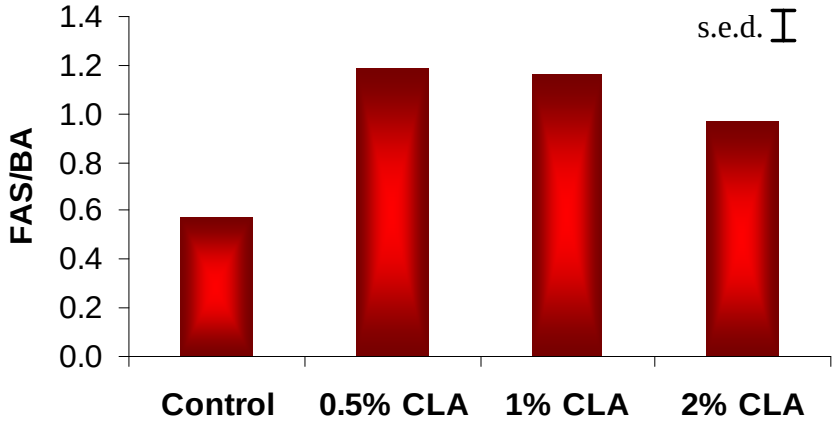
ACC gene expression was increased with CLA supplementation at the 0.5% level (Figure 5.16b), but increasing doses do not sustain this effect, with 2% CLA returning expression levels to those of control animals. FAS gene expression was increased in epididymal and perirenal adipose tissue with CLA supplementation (Figure 5.17). There was not a dose response relationship, but 0.5% dietary CLA significantly raises expression levels above those of control animals and increasing doses of CLA maintained this expression level.

Figure 5.17 FAS gene expression in hamster adipose tissue

a) Epididymal adipose tissue



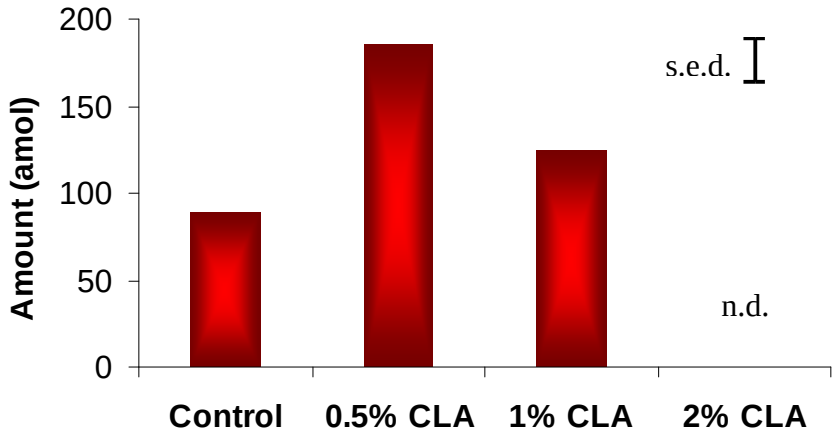
b) Perirenal adipose tissue



6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Samples of adipose tissue were taken at sacrifice for RNA extraction and cDNA synthesis, which was then quantified by real-time PCR. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). Level of significance p = 0.004.

SREBP-1c gene expression was kindly measured by Dr. Scott Cooper of the University of Nottingham, using a ribonuclease protection assay. SREBP-1c mRNA in perirenal adipose tissue significantly increased with CLA feeding (Figure 5.18), but was not dose dependent as the highest level of mRNA was seen at 0.5% supplementation.

Figure 5.18 SREBP-1c gene expression in hamster perirenal adipose tissue

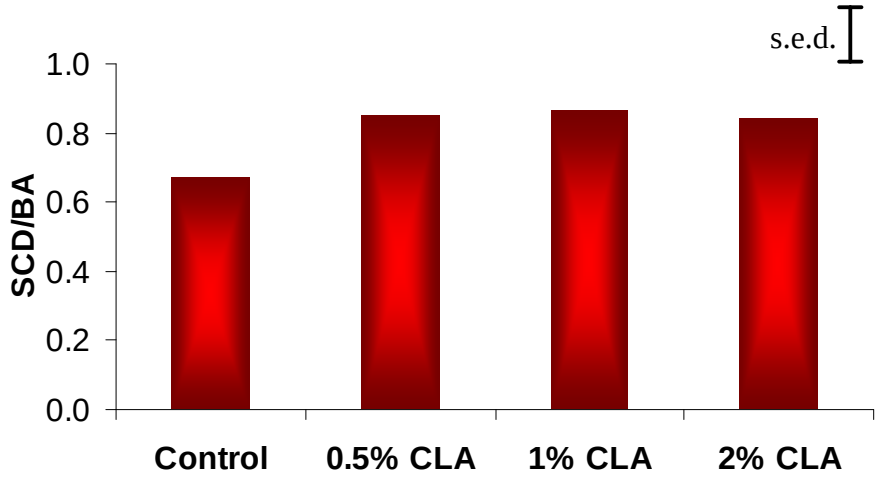


6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Samples of adipose tissue were taken at sacrifice for RNA extraction and ribo-nuclease protection assay. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). Level of significance for perirenal adipose tissue p = 0.01. n.d. = not determined

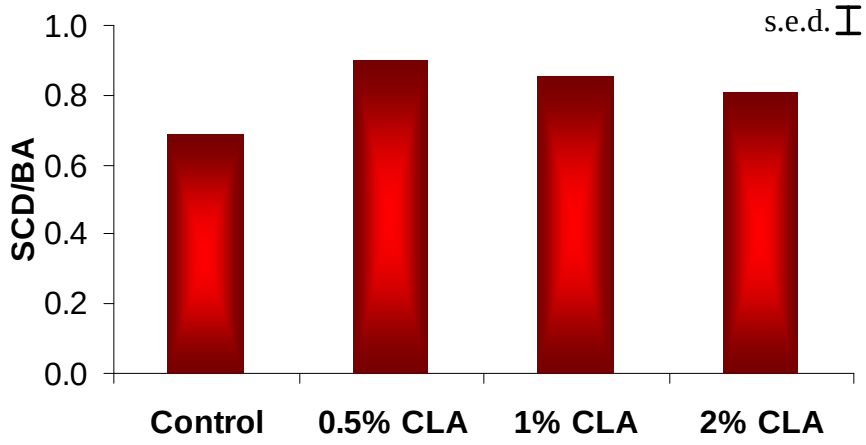
SCD mRNA levels remained the same independent of doses of CLA in epididymal and perirenal adipose tissue (Figure 5.19).

Figure 5.19 SCD mRNA levels in hamster adipose tissue

a) Epididymal adipose tissue



b) Perirenal adipose tissue

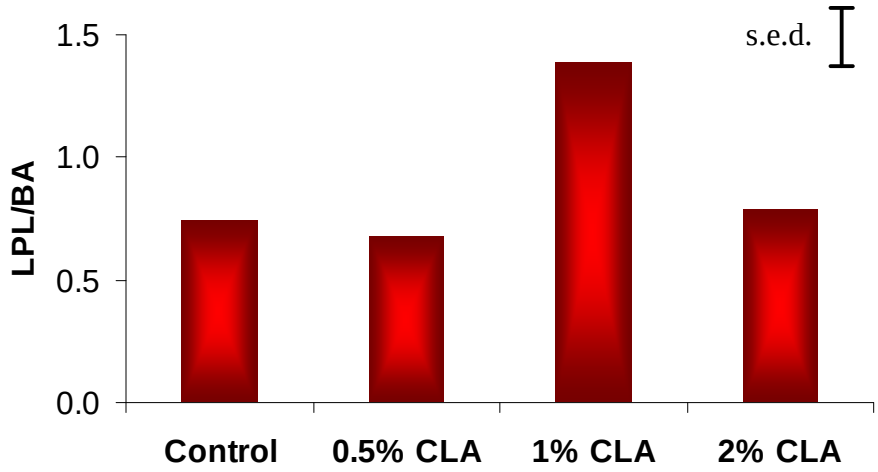


6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Samples of adipose tissue were taken at sacrifice for RNA extraction and cDNA synthesis, which was then quantified by real-time PCR. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). Level of significance for EP $p = 0.757$; PR $p = 0.317$.

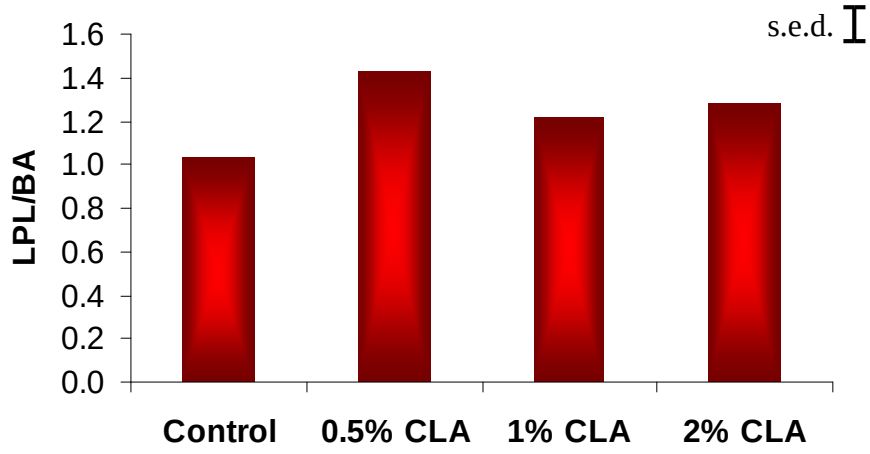
CLA supplementation did not change LPL mRNA levels in any of the fat depots (Figure 5.20).

Figure 5.20 LPL gene expression in hamster adipose tissue

a) Epididymal adipose tissue



b) Perirenal adipose tissue



6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Samples of adipose tissue were taken at sacrifice for RNA extraction and cDNA synthesis, which was then quantified by real-time PCR. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). EP s.e.d. 0.290; PR s.e.d. 0.2039. Level of significance for EP and PR p = 0.087 and 0.287 respectively.

5.1.6.2. Liver

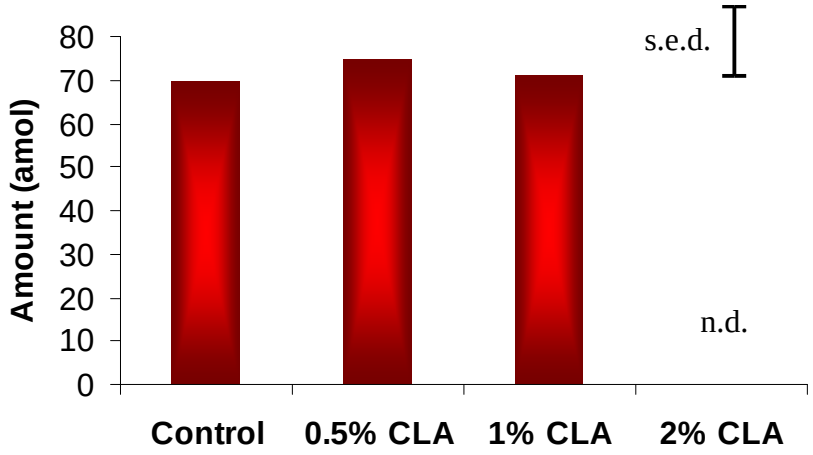
There was no effect of dietary CLA on ACC, FAS, LPL or SCD mRNA levels in hamster liver (Table 5.4). The general analysis of variance test performed tested for a linear relationship, but no evidence of a dose-response relationship was found. There was no effect of CLA feeding on SREBP-1c mRNA levels measured by ribonuclease protection assay in liver (Figure 5.21).

Table 5.4 Gene expression in hamster liver

CLA %	0% CLA	0.5% CLA	1% CLA	2% CLA	s.e.d.	p-value
ACC	0.882	1.014	0.835	0.871	0.1817	0.773
FAS	0.944	0.900	1.046	0.901	0.2430	0.923
LPL	0.324	0.602	0.474	0.765	0.1989	0.180
SCD	0.968	0.932	1.030	0.935	0.1489	0.904

6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Samples of liver were taken at sacrifice for RNA extraction and cDNA synthesis, which was then quantified by real-time PCR. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.).

Figure 5.21 SREBP-1c gene expression in liver



6 week old male Golden Syrian hamsters were fed 2% diets supplemented with 0.5, 1 and 2% CLA for 11 weeks. Total fat fed was constant for all diets. CLA was replaced by rapeseed oil. Samples of liver were taken at sacrifice for RNA extraction and ribo-nuclease protection assay. Data is presented as mean group values as generated by ANOVA statistical analysis (Genstat). Errors are presented as the standard error of the difference between the means (s.e.d.). Level of significance for liver $p = 0.954$.

5.2. DISCUSSION

5.2.1. Background to the study

There was no evidence of a reduction in fat deposition in sheep fed CLA (Wynn *et al*, 2004) or an effect of CLA on total lipogenesis or lipogenic enzymes (Chapter 3). The aim of the experiment was to identify possible mechanisms of action of CLA in an animal which has been shown to respond to CLA treatment. Many studies have been done in the mouse both with individual CLA isomers and mixtures of isomers, and these have demonstrated a huge response compared to other animals, suggesting that this particular species responds much better to CLA treatment. Studies in pigs, rats and hamsters show a much more conservative response to CLA treatment (Table 5.5). Previous work in the hamster has shown reduced body fat with CLA feeding (Bouthegourd *et al*, 2002; Kim *et al*, 2002; Gavino *et al*, 2000) but no effect on body weight or body weight gain except in two studies where hamsters fed a mixture of CLA isomers showed reduced body weight gain (Gavino *et al*, 2000) and reduced perirenal fat pad weights (Sher *et al*, 2003). One study has failed to demonstrate an effect of CLA feeding on fat pad weight (de Deckere *et al*, 1999). *Trans-10, cis-12* CLA and a mixture of CLA isomers have been shown to increase plasma triglyceride concentrations (de Deckere *et al*, 1999), indicating an effect on LPL activity, and reduce plasma cholesterol with up to 4 weeks of CLA treatment. More prolonged treatment did not significantly affect cholesterol concentrations. However in hamsters fed a high PUFA diet, supplemented with a CLA mix for 12 weeks, there was no effect on plasma triglyceride or cholesterol (Sher *et al*, 2003). These studies would tend to suggest an acute response to CLA in terms of plasma cholesterol and triglyceride which is normalised after 4 weeks of treatment. In hamsters fed an atherogenic diet, feeding a CLA mix dramatically reduced plasma triglycerides and cholesterol (Gavino *et al*, 2000) compared to animals fed *cis-9, trans-11* CLA and LA and this effect was seen at 2 and 6 weeks of feeding. Tissue fatty acid composition has been less well studied in the hamster in response to CLA feeding. In different groups of hamsters fed *cis-9, trans-11* CLA, *trans-10, cis-12* CLA and an isomeric mix, hamsters fed *trans-10, cis-12* CLA had significantly lower levels of linoleic acid in fat pad TAG than those fed an isomeric mix of *cis-9, trans-11* CLA. Levels of linolenic acid were also

reduced in hamsters fed the CLA mix or *trans-10, cis-12* CLA. Liver TAG was much more resistant to change, with no effect of CLA being seen on levels of linoleic or linolenic acids (de Deckere *et al*, 1999). There was no effect of CLA feeding on levels of oleic acid in liver or fat TAG. To date, there have been no studies investigating gene expression in the hamster and effects of CLA on lipid metabolism in terms of gene expression have been inferred from fat pad and liver weights and evidence from studies in other rodent species. The effect of CLA on fatty acid oxidation in the liver has been examined, by measuring the activity of carnitine palmitoyl transferase-1 (CPT-1) the rate limiting enzyme in fatty acid oxidation. Enzyme activity was significantly elevated in hamsters fed *cis-9, trans-11* CLA or an isomeric mix (Bouthegourd *et al*, 2002), suggesting that the fat reducing effect of CLA may be due to increased hepatic fatty acid oxidation, although in this study, there was no effect of CLA on white adipose tissue weights. *Trans-10, cis-12* CLA and a CLA mix increased liver weight (de Deckere *et al*, 1999) in hamsters fed for 8 weeks, but a CLA mix failed to change liver weights in hamsters fed for 12 weeks (Sher *et al*, 2003). A previous study in mice had suggested that the increased liver weight seen was due to lipid filling (West *et al*, 1998), but liver lipid was not measured so this could not be determined. Belury *et al* (1997) found CLA feeding increased hepatic lipids and suggested that peroxisome proliferation, causing hypertrophy of the liver may occur. There was no effect on key enzymes involved in peroxisome proliferation (de Deckere *et al*, 1999), so it is not likely that this is the mechanism by which liver weight is increasing. This is the first study to examine fatty acid composition and lipogenic gene expression in response to CLA feeding.

5.3. MAJOR FINDINGS FROM THIS STUDY

There was no effect on body weight or food intake and no change in plasma lipids. A 10% decrease in body fat was seen, with variable results in different adipose tissue depots. There was no effect on hepatic gene expression, but an increase in ACC and FAS mRNA in adipose tissue, associated with an increase in SREBP-1c was seen. SCD enzyme activity decreased but there was no change in gene expression. The following section discusses these results.

5.3.1. Body fat, weight gain and feed efficiency

There was no change in final body weights with CLA feeding. This has been shown in a number of studies with rats (Yamasaki, 2003; Sisk *et al*, 2001; Azain, 2000; Stangl, 2000), mice (Park *et al*, 1997), hamsters (see Section 5.2.1) and pigs (Weber *et al*, 2001). There are few studies in mice that have failed to demonstrate an effect of CLA on body weight; indeed many studies have shown a reduction in response to CLA feeding (Park *et al*, 1999; West *et al*, 1998). There was no change in feed intake in hamsters fed different levels of CLA, and this is supported by the majority of studies done in rats (Terpstra *et al*, 2002; Sisk *et al*, 2001; Azain *et al*, 2000), hamsters (Bouthegourd *et al*, 2002; Kim *et al*, 2002) and pigs (Weber *et al*, 2001; Ostrowska *et al*, 1999). Consequently, there was no significant effect of CLA on feed conversion efficiency in hamsters. This has also been demonstrated in rats (Yamasaki *et al*, 2003; Sisk *et al*, 2001; Azain *et al*, 2000), and one hamster study claimed that feed intake and body weight was not changed in animals fed CLA (Bouthegourd *et al*, 2002). In contrast to the hamster, pigs seem to be more responsive to CLA in terms of feed efficiency, with CLA feeding increasing feed efficiency in a number of studies (Wiegand *et al*, 2001; Ostrowska *et al*, 1999; Dugan *et al*, 1997). The proportion of carcass fat was reduced by 10% in hamsters fed CLA. This is in line with findings in mice (Terpstra *et al*, 2002; Peters *et al*, 2001; Park *et al*, 1999; 1997; West *et al*, 1998), rats (Azain *et al*, 2002; Stangl, 2000), pigs (Ostrowska *et al*, 1999; Dugan *et al*, 1997) and other studies in hamsters (Bouthegourd *et al*, 2002; Kim *et al*, 2002; Gavino *et al*, 2000). It is clear from previous studies (see Section 1.7.2 for a review) that the *trans-10, cis-12* isomer is likely to be responsible for the effects on body composition because reductions in body weight have been seen in mice fed

trans-10, cis-12 CLA and feed intake has been lower when mice were fed either *trans-10, cis-12* CLA or a CLA mixture (Park *et al*, 1999) and when rats fed a CLA mixture were shown to have reduced weight gains relative to those fed a CLA mixture (Gavino *et al*, 2000). Therefore it seems that hamsters do not respond well in terms of body weight, feed intake and feed conversion efficiency to CLA feeding. The 10% decrease in carcass fat with CLA feeding, however, indicates that CLA may change body composition via fat reduction at the expense of some other body component. Mice fed *trans-10, cis-12* CLA showed a reduction in body fat and a simultaneous increase in liver weight (Degrace *et al*, 2003). This is further evidence that the reduction in fat proportion seen in the current trial may be at the expense of fat partitioned into the liver. Although a mixture of both major CLA isomers was used in the current trial, it is likely from the literature reviewed that the *trans-10, cis-12* isomer is responsible for the changes in body fat between treatment groups and that there is no effect of the *cis-9, trans-11* isomer comparative to controls.

5.3.2. Adipose tissue depot weights

There was no significant difference in epididymal or perirenal fat pad weights from hamsters fed up to 2% CLA compared to control animals. There was a tendency for CLA to reduce perirenal fat pad weight, which was not statistically significant, but has been seen in hamsters fed CLA (Sher *et al*, 2003). This agrees partially with work done in rats (Yamasaki *et al*, 2003), where CLA had no effect on epididymal adipose tissue weight, but reduced perirenal adipose tissue depot weight; but the majority of work in rats demonstrates reductions in fat depot weights in response to CLA (Yamasaki *et al*, 2003; Kim *et al*, 2002; Sisk *et al*, 2001; Azain *et al*, 2000) where large reductions in fat depot weights have been seen. Peters *et al* (2001) showed that in mice fed 0.5% CLA, epididymal fat depots were significantly reduced compared to those of control animals, while in a study in rats conducted by Kim *et al* (2002) where CLA was added to high and low fat diets, retroperitoneal and omental fat was significantly reduced in animals fed the low fat diet, but only peritoneal fat was significantly reduced in animals fed the high fat diet. Azain *et al* (2000) showed that in female rats fed CLA at 0.25% and 0.5%, retroperitoneal and parametrial fat pad weight was significantly reduced compared to rats fed control diets containing soybean oil. These were

different fat pads to the ones measured in the current study, so this may reflect differential regulation between depots rather than a completely different response to CLA. It is interesting to note that one study (Kim *et al*, 2002) compared the responsiveness to CLA of rats and hamsters, and concluded, in terms of fat reduction, hamsters were the most responsive to CLA, and suggested that this was the best species to study effects of CLA on after the mouse.

Table 5.5 The effect of CLA on body fat in hamsters, rats and pigs

Author	Species	Treatment	Measurements	Conclusion
Alasnier *et al*, 2002	Rats	150mg *cis-9, trans-11*, trans-9, *trans-11*, *trans-10*, *cis-12* or *trans-10*, trans-12 CLA fed as part of a 5% fat diet	Body weight, carcass lipid content, fatty acid content of liver, muscle and perirenal adipose tissue	No effect of CLA on body weight or lipid content. Most incorporation of CLA in adipose tissue – mainly incorporated in TAG. No effect of cis or trans-9 isomers on FAC. t10,c12 reduced C16:1 and C18:1 in muscle. Double bonds at position 12 seem to be key to inhibit SCD activity. Wistar rats respond poorly to CLA diets in terms of lipogenic activities.
Bouthegourd *et al*, 2002	Hamster	9 week old hamsters fed control diet, control supp. with *cis-9, trans-11* CLA and control supp. with a CLA mixture	Whole body triglyceride, BMR, CPT-1, carcass parameters	No effect of CLA on food intake, body weight gain, white adipose tissue weight or carcass weights. CPT-1 activity in liver higher in c9, t11 CLA and CLA mix groups. Body triglyceride greater in c9, t11 CLA group. Plasma insulin higher with CLA mix feeding.
Kim *et al*, 2002	Hamster and rat	20% and 5% fat diet supplemented with 0.5 or 1% CLA at the expense of corn oil fed for 9 weeks	Body weight, fat pads and carcass composition	Significant reduction in % body fat in hamsters fed low fat (5%) diets; reduction in % body fat in hamsters fed 20% fat diets. Rat retroperitoneal and omental fat pad weights reduced in animals fed 5% fat; peritoneal fat pad weight reduced in rats fed 20% fat.

Azain *et al*, 2000	Rat	Diets supplemented with 0, 0.25 and 0.5% CLA fed for 5 weeks	Body weight, fat pad and liver weight, food intake, adipocyte size, plasma triglyceride and cholesterol	CLA feeding did not affect plasma triglyceride or cholesterol concentrations, food intake or body and liver weight. CLA at 0.5% reduced parametrial and retroperitoneal fat pad weights, suggesting a specific effect on fatty acid metabolism. CLA feeding reduced average adipocyte diameter suggesting that CLA inhibits lipid filling during growth of adipose tissue depots.
De Deckere *et al*, 1999	Hamster	11 week old hamsters fed control, CLA mix, c9, t11 and t10, c12 CLA-containing diets for 8 weeks	Plasma cholesterol, food intake, body weights, Liver and fat pad weights and FAC	Liver weights increased and epididymal fat pad weight reduced in t10, c12 fed group. Significantly lower plasma cholesterol and higher TAG in CLA mix and t10, c12 fed groups. T10, c12 fed groups stimulated C18 PUFA oxidation compared to other groups.
Gavino *et al*, 1999	Hamster	Mild atherogenic diet supplemented with 1% CLA as mixture of isomers, 0.2% *cis-9, trans-11* CLA and 0.2% LA	Plasma cholesterol and triglyceride, body weight, gain and food disappearance	Food disappearance greater in 1% CLA group. Mix of isomers reduced plasma triglycerides, body weight and fat despite hamsters eating more.
Ostrowska *et al*, 1999	Pig	Diets supplemented with 0, 1.25, 2.5, 5.0, 7.5 and 10.0 mg CLA-55 for 8 weeks	Feed intake, backfat thickness, body composition	CLA feeding significantly reduced carcass percentage fat but there was no significant effect on carcass protein. Reductions in backfat and increases in feed efficiency with CLA feeding seen after 3 wk.

5.3.3. Liver weight

There was a significant increase in liver weight with CLA feeding. This has been observed in a number of other studies in mice (Degrace *et al*, 2004; Clement *et al*, 2002; Terpstra *et al*, 2002; West *et al*, 1998) and in obese Zucker rats fed CLA (Sisk *et al*, 2001). Significant increases in liver weight in addition to reductions in body fat were seen in mice fed 0.5% CLA (Peters *et al*, 2001), and CLA was shown to increase liver weight when high (45%) and low (15%) fat diets were fed (West *et al*, 1998). Studies with individual isomers demonstrated that the *trans-10, cis-12* isomer was responsible for the increase in liver weight in mice (Degrace *et al*, 2004; Clement *et al*, 2002), and furthermore, that this was accompanied by a massive increase in hepatic TAG (Degrace *et al*, 2004) and lipid (Clement *et al*, 2002). It was suggested that the weight increase was due to hepatic lipid accumulation. CLA feeding increased liver weight by 30% in mice (Terpstra *et al* 2002). Loss of body fat and incidence of fatty liver is accompanied by hyperinsulinemia (Clement *et al* 2002). This is a health concern which has received little attention to date, set against the favourable effects of CLA on body fat. This study used mice fed both *trans-10, cis-12* CLA and *cis-9, trans-11* CLA and found that mice fed the *trans-10, cis-12* isomer displayed significantly heavier and paler livers, symptomatic of increased lipid storage. In addition, dramatic reductions in body fat were also observed. Hepatic steatosis has been shown to occur when there is an increase in lipogenesis above oxidation and secretion (Araya *et al* 2004). Although it is tempting to conclude that this is the method by which liver weight increased in the hamster with CLA feeding, this is unlikely to be the case in view of the mRNA data for ACC and FAS. Clement *et al* (2002) demonstrated that both isomers of CLA were equally efficient at activating peroxisome proliferator activated receptors α,β and γ, but only mice fed *trans-10,cis-12* CLA were hyperinsulinemic and had fatty livers. This led to the conclusion that the effect of *trans-10, cis-12* CLA is not likely to directly involve these nuclear receptors, but may act by inducing hyperinsulinemia which in turn induces fatty acid uptake and lipogenesis by stimulation of insulin sensitive genes such as ACC and FAS. However, there is evidence that there are species differences in terms of the liver response to CLA feeding. Mice fed CLA-supplemented diets developed enlarged livers symptomatic of

lipid accumulation (Tsuboyama-Kasaoka *et al*, 2000; Belury & Kempa-Stecko, 1997), but hamsters fed CLA developed hepatic hypertrophy, resulting in enlarged hepatocytes. This was shown to be independent of lipid accumulation (de Deckere, 1999). Clearly further work needs to be done, and awareness of species differences is needed in elucidating the effects of CLA on the liver. In support of the results presented here, a recent study by Kang *et al* (2004) showed that in mice fed 0.2% *trans-10, cis-12* CLA liver weight was increased but hepatic lipid content was not compared to control animals. Kang *et al* (2004) speculate that *trans-10, cis-12* CLA may be increasing energy expenditure as shown by West *et al* (2000) and Ryder *et al* (2001). It may be that the increase in liver weight can be explained by a repartitioning effect, and that the reduction in carcass fat seen in hamsters fed 2% CLA may have actually been repartitioned and present in the liver. However, as hepatic lipid content was not determined in this study, this remains speculative.

5.3.4. Blood cholesterol and triglyceride concentrations

There was no significant change in plasma cholesterol concentrations in hamsters fed varying amounts of CLA. A similar study, feeding 8% fat diets and 2% CLA to rats for 12 weeks showed that plasma cholesterol did not change (Yamasaki *et al*, 2000). There was a trend towards cholesterol increasing in hamsters fed 2% CLA but this was not significant. De Deckere *et al* (1999) fed 0.6% CLA to hamsters and found that plasma total cholesterol decreased in animals fed a 50/50 mix of both major isomers for 4 weeks, but that after 8 weeks there was no significant difference between treatment groups. However, unlike the current study hamsters were fasted for 16h before blood collection, which will have reduced lipoprotein formation and circulation. Plasma cholesterol levels in hamsters involved in the current trial were all between 3 and 4mmol/L, which is normal for hamsters (de Deckere *et al* 1993), so there is no evidence that CLA had any effect. There is evidence of a drop in plasma total cholesterol in hamsters fed CLA mixtures (Nicolosi *et al*, 1997, Lee *et al*, 1994), but the hamster model used had abnormally high plasma total cholesterol levels of 16mmol/L (Lee *et al*, 1994) so it may be that CLA is more efficacious at high concentrations of cholesterol. Sher *et al* (2003) fed hamsters 1% CLA for 7 weeks with and without 0.3% cholesterol and measured plasma total cholesterol and triglyceride. There was no effect of CLA

on total cholesterol in animals fed a cholesterol-free diet, in agreement with the current study, but CLA decreased cholesterol in hamsters fed 0.3% cholesterol. This has been seen in recent work done in our laboratory (unpublished results) where hamsters were fed a high cholesterol diet and butter enriched with *cis-9, trans-11* CLA. Plasma cholesterol concentrations were reduced in animals fed CLA compared to control animals. It has been suggested (Sher *et al*, 2003) that CLA did not affect plasma cholesterol in the absence of dietary cholesterol because the supposed impairment of cholesterol transport by CLA was not detectable under the analysis used. This study, combined with those of Nicolosi *et al* (1997) and Lee *et al* (1994) increases the evidence for an effect of CLA on plasma total cholesterol only when animals are fed diets high in cholesterol or who have high levels of circulating cholesterol. The beneficial effect of CLA on cholesterol therefore seems to require an abnormally high intake of cholesterol, the consumption of which has implications for other aspects of health, and the dose of CLA at which effects are seen is surplus to that which can be obtained from a natural food source. Hamsters fed up to 2% dietary CLA showed no change in plasma triglyceride concentrations; this is in line with observations in other studies (Sher *et al*, 2003). There was no effect of 1% (Sher *et al*, 2003) or 2% (Yamasaki, 2000) CLA feeding on circulating triglyceride levels in hamsters and rats. Feeding of the pure *trans-10, cis-12* CLA isomer and a CLA mixture increased plasma triglyceride concentrations, but *cis-9, trans-11* CLA failed to have an effect (de Deckere *et al*, 1999). Hamster livers were significantly larger in animals fed *trans-10, cis-12* CLA and the CLA mixture, so this may explain the higher plasma triglyceride concentrations, but increased free fatty acid levels in the CLA mix group (a major source of fatty acids for hepatic triglyceride synthesis under fasting conditions) were seen, making this unlikely. Plasma triglyceride levels can be affected by LPL activity; effects have been seen in adipocytes where CLA reduced LPL activity (Park *et al*, 1997). Paradoxically, Tsutsumi *et al* (1997) have found the opposite in hamsters; however the current results presented in this chapter show no effect of CLA on LPL mRNA. LPL is responsible for cleaving triglyceride-rich lipoproteins circulating in the blood, liberating fatty acids for esterification into adipocytes. From the results presented here it is unlikely that CLA is affecting LPL activity.

5.3.5. Fatty acid composition of hamster tissues

Analysis by gas chromatography identified the major fatty acids present in adipose tissue and liver. Analysis of the adipose tissue and liver showed that both isomers of CLA were present in detectable quantities and increased with the supplementation levels. There was slightly more *cis-9, trans-11* CLA than *trans-10, cis-12* which differed from the diet where approximately equal amounts were present. However it has been hypothesised that lower levels of the *trans-10, cis-12* isomer in both liver and adipose tissue could be due to more efficient metabolism of this isomer relative to its *cis-9, trans-11* counterpart (Alasnier *et al* 2002). In support of this Martin *et al* (2000) observed that the *trans-10, cis-12* isomer was preferentially driven through the beta-oxidation pathway over the *cis-9, trans-11* isomer due to its geometric and positional structure allowing it to escape four enzymatic steps that could be rate limiting. The *cis-9, trans-11* CLA isomer would only miss two, allowing accumulation in the tissues. In addition to this *cis-9, trans-11* CLA is the major isomer found in many tissues (Sehat *et al*, 1998; Parodi, 1997; Chin *et al*, 1992; Ha *et al*, 1989), so the abundance of this could merely be due to in vivo synthesis and not an imbalance between *trans-10, cis-12* and *cis-9, trans-11* in the diet. This has also been observed in cattle (Parodi, 1977). There was a dose dependent increase in levels of palmitic and stearic acids in both adipose tissue and liver with CLA feeding. SCD catalyses the desaturation of palmitic and stearic acids to form palmitoleic and oleic acids (Enoch *et al*, 1976), and levels of palmitoleic and oleic acids dropped with increasing CLA levels in a dose dependent manner. Taken together, these changes suggest that there is inhibition of SCD activity. The inhibitory effect was greater in adipose tissue than liver, with oleic acid dropping by 10% and 4% and palmitoleic acid dropping by 5% and 2% in adipose tissue and liver respectively, suggesting that there is a greater effect of CLA on sites of major fatty acid metabolism and storage. These decreases in monounsaturated fatty acids, coupled with the increases in the saturated fatty acids palmitic and stearic suggest that CLA may be having an effect on stearoyl-CoA desaturase (SCD) and that it is either acting at a transcriptional level by suppressing the SCD gene or directly inhibiting SCD enzyme activity. There was a dramatic reduction in oleic acid, making a strong case for the inhibition of SCD by CLA since its preferred

substrate is stearic acid (Enoch *et al*, 1976). Further evidence for an inhibition of desaturase is seen in the reduced desaturase ratios (C16:1/C16:0+C16:1 or C18:1/C18:0+C18:1) in both liver and adipose tissue, which reduced further with the degree of CLA supplementation. Both the increase in saturated fatty acids and the reduction in desaturase ratio with CLA feeding were seen in the adipose tissue of pigs fed CLA (Smith *et al*, 2002; Ramsay, 2001). These effects were most efficacious at 1-2% supplementation and were also dose dependent as shown in the current study. The dose dependent relationship suggests that CLA may be competitively inhibiting SCD as seen with sulphur-containing fatty acids, or sterculic acid (Hovik *et al*, 1997). Studies show that a *cis-12* double bond is needed to inhibit SCD activity (Park *et al*, 2000) and especially so when present with a *trans-10* double bond. The effect on fatty acid composition and SCD activity is likely to be due to the *trans-10, cis-12* isomer, as has been demonstrated in cell lines. SCD activity in MCF-7 cells treated for 48 hours with CLA isomers was significantly lower than in albumin treated control cells (Choi *et al* 2002). Studies using microsomal fractions from HepG2 cells treated with *trans-10, cis-12* and *cis-9, trans-11* CLA showed an inhibition of SCD activity of 60% with *trans-10, cis-12* CLA treatment, but not with *cis-9, trans-11* CLA (Choi *et al*, 2001). The effect on SCD activity was evident, indicating that CLA was directly acting on the enzyme and not via transcription of the gene. CLA added to hepatic microsomal fractions reduced incorporation of [1-^{14}C] palmitic acid into palmitoleic acid within 15 minutes (Park *et al*, 2000). The enzyme assay was backed up by fatty acid composition work which showed that levels of MUFAs were significantly lower in cells treated with the *trans-10, cis-12* isomer and that mRNA and protein levels were not affected by CLA treatment (Choi *et al*, 2002; Choi *et al*, 2001). Taken together, these studies show that CLA acts directly on the enzyme complex, rather than through transcription. There was an increase in levels of stearic and palmitic acids in adipose tissue and liver, but as with the monounsaturated fatty acids this change was not as pronounced in liver. This reflects the differences in composition of adipose tissue lipids and liver lipids. Liver contains more long chain fatty acids than adipose tissue, whereas adipose tissue contains 90% of its lipids as triglycerides, and of that 90%, 58% is present as oleic or palmitoleic acid (Kasturi & Joshi, 1982). Generally there is an increase in levels of long chain fatty acids in the liver tissue compared to those present in

adipose tissue. This is likely to be due to the extensive metabolism of cholesterol and lipoproteins which occurs in the liver. Conversely, a recent paper suggests that the effects of CLA are independent of effects on SCD gene expression and activity (Kang *et al* 2004). 2% CLA was fed in a high fat (20% provided as corn oil) diet to normal wild type mice and SCD1-null mice which possess a targeted disruption in the SCD1 gene (SCD-/-). Dietary *trans-10, cis-12* CLA feeding significantly reduced the 16:1/16:0 ratio in adipose tissue of wild type mice without changing SCD1 mRNA expression and this would seem to point to an inhibition of SCD activity; but the key finding of the study was that SCD1-null mice and wild type mice responded similarly to *trans-10, cis-12* CLA feeding. It is possible that the corn oil could have had an effect on SCD, with the high levels masking any effect of *trans-10, cis-12* CLA. Corn oil contains high levels of linoleic acid, oleic acid and other PUFAs (Chow, 1992) which are known to inhibit SCD in liver and adipocytes, casting doubt on the assertion that the anti-obesity effect of CLA is mediated through SCD1 gene expression and/or enzyme activity. However, with 4 SCD isoforms discovered to date in the mouse (Miyazaki *et al*, 2003), the possibility that CLA may have effects on the others cannot be ruled out. Ramsay *et al* (2001) fed CLA to pigs, and found that linoleic acid in tissues was reduced. They suggested that this may be due to a reduction in available linoleic acid in the diet as a result of replacement of corn oil with CLA. A reduction in levels of linoleic and linolenic acids was seen in hamster adipose tissue and liver; this may have been due to a lack of available linoleic acid in the diet due to replacement of linoleic acid-rich rapeseed oil with increased CLA supplementation. It is not possible for the desaturation steps from linoleic to linolenic to happen in animals, as the enzyme responsible for the conversion is only present in plants; therefore levels of linolenic acid in tissues are representative of dietary intake and will not be affected by endogenous CLA. The level and changes of linolenic acid concentrations were also very small and probably reflected the low amounts present in the diet. CLA can be metabolised to conjugated diene (CD) metabolites eg. CD18:3, CD20:3, so it is possible that the increase seen in these long chain fatty acids was due to metabolism of the dietary CLA rather than effects on a specific pathway. It is not possible from this analysis to tell which isomers have been formed and therefore by which pathway, so effects of CLA on specific enzymes remain speculative. However an increase in 22:6 was seen,

which is a metabolite of linolenic acid, and formed by beta oxidation. This may point to an activation of beta oxidation by CLA, which has already been suggested in rats as a potential body fat reducing mechanism (Sakano *et al* 1999) and also demonstrated using CLA isomers in 3T3-L1 adipocytes Evans *et al* (2002). Whether all of these effects are as a result of dietary fatty acid changes as mentioned by Ramsay *et al* (2001) or directly linked to CLA remains to be elucidated.

5.3.6. Steady-state mRNA levels in hamster tissues

5.3.6.1. ACC and FAS mRNA levels

Levels of ACC and FAS mRNA increased in adipose tissue of hamsters fed CLA. Studies have indicated that CLA may exert its effects on body composition through actions on lipogenic enzymes like ACC and FAS. There is no evidence that down-regulation of lipogenic gene expression is occurring in either adipose tissue or liver of hamsters fed CLA, so the reduction in carcass fat is not as a result of this. The lack of effect of CLA on fat pad weights is inconsistent with previous rodent studies (Table 5.5) which have shown reduced fat in animals fed CLA. Paradoxically the increase in adipose tissue ACC and FAS mRNA indicates that lipogenesis is being up-regulated, and yet there is no increase in fat pad weight as would be expected as a result of this. However, up-regulation of adipose tissue FAS has been seen in mice fed *trans-10, cis-12* CLA; but this was combined with a reduction in fat pad weight and an up-regulation of UCP-2, which increases energy consumption (Kang *et al,* 2004) and was cited as the reason for the change in fat pad weights. There was a significant reduction in intrascapular fat pad weight with 0.5% CLA feeding, but this effect was not sustained at 2% CLA, and mRNA levels for ACC and FAS showed the same pattern as for the other adipose tissue depots. The difficulties associated with dissection of white adipose tissue from the intrascapular fat pad and separation from the brown adipose tissue present may explain these results. Mice fed *trans-10, cis-12* CLA were found to have reduced FAS mRNA levels in white adipose tissue (Clement *et al,* 2002), but this was accompanied by an increase in hepatic FAS mRNA. FAS mRNA has been shown to increase in adipose tissue from wild type mice fed *trans-10, cis-12* CLA (Kang *et al*, 2004), supporting the idea that the effects

seen in the hamster are mediated through the actions of this isomer. There is also the possibility that the increase in lipogenic gene expression is a compensatory effect and that the adipose tissue is trying to respond to a drop in fat levels in the body by up-regulating ACC and FAS in the adipocytes. CLA feeding has been shown to increase hepatic FAS mRNA in wild type mice (Peters *et al* 2001). The current study showed no change in hepatic ACC or FAS mRNA in hamsters fed CLA, indicating that the effects on body composition were not mediated through genes expressed in the liver. Mice fed CLA were also shown to have levels of circulating insulin up to 10 times those seen in control mice. Clement *et al* (2002) suggest that CLA may affect FAS expression by causing hyperinsulinemia in animals fed CLA, via an up-regulation of SREBP-1c, present in adipose tissue, which then, combined with glucose and insulin, acts on the insulin responsive element in the FAS complex, causing up-regulation of the gene and increasing rates of fatty acid synthesis. This is consistent with the current study, which showed an increase in adipose tissue SREBP-1c with CLA feeding. In support of this, higher levels of insulin have also been seen in mice fed CLA (Tsuboyama-Kasaoka *et al*, 2000). Increased mRNA levels do not always mean that there will be increased transcription or activity of the enzymes for which they code (Peters *et al*, 2001), as there may be post-translational inhibition of the ACC and FAS enzyme complexes, resulting in reduced lipid synthesis, or simply reduced translation of the gene. Evans *et al* (2002a) demonstrated in 3T3-L1 cells that *trans-10, cis-12* CLA resulted in an increase in glucose and oleic acid incorporation into lipid, indicating that lipogenesis was being stimulated. However, treatment of the cells with *trans-10, cis-12* CLA resulted in increased oxidation of oleic acid, and combined with the suggestion made by Martin *et al* (2000) that *trans-10, cis-12* CLA is more efficiently oxidised by beta oxidation enzymes it suggests that CLA may stimulate lipogenic enzymes but also up-regulate those of the beta oxidation pathway. Although the activity of the rate limiting enzyme of beta oxidation, carnitine palmitoyl transferase-1 was not measured in this hamster study, Martin *et al* (2000) showed an increase in rats fed CLA, linking the effects of CLA with increased fatty acid oxidation. There is also evidence for an increase in energy expenditure with CLA feeding; metabolic rates in mice were significantly increased with CLA feeding, changing respiratory quotient and indicating an increase in lipid oxidation

(West *et al*, 1998). Saturated fatty acyl-CoAs are known to allosterically inhibit ACC, which reduces levels of malonyl CoA, which is required for fatty acid biosynthesis. It also has an inhibitory effect on the carnitine palmitoyl transferase shuttle system, which is the rate limiting step in mitochondrial import and oxidation of fatty acids. A reduction in intracellular malonyl-CoA leads to increased fatty acid transport into mitochondria for oxidation (Miyazaki & Ntambi, 2003) and may be the mechanism by which CLA increases energy expenditure. It has been reported that n-3 PUFAs reduce lipogenic gene expression (Jump *et al*, 1994) and stimulate beta oxidation. The increase in lipogenic enzymes seen both in this study and in cell lines fails to satisfy the hypothesis that CLA has its effects via an inhibition of lipogenesis and provides further evidence for a mild stimulatory effect of CLA on fatty acid synthesis and a larger stimulation of fatty acid oxidation. The current study provides no evidence to show that there is down-regulation of lipogenic gene expression in adipose tissue or liver as a result of CLA feeding, so the reduction in carcass fat cannot be explained by this mechanism. In contrast to the fatty acid data, none of the effects on ACC and FAS mRNA levels are dose dependent, and the largest effects are seen at the lowest dose. As supplementation increases ACC and FAS mRNA levels fall back to those seen in control animals. There may be an interaction between the two isomers which is concentration dependent; at low concentrations *cis-9, trans-11* CLA may act to increase lipogenesis via transcriptional up-regulation of ACC and FAS. The effect of CLA on SCD activity was dose dependent, with the most inhibition at the highest level of CLA, and this has been attributed to the *trans-10, cis-12* isomer. At higher concentrations therefore the inhibitory effect of *trans-10, cis-12* on SCD may become evident, reducing levels of oleic and palmitoleic acid. It has been shown that endogenously synthesised MUFAs are required for TAG synthesis (Miyazaki *et al*, 2001), and that if these are not present, levels of TAG are reduced by inhibition of ACC and FAS. This antagonistic effect of the two major isomers present in the CLA supplement could explain the change in lipogenic enzyme mRNA levels with different doses of CLA.

5.3.6.2. SCD mRNA levels and enzyme activity

The current study shows that there is no change in SCD gene expression in adipose tissue depots or liver of hamsters fed CLA. This has been shown in adipose tissue from mice fed *trans-10, cis-12* CLA (Kang *et al* 2004). This study also showed an increase in adipose tissue FAS mRNA levels as seen in the current study. There have been few studies where CLA has been fed to rodents and gene expression has been examined in adipose tissue; however a number of researchers have evaluated this in the 3T3-L1 adipocyte cell line. Choi *et al* (2000) treated 3T3-L1 pre-adipocytes with *trans-10, cis-12* CLA and examined levels of RNA during the differentiation period. SCD1 mRNA was shown to reduce in cells treated with *trans-10, cis-12* CLA in a dose dependent manner. SCD1 protein levels also followed this pattern and SCD1 activity was reduced by 15% and 60% in cells treated with *cis-9, trans-11* CLA and *trans-10, cis-12* CLA respectively. Levels of palmitoleic and oleic acids were also reduced, as seen in the current study, and this was consistent with the gene transcription, activity and protein results. *Trans-10, cis-12* reduced lipid within adipocytes; this may be due to an inhibitory effect on the SCD1 complex, because 16:1 and 18:1 make up 58% of the fatty acids in intracellular lipid droplets (Lee *et al*, 1995). The fatty acid composition data for hamster adipose tissue depots indicate that SCD activity is being inhibited as a result of CLA feeding, due to the drop in 16:1 and 18:1 and relative increase in 16:0 and 18:0. The lack of effect of CLA on SCD mRNA levels indicates that the inhibition of SCD is not at a transcriptional level and taken together with the fatty acid composition data suggests that CLA is reduces SCD enzyme activity. There is evidence that CLA, particularly the *trans-10, cis-12* isomer can inhibit adipogenesis in cultures of 3T3-L1 preadipocytes (Evans *et al*, 2000). The reducing effect of CLA on levels of 16:1 and 18:1 and increase in 16:0 and 18:0 may have increased fatty acid oxidation, by affecting intracellular intermediary factors.

5.3.6.3. LPL mRNA levels and enzyme activity

It is known that LPL is expressed differentially between tissues, and the findings from this study that LPL gene expression in liver was generally lower than that in epididymal and perirenal adipose tissue support this. There was no significant difference in expression of LPL with CLA feeding in all tissues studied. This is consistent with the plasma data which showed no changes in circulating cholesterol or triglycerides, which LPL removes from the circulation for esterification and storage in adipocytes. This is consistent with work done by Degrace *et al* (2003) who fed individual CLA isomers to mice at 1% in the diet and used RT-PCR techniques to measure hepatic LPL gene expression. There was no effect of CLA feeding on LPL gene expression in mice. In addition, mice were found to have significantly enlarged livers as in the current hamster study. Degrace *et al* (2003) studied very low density lipoprotein (VLDL) secretion by liver in CLA fed mice injected with 10% Triton to inhibit LPL activity, preventing cell breakdown and uptake of VLDL. Plasma concentrations of VLDL-apolipoproteinB-100 were therefore proportional to their secretion rates. Livers from mice fed CLA *trans-10, cis-12* showed a 20% increase per gram of tissue in the rate of VLDL clearance. These findings show that the liver triglyceride accumulation in mice fed *trans-10, cis-12* CLA was not due to a decrease in the VLDL secretion, despite overproduction because there was an increased influx of fatty acids entering the lipoprotein assembly process (Degrace *et al*, 2003). Lower plasma LDL concentrations and higher plasma TAG were observed in hamsters fed CLA (de Deckere *et al*, 2003); this is associated with inhibition of LPL activity (Park *et al*, 1997). In non-fasted animals LPL is active in white adipose tissue, resulting in a flux of fatty acids to the adipose tissue where their fate is esterification and storage. Therefore in the current trial where animals were in the fed state, LPL expression was expected to be high in adipose tissue, thus allowing possible effects of CLA to be seen clearly. However, many of the studies have fed high fat diets resulting in an increase in circulating chylomicrons and increased LPL activity to extract triglyceride from the lipoproteins. The current study fed a relatively low fat diet with 4% total fat, so LPL expression may have been low due to low circulating levels of triglyceride-rich lipoproteins. Degrace *et al* (2003) also only fed 5% fat in total in the study in mice, so this may be the

reason that no effects of CLA were observed. Kang *et al* (2003) fed a 20% fat diet inclusive of 5% CLA to mice and measured LPL gene expression. CLA was shown to reduce LPL gene expression in adipose tissue although there was no effect on circulating triglyceride. Few studies have examined the effect of CLA on LPL gene expression in animals, although a number of researchers have evaluated expression and enzyme activity in 3T3-L1 cells. 3T3-L1 cells are from a pre-adipocyte cell line and are grown to confluence in culture. However, only relatively short term studies (6-7 days post-differentiation) are possible as when the cells mature they lose their adhesion to the plate, so effects on mature cells are difficult to study. In addition, 100% differentiation is never achieved, so some reported effects will be due to reductions or increases in adipogenesis. Lin *et al* (2001a) treated 3T3-L1 cells with *cis-9, trans-11* CLA, *trans-10, cis-12* CLA and linoleic acid at concentrations up to 1000µmol/L. Intracellular LPL activity was increased with all treatments up to a concentration of 30µmol/L but then dropped back to initial levels. Park *et al* (1997) also noted that on addition of 5µmol/L CLA to cell cultures an increase in LPL activity was seen prior to a reduction above this concentration. Whether this is due to the initial increase in supply of fatty acids is not clear and requires further investigation. Heparin-releasable LPL (LPL secreted from adipocytes and anchored to the luminal surface of capillary endothelial cells) activity was reduced at all concentrations by *trans-10, cis-12* CLA, but increased with *cis-9, trans-11* CLA and linoleic acid treatment at concentrations up to 100µmol/L (Park *et al*, 1997). Activity declined again with concentrations greater than 100µmol/L. Park *et al* (1999, 1997) also saw this in isolated rat adipocytes. Lin *et al* (2001a) introduce the idea that *trans-10, cis-12* CLA may interfere with the transport of intracellular LPL to the cell membrane and therefore its attachment to the endothelial cell surface. The current study in hamsters measured total LPL gene expression however, and the only indication of activity measured was the plasma triglyceride assay. A drop in circulating triglyceride would indicate an increase in the activity of LPL. There was no effect of CLA feeding on plasma triglyceride in hamsters, therefore this argues against an effect of CLA on either LPL gene expression or activity and does not support the hypothesis of interference of *trans-10, cis-12* CLA with LPL transport to the cell membrane (Lin *et al*, 2001a). As mentioned previously though, it may be that there was a change in activity and/or gene expression

which was too small to be detected, due to the low flux of fatty acids through the lipid metabolism pathways as a result of the low fat diet. It is not clear what is happening in the current study as CLA did not change gene expression, and there was no change in plasma triglyceride levels, which tends to rule out an effect on LPL enzyme activity. However the effect on LPL with CLA may only be seen with high fat diets. Certainly much of the previous work seems to point towards a CLA-mediated effect on LPL enzyme activity rather than transcriptional change, but more work will be needed to clarify this. The current study suggests than LPL is not responsible for the change in carcass fat of these animals. Another possible mechanism for fat reduction is via an increase in lipolysis.

5.4. SUMMARY

Both isomers of CLA were incorporated into the adipose and liver tissues dose dependently, with less *trans-10, cis-12* CLA compared to *cis-9, trans-11* CLA; this is likely to be due to preferential metabolism of the former. There is no evidence that CLA supplementation has any effect on body weight, feed intake or feed efficiency in hamsters fed low fat diets. Epididymal and perirenal fat pad weights were not affected with CLA feeding, although a reduction in intrascapular fat pad weight was seen with low levels of CLA feeding, in spite of an increase in ACC and FAS mRNA levels in adipose tissue. This was not dose dependent and was greatest at the lowest level of CLA supplementation suggesting a dose dependent interaction between the two isomers with the effects of *cis-9, trans-11* CLA prevailing at low concentrations and those of *trans-10, cis-12* CLA prevailing at high concentrations. 0.5% CLA supplementation reduced carcass fat by 10%, but there was no further effect with 1 and 2% supplementation, as with the ACC and FAS mRNA levels, raising the possibility that lipogenic gene expression in adipose tissue was stimulated in response to a reduction in overall body fat. Liver weight was increased with CLA feeding; this may be due to lipid filling as there is no evidence to suggest that CLA acts on liver gene expression. It is likely that the effects of CLA are mediated through the adipose tissue. There is no evidence of an effect of CLA on LPL mRNA levels, or plasma total cholesterol and triglyceride data, which was not different with treatments and was physiologically normal. CLA inhibits SCD activity dose dependently,

evidenced by the reduction in MUFAs and increase in SFAs, and the reduction in the SCD index. There was no effect of CLA on SCD gene expression, indicating that CLA acts directly on the enzyme complex rather than through transcription. This study indicates that CLA has different effects at different concentrations and that the SCD inhibition and fat reducing effects that have been attributed to *trans-10, cis-12* CLA, are more evident at high concentrations, with the anti-carcinogenic effects of *cis-9, trans-11* CLA having a greater effect at lower concentrations. The two isomers may act antagonistically, with effects of the two isomers becoming evident at different concentrations. Clearly this has consequences for the control of lipid metabolism in response to CLA.

5.4.1. Conclusions

There is no evidence that the reduction in body fat is associated with decreased lipogenesis as both epididymal and perirenal depots were examined and did not change significantly.

There is a potentially adverse effect of CLA on fatty acid composition; this is likely to be due to inhibition of SCD activity rather than SCD expression.

6. CONCLUSIONS

Lamb consumption has shown a downward trend for a number of years (see Sections 1.1 and 1.2), at least partly due to the high levels of saturated fat associated with the meat, in conjunction with government advice to reduce intakes of this type of fat. In addition, obesity and associated medical conditions are on the increase. CLA has been shown to reduce fat deposition in a number of animals; however the mechanisms are as yet unclear. This project initially investigated fat synthesis in the sheep in response to CLA in an attempt to determine the factors in the process affected by CLA. Although there has been much work done on the effects of CLA in small animals, the effects of CLA on sheep have not been well investigated and much of the work done to date in ruminant animals has centred on dairy cows and the effects of CLA on milk fat synthesis. Work in small animal models has demonstrated that CLA seems to consistently act on the liver, the major site of fatty acid biosynthesis in rodents. The major site of fatty acid biosynthesis in ruminants is the adipose tissue, and the adipose tissue explant technique was used to study this as it was anticipated that CLA may also target this tissue. In the absence of an effect of CLA on lipogenesis or lipogenic gene expression in the sheep (both in explants and *in vivo*), feeding studies were performed in the hamster, a monogastric species previously shown to reduce body fat in response to CLA.

6.1. TISSUE CLA CONTENT

Feeding CLA to both sheep and hamsters resulted in an increase in the proportions of *cis-9, trans-11* and *trans-10, cis-12* CLA incorporated into adipose tissue and liver (Chapters 4 & 5). This demonstrated that it was possible to protect dietary CLA from hydrogenation in the rumen of the sheep. The CLA fed to sheep was approximately 66% protected (Wynn, 2003), and was clearly present in the small intestine for absorption into tissues. In both species and tissues studied, the proportion of *trans-10, cis-12* CLA was lower than that of *cis-9, trans-11* CLA, even when accounting for endogenous synthesis seen in sheep fed the control diet. This is consistent with a number of other studies (Sehat *et al*, 1998; Parodi, 1997; Chin *et al*, 1992; Ha *et al*, 1989)

There has been some support for the idea that the *trans-10, cis-12* isomer is metabolised in preference to *cis-9, trans-11* CLA (Alasnier *et al*, 2002; Martin *et al*, 2000), however it was not possible from the present studies to say whether this was the case.

6.2. EFFECTS OF CLA ON LIPOGENESIS

Studies in mice, rats and hamsters had demonstrated that CLA had favourable effects on body composition, with animals showing significant reductions in fat mass and more modest increases in lean tissue mass (Kim *et al*, 2002; Azain *et al*, 2000; Gavino *et al*, 1999; Park *et al*, 1999 Park *et al*, 1997). Studies on lipogenesis in ovine adipose tissue explants treated with CLA and other fatty acids (Chapter 3) showed variability between tissue preparations, evident by the different responses to insulin and dexamethasone treatment. Insulin is known to increase lipogenesis in ruminant adipose tissue (Chilliard & Faulconnier, 1995; Faulconnier *et al*, 1994; Miller *et al*, 1991; Etherton & Evock, 1986; Vernon, 1979), so increased lipogenesis with inclusion of this hormone indicated that the tissue was metabolically active and capable of responding to stimuli. Dexamethasone, a synthetic glucocorticoid has been shown to reduce lipogenesis in ruminant adipose tissue (Daniel *et al*, 2004; Faulconnier *et al*, 1997; Chilliard & Faulconnier, 1995; Vernon *et al*, 1991; Plested *et al*, 1987). Maximal stimulation of lipogenesis and *de novo* fatty acid synthesis with insulin and inhibition with dexamethasone demonstrated that the tissue was responsive to treatments. In addition, insulin maintains tissue viability in culture (Vernon, 1979), making it possible to study responses to treatments over several days. There was no consistent effect of CLA on lipogenesis in ovine adipose tissue explants, but as insulin was included in the culture there was the possibility that the potential inhibitory effect of the fatty acid could not overcome the stimulatory effect of insulin, as seen in rat adipose tissue explants cultured with fatty acid and insulin (Vernon, 1975). However, studies in ovine adipose tissue explants treated with fatty acids with and without insulin showed that insulin could not overcome the fatty acid induced inhibition of lipogenesis (Vernon, 1975). There was no evidence for a dose dependent inhibition of lipogenesis with oleic acid or CLA in the studies reported here; although there was a decrease in lipogenesis with the highest concentrations of fatty acids this may have been due to the cytotoxic effects of

the fatty acid. The availability of fatty acids to all of the cells within the tissue may also have been impaired. Small water soluble hormones may diffuse easily through the tissue to reach cells in the centre of the explants, but this may not be the case for hydrophobic fatty acids, even when complexed to albumin. Any direct effects of fatty acids on lipogenesis seem not to be of a magnitude that can be detected using the adipose tissue explant system.

6.2.1. ACC mRNA levels in response to CLA treatment

In adipose tissue of hamsters CLA seemed to stimulate lipogenesis, measured by increases in ACC and FAS mRNA. There was no evidence to suggest that CLA had any effect on lipogenesis in sheep in explants or *in vivo*. It was noted that in the hamster levels of ACC and FAS mRNA did not differ from controls with levels of CLA above 1% in the diet. There was no significant effect of CLA on ACC mRNA levels in sheep fed PCLA. The hamster showed a 10% reduction in body fat with CLA feeding, despite the increase in adipose tissue lipogenic mRNA levels at 0.5% CLA. Taking all these results together, it is significant that the highest level of mRNA for both ACC and FAS was seen in conjunction with the drop in body fat and a non-significant drop in perirenal adipose tissue weight. This was all seen at the lowest level of supplementation and suggests that there is increased oxidation of fat as well as increased lipogenesis. The two major isomers present in the CLA mix used for both the explant and hamster studies contained 90% CLA, with approximately 98% of this being either *cis-9, trans-11* or *trans-10, cis-12* CLA, in equal proportions. It has been demonstrated that *trans-10, cis-12* CLA is the isomer responsible for the changes in body composition (Baumgard *et al*, 2002; Brown *et al*, 2001; Park *et al*, 1999), but there are no reports of effects of the two isomers at different concentrations. It is possible that the effects of the individual isomers are manifested at different concentrations; *cis-9, trans-11* CLA may increase ACC and FAS mRNA levels at low concentrations, while *trans-10, cis-12* CLA may reduce or prevent further increase in gene transcription at higher concentrations, resulting in a reduction in lipogenesis. However, studies in ovine adipose tissue explants with individual isomers showed no effect of *cis-9, trans-11* CLA on ACC mRNA levels; it would be interesting to see whether this is the case in hamsters fed different levels of individual isomers. There was no effect of CLA feeding on ACC mRNA levels in liver of sheep or hamsters,

suggesting that there is more effect of dietary fat on the adipose tissue, which is the predominant site of lipid metabolism.

6.3. EFFECTS OF CLA ON SCD

There was no apparent effect of CLA on synthesis of saturated and monounsaturated fatty acids in ovine adipose tissue explants. However this was measured by incorporation of acetate into the tissue, and not by fatty acid composition, as in hamsters and sheep fed CLA. It would have been interesting to look at fatty acid composition of the tissue in addition to fatty acid formation to see if acetate incorporation was a good indicator of fatty acid composition. However, this would be difficult to study in the time frame used in these experiments, as the tissue would not turn over rapidly enough for any changes to be measured. Additionally, there are problems associated with long term culture of explants. Conversely, in sheep and hamsters fed CLA there were significant differences in fatty acid composition, with lower levels of palmitoleic and oleic acids in animals fed CLA than those fed control diets. The type of fat fed significantly affected desaturase ratios in sheep, but there was no significant dose effect, only a trend towards lower values with increased CLA. Conversely, the effect of CLA on hamster desaturase activity was dose dependent with the greatest degree of SCD inhibition being seen at the highest level of CLA. CLA treatment had most effect on adipose tissue SCD, with liver SCD activity changing very little in both sheep and hamsters relative to CLA dose. This is likely to be due to the different constitutions of adipose tissue and liver. Liver contains a larger proportion of phospholipids than adipose tissue, which is mainly made up of triacylglyceride. Phospholipids appear to be highly resistant to changes in their fatty acid composition (Ip & Scimeca, 1997); this is consistent with the results seen in sheep and hamsters. There was no effect of CLA treatment on SCD mRNA levels in ovine adipose tissue explants, ovine adipose tissue or hamster adipose tissue. Taken together with the fatty acid composition data, this strongly indicates that CLA inhibits SCD enzyme activity and does not have any effect on transcription of the SCD gene.

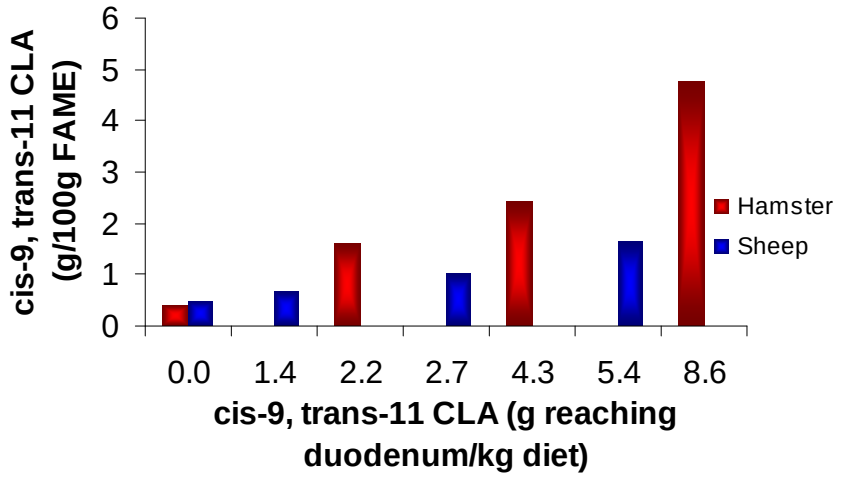
6.4. EFFECTS OF CLA ON HAMSTERS AND SHEEP

In the present studies, the hamster was more responsive to CLA feeding than the sheep in terms of reductions in body fat, SCD activity and lipogenesis (indicated by ACC and FAS mRNA levels) and changes in these with different levels of CLA feeding. Clearly there are difficulties in the ruminant due to the hydrogenation of fats in the rumen, necessitating the protection of supplements from breakdown by the rumen micro-organisms. Feeding a fat supplement to the monogastric like the hamster is simpler in that there are few micro-organisms present in the digestive tract until after the small intestine, the site of fat absorption. However, protection of the CLA was achieved sufficiently to allow changes in fatty acid composition to be seen. In an attempt to explain a difference in the responses of the two species, the amounts of CLA available for absorption at the level of the small intestines of the sheep and the hamster were calculated, and compared with the amounts of both CLA isomers in perirenal adipose tissue depots. There was significantly more CLA reaching the adipose tissue of hamsters than sheep (Figure 6.1). This was the case even when there was more CLA available for absorption at the level of the duodenum. However, the calculation of the amount of CLA available for absorption was based on the proportions of the supplements present as *cis-9, trans-11* and *trans-10, cis-12* CLA, and was not confirmed by levels of CLA present in the digestive tract of the hamster. CLA present in the abomasum of sheep was shown to increase with supplementation levels (Wynn, 2003) in the same way, but without looking at flow rates, absolute values for an amount of supplement could not be determined. However, looking at the levels of CLA available for absorption it appears that the greater responses seen in hamsters may be due to the increased availability of CLA for absorption at the level of the small intestine. There my not have been enough CLA to elicit an effect on the parameters measured, however there were similar amounts of both CLA isomers in tissues of sheep (Figure 6.1) fed the highest amount of CLA (100g/kg) and hamsters fed the lowest amount of CLA (50g/kg) so this seems unlikely. Additionally, Ostrowska *et al* (1999) fed pigs 10g CLA/kg diet and observed significant reductions in back fat, indicative of reduced lipogenesis. Taken together, these observations suggest that the sheep does not respond to dietary CLA. It may be that levels of CLA available at the duodenum in

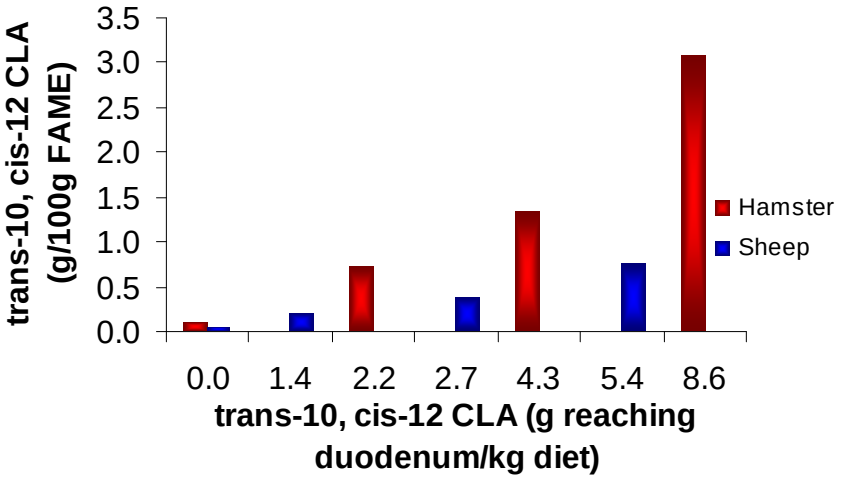
practice were much lower than those calculated despite feeding larger amounts of CLA. There was no effect of CLA on hepatic ACC or SCD mRNA in hamsters or sheep fed CLA. While there is little lipogenesis occurring in the liver of ruminants, with the majority of fatty acid synthesis occurring in the adipose tissue, lipogenesis is split between the liver and adipose tissue in hamsters. The results suggest that CLA targets adipose tissue, rather than liver in the instigation of its effects.

Figure 6.1 Proportions of CLA isomers in perirenal adipose tissue from hamsters and sheep fed different levels of CLA

a) *Cis-9, trans-11* CLA



b) *Trans-10, cis-12* CLA



6.5. FUTURE WORK

Although there was no effect on lipogenesis in ruminants fed a mixture of *cis-9, trans-11* and *trans-10, cis-12* CLA at the levels of supplementation used in the present study, it would be interesting to examine if supplementation of diets with pure *cis-9, trans-11* and *trans-10, cis-12* CLA affected the lipogenic enzymes and fatty acid composition studied here. Another line of investigation could focus on the effects of feeding CLA to breeding ewes and the effects on the lambs in terms of fat deposition, enzyme activities and fatty acid composition. There is already evidence to suggest that feeding CLA during gestation to rats and pigs has long term effects on the offspring in terms of body weight, body composition and tissue fatty acid composition (Poulos *et al*, 2001; Bee, 2000). Production of lambs with less fat would appeal to the health-conscious consumer and may have commercial relevance. Ruminant mammary tissue clearly responds to *trans-10, cis-12* CLA in terms of a reduction in milk fat synthesis (Baumgard et al, 2002; Baumgard et al, 2000) and CLA has been shown to reduce milk fat in lactating sheep (Sinclair, personal communication). Comparative studies examining the effect of CLA on mammary and adipose tissues would be interesting to investigate whether the effect of CLA on ruminant mammary tissue is tissue specific. The studies in hamsters fed CLA revealed that the effects of CLA were different with different levels of CLA feeding. This suggested an antagonistic role for the two isomers, with the possibility that the effects of *cis-9, trans-11* CLA predominate over those of *trans-10, cis-12* CLA at a low concentration and *trans-10, cis-12* becomes dominant at high concentrations. Future studies feeding individual isomer supplements would help to determine whether this is the case, and further studies are still required to explain the decrease in carcass fat seen with CLA feeding.

Investigation of the effects of the individual isomers of CLA on aspects of lipid metabolism, the concentrations at which they are effective, the tissues that they target and the degree of efficacy will be a key aim for future work.

6.6. CONCLUSION

The effects of CLA on lipid metabolism have been investigated in sheep and hamsters. This is the first report on the effects of CLA on ovine adipose tissue lipogenesis. There was no effect of CLA on total lipogenesis, but an inhibitory effect of increasing concentrations of fatty acids. CLA had no effect on proportions of saturated and monounsaturated fatty acids formed in ovine adipose tissue, suggesting that CLA failed to alter fatty acid composition via inhibition of SCD. Finally, the effect of CLA on ACC and SCD mRNA was measured. There was no effect of CLA on either ACC or SCD mRNA levels consistent with the previous experiments on total lipogenesis and fatty acid formation. The use of tissue culture models is limiting, however, due to the absence of many hormones and processes present in the whole body, therefore the work with CLA in adipose tissue was followed by a feeding trial in sheep. Protection of the CLA from ruminal degradation was essential, to ensure that it was available for absorption at the level of the small intestine, and this was achieved successfully, with the CLA being 66% protected and significant quantities of both isomers being found in the adipose tissue depots and liver to demonstrate this. The decrease in monounsaturated fatty acids with CLA feeding was indicative of an inhibition of SCD enzyme activity; this is likely to be due to direct action of CLA on the SCD enzyme complex and not to be mediated through transcription of the SCD gene, as levels of SCD mRNA were shown not to be affected by CLA feeding. The results presented in this thesis show that sheep do not respond to CLA in terms of a reduction in body fat in the same way as other species such as rodents and pigs.

A CLA feeding trial in hamsters demonstrated changes in fatty acid composition indicative of inhibition of SCD activity, but again no change in SCD mRNA levels. This is consistent with the sheep, and suggests the inhibition of SCD activity by CLA is an effect common across species. However, the hamster seems to respond differently in that adipose tissue ACC and FAS mRNA was increased with CLA feeding, but there was no increase in fat pad weights suggesting that fat oxidation was also increased. Carcass fat was decreased by 10% with CLA feeding; however there was an increase in liver weight, so it is possible that the fat was being mobilised from the carcass and stored in the liver in response to CLA feeding. There was no effect of CLA

on liver ACC or FAS mRNA, ruling out the possibility of increased hepatic lipogenesis being responsible for the increase in liver weight. Adipose tissue SREBP-1c mRNA is increased by CLA feeding, suggesting that ACC and FAS are not directly affected by CLA but up-regulated by the increase in availability of this transcription factor.

This thesis demonstrates that CLA has no effect on ovine lipogenesis, so is unlikely to be useful as a method of reducing fat associated with lamb, and has unfavourable effects on the fatty acid profile, due to the inhibition of SCD enzyme activity. The amount of CLA in the tissues is not increased to any large degree, therefore to significantly increase dietary intake of CLA it would be necessary to take in more saturated fatty acid, present as a consequence of CLA enrichment, which from a health point of view is not desirable. Taken together with the negligible repartitioning effects seen in growing sheep (Wynn, 2003); there is no commercial reason for feeding CLA to meat producing animals.

On the other hand, work in the hamster, a species shown to respond to CLA demonstrates that the effects of CLA act on SREBP-1c, a transcription factor responsible for the up-regulation of many lipogenic genes, including ACC and FAS. This may be activated in response to the drop in total carcass fat seen with CLA feeding. The reason for the lack of effect of CLA on fat pad weights is more perplexing; it may be that CLA increases fatty acid oxidation, and the resultant decrease in body fat stores activates SREBP-1c. The mechanism by which CLA works is far from elucidated, and the results in this thesis and in the literature demonstrate that it has variable effects both within and between species, and that results require careful interpretation in an attempt to verify how CLA has its effects on lipid metabolism. However, in neither species was there any evidence to suggest that CLA works through inhibiting lipogenesis.

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