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# **The effect of synthetic and bovine conjugated linoleic acid on energy balance**

A thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Nutritional Science at Massey University, Palmerston North, New Zealand

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1999**

## ABSTRACT

Conjugated linoleic acid (CLA) is biologically active and has altered body composition in experimental animals. Dietary supplementation with synthetic CLA reduced body fat in mice and rats in a number of studies. The CLA used in previously published research contained mixed isomers, the majority of which were 9c11t-CLA and 10t12c-CLA. The biologically active isomer at the time of starting the trials described in this thesis was assumed to be 9c11t-CLA, due to the prevalence of this isomer in biological tissues.

The two trials in this thesis were designed to investigate the effect of dietary CLA on energy balance. In the first (refer Abstract, section 2.1), synthetic CLA reduced body fat in male BALB/c mice in a dose response manner, over the range 0.25 to 1.0 % w/w CLA in the diet. High levels (1.0 % and 2.0 %) caused a reduction in growth. In the second (refer Abstract, section 3.1) dietary treatments supplemented with synthetic CLA, or bovine CLA in milk fat, at levels similar to the 0.25 % w/w synthetic CLA treatment found to be effective in reducing body fat in mice, had no effect on energy balance in female Sprague-Dawley rats.

The CLA in milk fat contains approximately 86 % of the 9c11t-CLA isomer while synthetic CLA contains approximately 37 % 9c11t-CLA and 46 % 10t12c-CLA. Results from these two trials support recent evidence from research demonstrating 10t12c-CLA is the biologically active isomer, in relation to energy metabolism and body composition.

9c11t-CLA is the prevalent isomer of CLA found in the human diet. The CLA used in previously published research was chemically synthesised and contained a considerably higher proportion of 10t12c-CLA than found in human food sources.

**PREVIOUS PUBLICATION:** The study described in Chapter 2 has been previously published as an abstract and displayed as a poster presentation at the Pacific Partners in Nutrition Conference, held at Auckland, New Zealand, September, 1999 (Hayman, *et al.*, 1999).

## ACKNOWLEDGEMENTS

I wish to thank my family for support, interest in my work, and for willingly providing me with time to complete this work. Particularly my husband Neil, and Tim and Craig. Thanks also to my parents, for company and meals, during some of the time at Massey University.

Sincere thanks are given to my supervisors Dr Hilary Green (Milk and Health Research Centre) and Dr Rodger Pack (Massey University) for their valued opinions, encouragement, commitment and sharing their many years of experience. Special thanks to Hilary for assistance with experimental protocol, guidance during bench work and comments during draft writing.

The Milk and Health Research Centre (MHRC), which is jointly funded by the New Zealand Dairy Board and New Zealand Dairy Research Institute, funded this work. My employers, the New Zealand Dairy Board, granted study leave. Massey University assisted financially with a Massey Masterate scholarship, as did M&HRC with a scholarship in Milk and Health. I would like to express my appreciation to the M&HRC, NZDB and Massey University for making the opportunity to complete a higher degree possible.

I wish to thank Marie Russel and Florence Chung in the Nutrition Laboratory of the Institute of Food, Nutrition and Human Health (IFN&HH), Massey University, for analytical services in determining fat, protein, ash and moisture.

The mice in the energy balance study described in Chapter 2 were also used in a separate study investigating the effects of CLA on immune function (Zhao, 1999). Thanks are due to Dr Kay Rutherford and Hui Zhao for sharing animals and facilities during the mouse CLA feeding trial.

Thanks are due to Bertram Fong and Dr Alastair MacGibbon for CLA analysis and method development carried out at New Zealand Dairy Research Institute.

I would also like to thank the following people;

Dr Linda Schollum, Chris Booth and Anne Broomfield of M&HRC for advise and assistance in working with animals.

The staff at the Small Animal Production Unit for use of facilities.

Margaret Scott and Justine Shoemark at the Food Evaluation Unit of NZ Institute Crop and Food Research Ltd for use of facilities.

Brett Guthrie and John Pedley in the Physiology Laboratory (IFN&HH) for use of facilities and assistance with calorimetry. The musical and humorous working atmosphere in the Laboratory was also much appreciated.

Dr Kay Rutherford, for assistance with Animal Ethics approval, and staff in the Immune Laboratory of the M&HRC for assistance in obtaining blood and body composition samples.

Nicky Frearson and Sally Robinson in the Nutrition Laboratory (M&HRC) for assistance with animal and laboratory work.

Dr Phil Pearce (IFN&HH) for the free fatty acid analytical service.

Don Thomas for use of sample preparation facilities at The Poultry Research Centre (IFN&HH).

Roger Kissling (NZDB) and Dr Barbara Kuhn-Sherlock (M&HRC) for statistical advise.

## TABLE OF CONTENTS

	Page
Abstract	iii
Previous Publication	iii
Acknowledgements	iv
Table of contents	vi
List of tables	xi
List of figures	xiii

### Chapter 1 Review of the Literature

<b>1.1</b>	<b>Conjugated linoleic acid in the diet</b>	1
1.1.1	Introduction	1
1.1.2	CLA content of food	2
1.1.3	Dietary CLA intake	3
1.1.4	CLA content in the infant diet	4
1.1.5	CLA content of cow's milk	6
1.1.6	Effect of processing and storage on dairy product CLA level	9
1.1.7	Effect of dietary CLA on human blood CLA level	12
1.1.8	CLA derived from linoleic acid in non-ruminants	12
1.1.9	CLA in milk from other mammals	14
<b>1.2</b>	<b>Biological activity of CLA</b>	15
1.2.1	Biologically active isomer	15
1.2.2	Cancer	16
1.2.3	Immune	18
1.2.4	Atherosclerosis	18
1.2.5	Bone formation and resorption	19
1.2.6	Energy partitioning and metabolism	20
1.2.6.1	Body weight and food intake	20
1.2.6.2	Body composition	21
1.2.6.3	Energy metabolism	23

<b>1.3</b>	<b>Measurement of energy balance</b>	<b>25</b>
1.3.1	Energy balance equation	25
1.3.1.1	Measurement of energy expenditure	26
1.3.2	The role of brown adipose tissue in diet induced thermogenesis	27
<b>1.4</b>	<b>Summary</b>	<b>29</b>

## **Chapter 2 Trial 1; Investigation of the Effect of Synthetic Conjugated Linoleic Acid on Energy Balance in Mice**

<b>2.1</b>	<b>Abstract</b>	<b>32</b>
<b>2.2</b>	<b>Experimental materials and procedures</b>	<b>34</b>
2.2.1	Animals and housing	34
2.2.2	Mouse diets	34
2.2.2.1	Relationship of mouse diet CLA level to the New Zealand and Australian dietary CLA level	35
2.2.3	Experimental procedure	36
<b>2.3</b>	<b>Physical and chemical methods</b>	<b>37</b>
2.3.1	Intake of food and energy	37
2.3.1.1	Food intake	37
2.3.1.2	Feed efficiency	38
2.3.1.3	Mouse diet composition	38
2.3.1.31	Preparation of mouse diets for chemical analysis	38
2.3.1.23	Compositional analysis	38
2.3.1.33	Conjugated linoleic acid	38
2.3.1.34	Fatty acid profile of Tonalin and corn oil	40
2.3.1.4	Energy intake	40
2.3.2	Energy Expenditure	40
2.3.2.1	Calorimetry	40
2.3.2.2	Activity	42
2.3.2.3	Sleep/wake pattern	43
2.3.2.4	Serum free fatty acid content	43
2.3.3	Body composition	44
2.3.3.1	Live weight	44

2.3.3.2	Measurement of body composition	44
2.3.3.21	Body composition by dissection	44
2.3.3.22	Body composition by chemical analysis	45
2.3.3.22.1	Preparation of mouse samples for chemical analysis	45
2.3.3.22.2	Fat determination	46
2.3.3.22.3	Nitrogen determination	46
2.3.3.22.4	Dry matter determination	46
2.3.3.22.5	Ash determination	46
2.3.3.22.6	Gross energy determination	46
2.3.3.22.7	Formula used to calculate mouse body composition	47
<b>2.4</b>	<b>Results</b>	50
2.4.1	Data analysis	51
2.4.1.1	Data excluded from analysis	51
2.4.2	Mouse deaths	51
2.4.3	Food intake	52
2.4.4	Energy expenditure	54
2.4.5	Body composition	59
<b>2.5</b>	<b>Discussion</b>	71
2.5.1	Mouse deaths	71
2.5.2	Food intake	71
2.5.3	Energy expenditure	72
2.5.4	Body composition	73
2.5.5	Isomers	75
2.5.6	Further research	75

**Chapter 3 Trial 2; Investigation of the Effect of Bovine Conjugated Linoleic Acid on Energy Balance in Rats**

<b>3.1</b>	<b>Abstract</b>	76
<b>3.2</b>	<b>Experimental materials and procedures</b>	78
3.2.1	Animals and housing	78
3.2.2	Rat diets	78
3.2.3	Experimental procedure	81

<b>3.3</b>	<b>Physical and chemical methods</b>	83
3.3.1	Intake of food and energy	83
3.3.1.1	Food intake	83
3.3.1.2	Feed efficiency	83
3.3.1.3	Rat diet composition	83
3.3.1.31	Preparation of rat diet samples for chemical analysis	83
3.3.1.32	Compositional analysis	84
3.3.1.33	Fatty acid profile, including conjugated linoleic acid	84
3.3.1.4	Energy intake	84
3.3.1.5	Faecal gross energy	84
3.3.1.6	Digestible energy	84
3.3.2	Energy expenditure	85
3.3.2.1	Calorimetry	85
3.3.2.11	Preliminary studies	85
3.3.2.12	Calorimetry measurements on trial rats	87
3.3.2.13	Activity	88
3.3.3	Body composition	88
3.3.3.1	Live Weight	88
3.3.3.2	Measurement of body composition	89
3.3.3.21	Body composition by dissection	89
3.3.3.22	Body composition by chemical analysis	89
3.3.3.22.1	Preparation of samples for chemical analysis	89
3.3.3.22.2	Fat determination	90
3.3.3.22.3	Nitrogen determination	90
3.3.3.22.4	Dry matter determination	90
3.3.3.22.5	Ash determination	90
3.3.3.22.6	Formula used to calculate rat body composition	90
<b>3.4</b>	<b>Results</b>	92
3.4.1	Data analysis	92
3.4.1.1	Data excluded from analysis	93
3.4.2	Food intake	93
3.4.3	Energy expenditure	96

3.4.3.1	Diet induced thermogenesis	97
3.4.4	Body composition	98
<b>3.5</b>	<b>Discussion</b>	103
3.5.1	Food intake	103
3.5.2	Energy expenditure	105
3.5.3	Body composition	106
3.5.4	CLA isomers	106
3.5.5	Milk samples	107
<b>Chapter 4 General discussion</b>		109
<b>References</b>		114
<b>Appendices</b>		122

## LIST OF TABLES

Table		Page
2.1	Ingredient composition of mouse diets	35
2.2	Protocol for trial 1	36
2.3	Mouse activity score	43
2.4	Fatty acid composition of corn oil and Tonalin	52
2.5	Composition of mouse diets	52
2.6	Feed intake, weight gain and feed efficiency	53
2.7	Oxygen consumption, oxygen consumption/bodyweight, carbon dioxide production, respiratory quotient, energy equivalence of oxygen, metabolic rate and activity score for mice after two weeks of CLA feeding trial	56
2.8	Oxygen consumption, oxygen consumption/body weight, carbon dioxide production, respiratory quotient, energy equivalence of oxygen, metabolic rate and activity score and serum free fatty acid for mice at the end of CLA feeding trial	57
2.9	Live weight, weight gain, carcass weight and comparison of group means	62
2.10	Body fat, relative and absolute, by dissection and comparison of group means	63
2.11	Body fat, relative and absolute, by chemical analysis, and gross energy content and comparison of group means	64
2.12	Lean body mass; moisture, protein and ash and comparison of group means	69
2.13	Lean body mass and comparison of group means	70
3.1	Ingredient composition of rat diets	79
3.2	Protocol for trial 2	81
3.3	Rat activity score	88
3.4	Trial diet composition	93
3.5	Diet fatty acid profile	94

3.6	Feed intake, weight gain and feed efficiency for whole trial	95
3.7	Energy intake, faecal energy and digestible energy in week four of trial	95
3.8	Rate of oxygen consumption, rate of carbon dioxide production, respiratory quotient, energy equivalence of oxygen and metabolic rate for fasted rats	96
3.9	Rate of oxygen consumption, rate of carbon dioxide production, respiratory quotient, energy equivalence of oxygen and metabolic rate for fed rats	96
3.10	Difference in fed and fasted respiratory quotient, energy equivalence of oxygen and metabolic rate between fed and fasted rats	97
3.11	Mean oxygen consumption, carbon dioxide, respiratory quotient and metabolic rate for all rats grouped together when fed or fasted	98
3.12	Live weight and body fat, relative and absolute, by dissection and chemical analysis	101
3.13	Components of lean body mass; moisture, protein, ash, lean body mass relative and absolute, by chemical analysis	102
Appendix Table 1.1	CLA content of food	122
Appendix Table 1.2	CLA content of dairy products	124
Appendix Table 1.3	CLA content of breast milk	126
Appendix Table 1.4	CLA content of infant formula	126
Appendix Table 1.5	Feeding trials investigating the CLA content of cow's milk	127
Appendix Table 1.6	Effect of CLA supplemented diet on body weight, food intake and feed efficiency in animal feeding trials	129
Appendix Table 2.1	Ingredient composition of pre-acclimatisation and acclimatisation diets	133

## LIST OF FIGURES

<b>Figure</b>		<b>Page</b>
Fig 1.1	Chemical structure of linoleic acid and 9c11t-CLA	1
Graph 2.1	Metabolic rate versus activity	58
Graph 2.2	Body fat (dissection) % versus diet CLA %	65
Graph 2.3	Body fat (chemical) % versus diet CLA %	66
Graph 2.4	Gross energy versus diet CLA %	67
Graph 2.5	Body fat (dissection) % versus body fat (chemical) %	68

# CHAPTER 1

## Review of the Literature

### 1.1 Conjugated Linoleic Acid in the Diet

#### 1.1.1 Introduction

Linoleic acid is an essential polyunsaturated 18-carbon fatty acid containing two double bonds in the 9 and 12 positions (*cis*-9,*cis*-12 (*c9,c12*)-octadecadienoic acid). Conjugated linoleic acid (CLA) is a collective term referring to a group of linoleate derivatives with conjugated double bonds in positions 9 and 11 or 10 and 12, and geometric (*cis* or *trans*) isomers. There are eight possible positional and geometric isomers of 9,11- and 10,12-CLA (9,11:8,10 ct/tc, 11,13:10,12 ct/tc, cc, tt).

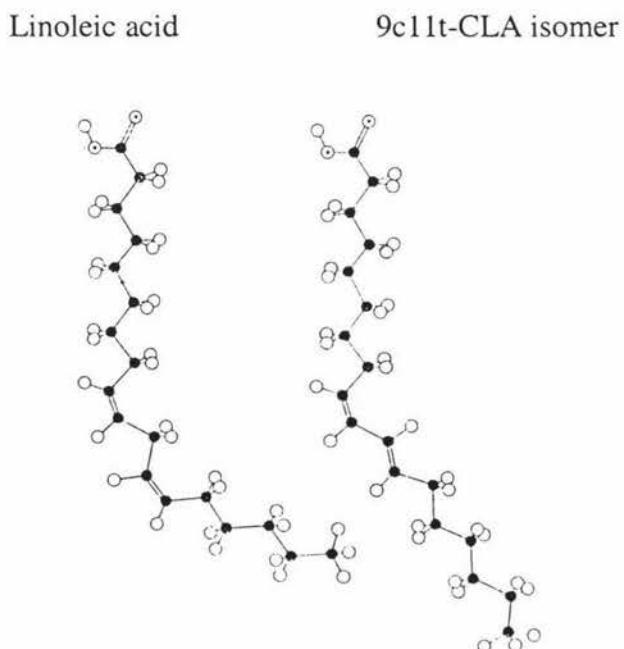


Fig 1.1. The chemical structure of linoleic acid (*c*-9,*c*-12-octadecadienoic acid) and the 9c11t-CLA isomer (*c*-9,*t*-11-octadecadienoic acid) (Chin *et al.*, 1992).

Dietary CLA is produced in ruminants and is present in their meat and milk. The ruminant bacteria *Butyrivibrio fibrisolvens* utilises a linoleic acid isomerase to produce 9c11t-CLA as a first intermediate in the biohydrogenation of dietary linoleic acid. CLA produced in the rumen pass to the small intestine where they are absorbed, re-esterified and circulated to all parts of the body (Parodi, 1994).

### 1.1.2 CLA Content of Food

Products containing fat from ruminant animals are the major sources of natural CLA in the diet. These include dairy products which contain milk fat, and meat and meat products from beef and sheep. Minor sources of CLA include vegetable oils, and non-ruminants, turkey and pork.

The CLA content in the fat of dairy products and a range of other foods has been reported in detail by (Chin *et al.*, 1992; Shantha *et al.*, 1994 & 1995; NZDRI, Fong, 1998). The total fat content of the foods surveyed is reported by Shantha *et al.* (1994 & 1995), and NZDRI, Fong (1998), but not by Chin *et al.* (1992). The CLA content of food as consumed, can be calculated from the work of the first two researchers. Chin *et al.* (1992) and Shantha *et al.* (1994 & 1995) have reported the proportion of the 9c11t-CLA isomer in CLA in food. Appendix Table 1.1 shows the CLA content of food, excluding dairy products, and Appendix Table 1.2 the CLA content of dairy products.

Dairy products are one of the richest sources of CLA. Additional researchers have published the CLA content of dairy products (Ha *et al.*, 1989, Lin *et al.*, 1995).

The amount of CLA in the milkfat of products analysed ranges from 8 - 12 mg / g fat. The 9c11t-CLA isomer accounts for approximately 80 - 90 % of the total CLA. Dairy products with high fat content are the main foods with high CLA levels. These levels range from 649 - 993 mg CLA / 100 g butter, 136 - 354 mg CLA / 100 g cheddar cheese, 76 - 140 mg CLA / 100 g sour cream, 50 mg CLA / 100 g icecream and 24 mg CLA / 100 g milk.

A number of factors influence the CLA content in dairy products. The most important are the CLA content of the raw milk (refer section 1.1.5) and the fat content in the product. Processing is less important, but may enhance CLA content (refer section 1.1.6).

Dairy products from New Zealand have a high CLA content in comparison to products from the USA because of the high CLA content in New Zealand milk eg Cheddar cheese from USA has 160 mg CLA / 100 g cheese compared with 354 mg CLA / 100g cheese in New Zealand cheddar. The country of origin of dairy food samples is included in these tables since milk from different countries has different CLA content.

Other foods containing high amounts of CLA are beef (approximately 6 mg / g fat), shortening (460 mg / 100 g shortening), and processed foods such as meat patty (170 mg / 100 g patty), luncheon meat (98 mg / 100 g luncheon) and mince pie (50 mg / 100 g pie).

### 1.1.3 Dietary CLA Intake

Typical dietary levels of CLA have been reported in New Zealand and Australia.

The New Zealand daily dietary intake for males is 570 mg CLA and for females 460 mg CLA (NZDRI, Fong, 1998). For Australia the daily intake reported is 500 to 1500 mg CLA per day (Parodi, 1994). On a body weight basis (70 kg person) the Australian daily CLA intake is equivalent to 7 to 21 mg / kg.

The estimate of dietary intake of CLA in New Zealand was derived from the CLA content of a limited number of foods – milk, butter, cheddar cheese, pastry, shortening and mince pie. Consequently the estimate is probably lower than actual intake. More data is needed on the CLA content of other important CLA containing foods such as New Zealand beef and sheep meat, a wider range of cheeses, processed cheese, yoghurt, icecream and dairy desserts. For people who eat these foods, the CLA intake may be closer to 1000 mg each day. The range of dietary intake in Australia is explained by individual dietary preferences (Parodi, 1994).

Dietary CLA is mainly obtained from ruminant meat and dairy products. Consumption of these foods by individuals will affect daily CLA intake, and wide individual variation can be expected. For instance, vegetarians and lactovegetarians will have considerably lower intakes than individuals consuming beef, sheep meat or dairy products daily. Additionally, in countries where the CLA content of milk is low (refer section 1.1.5), dietary CLA intake will generally be lower than in a country such as New Zealand where milk CLA levels are relatively high.

#### 1.1.4 CLA Content in the Infant Diet

The CLA content in the infant diet will depend on whether the infant is breast or formula fed, and the CLA content of breast milk or formula.

##### (a) Breast milk

McGuire *et al.* (1997) and Jensen *et al.* (1998) have analysed the content of CLA in breast milk. Appendix Table 1.3 contains the CLA content of breast milk.

Studies show that fatty acids in the maternal diet appear in milk. The amount of CLA in milk will depend on the amount in the maternal diet (Jensen *et al.*, 1998). The majority of CLA in the maternal diet is from ruminant derived products and consequently maternal consumption of these products will effect the CLA content of breast milk.

Studies showing that fatty acids in the maternal diet appear in breast milk do not explain the higher concentration of the 9c11t isomer of CLA in maternal milk CLA, compared with the maternal diet. The major contributors to CLA in the maternal diet – ruminant derived products such as dairy products contain approximately 80 – 90% of this isomer. In maternal milk, the samples contained 83 % to 100 % of this isomer (28 out of 34 samples contained 100 % 9c11t-CLA). Assuming analytical techniques are accurate, this data suggests dietary 9c11t-CLA is selectively concentrated in breast milk.

Jensen *et al.* (1998) suggest small quantities of CLA might be found in milks from women who consume no ruminant foods. They suggest the CLA could be produced by

the action of intestinal flora upon linoleic acid, as seen in rats (refer section 1.1.8). More research is required to test this hypothesis.

Although data on human milk CLA levels is scarce, information to date indicates it is universally present. This will mean breast feed infants consume CLA on a daily basis during the entire nursing period. An estimate of the amount of CLA consumed can be calculated from levels in breast milk (refer Table 1.3). Jensen *et al.* (1998) found 14 to 28 mg CLA / 100 g milk during the period three weeks postpartum. Based on the consumption of 700 ml of breast milk per day, the breast fed infant will consume daily from 98 to 196 mg CLA. This is equivalent to 26 to 53 mg CLA / kg body weight for a 3.7 kg baby (breast milk intake and baby weight from Linder, 1991, modified from WHO, 1985e).

More research is required investigating the effect of maternal intake of CLA on breast milk levels. Published data on breast milk CLA levels is from the U.S.A. population, and we can expect other populations to have a greater daily CLA intake eg Australia, New Zealand. It would be interesting to find out if high and low levels of maternal dietary CLA intake are directly related to breast milk levels, or if the body has a mechanism of ensuring at least a minimum is produced and/or restricting the upper level.

(b) Infant formulae

The CLA content of infant formula is determined by the level in constituent ingredients and the proportion of these ingredients in the formula. Chin *et al.* (1992), Shantha *et al.* (1995), McGuire *et al.* (1997), have analysed the CLA content of fat in infant formula. The CLA content of fat in infant formula is shown in Appendix Table 1.4.

The total daily intake of CLA for the formula feed infant will depend on the CLA content of the formula, and the volume of formula consumed. Of the milk based infant formulae analysed, two contained undetectable amounts of CLA, one contained 0.3 mg CLA / g fat, and one contained 2.2 mg CLA / g fat. Variation in the CLA content of infant formula is influenced by the source of fat, which is generally plant oil. Sometimes

milk fat is also a component. Plant oils contain very little CLA. Daily CLA intake obtained from the formula containing the most CLA, 2.2 mg / g fat, assuming 3.5 % fat formula when consumed, and consumed at the rate of 700 ml formula / day, will provide 54 mg CLA per day. For a 3.7 kg baby this is 15 mg CLA / kg body weight (breast milk intake and baby weight from Linder, 1991, modified from WHO, 1985e). Infants consuming the infant formulae containing undetectable amounts of CLA will obtain no CLA. The CLA consumption for infants consuming these infant formulae will range from zero to approximately 54 mg daily. CLA intake for formula fed babies is considerably less than breast-fed babies.

(c) Comparison between Adult and Infant CLA intake

Data on the typical adult and infant daily CLA intake shows a considerable range for both adults and infants. From the data available, the breast-fed infant receives from one to seven times as much CLA per kg body weight as an adult. The infant consuming formula with undetectable amounts of CLA obtains considerably less than adults, while infants consuming the infant formula containing the highest level of CLA (2.2 mg / g fat) will have an intake in the middle of the adult range (on a per kg body weight basis).

#### 1.1.5 CLA Content of Cow's Milk

The CLA content of cows milk has been studied widely and the results from recent work (Jiang *et al.*, 1996; NZDRI, MacGibbon and Hill, 1997; Stanton *et al.*, 1997; Jahreis *et al.*, 1997; Kelly *et al.*, 1998) are summarised in Appendix Table 1.5. CLA is present in the milk fat fraction, and the content reported in these articles, in some cases, does not distinguish the proportion of isomers. Chin *et al* (1992) found the prevalent 9c11t-CLA isomer in milk fat to account for 92 %, and Stanton *et al.* (1997) found only this isomer, in levels sufficient for quantification in milk fat samples.

Typical levels of CLA in milk fat have been reported as 3-6 mg / g fat (Kelly *et al.*, 1998). Worldwide, it is probably not appropriate to consider the level of CLA in milk fat to belong within a narrow typical range. Throughout the world, farming factors affecting CLA levels are too numerous and varied to produce this typical milk. When

cow diets are not manipulated to a major extent, levels of CLA, across several countries, ranged from approximately 3.5 to 19 mg / g fat. With cow diet supplementation, the level reached up to 24 mg / g fat.

Evaluation of the importance of factors affecting milk fat CLA levels from different studies (Jiang *et al.*, 1996; NZDRI, MacGibbon and Hill, 1997; Stanton *et al.*, 1997; Jahreis *et al.*, 1997; Kelly *et al.*, 1998) is complicated by a wide variety of trial conditions. These have included two-week diet treatment periods with cows in individual tie stalls, all year round outdoor grazing, in five different countries (New Zealand, Ireland, Germany, Finland, Sweden), using different cow breeds (Friesian, Jersey, Holstein, Swedish Red and White). In addition, it is probably inappropriate to consider some of the milk fat CLA levels achieved in these trials as commercially practical when production cost and milk yield are considered.

Dietary regimen is an important factor, with the suggestion that diets rich in polyunsaturated fatty acids (Jahreis *et al.*, 1997), and particularly linoleic acid (Kelly *et al.*, 1998) – either from supplemented plant oils or green fodder and grass/legume silages – enhanced CLA levels. It is likely that this provides the substrate for biohydrogenation and formation of CLA in the rumen.

Although dietary supplementation may increase CLA content it is also important to measure the effect of supplementation on other components of milk composition. Unfortunately, details of the effect of these dietary regimens on milk yield, protein and fat in comparison to relevant controls are not reported in detail by most researchers. One researcher, Kelly *et al.* (1998) did report low milk fat levels (mean 2.25 %) for all plant oil supplemented diets, compared with pre-treatment levels (mean 3.38 %).

One study showed no effect due to stage of lactation (Stanton *et al.*, 1997), although milk samples from extremes of lactation were not measured. The New Zealand study (NZDRI, MacGibbon and Hill, 1997) where stage of lactation and season coincide, showed a seasonal effect, which may be due to seasonal feed composition changes and/or stage of lactation. Summer or mid-lactation milk CLA values tended to be lower.

Cow breed was important in the one study designed to investigate this effect. The New Zealand study (NZDRI, MacGibbon and Hill, 1997) showed Friesian and Jersey breeds produce milk fat containing significantly different levels of CLA. Friesian milk typically contained 9 – 19 mg CLA / g fat and Jersey milk 6 – 13 mg CLA / g fat.

In all studies, a substantial individual cow variation was observed, indicating that many factors, including individual genetics, may be affecting ruminal production and subsequent milk fat CLA concentrations.

The CLA in milk fat from New Zealand is considerably higher than most other milk fat samples. In the New Zealand studies, milk fat samples were obtained from typical factory butter or the milk of herds supplying a dairy factory. The mean CLA content of milk fat from butter was 11 mg / g with a range of 7 – 15 mg / g. In studies conducted in other countries, the diets of cows were manipulated before measuring the CLA in milkfat. This milk was not typical of that used to make local dairy products. Holstein cows on a diet formulated with corn silage, hay, corn grain, soybean meal and protein supplement, supplemented with peanut oil, sunflower oil or linseed oil produced milk fat with the highest CLA levels (means 13, 24, 17 mg / g respectively) (Kelly *et al.*, 1998). In this study, total milk fat was reduced by approximately one third. One trial group of Swedish Red and White cows on a restricted 35:65 forage to concentrate ration produced 11 mg / g CLA in milkfat (Jiang *et al.*, 1996). The levels obtained in trials using supplementary feeding in Ireland (means 3.9, 5.2, 5.9, 7.9 mg CLA / g fat) (Stanton *et al.*, 1997) and Germany (means 3.4, 6.1, 8.0 mg CLA / g fat) and two trials in Sweden (means 5.0, 6.6 mg CLA / g fat) (Jiang *et al.*, 1996) were less than the “typical” New Zealand levels.

The conclusions from these trials are;

- (i) There is considerable individual cow variation in milk CLA level.
- (ii) Milk CLA levels can be elevated by manipulating cow diet.
- (iii) Additional factors influencing milk CLA levels may include cow breed, lactation number of cow, time of season and/or stage of lactation.
- (iv) Milk from New Zealand cows typically contains high CLA levels. This is believed to be a result of pasture feeding.

### 1.1.6 Effect of Processing and Storage on Dairy Product CLA Content

The CLA content of dairy products is dependent on three major factors;

- (i) The CLA content of the raw milk (refer section 1.1.5), formed through microbial enzymatic reactions, converting linoleic acid to CLA in the rumen. The CLA is digested and absorbed into the blood and incorporated into milk in the mammary gland.
- (ii) The fat content of the product, as CLA is contained in the fat fraction of milk.
- (iii) Further isomerization reactions during processing (Lin *et al.*, 1995).

There are a number of papers (Ha *et al.*, 1989; Chin *et al.*, 1992; Shantha *et al.*, 1992; Shantha & Decker, 1993; Lin *et al.*, 1995; Shantha *et al.*, 1995) investigating the effect of processing on CLA content in dairy products.

The early work of Ha *et al.* (1989) and Chin *et al.* (1992) showed some dairy products contain more CLA, on a fat basis, than others. The conclusion, based on very few samples of each type of dairy product, was that processing enhances product CLA levels. As more data became available it showed the differences in CLA content between alternate brands of the same product was often larger than the differences between alternate types of products (Lin *et al.*, 1995). This suggests variation in the CLA content of the raw milk and variation in the fat content of the product is more important than any processing effect.

Most of the literature discussing the effect of processing on CLA levels uses as a basis, the CLA content in milk fat of various surveyed dairy products. Processing factors, which may effect these levels, are retrospectively suggested.

Controlled studies examining the effect of a processing condition on product CLA level are rare. Shantha and colleagues investigated CLA concentrations in processed cheese in a series of experiments (Shantha *et al.*, 1992; Shantha & Decker, 1993). In this work they used bench scale conditions, mixing ingredients in a glass beaker, to manufacture

processed cheese from cheddar cheese. In their first set of experiments, they found that processing cheddar cheese at temperatures of 80 °C and 90 °C under atmospheric conditions significantly increased CLA content, while processing under nitrogen (70°C, 85°C), had no effect. Increasing concentrations of whey protein concentrate (WPC), from 0 to 6% increased CLA formation. They suggested the presence of air during processing could result in the formation of oxygen radicals, such as the hydroxyl radical. This is known to initiate lipid oxidation by causing the formation of lipid free radicals eg formation of the linoleic acid radical (possibly enhanced by heat), which rearranges to form the conjugated dienyl radical. Components in the WPC fraction may interact with the conjugated dienyl radical, eg by donating hydrogen, to give rise to CLA.

In their next set of experiments, the effect of hydrogen donors and a free radical initiating system on CLA concentrations were investigated. Hydrogen donors – butylated hydroxytoluene (BHT), ascorbic acid, cysteine and dairy additives (sodium caseinate, skim milk powder, whey powder), and a free radical initiating system  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$ , all significantly increased the level of total CLA. The greatest increase, of 1.65-fold total CLA compared to process cheese with no additive, occurred when sodium caseinate was added at a concentration of 6 %. The 9c11t-CLA isomer proportion was 64% compared with 80% in the control. High levels of 9c11t-CLA occur in milk fat through enzymatic isomerisation of linoleic acid by rumen bacteria. The formation of CLA by the various processing methods is chemical in nature, and so produces an increase in all isomers of CLA, not necessarily the 9c11t isomer (Shantha and Decker, 1993). There was no mention of flavour acceptability of cheeses made under these conditions, and it is likely that deliberate free radical initiation in products containing milk fat is not desirable.

Another series of controlled experiments investigated the effect of processing and storage on the level of CLA in dairy products (Shantha *et al.*, 1995). They measured the 9c11t-CLA and total CLA content in unprocessed raw material and finished product for salted and unsalted butter, yoghurts, sour cream, ice cream, and three types of cheeses – Mozzarella, Gouda, and Cheddar. All of the products were manufactured under normal factory conditions, except the butters, which were made in a pilot plant. Products were

stored for a period of time that was similar to their expected shelf life. They stored the yoghurts, sour cream, and butter at 4 °C for 6 weeks, the cheese at 4 °C for 8 months and the icecream and more butter at -20 °C for 6 months. The CLA content of these products during storage was stable.

Shantha *et al.* (1995) found that processing milk or cream into low fat and regular yoghurt, sour cream, cheese, and ice cream did not substantially enhance the CLA concentration. Skim milk processed into nonfat yoghurt resulted in a 1.2 fold increase, and cream into butter a 1.3 fold increase. The nonfat yoghurt contained very little CLA, and a 20 % increase represented very little additional CLA. Conversely, butter contains substantial amounts of CLA. In the study made by Shantha *et al.* (1995), the CLA content in butter ranged from 554 - 649 mg CLA / 100 g butter and in nonfat yoghurt 0.2 - 0.3 mg CLA / 100 g yoghurt. The absolute increase in butter CLA represented by a 1.3 fold increase (190 mg CLA / 100 g butter) is many times greater than the 1.2 fold increase in nonfat yoghurt (0.06 mg CLA / 100 g nonfat yoghurt). The increase in CLA observed in these products was not as great as the 3-fold variation in CLA in unprocessed milk and cream. This unprocessed milk was all obtained from the same dairy factory.

Because of the large variation in CLA content of raw milk, it is important that future experiments investigating effects of a process or storage on product CLA content, control raw milk CLA content.

The influence of aging on CLA formation was originally controversial. Lin *et al.* (1995) suggested aging is responsible for the increased CLA in sharp cheddar, compared with medium cheddar, across three brands of each. Chin *et al.* (1992) observed the opposite effect between aged and fresh cheeses. They found cheeses such as Parmesan and Romano, which were aged or ripened more than 10 months contained 3.0 and 2.9 mg CLA / g fat respectively. Bacterial surface ripened cheeses such as Brick and Muenstre, aged 4 to 8 weeks, contained 7.1 and 6.6 mg CLA / g fat respectively. It is highly likely that variation in the milk CLA content before these cheeses were made was greater than an aging effect.

It is likely that several different mechanisms exist for CLA enhancement during processing. These include heat, oxygen and antioxidants (hydrogen donors) encouraging formation of the linoleic acid free radical and subsequent formation of CLA. This may occur in processes such as churning milkfat into butter, or casein formation in cheese production. In fermented products, such as yoghurt, bacteria themselves may be responsible for biohydrogenation of linoleic acid to form CLA.

Although CLA levels may be significantly enhanced during processing, the effect on total dietary intake may not be important. A process that enhances CLA levels is most likely to make a difference to dietary intake of CLA when there is a large absolute increase in the CLA content of a product consumed on a regular basis eg butter and cheese.

#### 1.1.7 Effect of Dietary CLA on Human Blood CLA Level

A number of studies have confirmed that dietary CLA effects blood CLA level. Britton *et al.* (1992) measured 9,11-CLA in venous serum obtained from fasted human subjects who ate a "high CLA diet" including more foods rich in 9,11-CLA, or "low CLA diet", with less of these foods compared with base level. They found the concentration of 9,11-CLA in serum phospholipids was influenced by diet, increasing with increased dietary consumption.

Huang (1994) investigated the effect of Cheddar cheese consumption on plasma CLA. Nine male subjects were involved in the four week dietary intervention trial (112 g / day Cheddar cheese). The plasma phospholipid-esterified CLA concentration and plasma CLA/linoleic acid molar ratio increased during dietary intervention compared with baseline and post-dietary intervention.

#### 1.1.8 CLA Derived from Linoleic Acid in Non-ruminants

It is generally believed that CLA found in the tissues of non-ruminants is a consequence of dietary intake. Another possibility, that part may result from the conversion of linoleic acid by intestinal bacteria to CLA was investigated by Chin *et al.* (1994a). They

fed conventional or germ free rats diet fortified with free linoleic acid. Lipids extracted from liver, lung, kidney, skeletal muscle and abdominal adipose tissues were analysed for CLA. They found that CLA concentrations in the tissues of germ-free rats were not affected by diet. In conventional rats they were 5-10 times higher than controls (controls were conventional rats, diet not supplemented with free linoleic acid). In addition they included a group of conventional rats fed linoleic acid in triglyceride form – from corn oil. Feeding the corn oil-fortified diet to conventional rats did not increase tissue CLA. These results indicate that in the rat, bacteria are capable of converting free linoleic acid to CLA. These bacteria do not appear capable of converting esterified linoleic acid to CLA. The experiment did not prohibit coprophagy. The researchers were not able to determine whether the responsible microbial flora was located in the stomach, small or large intestine. Long-chain unsaturated fatty acids are not absorbed in the large intestine. If this were the location of these bacteria, absorption of CLA into tissues would occur after coprophagy.

The finding that intestinal microorganisms do not supply conjugated linoleic acid was confirmed in another rat study (Kamlage *et al.*, 1999). A linoleic acid-conjugating bacterial community isolated from human intestine was established in germ free rats. The rats were fed a diet rich in sunflower seed oil which contains esterified linoleic acid. Control rats were fed the same diet, but remained germ free. Although CLA was found in faeces and lower intestinal tract samples of the bacterial colonised rats and not the controls, CLA accumulation in tissues was not significantly different.

Free linoleic acid is not a common component of the human diet, whereas the esterified form is a major component in many plant oils, including corn and safflower oil. Herbel *et al.* (1998) investigated the effect of increased consumption of linoleic acid, in triglyceride form, on human plasma CLA levels. The experiment involved dietary intervention for a period of 6 weeks, with six men and six women in a crossover experiment. They consumed an extra 16 g / day of esterified linoleic acid, by consumption of a salad dressing made with safflower oil. Linoleic acid intake increased significantly during the dietary intervention, and there was no change in plasma CLA concentrations.

The experiments outlined in sections 1.1.7 and 1.1.8 show that dietary CLA increases content of CLA in the blood. They show that free linoleic acid, not common in the human diet, can be converted to CLA and accumulate in body tissue in the rat. The triglyceride form of linoleic acid, common in the human diet, does not increase rat tissue CLA level or human plasma CLA concentrations. From these experiments it seems blood CLA levels and body tissue CLA levels are increased by dietary CLA, but not from dietary linoleic acid (in triglyceride form).

#### 1.1.9 CLA in Milk from other Mammals

CLA is found in both human and cow milk. It is also found in milk from the rat (Chin *et al.*, 1994a).

Chin *et al.* (1994a) found that feeding a synthetic mixture of CLA isomers to rat dams during gestation and lactation increased the level of CLA in milk. In one experiment the milk of the CLA fed dams contained 46.7 µmol CLA / g milk fat, compared with 1.68 µmol CLA / g milk fat in the control group. Unfortunately details of the isomer content of CLA in the milk are not published. In a second experiment, the CLA content of milk increased as dietary CLA increased during lactation (control 0.96 µmol / g milk fat, 0.25 % CLA diet 22.3 µmol / g milk fat, 0.5 % CLA diet 46.8 µmol / g milk fat, 0 % CLA during gestation and 0.5 % during lactation 42.5 µmol / g milk fat). In these rats, dietary supplementation with CLA during lactation produced similar milk CLA levels to supplementation during both gestation and lactation

The presence of CLA in mammalian milk suggests the possibility of nutritional significance for the neonate. There is no doubt that it has always been a component in ruminant milk and a normal part of the nutritional intake for neonates from these species. The presence of CLA in the milk of other mammals appears to result from maternal consumption of CLA. CLA in human milk contains from 83 % to 100 % 9c11t-CLA (refer section 1.1.4). The biological significance of CLA to the neonate of these species is not known.

## 1.2 Biological Activity of CLA

The biological activity of CLA and the effect on cancer, immune function, atherosclerosis, bone formation, energy metabolism, and body composition is an active current field of worldwide research. The number of citations listed in a PubMed query for conjugated linoleic acid; and cancer and immune function was 49; and atherosclerosis 14; and bone formation 1; and energy metabolism 76; and body fat and body composition 15.

All of the research to date describes the positive benefits of CLA consumption<sup>1</sup>. There are no indications of adverse effects. One published toxicological evaluation reports no adverse effects in rats during a 36-week study fed a diet supplemented with 1.5 % CLA (Scimeca, 1998).

The *in vivo* CLA research has used animal models - mammals – in which CLA is consumed as a dietary supplement. In these studies, the levels of dietary CLA are reported as the proportion (%) of CLA in the diet (weight CLA / weight diet basis). The CLA isomers used, in all but the most recent research (Deckere and Rudrum, 1999, Park *et al.*, 1999a and 1999b) (refer section 1.2.1), consisted principally of approximately similar amounts of two isomers, 9c11t-CLA and 10t12c-CLA.

### 1.2.1 Biologically Active CLA Isomer

The biological activities of particular CLA isomers remain unresolved in the literature. Nearly all publications suggest the 9c11t isomer is responsible for the observed biological activities. This is based on observations of the presence of this isomer in biological tissues. It is the prevalent isomer (80 to 90% of the total CLA) found in ruminant animal fat and milk, the major natural source of CLA (Chin *et al.*, 1992, Lin *et al.*, 1994, Shantha *et al.*, 1995, NZDRI, Fong, 1998, refer section 1.1.2). It is

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<sup>1</sup> Note; three of the principle researchers (Park, Y., Pariza, M.W., and Cook, M.E.), are inventors of CLA use patents.

incorporated into the phospholipid fraction of tissues of animals fed a mixture of CLA isomers (Ha *et al.*, 1990, Ip *et al.*, 1991). It makes up 83 to 100 % of the CLA isomer found in human breast milk (McGuire *et al.*, 1997, Jensen *et al.*, 1998)).

There is no published research using CLA from a natural dietary source – milk fat or ruminant meat fat. The CLA in milk fat contains approximately 80 % to 90 % of 9c11t-CLA whereas CLA synthesised by base isomerisation of linoleic acid generally contains approximately 37 % 9c11t-CLA and 46 % 10t12c-CLA. A synthetic mixture of CLA isomers containing principally 9c11t-CLA and 10t12c-CLA has been used in all but the most recent research investigating the biological activities of CLA. It is important to be aware of the CLA isomer ratio, as well as the total CLA content, in any research investigating a dietary effect from CLA. Further research is required to elucidate the biological activity of individual CLA isomers.

Recent research, indicates that the 10t12c-CLA isomer may be the biologically active form with respect to metabolism of blood lipids – LDL, HDL, VLDL cholesterol (Deckere and Rudrum, 1999) (refer section 1.2.4). It is also indicated as the biologically active form effecting body composition changes (Park *et al.*, 1999b) (refer section 1.2.6.2), and energy metabolism (Park *et al.*, 1999a) (refer section 1.2.6.3). Deckere and Rudrum (1999) used purified CLA isomers in their studies, and Park *et al.* (1999b) used both purified CLA isomers or CLA isomer mixtures enriched with 9c11t-CLA or 10t12c-CLA.

### 1.2.2 Cancer

The effects of CLA on cancer have been extensively studied (Ha *et al.*, 1990; Ip *et al.*, 1991; Ip *et al.*, 1994; Ip *et al.*, 1995; Ip *et al.*, 1996; Shultz *et al.*, 1992; Belury *et al.*, 1996; Visonneau, Cesano *et al.*, 1996; Visonneau, Cesano *et al.*, 1997; Thompson *et al.*, 1997; Wong *et al.*, 1997; Cesano, Visonneau *et al.*, 1998). The research provides a strong body of evidence demonstrating the protection of dietary CLA against chemically induced tumour formation and promotion, in animal cancer models.

Protection by dietary CLA in several chemically induced animal tumour models was demonstrated by several researchers (Ha *et al.*, 1990; Ip *et al.*, 1991; Ip *et al.*, 1994; Ip *et al.*, 1995; Belury *et al.*, 1996; ), at levels up to 1.0 % w/w CLA in the diet. Ip *et al.* (1994) observed a dose-dependent inhibition of mammary carcinogenesis by CLA in the range 0.05 % to 0.5 % CLA in the diet. They also demonstrated that consumption of CLA during the short period of maturation of the mammary gland (weaning to 50 days) inhibited chemically induced mammary carcinogenesis in rats. This was also demonstrated by Thompson *et al.* (1997). In 1995, Ip *et al.* verified that the anticancer activities of free fatty acid-CLA and triglyceride-CLA were the same. Ip *et al.* (1996) showed that the magnitude of tumour inhibition in rats, by 1.0 % dietary CLA, was independent of the level and type of fat in the diet. Belury *et al.* (1996) showed that dietary CLA inhibited tumour promotion in the mouse epidermal cancer model.

CLA was not effective in a transplantable tumour model, where the tumour had already been initiated (Wong *et al.*, 1997) suggesting CLA may exert its effect by blocking the initiation and promotion events in tumour development

Shultz *et al.* (1992) showed CLA was cytotoxic to human cancer cells. Malignant melanoma, colorectal and breast cancer cells were incubated with CLA at a concentration likely to be present in human blood. There was a significant reduction in cell proliferation compared to control cultures. Visonneau, Cesano *et al.* (1996) also demonstrated CLA to be cytostatic and cytotoxic *in vitro* to a variety of human cancers, including melanoma, colorectal, prostate, ovarian, and breast carcinomas, and leukemia. Visonneau, Cesano *et al.* (1997) examined the effect of 1 % dietary CLA on the growth of human breast adenocarcinoma cells in severe combined immunodeficient (SCID) mice. In this model, CLA was able to block both the local growth and systemic spread of human breast cancer. Cesano, Visonneau *et al.* (1998) demonstrated dietary CLA inhibited the growth of human prostatic cancer cells and metastasis in SCID mice, while an opposite effect was observed for linoleic acid.

The CLA used in anticarcinogen studies was a synthetic mixture of isomers, mainly 9c11t and 10t12c. The efficacy of individual isomers remains to be investigated (O'Shea *et al.*, 1998).

### 1.2.3 Immune

Cook *et al.* (1993) found CLA is effective in preventing the catabolic effects of immune stimulation in both chicks and rats. Later, the same researchers found the same effect in mice, and repeated the experiment in rats and chicks (Miller *et al.*, 1994). It is known that stimulation of cells of the immune system can trigger systemic metabolic changes, which include fever, anorexia, decreased net skeletal muscle protein accretion, and thus, growth. These catabolic effects of immune stimulation may occur regularly during animal growth, particularly following vaccination. Effective reduction by dietary means could benefit animal growth and production.

In these experiments animals were injected with *Escherichia coli* endotoxin, following consumption of a diet containing 0.5% CLA, or various alternatives including 0.5% fish oil, 0.5% stearic acid, or the appropriate basal diet as control. In all animals injected with endotoxin, the group consuming dietary CLA had less weight loss at 24 hours after injection, faster rate of return of weight to the pre-injection level, and less reduction of feed consumption in the 24 hours following injection.

The CLA isomer composition of the CLA used in this experiment is not reported. The method of preparation was described by (Chin *et al.*, 1992), and in subsequent experiments reported to contain 34.5 % 9c11t-CLA, 38.8 % 10t12c-CLA and 6.9 % minor CLA isomers (Wong *et al.*, 1997)

### 1.2.4 Atherosclerosis

CLA has been reported to exhibit antiatherogenic effects. In a preliminary study, Lee *et al.* (1994), fed 12 rabbits a high fat diet containing 14 % fat (mainly coconut oil) and 0.1 % cholesterol for a 22 week experimental period. The 6 rabbits in the CLA-treatment group were each given an additional 0.5 g CLA per day. Histological examination of the aortas showed less atherosclerosis in the abdominal aortas (approaching significance  $p < 0.09$ , for plaque to wall volume ratio) and a small, but not significant reduction in the thoracic aorta of CLA-fed animals. While the results from this preliminary experiment are encouraging, the only statistically significant result was

reduction in LDL cholesterol and LDL / HDL cholesterol ratio in the CLA fed group. The CLA isomer composition was not reported.

Nicolosi *et al.* (1997) showed that dietary CLA in a high saturated fat and cholesterol diet, suppresses plasma lipoprotein cholesterol and lipid levels, and aortic fatty streak formation in hamsters. It is more effective than linoleic acid at reducing plasma total cholesterol and non-HDL cholesterol. Remarkably, very low levels of dietary CLA (0.025 %) had a similar effect, and in the case of lipid levels, a greater effect, than a 20-fold higher dose. The CLA was composed of a mixture of isomers, the majority of which were 9c11t-CLA and 10t12c-CLA.

Effects of CLA isomers on lipid metabolism were investigated in hamsters whose diet was supplemented with CLA (Deckere and Rudrum, 1999). A CLA isomer mix containing equal amounts of 9c11t-CLA and 10t12c-CLA, and 10t12c-CLA alone, decreased LDL-cholesterol and HDL-cholesterol, and increased VLDL cholesterol, compared with controls. 9c11t-CLA alone had no significant effects. These results suggest 10t12c-CLA is likely to be the biologically active isomer in metabolism of these lipids.

#### 1.2.5 Bone formation and resorption

As milk is the source of the major bone building block, calcium, in mammals prior to weaning, it is likely that other components in milk assist in bone formation and growth.

CLA supplementation was observed to enhance bone ash in chicks in two experiments (Cook *et al.*, 1997).

Li and Watkins (1998) supplemented the diets of rats with 1.0 % CLA, and observed differences in CLA enrichment in various tissues, with bone tissue containing the highest concentrations of CLA. The CLA isomer mixture contained approximately equal proportions of major isomers 9c11t-CLA and 10t12c-CLA. They observed CLA altered the fatty acid composition of a range of rat tissues and that CLA depressed *ex vivo* prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production in bone organ culture. PGE<sub>2</sub> is an agent that

stimulates both bone formation and resorption, essential processes in bone growth. They observed that CLA depressed *ex vivo* PGE<sub>2</sub> production in bone organ culture. The effects of the observed PGE<sub>2</sub> depression on either bone formation or resorption are not known.

### 1.2.6 Energy Partitioning and Metabolism

#### 1.2.6.1 Body Weight and Food Intake

There are a number of publications recording the effects of dietary CLA on body weight, food intake and feed efficiency (Chin *et al.*, 1994b; Belury and Kempa-Stecko, 1997; Park *et al.*, 1997; West *et al.*, 1998). In addition, trials investigating the effects of CLA on atherosclerosis, immune function, and CLA uptake into body tissues, often include measurements of body weight and food intake (Lee *et al.*, 1994; Nicolosi *et al.*, 1997; Wong *et al.*, 1997; Sugano *et al.*, 1998; Li and Watkins, 1998). A summary of the results from ten CLA feeding trials is in Appendix Table 1.6. The isomer composition of CLA used in these feeding trials is detailed in Table 1.6. In all trials, synthetic mixtures of CLA contained approximately equal proportions of major CLA isomers 9c11t-CLA and 10t12c-CLA.

The majority of trials found no difference in body weight, food intake or feed efficiency due to CLA feeding. Body weight decreased in three trials and increased in two. Food intake decreased in one trial. Feed efficiency decreased in two trials and increased in two trials. The two trials showing an increase in body weight involved suckling or weanling rats. The dams of the suckling rats were fed diets containing CLA (refer section 1.1.9). The weanling rats had also received CLA enhanced milk during suckling from dams fed with CLA supplemented diets. Another trial with weanling mice fed a CLA enriched diet did not show a difference in body weight. The two trials showing increased feed efficiency involved weanling rats (including one trial where rats previously received CLA enhanced milk during suckling). Animals showing weight reduction, or reduced feed efficiency were older (still growing, but not mature at start of feeding trial). There were no trials using mature animals at the start of CLA feeding, and so the effect of CLA on body weight in mature subjects was not investigated.

These trials do not demonstrate a universal effect of CLA on body weight and feed intake. In the majority of cases, there is no significant effect. Suckling and weanling rats showed increased body weight and feed efficiency at this important early stage of development when milk or food was enriched with CLA. Some trials with growing animals showed reduced weight gain, which may be an adverse effect of CLA consumption during this developmental stage.

#### 1.2.6.2 Body Composition

Changes in body composition are not necessarily related to changes in body weight. For instance, a reduction in either body fat and/or lean body mass will show a weight reduction. A reduction in body fat combined with an increase in lean body mass may show no net weight change. Further increase in lean body mass will show a weight increase. Park *et al.* (1997)<sup>2</sup>, Sugano *et al.* (1998)<sup>3</sup>, West *et al.* (1998)<sup>4</sup> and DeLany *et al.* (1999) all found that dietary supplementation with CLA reduced the amount of body fat in experimental mice or rats.

Park *et al.* (1997) investigated the effects of CLA on body composition in mice and observed reduced fat deposition. In their experiments, weanling male or 7 week old female ICR mice were fed a control diet containing 5.5 % corn oil or a CLA supplemented diet (5.0 % corn oil and 0.5 % CLA). The mice were fed *ad libitum* for 4 weeks. Male mice fed the CLA-diet had body weights indistinguishable from controls. Females appeared to have a reduced body weight although it was not statistically significant. Food intakes in both groups appeared lower, but again this was not statistically significant.

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<sup>2</sup> Mixture of CLA isomers synthesised from linoleic acid as described (Chin *et al.*, 1992). The CLA isomer composition was not reported. In a subsequent study (West *et al.*, 1997), the same method of preparation was used, and the isomer composition reported was 34.5 % 9c11t-CLA, 38.8 % 10t12c-CLA and 6.9 % minor CLA isomers.

<sup>3</sup> The CLA isomer composition was 29.8 % 9c11t-CLA, 29.6 % 10t12c-CLA, 18.6 % 9t11t-CLA and 2.7 % minor CLA isomers.

<sup>4</sup> The CLA isomer composition was 39.1 % 9c11t-CLA, 40.7 % 10t12c-CLA and 4.2 % minor CLA isomers.

The major difference observed in the CLA-fed mice of both sexes was a reduction in body fat. Relative to their respective controls, the body fat percentage in males was reduced by 57 % (control diet  $10.13 \pm 1.17$  % and CLA diet  $4.34 \pm 0.40$  % body fat) and females 60 % (control diet  $18.68 \pm 3.08$  % and CLA diet  $7.47 \pm 0.59$  % body fat) (values are mean  $\pm$  SE). Despite similar body weights, dietary CLA supplementation resulted in significantly less body fat in both male and female mice.

In addition, the results showed the percentages of whole body protein and carcass water were enhanced for CLA-fed mice. These results need to be treated with some caution, as whole body protein mass for controls and CLA-fed groups, respectively, were 5.7 g and 6.0 g in males and 4.4 g and 4.6 g in females, and were not significantly different. The authors state that combined data from this and 8 more unpublished mice CLA feeding trials indicates that mice fed CLA-supplemented diets have increased whole body protein relative to controls ( $p = 0.04$ ).

Sugano *et al.* (1998) fed 4 week old male Sprague-Dawley rats a 10.0 % soybean oil diet supplemented with either 1.0 % linoleic acid, 0.5 % of both linoleic acid and CLA, or 1.0 % CLA for a three week trial period. The weight of perirenal adipose tissue in the 1.0 % CLA group (0.97 g / 100 g body weight) was significantly less than the weight in the 1.0 % linoleic acid group (1.41 g / 100 g body weight).

West *et al.* (1998) fed 6 week old male AKR/J mice a high-fat (24 %) or low-fat (6.7 %) diet with or without CLA for a six week trial period. CLA significantly reduced energy intake, growth rate, body weight, adipose tissue weight, carcass lipid and carcass protein content in both high and low fat CLA diets. CLA had the largest effect on retroperitoneal adipose depot, and less on the epididymal adipose depot. The total carcass lipid in high-fat control animals weighed  $10.77 \pm 0.67$  g while in the high-fat CLA fed animals it was  $4.08 \pm 0.26$  g. The total carcass lipid in low-fat control animals weighed  $6.28 \pm 0.56$  g, while in the low-fat CLA fed animals it was  $2.08 \pm 0.11$  g (values are mean  $\pm$  SE).

DeLany *et al.* (1999) fed 6 week old male mice maintained on a high fat diet, five doses of CLA (maximum dose 1.0 %). Body fat reduction was first noticed in the 0.5% group

compared with controls. Carcass protein content was significantly increased at high CLA levels.

CLA reduced body fat in all of these studies. In two studies (Park *et al.*, 1997; DeLany *et al.*, 1999) found carcass protein increased, while one study (West *et al.*, 1998) found a reduction in carcass protein.

There is one report of a trial investigating the effect of CLA on body fat and weight in humans. The MEDSTAT report, 1997, describes a small pilot scale human study in which two groups of ten people, consumed either 0.6 g pure CLA (2 x 500 mg capsules as Tonalin) 3 times per day (dosage of 1.6 g CLA per day), or a placebo. The groups were both five females and five males, with an average age of 28 years. The body weight of participants was weight stable during the three month study period. Body fat was measured using Futrex near infrared (NIR) technology. Although subjects were asked not to change their diet or lifestyle while participating in the study, there are no results of measurements of dietary intake or energy expenditure in the report. There was a significant reduction in body fat ( $p < 0.05$ ) in the Tonalin group from 21.3 % body fat to 17.0 % body fat, and not in the placebo group. Two out of ten in the Tonalin group withdrew due to unpleasant gastrointestinal upsets. The groups in this study were small, and it has not been reported in the published literature. The report indicated that more studies would be done, and there are no results available to date.

Recent research indicates that 10t12c-CLA is the biologically active isomer with respect to body composition (Park *et al.*, 1999b). By using semi-purified CLA isomers or CLA isomer mixtures enriched with 9c11t-CLA or 10t12c-CLA, they concluded body composition changes in mice (reduced body fat, enhanced body water, enhanced body protein and enhanced body ash) were associated with dietary 10t12c-CLA. They found no effects from the semi-purified 9c11t-CLA.

#### 1.2.6.3 Energy Metabolism

The effect of CLA on energy metabolism has been studied by Belury *et al.* (1997), Park *et al.* (1997), West *et al.* (1998) and Park *et al.* (1999a).

Belury *et al.* (1997) studied the role of dietary CLA in modulating fatty acid composition and metabolism in the liver. The liver is the major organ for lipid metabolism. They hypothesised the wide range of chemoprotective actions of dietary CLA in extrahepatic tissues are dependent upon its role in modulating fatty acid composition and metabolism in the liver. They looked at the fatty acid composition of liver from female SENCAR mice fed increasing levels of dietary CLA (0, 0.5, 1.0, 1.5 % CLA) for a period of 6 weeks. The CLA was added to a 5.0 % corn oil diet at the expense of dextrose. The CLA fed mice had lower body weights, and more extractable total lipid in livers. They found differences in the fatty acid composition of lipid extracted from the liver. CLA was incorporated into hepatic neutral and phospholipids at the expense of linoleate. In neutral lipids of CLA fed groups, oleate increased and arachidonate decreased. In phospholipids of CLA fed groups, linoleate decreased. In an *in vitro* assay, CLA was desaturated to an unidentified 18:3 product to a similar extent as linoleate conversion to  $\gamma$ -linolenate. These data suggest that CLA may affect metabolic interconversion of fatty acids in the liver. The authors postulate extrahepatic tissue fatty acid composition may be altered and consequently arachidonate-derived eicosanoid production may be modified.

Park *et al.* (1997) conducted *in vitro* experiments to measure the activity of two key enzymes in lipid metabolism, adipocyte lipoprotein lipase (LPL) and carnitine palmitoyltransferase (CPT). LPL hydrolyses free fatty acids from circulating triglycerides. Following hydrolysis the free fatty acids are taken up by the adipocytes and re-esterified. CPT is rate-limiting for fatty acid  $\beta$ -oxidation and was used as an indicator of fatty acid oxidation in cell cultures. CLA was added to the culture medium of mouse adipocytes and produced a dose-dependent reduction in LPL activity. Measurements of intracellular esterified and free glycerol, and free glycerol in the adipocyte culture media indicated increased lipolysis within cells. The activity of CPT in various cells from mice fed CLA-supplemented or control diet for one week was measured. Skeletal muscle and adipocyte cells exhibited elevated CPT activity. Overall, results suggested CLA supplementation reduced fat deposition from circulating triglycerides, increased lipolysis in adipocytes and enhanced fatty acid oxidation in muscle cells and adipocytes.

West *et al.* (1998) investigated the effects of CLA feeding on energy metabolism in mice fed high and low fat diets. They found a significant increase in the total 24 hour energy expenditure in CLA-treated animals. There was no overall effect of CLA on respiratory quotient (R) measured over 24 hours. Respiratory quotient is an index of substrate oxidation (refer 1.3.1.1). When day and night time R were compared there was a difference between the CLA and control groups. They found in animals not treated with CLA, the night time R was significantly higher than daytime R. In CLA-treated animals there was no significant day versus night R difference. CLA appeared to block the normal day-night difference in R. Mice are nocturnally more active and the observed lower day time R in the control group was explained by the mobilisation of stored lipid during the day while sleeping. The decreased diurnal R difference in CLA fed mice is explained by CLA promoting fat oxidation during the night either through mobilisation of stored lipid or impairment of incorporation of ingested lipid into adipocytes.

Recent research suggests 10t12c-CLA may be the biologically active form with respect to energy metabolism (Park *et al.*, 1999a). They recorded depletion of CLA from mouse tissue following withdrawal of mixed isomer CLA supplementation in the diet. The depletion pattern in muscle tissue showed 10t12c-CLA was depleted from tissue at a greater rate than the 9c11t-CLA. From this, the authors concluded preferential metabolism of 10t12c-CLA by skeletal muscle.

### 1.3 Measurement of Energy Balance

#### 1.3.1 Energy Balance Equation

The energy balance equation is;

$$\text{Energy Intake} - \text{Energy Expenditure} = \text{Energy Stored}$$

The components of the equation can be measured as follows; energy intake from food consumption and composition, energy expenditure from indirect calorimetry where respiratory gas exchanges are measured, and energy stored from body composition

analysis. Details of the methods used are in the methodology sections in Chapters 2 and 3 (refer sections 2.3 and 3.3).

### 1.3.1.1 Measurement of Energy Expenditure

The following is a brief outline of the theory behind the measurement of energy expenditure by indirect calorimetry (Montoye *et al.*, 1996). Warm-blooded animals use energy in three ways. Energy expenditure is the sum of;

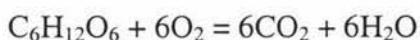
- (i) Resting metabolic rate – the energy required at rest to maintain body temperature and involuntary muscular work for functions including circulation and respiration.
- (ii) Diet induced thermogenesis (DIT) – the increase in metabolic rate that follows the ingestion of food. It has two components; obligatory DIT (the cost of absorption, digestion and storage of nutrients) and facultative DIT (the thermogenic effect of nutrients, mainly achieved by brown adipose tissue, believed to be important in some mammals (refer section 1.3.2))
- (iii) Muscular activity – the energy required for physical activity.

Indirect calorimetry techniques utilise measurement of the rate of oxygen consumption ( $\text{VO}_2$ ) and the rate of carbon dioxide production ( $\text{VCO}_2$ ) to calculate energy expenditure (refer section 2.3.2.1). This type of calorimetry is “indirect” because heat production is not measured directly. The amount of heat produced by the utilisation of 1 litre of oxygen varies with the foodstuff consumed and combusted. When fat alone is combusted, 1 litre of oxygen yields 19.59 kJ, and when carbohydrate alone is combusted 21.18 kJ. The respiratory quotient (R) is the ratio of the rate of carbon dioxide produced to the rate of oxygen consumed. In a “steady-state” the ratio is 0.70 when pure fat is burned, and 1.00 when pure carbohydrate is the source of energy.

Two major assumptions are made when using R to calculate energy expenditure. Firstly, a “steady state” is assumed – when the oxygen uptake of tissues is proportional to

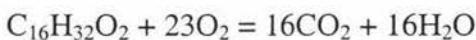
energy expenditure, and there is no anaerobic energy production ie accumulation of lactic acid or excess CO<sub>2</sub> production. Secondly, the percentage of protein being utilised for energy is ignored, because in most instances the energy equivalents of oxygen are similar for carbohydrates and protein.

(i) Respiratory quotient during carbohydrate combustion



$$R = \dot{V}\text{CO}_2 / \dot{V}\text{O}_2 = 6\text{CO}_2 / 6\text{O}_2 = 1$$

(ii) Respiratory quotient during fat combustion



$$R = 16\text{CO}_2 / 23\text{O}_2 = 0.70$$

### 1.3.2 The Role of Brown Adipose Tissue in Diet Induced Thermogenesis

Brown adipose tissue (BAT) was originally distinguished from white adipose tissue by its yellowish-brown colour, resulting from multilocular lipid droplets containing large numbers of mitochondria. In more recent times colour has become less important and the criterion for distinguishing the two forms of adipose tissue is the presence or absence of the BAT-specific mitochondrial uncoupling protein, which can be determined immunologically. BAT is capable of thermogenesis through uncoupling of oxidative phosphorylation by this 32,000-dalton uncoupling protein. BAT is specialised for facultative heat production by non-shivering means. It is believed to be important in controlling body temperature, and during maximal rates of thermogenesis eg mice adapted to cold temperatures, BAT may make a substantial contribution to energy expenditure (Trayhurn, 1995).

BAT tissue is prevalent in small animals, especially those that undergo hibernation (eg ground squirrels, hamsters), and adult rodents adapted to cold environments (mice, rats, rabbits). In these animals, BAT, in characteristic locations (eg between the scapulae in rats), is easily distinguishable from other fat deposits by its colour. It is present in the

young of many species, including human infants where it is estimated to weigh 30g in total (approximately 8 % of body fat in a 3.0 kg new born with 12 % body fat (body weight and body fat figures from Linder, 1991)). The site of BAT in humans is all central and internal (the largest sites are axillary-deep cervical and perirenal adipose tissue), distributed like a "high-collared vest". This location means heat generated warms the blood to vital organs – brain, heart and kidneys. It is thought that the BAT present at infancy becomes diluted with lipid, so that the proportion of cells showing multilocular lipid decreases, and ultimately becomes histologically indistinguishable from subcutaneous white adipose tissue. As the infant grows, lipid accumulation reduces demands for thermogenesis (Lean, 1989). Until recently, using histological techniques it was believed that adult humans had only traces of BAT. With the development of immunological identification techniques for the mitochondrial uncoupling protein it is known that adults possess brown fat, although at levels greatly reduced compared with neonates.

Although the functional significance of brown fat in humans is not clear (Lowell & Flier, 1997), the role of BAT in small animals is better understood. It has been demonstrated in animal models that BAT is the site of facultative DIT, following ingestion of high-energy food (Rothwell and Stock, 1979; Richard *et al.*, 1988; Oudart *et al.*, 1997). With this tissue, laboratory animals such as mice and rats, are able to partially compensate for excess energy intake, by increased energy expenditure. This increase plays an important role in the regulation of energy balance in these animals. Furthermore, a number of researchers have demonstrated that the stimulation of BAT thermogenic activity or the post-prandial increase in DIT, is influenced by dietary composition. Richard *et al.* (1988) showed that facultative DIT develops in mice made hyperphagic by an energy-dense high fat diet and that BAT mitochondrial guanosine diphosphate (GDP) binding (an indication of the activity of the BAT-specific uncoupling protein) also increased.

Other researchers have shown DIT to be effected by the composition of fatty acids in isocalorific diets. DIT, measured by post-prandial whole-body oxygen consumption, decreased in rats fed isocalorific diets rich in saturated fats (lard), compared with diets rich in mono or polyunsaturated fats (plant oils) (Shimomura *et al.*, 1990; Takeuchi *et*

*al.*, 1994). Shimomura *et al.* (1990) also assessed respiratory quotient and found fat oxidation rate was lower in rats fed diets rich in saturated fats. In both studies, body composition was affected and carcass fat content was significantly higher in the lard diet group, although energy intake was the same. They suggested increased DIT and fat oxidation accounted for the difference. Oudart *et al.* (1997), using rats, found a diet rich in n-3 PUFA induced a marked stimulation of BAT thermogenic activity. They suggested PUFA in general, both n-3 and n-6, induce a stimulation of BAT thermogenic activity during high-fat feeding in the rat.

The mitochondrial uncoupling protein necessary for regulated non-shivering thermogenesis can be demonstrated in the "brown compartment" of human adipose tissue, and it seems biochemically homologous and functionally the same as that in experimental animals. There is the biochemical potential in human adipose tissue BAT sites, for the same thermogenic function as the BAT of experimental animals. While the concept of a more metabolically active 'brown' tissue compartment in human adults, as distinct from subcutaneous white adipose tissue, is attractive, the grounds for human BAT thermogenesis are currently speculative and analogies with animal models have yet to be established (Lean, 1989). Never the less, the importance of a small amount of "fuel wasting" active BAT in human body weight regulation is given credibility in textbooks such as Linder (1991). BAT needs to be considered when mice and rat models are used for human energy balance studies.

#### 1.4 Summary

Dietary CLA is present in greatest amounts in meat and dairy products derived from ruminant animals. It is present in mammalian milk, including bovine milk as a result of biohydrogenation of linoleic acid in the rumen, in human breast milk most likely as a result of dietary consumption of CLA, and in milk from the rat following consumption of dietary CLA.

The level of CLA in bovine milk from New Zealand is higher than other countries. This is believed to be due to pasture feeding, although other factors such as cow breed, time of season and/or stage of lactation may also be important. Processing of dairy products

may moderately enhance CLA levels, although the level of enhancement is less than the variation seen between CLA levels in raw milk. CLA is stable during the normal shelf life of dairy products. Products highest in CLA include butter and cheddar cheese.

Research investigating the biological activities of a synthetic mixture of CLA has been the subject of much recent research. Dietary CLA has been demonstrated to effect energy partitioning and metabolism in several animal models. Growth and feed efficiency were enhanced by dietary CLA in suckling or weanling rats. In contrast, older, but still growing animals showed weight reduction and reduced feed efficiency in some trials, and in other trials, no effect from CLA consumption. Conclusions about the effect of CLA on growth and feed efficiency can not be made from these conflicting results across a range of trial animals at different stages of maturity. Results suggest possible growth enhancement in suckling and weanling rats, and growth or weight reduction in more mature, but not fully grown, animals.

Three recent researchers found dietary supplementation with CLA reduced the amount of body fat in experimental mice or rats. The reduction was highly significant and body fat levels in animals fed 0.5 – 1.0 % w/w CLA were less than half the level in control animals. Another researcher found CLA fed mice had altered fatty acid composition in hepatic lipid extracts. One of the researchers finding body fat reduction also used *in vitro* experiments to measure the activity of key enzymes in lipid metabolism. Results suggested reduced fat deposition from circulating triglycerides, increased lipolysis in adipocytes and enhanced fatty acid oxidation in muscle cells and adipocytes. Another of the researchers finding body fat reduction demonstrated increased 24 hour energy expenditure.

The synthetic CLA mixture used in the majority of research to date contained two major isomers, often approximately 37 % 9c11t-CLA and 46 % 10t12c-CLA. Naturally occurring CLA, found in ruminant meat and dairy products, contains a different isomer ratio to synthetic CLA. It contains 80 – 90 % of 9c11t-CLA, approximately double the amount per unit weight of total CLA. Up until recent times, the 9c11t-CLA isomer has been considered the biologically active isomer, due to its prevalence in biological tissues. Three very recent studies support biological activity from 10t12c-CLA (and not

9c11t-CLA) in relation to metabolism of blood lipids, energy metabolism and body composition.

This thesis investigates the effect of CLA on energy balance - energy intake, energy expenditure and body composition. A reduction in body fat, as reported in the literature, is hypothesised to be balanced by a change in energy intake and/or energy expenditure. Synthetic CLA, similar to that used in the published research, is used in the first mouse feeding trial. A natural CLA, from bovine milk, has not been used in previous research, and is used in the second rat feeding trial.

## **CHAPTER 2**

### **Trial 1; Investigation of the Effect of Synthetic Conjugated Linoleic Acid on Energy Balance in Mice**

#### **2.1 Abstract**

##### Introduction

Dietary CLA is reported to reduce body fat in experimental animal feeding trials (Park *et al*, 1997, Sugano *et al* 1998, West *et al* 1998, Park *et al.*, 1999a and 1999b) (refer section 1.2.6.2). Conflicting results report CLA to both reduce body weight in animal feeding trials (West *et al*, 1998, Belury and Kempa-Stecko, 1997, Nicolosi *et al*, 1997), and not effect body weight (Park *et al*, 1997, Sugano *et al*, 1998, Li and Watkins, 1998, Wong *et al*, 1997) (refer section 1.2.6.1).

This dose response study was conducted to investigate the effect of consumption of synthetic dietary CLA (37 % 9c11t-CLA and 46 % 10t12c-CLA isomer) on energy balance in mice.

##### Methods

Six groups of weaned, but growing, male BALB/c mice, aged 7 to 8 weeks, were fed standard isoenergetic diets supplemented with 0.0, 0.1, 0.25, 0.5, 1.0 and 2.0 % w/w CLA for a period of four weeks.

The components of energy expenditure measured in this trial were;

- (i) Food intake and energy intake.
- (ii) Energy expenditure by indirect calorimetry, activity by observation, and serum free fatty acid, an indicator of fatty acid metabolism.
- (iii) Stored energy, measured by chemical analysis of body fat, protein, moisture and ash, and weight of dissected adipose tissue deposits.

### Results

Data was analysed using a one way analysis of variance and Tukey comparison. We found a highly significant ( $p < 0.001$ ) dose response reduction in body fat and gross energy content. The 0.25 % group was the first to show reduced body fat. There was no significant difference in body fat between the 1.0 and 2.0 % CLA groups. Control animals had 8.65 % body fat and the 1.0 % CLA group 2.90 % body fat. Carcass gross energy showed a similar pattern of reduction (refer Table 2.10 and Table 2.11 and Graphs 2.2, 2.3 and 2.4). The percent of lean body mass increased as body fat decreased ( $p < 0.001$ ).

Weight gain of the 2.0 % CLA group was less than the control, 0.1, 0.25 and 0.5 % groups. Weight of lean body mass (LBM) in the 1.0 and 2.0 % CLA group was less than the control, 0.1 % group (2.0 % CLA group only) and 0.5 % group.

There was no difference in energy expenditure or activity between the six groups consuming different levels of dietary CLA. Metabolic rate increased when activity increased and was not affected by diet treatment.

### Conclusions

CLA caused a dose response reduction in body fat, effective over the range 0.25 % CLA to 1.0 % CLA. Reduction in weight gain and LBM occurred in groups of mice consuming 1.0 % and 2.0 % CLA, in comparison to controls and groups consuming lower levels of CLA. High levels of dietary CLA caused growth reduction in these animals.

## 2.2 Experimental materials and procedures

The Massey University Ethics Committee approved all aspects of this study.

### 2.1.1 Animals and housing

One hundred and twenty male BALB/c mice aged seven to eight weeks were used in this study. They were housed in pairs at the Small Animal Production Unit at Massey University. A single ear clip tagged one mouse from each pair. The animals were kept in stainless steel cages, which were enclosed on three sides and the roof, and had a mesh front and floor. A sliding tray collected spilt food and waste 2 cm beneath the floor. Cages were hung on two trolleys in a room maintained at  $22 \pm 2^\circ\text{C}$  with a constant 12 hours light 6.30am to 6.30pm, and 12 hours dark.

### 2.1.2 Mouse diets

Mice were divided into six groups of twenty. They were fed one of six skim milk powder based powdered diets, *ad lib*, during the four-week trial. The powdered diets were controlled for composition except for the amount of Tonalin added to each diet (Tonalin supplied by PHARMANUTRIENTS™, 918 Sherwood Drive, Lake Bluff, Illinois, 60044, USA). Corn oil was substituted with Tonalin and total fat content was controlled. The trial diet was skim milk powder based, with added minerals, vitamins, cellulose, cornflour and corn oil. CLA was added to the diets by addition of commercially available Tonalin.

The pre-acclimatisation diet was a basic rodent chow pellet. The acclimatisation diet, which was fed for a period of one week before the trial, was a ground, milk-free pellet. Sharpes Grain and Seed, Palmerston North, supplied these two diets and their composition was verified at Massey University (refer Table 2.5).

The ingredients in the acclimatisation diets are shown in Appendix 2.1. The ingredients used to make trial diets are shown in Table 2.1.

**Table 2.1 Ingredient composition of mouse diets**

<b>Ingredient g/ 100 g diet</b>	<b>Diet treatments</b>					
	Control	0.1% CLA	0.25% CLA	0.5% CLA	1.0% CLA	2.0% CLA
Skim milk powder (bovine)	54.32	54.32	54.32	54.32	54.32	54.32
Mineral mix <sup>1</sup>	5.00	5.00	5.00	5.00	5.00	5.00
Vitamin mix <sup>2</sup>	5.00	5.00	5.00	5.00	5.00	5.00
Cellulose	1.00	1.00	1.00	1.00	1.00	1.00
Cornflour	29.38	29.38	29.38	29.38	29.38	29.38
Corn oil	5.300	5.146	4.915	4.531	3.762	2.223
Tonalin <sup>3</sup>	-	0.154	0.385	0.769	1.538	3.077
Total	100g	100g	100g	100g	100g	100g

1 Mineral mix added to supply an additional:  
calcium, 630; chloride, 780; potassium, 520; phosphorus, 490; sodium, 200;  
magnesium, 110; iron, 42; manganese, 7.8; zinc, 4.8; cobalt, 2.9; copper, 1.0;  
(all mg / 100g diet);  
chromium, 200; molybdenum, 180; iodine, 180; selenium, 15; (all µg / 100g diet).

2 Vitamin mix added to supply an additional:  
choline, 150; inositol, 20; tocopherol, 20; pantothenic acid (B<sub>5</sub>), 2; niacin, 2;  
(all mg / 100g diet);  
pyridoxine (B<sub>6</sub>), 800; riboflavin (B<sub>2</sub>), 700; retinol (A), 500; thiamine (B<sub>1</sub>), 500;  
menadione (vitamin K<sub>3</sub>), 300; folic acid, 200; biotin, 100; cyanocobalamin (B<sub>12</sub>), 5;  
ergocalciferol (D<sub>2</sub>), 2.5; (all µg / 100g diet).

3 Tonalin, in free fatty acid form.

Experimental diets were formulated to contain 0.0, 0.1, 0.25, 0.5, 1.0, and 2.0 % w/w CLA. These levels of CLA in diets provided dose response treatments over a realistic range of CLA (refer section 2.2.2.1). Additionally, the 0.5 % and 1.0 % CLA levels have been reported to effect body fat in mice (Park *et al.*, 1997, Sugano *et al.*, 1998) (refer section 1.2.6.2).

The mouse diets were analysed for protein, fat, ash, moisture, and total energy by the Analytical Laboratory of the Institute of Food Nutrition and Human Health at Massey University. CLA content was analysed at New Zealand Dairy Research Institute (NZDRI). Diet composition is shown in Table 2.5.

#### 2.2.2.1.1 Relationship of mouse diet CLA level to the New Zealand and Australian dietary CLA level

Daily dietary consumption of CLA in human diets has been reported to range from 460 mg CLA / day (NZDRI, Fong, 1998) to 1500 mg / day (Parodi, 1994). These are

approximately 0.1 % to 0.3 % w/w (dry food basis) CLA in the New Zealand or Australian diet (refer Appendix 2.2 for calculation). These data suggest that a level of 1.0 % CLA in the human diet would be unlikely, although possible with dietary supplementation, eg Tonalin capsules. The mouse treatment diets (0 to 2.0 % w/w CLA) were in the range expected for human consumption on a weight of CLA per weight of diet basis.

### 2.2.3 Experimental procedure

The experimental protocol for the CLA dose response trial is summarised in Table 2.2.

**Table 2.2 Protocol for Trial 1<sup>1</sup>**

Number of male BALB/c mice	120 mice fed experimental diet.	
Mouse supplier	Small Animal Production Unit, Massey University.	
% of CLA in 6 diet treatments	0.0, 0.1, 0.25, 0.5, 1.0, 2.0 %w/w	
Number of mice on each diet treatment	20	
Number and content of groups	5 groups of 24 mice, comprised of 4 mice on each of 6 diet treatments	
Acclimatisation period	1 week (days -7 to -1)	
Age of mice at start of trial (day 0)	7 and 8 weeks (55 – 62 days)	
Trial start dates	Group 1 Group 2 Group 3 Group 4 Group 5	25/05/98 29/05/98 1/06/98 5/06/98 8/06/98
Trial period	4 weeks (days 0 to 28)	
<b>Measurements</b>		
Live weight	Weekly	
Food consumption	Weekly	
<b>Calorimetry and activity</b>		
Base line calorimetry	Day 4 or 5 of acclimatisation week (trial days - 4 , or - 3)	
Middle calorimetry	Day 9,10 or11	
End calorimetry	Day 24 or 25	
Body composition and blood samples	Carcass and blood plasma sample frozen after mouse euthanased on day 28	
Body fat by dissection	Upper body and lower body adipose tissue deposits dissected and weighed from all carcasses.	
Body composition	Chemical analyses of fat, dry matter, ash and nitrogen.	

<sup>1</sup> This was part of a study on immune function on the same mice. Mice were vaccinated on day 7 (1° oral polio and subcutaneous tox / fluvax) and day 21 (2° oral polio and subcutaneous tox/fluvax).

The mice were divided into six groups, each of twenty. Each group began the acclimatisation period on consecutive Mondays or Fridays over a period of fifteen days. Following a one week acclimatisation period on milk-free pellets, 2 pairs of mice in each group were randomly allocated one of the six diets. The experimental diets began on day zero of the 28 day trial and mice were fed *ad lib.*

The six diets were colour coded. Researchers doing the energy expenditure and chemical analysis were blind to the level of CLA in each diet treatment, but were able to identify which animals were on the same diet.

We had sufficient Tonalin to make up the control, 0.1, 0.25 and 0.5 % CLA group diets. It was not possible to obtain sufficient CLA to make large quantities of the 1.0 and 2.0 % CLA diets. It was necessary to conserve the 1.0 % and 2.0 % CLA mouse diets by sieving and recycling spilt powder. In addition, there was insufficient 2.0 % CLA diet for the four mice in group six during the last week. These four mice were not included in trial results.

The powdered diet was placed in a vertical feeding cylinder, with a perforated loose fitting top held in place by a central vertical shaft. The lid moved down into the feeder as the mice consumed the diet. The feeders and cages were cleaned weekly, and diet topped up as required. Water was fed from a vertical dispenser attached to the outside of the cage.

On day 28 mice were euthanased by inhaling isoflurane overdose. At the time of death a blood sample was taken by cardiac puncture to obtain 1-2 ml of blood.

### **2.3 Physical and chemical methods**

#### **2.3.1 Intake of food and energy**

##### **2.3.1.1 Food intake**

Food intake was determined on a weekly basis, for each pair of mice housed together. It was the difference between food offered and food remaining for the pair. All food offered was weighed after placing in the feeder. Food remaining in the feeder was weighed before the weekly clean of feeder and cage. Spilt food was collected on a paper in the tray beneath the cage. It was sieved once to separate from faeces, and weighed.

Weekly food intake was the sum of food offered minus spilt food and food remaining in the feeder. This was measured for each of four weeks during the trial, and summed to obtain the weight of food intake during the trial. To obtain feed intake per day this was divided by 28, the number of days the mice were in the feeding trial.

#### 2.3.1.2 Feed efficiency

Feed efficiency was;  $\frac{\text{weight gain of a rat over four weeks}}{\text{food intake of a rat over four weeks}}$

#### 2.3.1.3 Mouse diet composition

##### 2.3.1.31 Preparation of mouse diet samples for chemical analyses

The powdered mouse diet sample was taken at the time of the trial and placed in a sealed plastic container in the freezer. Samples for analysis were weighed directly from these containers.

##### 2.3.1.32 Compositional analysis

Fat, nitrogen, dry matter, ash and energy was determined for the six diets by the methods described in sections 2.3.3.22. Analytical results for fat %, ash % and energy (kJ/g) were used directly. Protein % was obtained by multiplying nitrogen % by 6.38<sup>5</sup>. Percent moisture was obtained by subtracting dry matter % from 100 %.

##### 2.3.1.33 Conjugated linoleic acid

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<sup>5</sup> (McDonald *et al*, 1996) An average conversion factor of 6.38 often used for milk protein has been selected since skim milk powder is the major protein-containing component in the diets.

CLA was analysed at NZDRI. Difficulty was experienced recovering CLA and method development was undertaken.

In the first method, fat was extracted gravimetrically by accelerated solvent extraction (ASE) prior to fatty acid methyl analysis (FAME) by capillary gas chromatography. Approximately 50 % of the CLA was recovered by this method, although fat appeared to be fully recovered. Although the absolute amount of CLA recovered was lower than expected, the rank order and relative amounts of CLA in the six diets was as expected. The control diet contained no CLA.

The second method used Soxhlet fat extraction, before the FAME and fat content were determined, using the same GC analysis as the first method. This time an internal standard, tridecanoic acid, was added to the extracted sample. Control diet samples spiked with Tonalin were used in this analysis. Again, recoveries were low, with fat recovery of two samples at 78 % and 79 %, and CLA recovery at 58 % and 59 % of expected amounts. The method was not used to analyse further diet samples.

The third method used ASE followed by FAME, as in the first method. This time, tridecanoic acid internal standard and antioxidant 2,6, *Di tert*-butyl 4 methylphenol (BHT) were also added into the sample before extraction. The antioxidant was added to protect oxidation of the CLA. Control diet spiked with Tonalin, obtained 99 %, 101 %, 103 % and 103 % of expected CLA levels. The 2.0 % CLA diet was analysed by this method, and the result was 1.32 % and 1.38 % CLA. No further diets were analysed.

The reason for the lower than expected result, using method 3, for the 2.0 % CLA diet from this trial was not known. Possible sources of error may be homogeneity of the blended diets or samples. Another possible explanation for the discrepancy was long term stability of CLA in the feed mix. The ingredient weight data obtained when making the diets was used to calculate the CLA content of dietary treatments.

The third method was later used for the determination of CLA content in the diets used in the trial described in Chapter 3. Results generated were in line with feed formulations for that trial.

### 2.3.1.34 *Fatty acid profile of Tonalin and corn oil*

The fatty acid profile of Tonalin was determined by capillary gas chromatography at DRI. The fatty acid profile of corn oil was obtained from published DSIR data (DSIR, 1989).

### 2.3.1.4 Energy Intake

Energy intake for each pair of mice was obtained by multiplying food consumed (g) by the gross energy content (kJ/g) of each diet. The energy intake was obtained for each week of four weeks during the trial, summed to obtain the total energy intake during the trial, and divided by 28 to obtain energy intake per day.

### 2.3.2 Energy Expenditure

#### 2.3.2.1 Calorimetry

The calorimeter system and operating method described in Appendix 2.4 was used to measure respiratory exchange gases. The calorimeter was located in the Physiology Laboratory in the Institute of Food, Nutrition and Human Health at Massey University and animals were transported from the housing unit.

Respiratory gases were measured in the calorimeter during the acclimatisation week, the middle and end of the trial. On each occasion, pairs of animals were removed from their cages in the Small Animal Production Unit, individually weighed and placed in labelled plastic cages, 230 mm long x 135 mm high x 100 mm wide. The plastic cages had a small handful of wood shavings on the floor and clip-on wire rod lids. Feeders and water were placed with the plastic cage. These plastic cages were taken, in an enclosed box, in a five minute car journey, to the Physiology laboratory. The temperature in this laboratory was not controlled and ranged from 16.7 °C to 25.8 °C over the six week calorimetry period, and between 0.8 °C and 4.4 °C on any single day.

Following transportation and placing in a new environment, mice were initially active, and generally settled after a short period. The sleep/wake pattern of mice in the Physiology Laboratory was recorded by the method described in 2.3.2.3. It is not known if the sleep/wake pattern observed was a natural rhythm in these nocturnal animals, or a reaction to a changed environment. Mice were removed from the Small Animal Production Unit by 8.30 a.m. and returned by 6.00 p.m.

Two cages of paired mice, a total of four mice, on the same diet and in the same group, were placed in the calorimeter for a 45 minute experimental period while respiratory exchange gases were measured (refer to appendix 2.3). It was necessary to place four mice at a time in the calorimeter to obtain sufficient quantities of respiratory exchange gas for analysis. Four mice consumed approximately 6.0 ml of oxygen.

All groups of four mice were placed in the calorimeter for a 45 minute period on three separate occasions. The base line calorimetry measured respiratory exchange gases during the acclimatisation period, the middle calorimetry after the experimental diets had been fed for 9 to 11 days, and the end calorimetry, near the end of the feeding trial on day 24 or 25.

Groups of mice were tested in the calorimeter in group numerical order, after being on the trial diet for an equivalent number of days. This meant animals were all a similar age at time of calorimetry. The order of selection of a diet colour was random within each group. This removed possible bias from sleep/wake activity patterns. During the 45 minute calorimetry experiment, respiratory exchange gases were analysed for carbon dioxide and oxygen content continuously for the whole period at six second intervals. Flowrate of air through the calorimeter was measured at the same time. The result was recorded on MACLAB chart 3.5.6/s software. The mean rate of oxygen consumption and carbon dioxide production for the last recorded ten minutes (from 35 to 45 minutes in the calorimeter) was used in subsequent calculations. After 45 minutes the cages were removed from under the calorimeter lid. Mice were able to feed *ad libitum* during calorimetry.

The procedure of checking the calorimeter calibration (refer Appendix 2.4) with a five minute "no mice" test before and after the 45 minute calorimeter period was not instigated until the start of the middle calorimeter measurements. For this reason, it was decided not to include the base line calorimeter measurement results in further analysis of energy expenditure.

At the same time as respiratory gas measurements were made, mouse activity, the time of day, and temperature were recorded. These were measured because activity, sleep/wake patterns and maintenance of body temperature effect energy expenditure.

From calorimeter respiratory exchange gas measurements of the rate of oxygen consumption and the rate of carbon dioxide production, energy expenditure was calculated (refer section 1.3.1.1). The formulae used to calculate respiratory quotient (R), energy equivalence of oxygen and metabolic rate were;

Respiratory quotient;

$$R = \dot{V}CO_2 / \dot{V}O_2 = \frac{\text{CO}_2 \text{ produced ml / minute}}{\text{O}_2 \text{ consumed ml / minute}}$$

Energy equivalence of oxygen;

$$\text{kJ / l} = (5.156 \times R) + 15.969 \text{ (Andrew, 1965).}$$

Metabolic rate;

$$\text{kJ / minute} = (\text{O}_2 \text{ consumed ml / minute} / 1000) \times \text{energy equivalence of O}_2 \text{ (Andrew, 1965).}$$

### 2.3.2.2 Activity

Activity is an important determinant of energy expenditure. Because we were investigating the effect of diet on energy expenditure, it was important to know the activity level at the time of energy expenditure measurement.

Mouse activity was recorded for each individual mouse by observation of mice at five minute intervals during the last 20 minutes in the calorimeter. A mean activity score

was calculated for the four mice in the calorimeter over this time period. The activity scoring scale is shown in Table 2.3.

**Table 2.3 Mouse activity score**

Score	Description of mouse activity
1	Asleep.
2	Drowsy, shuffling feet, not moving, cleaning, grooming.
3	A little movement around cage, feeding, tunnelling in wood shavings.
4	Moderate movement around cage, climbing on feeder, playing vigorously with wood shavings, picking up and moving shavings.
5	Vigorous movement around cage, running, jumping, climbing/hanging with all four feet on roof bars, hanging with front feet on roof bars.

#### 2.3.2.3 Sleep/wake pattern.

Diurnal rhythm may be a determinant of activity, which effects energy expenditure. The sleep pattern of mice was recorded by observation while mice were in the Physiology Laboratory containing the calorimeter system. All mice in the laboratory were observed at fifteen minute intervals throughout the day and the count of sleeping mice recorded. The total number of mice in the laboratory on each day was also recorded. A total of 263 mice were observed over a period of twenty days. A frequency histogram of mice sleeping at 15 minute intervals showed the sleep pattern throughout the day.

#### 2.3.2.4 Serum free fatty acid content

On day 28 mice were blood sampled under isoflurane anaesthesia by cardiac puncture to obtain 1-2 ml of blood. Blood was coagulated and centrifuged, and kept on ice. The serum was pooled from four mice on the same diet in each group, frozen and a 50 $\mu$ l sample subsequently analysed for free fatty acid content. These were the same mice groupings that were used in the calorimeter experiment.

The Wako test kit was used to quantify non-esterified fatty acids (NEFA) in serum. This utilises an *in vitro* enzymatic colourimetric method. The acylation of coenzyme A (CoA) by the fatty acids occurs in the presence of added acyl-CoA synthetase (ACS). The acyl-CoA is then oxidised by added acyl-CoA oxidase, with generation of hydrogen

peroxide. Hydrogen peroxide, in the presence of peroxidase permits the oxidative condensation of 3-methyl-N-ethyl-N-( $\beta$ -hydro-xyethyl)-aniline with 4-aminoantipyrine to form a purple coloured adduct which can be measured colourimetrically at 550 nm.

### 2.3.3 Body Composition

#### 2.3.3.1 Live Weight

Individual mice were weighed on day one, seven, fourteen, twenty one, twenty eight and on the morning of each of three calorimetry experiments. A mouse was placed on the scales (Watson-Victor Mettler Ltd, PM6000) in a tared 500 ml plastic beaker, and a second tared beaker placed loosely on top to restrain the mouse. The scales were set to the animal weigh function, which recorded the average of 10 individual consecutive measurements

#### 2.3.3.2 Measurement of body composition

Immediately after blood sampling and euthanasia, the intestine was cut below the stomach, between the pylorus and duodenum, and the remaining gastrointestinal tract removed. The small amount of adipose tissue adhering to the intestine was removed by dissection and kept with the carcass, and the intestine was discarded.

Carcasses were placed individually into labelled plastic bags, frozen and subsequently analysed for body composition.

#### 2.3.3.21 *Body composition by dissection*

The frozen mouse carcass, still in a plastic bag was thawed at room temperature and placed on ice. The carcass was weighed, the stomach removed, and the carcass weighed again. This carcass weight which had the stomach, intestine, and 1-2 ml of blood removed, was used in subsequent calculations of body fat by dissection. Measurements were obtained for individual mice.

The upper body fat was removed and weighed. It comprised interscapular brown adipose tissue, and in fatter animals, subcutaneous adipose tissue located on the upper back and in the region where the thoracic limbs join the body.

The lower body fat was removed and weighed. It was located in the abdominal region, the genital region, surrounding the kidneys and lining the anterior of the abdominal cavity, and on the pelvic limbs.

The dissected adipose deposits and the carcass (without stomach and intestine) were combined, weighed again, and used for subsequent chemical analysis. The total body fat by dissection was the sum of the upper and lower body fat (g).

Body fat % by dissection was calculated relative to the carcass weight at the start of dissection.

#### 2.3.3.22      *Body composition by chemical analyses*

##### 2.3.3.22.1      *Preparation of mouse samples for chemical analyses*

Samples were prepared for chemical analysis in groups of four, from the same diet treatment and group. These were the same mice groupings used in the calorimeter experiment. The animals were analysed in groups of four. The four carcasses were homogenised in a food blender (Waring), placed in a previously weighed plastic bag, and weighed. The weight of the bag was subtracted to give the combined weight of four mice carcasses. This weight is referred to in subsequent calculations as weight A. The blender was wet washed and dried between each sample preparation.

The bags of sample were frozen, freeze dried (Cuddon Model 0610 Freeze Drier, W.G.G. Cuddon Ltd., Blenheim, New Zealand) and weighed. The weight of the bag was subtracted to give the combined weight of four freeze dried mouse carcasses. This weight is referred to in subsequent calculations as weight FD (freeze-dried).

The freeze dried material was ground in a rotating hammer mill (Thomas Scientific, USA), and passed through a 2 mm screen. A small amount of oversize material was retained in the mill. This was brushed from the screen and inside of the mill and

included with the sample for analysis. The mill was dry cleaned using a vacuum cleaner and brush between each sample preparation.

#### *Fat Determination*

A Soxhlet apparatus was used to extract fat from the freeze dried sample. The solvent used was diethyl ether, as previously described (Park *et al*, 1997). Solubilised fat was collected in the distillation flask and the increase in weight of the flask was the fat content.

#### **2.3.3.22.3                  Nitrogen Determination**

The Leco® autoanalysis technique (Anon, 1994), was used to determine the nitrogen content of samples. Duplicate weighed samples were analysed in the Leco® autoanalyser (Leco® CNS-2000, Leco Corporation). Samples were combusted in a resistance furnace at 600°C and a Thermal Conductivity cell determined the nitrogen content.

#### **2.3.3.22.4                  Dry Matter Determination**

The dry matter of weighed duplicate samples was determined by drying overnight (16 hours) at 105°C. Samples were cooled in a desiccator and weighed.

#### **2.3.3.22.5                  Ash Determination**

The ash content of weighed duplicate samples was determined by burning overnight in a muffle furnace at 500°C.

#### **2.3.3.22.6                  Gross Energy Determination**

Gross energy was determined using bomb calorimetry (Gallentramp Autobomb, Watson Victor Ltd., Wellington, New Zealand). Duplicate samples using 0.5 – 1.0 g were compressed to give a solid pellet, weighed, and placed inside the bomb.

The calorimeter vessel was filled with water to a total weight of 3000 within 0.1 g and the temperature adjusted to approximately 24.5 °C. The bomb was placed in the calorimeter and the initial temperature recorded after 5 - 10 minute's stabilisation. The

sample was combusted and after 10 minutes the final, stabilised temperature was recorded. The bomb was removed from the calorimeter and the vessel reweighed to 3000 g. A daily standard of duplicate benzoic acid pellets was analysed and checked to be within the specification (ie 26431.6 to 26438.6 J / g ).

The temperature difference was multiplied by the water equivalent (10.873 kJ / °C) to give a calorific value for the sample. This was converted to a value per gram of sample (kJ / g).

#### **2.3.3.22.7                  *Formula used to calculate mouse body composition***

The analytical results for fat, nitrogen, dry matter, organic matter and gross energy content of the freeze-dried sample were used to calculate mouse body composition. Body fat, protein, moisture, ash, lean body mass, and gross energy content were calculated according to the following formula. Body composition was calculated relative to total body mass (%) and as an absolute weight in grams. In these formula FD is freeze-dried and weight A is the weight of a group of four homogenised mice carcasses (refer 2.3.3.22.1).

Worked examples of calculations are shown for the following set of hypothetical results; The four hypothetical homogenised mice carcasses in a group consuming the 0.5 % CLA diet weighed 77.68 g. After freeze drying the weight was 25.26 g, the fat was 14.86 %, nitrogen 10.42 %, dry matter 94.33 %, ash 12.28 %, and gross energy value 22.39 kJ / g.

##### **(i)                  Fat**

The percent fat in a group of four homogenised mice carcasses was calculated from the fat in the freeze-dried sample using the following formula;

$$\text{Fat \%} = \frac{\text{fat \% in FD sample} \times \text{weight FD sample g}}{\text{weight A g}}$$

$$\text{Fat \%} = \frac{14.86 \% \times 25.26 \text{ g}}{77.68 \text{ g}} = 4.83 \% \text{ fat}$$

The weight of fat in the four hypothetical mice was  $4.83\% \times 77.682\text{ g} / 100 = 3.75\text{ g}$ .

The average weight of fat in an individual mouse from this group was  $3.75\text{ g} / 4 = 0.94\text{ g}$ .

(ii) Protein

The percent protein in a group of four homogenised mice carcasses was calculated from the nitrogen in the freeze-dried sample using the following formula;

$$\text{Protein \%} = \frac{\text{nitrogen \% in FD sample} \times 6.25^6 \times \text{weight FD sample g}}{\text{weight A g}}$$

$$\text{Protein \%} = \frac{10.42\% \times 6.25 \times 25.26\text{g}}{77.68\text{ g}} = 21.18\text{ protein \%}$$

The weight of protein in the four hypothetical mice was  $21.18\% \times 77.68\text{ g} / 100 = 16.45\text{g}$ .

The average weight of protein in an individual mouse from this group was  $16.45\text{ g} / 4 = 4.11\text{ g}$ .

(iii) Moisture

The percent moisture in a group of four homogenised mice carcasses was calculated from the moisture in the freeze-dried sample and the moisture lost during the freeze drying process, using the following formula;

$$\text{Moisture \%} = \frac{\text{weight A g} - (\text{dry matter \% in FD sample} / 100 \times \text{weight FD sample g}) \times 100}{\text{weight A g}}$$

$$\text{Moisture \%} = \frac{77.68\text{ g} - (94.33\% / 100 \times 25.26\text{ g}) \times 100}{77.68\text{ g}} = 69.32\text{ \% moisture}$$

<sup>6</sup> (McDonald et al, 1996). An average conversion factor often used for meat protein has been selected. The protein value calculated from nitrogen using this conversion factor will differ from true protein because of the assumptions that all nitrogen is present as protein and all protein contains 160g N/kg.

The weight of moisture in the four hypothetical mice was  $69.32\% \times 77.68\text{ g} / 100 = 53.85\text{ g}$ .

The average weight of moisture in an individual mouse from this group was  $53.85\text{ g} / 4 = 13.46\text{ g}$ .

(iv) Ash

The percent ash in a group of four homogenised mice carcasses was calculated from the ash in the freeze-dried sample using the following formula;

$$\text{Ash \%} = \frac{\text{ash \% in FD sample} \times \text{weight FD sample g}}{\text{weight A g}}$$

$$\text{Ash \%} = \frac{12.28\% \times 25.26\text{ g}}{77.68\text{ g}} = 3.99\%$$

The weight of ash in the four hypothetical mice was  $3.99\% \times 77.68\text{ g} / 100 = 3.10\text{ g}$ .

The weight of moisture in an individual mouse from this group was  $3.10\text{ g} / 4 = 0.78\text{ g}$ .

(v) Lean body Mass

The percent lean body mass (LBM) in a group of four homogenised mice carcasses was calculated by two methods. The first method provided a direct measurement of LBM by summing the percent protein, moisture and ash for the group of four mice. The second method calculated LBM indirectly by subtracting body fat %, by dissection, from 100 %. The first method had less data since mice body composition was analysed in groups of four ( $n = 5$ ) and used results from chemical analysis, while the second method used individual data ( $n=20$ ) and results from dissections. The first method used the following formula;

$$\text{Lean body mass \%} = \text{protein \%} + \text{moisture \%} + \text{ash \%}$$

$$\text{Lean body mass \%} = 21.18\% + 69.32\% + 3.99\% = 94.49\%$$

The weight of LBM in the four hypothetical mice was  $94.50\% \times 77.68\text{ g} / 100 = 73.40\text{ g}$

The average weight of moisture in an individual mouse from this group was 73.40 g / 4 = 18.35 g.

It is acknowledged that this value for lean body mass will not include the small amount of carbohydrate in the body.

The second method used the following formula;

$$\text{Lean body mass \%} = 100 \% - \text{fat \%}$$

e.g. The first hypothetical mouse carcass in group one consuming the 0.50 % CLA diet weighed 19.06 g and had a fat content, by dissection, of 3.67 %.

$$\text{Lean body mass \%} = 100 \% - 3.67 \% = 96.33 \%$$

The weight of LBM in this mouse was  $96.33 \% \times 19.06 \text{ g} / 100 = 18.36 \text{ g}$ .

This LBM calculation includes a component of fat in the LBM that could not be dissected. This is inter and intra cellular fat dispersed throughout muscle tissue. Dissectible adipose tissue deposits have been removed.

#### (vi) Gross energy

The gross energy per gram in a group of hypothetical homogenised mice carcasses was calculated from the gross energy in the freeze-dried sample using the following formula;

$$\text{Gross energy / g} = \frac{\text{gross energy kJ / g FD sample} \times \text{g FD sample}}{\text{weight A}}$$

$$\text{Gross energy / g} = \frac{22.39 \text{ kJ / g} \times 25.26 \text{ g}}{77.68 \text{ g}} = 7.28 \text{ kJ / g}$$

## 2.4 Results

#### 2.4.1 Data analysis

Individual measurements were recorded for live weight, carcass weight, upper and lower body fat weights, and activity during calorimetry. Although data was recorded for individual mice, experimental units were pairs of mice. The individual data was grouped and analysed as pairs of mice. Measurements for pairs of mice were recorded for food intake, feed efficiency, and energy intake. One set of data for each pair was analysed.

Measurements for groups of four mice, from the same dietary treatment, in the same group, were recorded for the rate of oxygen consumption and carbon dioxide production in the calorimetry experiment, and chemical analysis of body composition - fat, protein, ash, moisture and energy. One set of data for each group of four was analysed.

Results were analysed using the statistical package MINITAB version 12.1. A one-way ANOVA analysed each experimental response for mice grouped according to the level of CLA in each dietary treatment group. Where the F test showed a significant difference between means, a Tukey comparison was used to compare which means differed and the level of significance.

##### 2.4.1.1 Data excluded from analysis

Five mice died during the trial. Since mice were housed in pairs, when one animal died, the experimental conditions for the surviving individual were no longer the same as for a pair. For this reason, when a mouse died, individual data for the surviving individual, and paired and four mouse data pertaining to the dead mouse were removed before data analysis.

As previously mentioned, there was insufficient 2.0 % CLA diet for the four mice in group 5 during the last week. These mice were not included in the data analysis.

#### 2.4.2 Mouse deaths

Five mice died during the trial. The diet groups and causes of death were; one from the

Five mice died during the trial. The diet groups and causes of death were; one from the 0.25 % CLA diet on day 15, following a week of sickness; two from the 0.5 % CLA diet on day 5 and day 14 found dead, both with the head partially eaten; one from the 1.0 % diet on day 13 which had been injured in a fight two days before; and one from the 2.0% diet euthanased following a serious head injury received in a fight.

#### 2.4.3 Food Intake

The fatty acid composition of corn oil and Tonalin is shown in table 2.4. Cornoil was substituted with Tonalin in experimental diets to obtain the required CLA level.

**Table 2.4 Fatty acid composition of corn oil and Tonalin**

Fatty acid	Corn oil % (DSIR, 1989)	Tonalin % (NZDRI, 1998)
14:0	0.57	-
16:0 palmitic acid	13.37	6.47
18:0 stearic acid	2.20	4.14
20:0	0.29	-
16:1	0.29	-
18:1 oleic acid	28.65	22.19
20:1	0.19	-
22:1	0.19	-
18:2 linoleic acid	47.75	4.53
Conjugated 18:2 <sup>1</sup>	-	59.14

1 (NZDRI, 1998).

Distribution of CLA isomers in Tonalin;

9,11; 8,10 ct/tc-CLA 37.1 %

11,13; 10,12 ct/tc-CLA 46.3 %

cc-CLA 9.2 %

tt-CLA 7.4 %

The composition of the trial diets is shown in Table 2.5

**Table 2.5 Composition of mouse diets**

Component	Acclimatisation diet	Trial diet treatments					
		0.0 % CLA (control)	0.1 % CLA	0.25 % CLA	0.5% CLA	1.0 % CLA	2.0 % CLA
Protein %	18.36	20.05	20.27	20.20	20.35	19.86	20.63
Fat %	2.71	5.40	5.37	5.65	5.44	5.10	4.75
Ash %	5.50	8.06	7.90	8.16	8.15	8.14	8.00
Moisture %	11.27	6.65	6.86	6.40	6.59	6.7	6.91
Total energy kJ/g	16.40	17.14	17.16	17.21	17.24	17.13	17.18
CLA % w/w	0.03 <sup>1</sup>	0.0 <sup>2</sup>	0.1 <sup>2</sup>	0.25 <sup>2</sup>	0.5 <sup>2</sup>	1.0 <sup>2</sup>	2.0 <sup>2</sup>

1 Gas chromatography analysis (NZDRI, 1999a)

2 Calculated from addition of Tonalin during diet formulation

The small amount of CLA in the acclimatisation diet (0.03%) may have been present in the meat bone meal or fat portion of the milk powder.

Total energy was similar for all mice trial diets. The 2.0 % CLA diet protein was a little higher and the fat a little lower than the other diets. This difference is minor and not expected to affect results. The CLA level in each of the six diet groups was 0.0, 0.1, 0.25, 0.5, 1.0, 2.0 % w/w. Since CLA originated from Tonalin, the proportion of the 9c11t-CLA isomer in each diet group was assumed to be 37.1 % and 10t12c-CLA was 46.3 %.

Feed intake, energy intake, weight gain and feed efficiency for pairs of mice are shown in Table 2.6. Results shown in this and subsequent tables are mean values  $\pm$  standard deviation.

**Table 2.6 Feed intake, weight gain and feed efficiency**

Diet CLA %	N	Feed intake g / day / pair	Energy intake kJ / day / pair	Weight gain g / four weeks/ pair	Feed efficiency <sup>1</sup> $\times 10^2$
0.0	10	8.42 $\pm$ 0.83	144 $\pm$ 14	7.81 $\pm$ 2.01	3.29 $\pm$ 0.70
0.1	10	8.72 $\pm$ 0.61	150 $\pm$ 10	7.75 $\pm$ 2.03	3.22 $\pm$ 1.01
0.25	9	9.50 $\pm$ 0.73 <sup>2</sup>	163 $\pm$ 13 <sup>3</sup>	7.51 $\pm$ 2.22	2.87 $\pm$ 0.97
0.5	8	8.46 $\pm$ 1.14	146 $\pm$ 20	8.17 $\pm$ 2.34	3.45 $\pm$ 0.83
1.0	9	8.12 $\pm$ 0.75	140 $\pm$ 13	7.15 $\pm$ 1.17	3.14 $\pm$ 0.40
2.0	7	8.52 $\pm$ 0.45	146 $\pm$ 8	5.88 $\pm$ 2.68	2.44 $\pm$ 1.07

$\pm$  standard deviation

1 feed efficiency = weight gain (g) / food intake (g), both over four weeks, for pairs of mice

2 0.25 % CLA diet feed intake > 0.0 & 1.0 % CLA diets, p < 0.05

3 0.25 % CLA diet energy intake > 0.0 & 1.0 % CLA diets, p < 0.05

Mice ate on average, 4.3 g of treatment diet each day of the trial (range 3.2 to 5.3 g).

Food intake and energy intake were the same for mice on all dietary treatments, except for the 0.25 % CLA diet treatment group which consumed significantly more than the control and 1.0 % group (p < 0.05).

Feed efficiency was determined for pairs of mice, since food intake was measured for pairs of mice housed together. Although weight gain was measured in individual mice, a figure for paired weight gain is shown in this table since it was required to calculate the feed efficiency value. There was no significant difference in feed efficiency between the diet groups.

#### 2.4.3 Energy Expenditure

Results for energy expenditure measurements made in the middle of the feeding trial are shown in Table 2.7, and measurements made at the end in Table 2.8.

Data was analysed to compare energy expenditure of groups of mice on the six dietary treatments. The energy expenditure data was analysed separately twice during the trial – the middle, after two weeks CLA feeding, and the end, after four weeks CLA feeding. There were no significant differences in rate of oxygen consumption and rate of carbon dioxide production between the six diet groups at either time during the trial. There were no significant differences in R, energy equivalence of oxygen, or metabolic rate between the diet groups. The variable in these isoenergetic diets was substitution of corn oil for Tonalin, containing mainly CLA. This dietary change did not effect energy expenditure.

Data from all mice was combined and analysed to compare energy expenditure of all mice in the middle of the trial, and the end. The volume of oxygen consumed by each group of four mice ranged was  $5.68 \pm 1.18$  ml/minute in the middle of trial and  $6.08 \pm 1.13$  ml/minute at the end of the trial (mean  $\pm$  standard deviation). A paired t-test showed no significant difference in these two oxygen consumption rates.

R values during the calorimeter experiment in the middle of the trial ranged from 0.90 to 0.94 (mean 0.92). At the end of the trial these ranged from 0.92 to 0.96 (mean 0.93). A paired t-test was used to compare all R values at the end of the trial, with R values in the middle of the trial. This comparison was made firstly with all of the mice, and in the

second instance with mice consuming diets containing 0.25 % CLA or greater. There was no significant difference in the R value during the two week time period.

Activity was not significantly different between any of the diet treatments, at the middle or end of the trial. Activity of individual groups of mice varied from 1.0 (four mice asleep throughout calorimetry experiment) to 4.0 (constant moderate and vigorous activity throughout). A paired t-test was used to compare all activity values at the end of the trial with activity values in the middle. They did not change significantly during this period.

**Table 2.7 Oxygen consumption, oxygen consumption / bodyweight, carbon dioxide production, respiratory quotient, energy equivalence of oxygen, metabolic rate and activity score for mice after two weeks of CLA feeding trial**

Diet CLA %	Weight g (two weeks)	n	$\dot{V}O_2^1$ ml/minute	$\dot{V}O_2^1$ ml/minute /g body weight	$\dot{V}CO_2^2$ ml/minute	Activity Score	R	Energy equivalence of oxygen kJ/l	Metabolic rate kJ/minute
0.0	23.79 ± 1.39	5	5.93 ± 1.02	0.250 ± .045	5.47 ± 1.16	2.2 ± 0.7	0.92 ± 0.06	20.7 ± 0.3	0.12 ± 0.02
0.1	23.04 ± 2.51	5	5.94 ± 1.67	0.263 ± 0.092	4.52 ± 2.80	2.4 ± 1.2	0.90 ± 0.08	20.6 ± 0.4	0.12 ± 0.04
0.25	22.25 ± 1.80	4	5.28 ± 0.70	0.237 ± 0.040	4.98 ± 0.81	2.2 ± 0.8	0.94 ± 0.03	20.8 ± 0.3	0.11 ± 0.02
0.5	23.24 ± 1.46	3	5.71 ± 1.11	0.243 ± 0.044	5.30 ± 1.26	2.1 ± 0.6	0.92 ± 0.05	20.7 ± 0.2	0.12 ± 0.02
1.0	21.67 ± 1.55	4	4.89 ± 0.91	0.224 ± 0.032	4.49 ± 0.87	1.5 ± 0.7	0.92 ± 0.03	20.7 ± 0.2	0.10 ± 0.02
2.0	22.43 ± 1.73	3	6.37 ± 1.60	0.281 ± 0.065	5.96 ± 1.67	2.7 ± 0.8	0.93 ± 0.03	20.8 ± 0.2	0.13 ± 0.03

± standard deviation

1 Rate of oxygen consumption measured in calorimeter

2 Rate of carbon dioxide production measured in calorimeter

Table 2.8 Oxygen consumption, oxygen consumption / body weight, carbon dioxide production, respiratory quotient, energy equivalence of oxygen, metabolic rate, and activity score and serum free fatty acid for mice at the end of CLA feeding trial

Diet CLA %	n	$\dot{V}O_2^1$ ml/minute	$\dot{V}O_2^1$ ml/minute/g body weight <sup>2</sup>	$\dot{V}CO_2^3$ ml/minute	Activity	R	Energy equivalence of oxygen kJ/l	Metabolic rate kJ/minute	Metabolic rate/g lean body mass <sup>4</sup> kJ/Min/g × 10 <sup>2</sup>	Serum free fatty acids mEq/l
0.00	5	6.18 ± 1.21	0.238 ± 0.044	5.72 ± 1.20	2.36 ± 1.04	0.92 ± 0.02	20.7 ± 0.1	0.13 ± 0.03	0.73 ± 0.16	1.56 ± 0.28
0.10	5	6.18 ± 1.28	0.244 ± 0.057	5.77 ± 1.51	2.84 ± 1.17	0.96 ± 0.06	20.7 ± 0.3	0.13 ± 0.03	0.75 ± 0.20	1.52 ± 0.16
0.25	4	5.74 ± 1.33	0.231 ± 0.057	5.29 ± 1.21	2.08 ± 0.62	0.92 ± 0.02	20.7 ± 0.1	0.12 ± 0.03	0.69 ± 0.15	1.49 ± 0.22
0.50	3	5.63 ± 1.19	0.217 ± 0.041	5.24 ± 1.33	1.97 ± 0.90	0.92 ± 0.05	20.7 ± 0.2	0.12 ± 0.03	0.66 ± 0.14	1.88 ± 0.17
1.00	4	6.07 ± 1.41	0.246 ± 0.051	5.82 ± 1.55	2.48 ± 0.85	0.94 ± 0.05	20.8 ± 0.2	0.13 ± 0.03	0.74 ± 0.16	1.29 ± 0.16 <sup>5</sup>
2.00	3	6.64 ± 0.53	0.271 ± 0.016	6.40 ± 0.57	2.60 ± 0.70	0.96 ± 0.01	20.9 ± 0.1	0.14 ± 0.01	0.84 ± 0.05	1.11 ± 0.18 <sup>5</sup>

± standard deviation

1 Rate of oxygen consumption measured in calorimeter

2 Body weight refer Table 2.9

3 Rate of carbon dioxide production measured in calorimeter

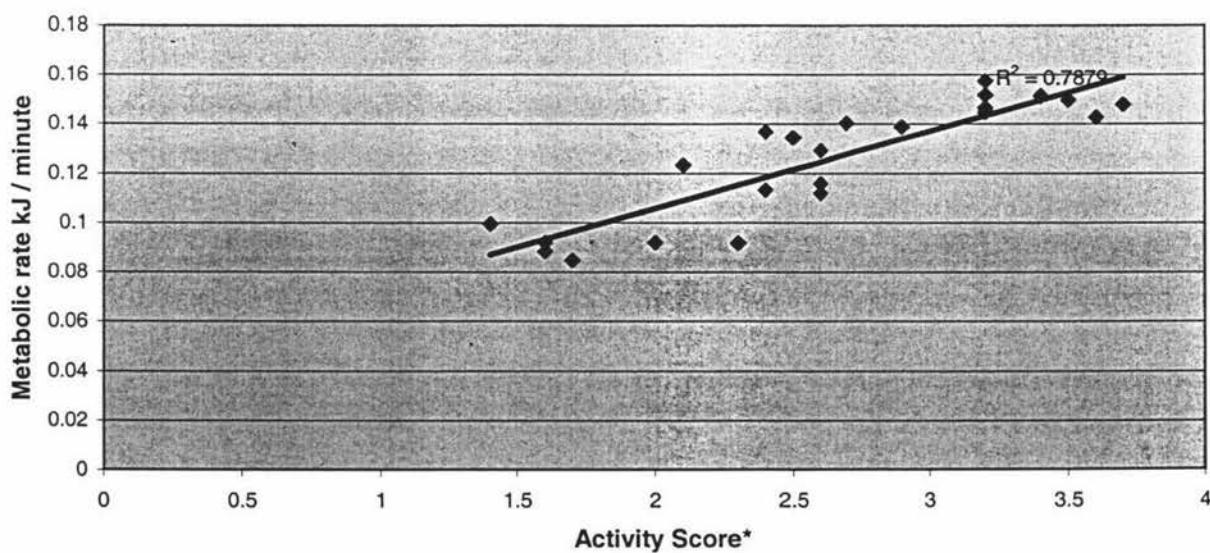
4 LBM = Sum protein g + water g + ash g

5 2.0 & 1.0 % CLA diet serum free fatty acid < 0.5 % CLA diets, p < 0.05

Rate of oxygen consumption / g body weight was compared between diets in the middle of the trial (after two weeks of trial feeding) and was not significantly different. Similarly, there was no difference at the end of the trial, after four weeks of feeding. The difference between rate of oxygen consumption / g body weight at two and four weeks was compared for each diet group and was not significantly different. Data from all mice was combined and analysed (paired t-test) to compare rate of oxygen consumption / g body weight after two and four weeks feeding. There was no significant difference between the middle and end of the trial.

Energy expenditure / g lean body weight was compared for each diet group at the end of the trial and was not significantly different. There was no correlation between oxygen consumption and laboratory temperature, or oxygen consumption and time of day.

**Graph 2.1 Metabolic Rate versus Activity**



\* Each point represents mean activity of four mice in the calorimeter

Metabolic rate was plotted against activity at the end of the feeding trial. This is shown in Graph 2.1. The scatter plot indicates a strong positive relationship between activity and metabolic rate, with a correlation coefficient of 0.89 ( $R^2 = 0.7879$ ). The correlation should be interpreted with caution since the activity scale was based on subjective non-

parametric observations. However, the positive relationship with metabolic rate suggests that activity is correlated with VO<sub>2</sub>.

The frequency histogram of diurnal activity showed mice were generally all awake until 11.00am. The proportion of mice sleeping increased gradually to approximately one half at 1.00 p.m., with a peak of two thirds sleeping at 2.30 p.m. From 4.30 p.m. until observations stopped at 6.00 p.m., approximately one third of the mice were asleep. We had selected mice randomly from within a group for calorimetry measurements to remove any possible bias from activity related to sleep/wake patterns.

We found the rate of oxygen consumption and energy expenditure of mice was related to activity at the time of measurement, with higher VO<sub>2</sub> and energy expenditures seen when mice were active. In addition, energy expenditure and activity were not related to the level of CLA in the diet, the time of day of measurement and the temperature in the laboratory.

Serum free fatty acids were lower for the 1.0 % and 2.0 % CLA diets compared with the 0.5 % diets ( $p < 0.05$ ).

#### 2.4.5 Body Composition

Live weight, carcass weight, and weight gain are shown in Table 2.9.

There was no significant difference in live weight at the start of the trial between mice in the six diet groups ( $p < 0.05$ ). Following four weeks on trial diets the live weight of the control was significantly more than the 1.0 % group ( $p < 0.05$ ), but not the 2.0 % group. The mean of the latter group was just 0.14 g more than the 1.0 % group (mean g  $\pm$  SD g; 1 % group  $24.55 \pm 1.53$ ; 2 % group  $24.69 \pm 2.12$ ).

Weight gains over four weeks on the diet for the control, 0.1, 0.25, and 0.5 % CLA groups were all significantly more than the 2.0 % group ( $p < 0.05$ ). For some groups the level of significance was greater ( $p < 0.01$  for the 0.1 % & 0.5 % groups).

Carcass weights for the control and 0.1 % CLA groups were significantly more than the 1.0 and 2.0 % group ( $p < 0.001$ ). The later were also less than the 0.5 % group and the 2.0 % group was less than the 0.25 % group. The carcass weight, in comparison to the live weight, had 1-2 ml blood sample removed, and the stomach, intestine and their contents removed. It was frozen and thawed before weighing, processes which produced a small amount of unmeasured drip-loss.

Body composition results for body fat by dissection are shown in Table 2.10, and body fat by chemical analysis and gross energy content in Table 2.11.

The dose response of body fat to diet CLA % is shown in Graph 2.2. Mean body fat and gross energy content for each diet group are shown in Graphs 2.3 and 2.4. Standard error bars are shown in Graphs 2.3 and 2.4.

Body fat decreased in a dose response relationship, as the amount of CLA increased in the diets. This occurred for both relative body fat (%) and absolute body fat (g). The difference was highly significant ( $p < 0.001$ ). The amount of body fat in mice fed the control diet was significantly more than the 0.25 % diet, but not the 0.1% diet. This was the case for body fat (%), total body fat (g) and lower body fat (g) by dissection. For this data, the number of mice ranged from 14 to 20 mice per group. For chemical analysis of body fat, where mice were analysed in groups of four, and the number of mice in groups ranged from 3 to 5, the amount of body fat in the control mice and 0.1 % CLA group was significantly more than mice fed 0.5 % or greater dietary CLA. Again, there were no differences in chemically analysed body fat between the 1.0 and 2.0 % CLA dietary treatment groups. The body fat (by chemical analysis) of control animals was 8.65 %. Animals consuming 1.0 % dietary CLA had 2.90 % body fat.

The gross energy content per gram of carcass decreased for mice, first noticed at 0.1 % CLA, and continued as the CLA % in the diet increased to 1.0 % ( $p < 0.001$ ). The control and 0.1 % CLA groups contained 8.45 and 8.24 kJ/g carcass weight respectively. The 1.0 % and 2.0 % CLA groups contained 6.42 and 6.56 kJ/g respectively. The carcass energy content of the control was greater than the 0.25 % group ( $p < 0.05$ ) and the 0.5 % group ( $p < 0.001$ ). The 0.5 % group carcass energy

content was greater than the 1.0 % group ( $p < 0.05$ ), and the 0.25 % group greater than the 1.0 % and 2.0 % group ( $p < 0.001$ ).

Body fat by dissection and chemical analysis are correlated with a correlation coefficient of 0.90 ( $R^2 = 81\%$ ). Graph 2.5 shows body fat (dissection) plotted against body fat (chemical). Each body fat by dissection data point, is the mean value of the group of four, corresponding to the chemically analysed body fat from a sample of four homogenised carcasses.

Components of lean body mass (LBM), moisture, protein and ash are shown in Table 2.12. Total lean body mass is shown in Table 2.13.

Lean body mass, relative to total body mass (LBM %), was significantly ( $p < 0.001$ ) higher as the amount of dietary CLA increased. This increase was a result of reduction in body fat.

LBM % was calculated in two ways (refer 2.3.3.22.7). The effect was highly significant and was seen both when LBM % was calculated as the sum of moisture %, protein % and ash %, and calculated as 100 % - fat %. There was no difference in LBM % between the control and the 0.10 % group, and no difference between the 1.0 % and 2.0 % CLA groups.

Weight of lean body mass, calculated as the sum of moisture g, protein g and ash g ( $n = 5$ ) was not significantly different. Weight of lean body mass, calculated as 100 % - fat % ( $n = 20$ ) was significantly different. The weight of LBM in the 1.0 % CLA group was significantly less than the control and the 0.5 % group ( $p < 0.05$ ). The weight of LBM in the 2.0 % CLA group was less than the control and 0.5 % CLA groups ( $p < 0.01$ ), and the 0.1 % CLA group ( $p < 0.05$ ). Analysed separately, weight of body moisture, weight of protein or weight of ash were not significantly different ( $n = 5$ ).

**Table 2.9 Live weight, weight gain, carcass weight and comparison of group means**

Diet CLA %	n	Live weight after 4 weeks on diet g					Weight gain during trial g				Carcass weight g					
0.0	20	25.88 ± 1.57					3.90 ± 1.07				21.05 ± 1.23					
0.1	20	25.49 ± 2.02					3.88 ± 1.20				20.77 ± 1.69					
0.25	18	24.88 ± 1.30					3.76 ± 1.20				20.09 ± 1.18					
0.5	16	25.72 ± 1.77					4.09 ± 1.19				20.69 ± 1.43					
1.0	18	24.55 ± 1.53					3.57 ± 0.74				19.18 ± 1.07					
2.0	14	24.69 ± 2.12					2.94 ± 1.43				18.71 ± 1.50					
Statistical significance <sup>1</sup>		0.1	0.25	0.5	1.0	2.0	0.1	0.25	0.5	1.0	2.0	0.1	0.25	0.5	1.0	2.0
0.0		NS	NS	NS	*	NS	NS	NS	NS	**	NS	NS	NS	***	***	
0.1		-	NS	NS	NS	NS	-	NS	NS	NS	**	-	NS	NS	***	***
0.25		-	NS	NS	NS	-	-	NS	NS	*	-	NS	NS	*		
0.5		-	NS	NS	-		-	NS	**		-	**	**	***		
1.0		-	NS	-			-	NS			-	NS	-			
2.0		-	-	-			-	-			-	-	-			

± standard deviation

1 Significance level

\* < 0.05

\*\* < 0.01

\*\*\* < 0.001

NS Not significant (ie no significant difference between means of the groups)

**Table 2.10 Body fat, relative and absolute, by dissection and comparison of group means**

Diet CLA %	n	Body fat (dissection) <sup>1</sup> %	Total body fat (dissection) <sup>1</sup> g	Upper body fat (dissection) <sup>1</sup> g	Lower body fat (dissection) <sup>1</sup> g
0.0	20	5.60 ± 1.27	1.19 ± 0.32	0.31 ± 0.08	0.88 ± 0.28
0.1	20	5.44 ± 1.27	1.14 ± 0.34	0.26 ± 0.08	0.88 ± 0.29
0.25	18	4.38 ± 0.92	0.88 ± 0.19	0.26 ± 0.07	0.63 ± 0.15
0.5	16	2.96 ± 0.68	0.61 ± 0.15	0.21 ± 0.04	0.40 ± 0.14
1.0	18	2.23 ± 0.82	0.42 ± 0.16	0.14 ± 0.02	0.29 ± 0.16
2.0	14	2.37 ± 0.64	0.45 ± 0.14	0.12 ± 0.05	0.32 ± 0.11
Statistical significance <sup>2</sup>		0.1 0.25 0.5 1.0 2.0	0.1 0.25 0.5 1.0 2.0	0.1 0.25 0.5 1.0 2.0	0.1 0.25 0.5 1.0 2.0
0.0		NS *** *** *** ***	NS *** *** *** ***	NS * *** *** ***	NS *** *** *** ***
0.1		- *** *** *** ***	- *** *** *** ***	- NS NS *** ***	- *** *** *** ***
0.25		- *** *** *** ***	- *** *** *** ***	- NS *** ***	- *** *** *** ***
0.5		- * NS	- ** *	- ** ***	- NS NS
1.0		- NS	- NS	- NS	- NS
2.0		-	-	-	-

± standard deviation

1 Based on individual carcass weight at start of dissection (refer table 2.9)

2 Significance level

\* < 0.05

\*\* < 0.01

\*\*\* < 0.001

NS Not significant (ie no significant difference between means of the groups)

**Table 2.11 Body fat, relative and absolute, by chemical analysis, and gross energy content and comparison of group means**

Diet CLA %	n <sup>1</sup>	Body fat (chemical) <sup>2</sup> %					Body fat (chemical) <sup>3</sup> g/mouse					Gross Energy <sup>2</sup> kJ/g				
0.0	5	8.65 ± 0.82					1.68 ± 0.20					8.45 ± 0.28				
0.1	5	8.13 ± 1.18					1.55 ± 0.32					8.24 ± 0.42				
0.25	4	6.79 ± 1.28					1.27 ± 0.24					7.66 ± 0.35				
0.5	3	4.77 ± 0.14					0.90 ± 0.05					7.18 ± 0.08				
1.0	4	2.90 ± 0.16					0.51 ± 0.03					6.42 ± 0.10				
2.0	3	2.70 ± 0.60					0.46 ± 0.10					6.56 ± 0.13				
Statistical significance <sup>4</sup>		0.1	0.25	0.5	1.0	2.0	0.1	0.25	0.5	1.0	2.0	0.1	0.25	0.5	1.0	2.0
0.0		NS	NS	***	***	***	NS	NS	***	***	***	NS	*	***	***	***
0.1		-	NS	***	***	***	-	NS	**	***	***	-	NS	***	***	***
0.25		-	NS	***	***		-	NS	***	***		-	NS	***	***	***
0.5		-	NS	NS			-	NS	NS	NS		-	*	NS		
1.0		-	NS				-	NS				-		-	NS	
2.0		-			-		-			-		-		-	-	

± standard deviation

1 Carcasses analysed in groups of four

2 Based on weight of group of four combined homogenised carcasses prior to freeze drying

3 Mean value for each mouse in group of four

4 Significance level

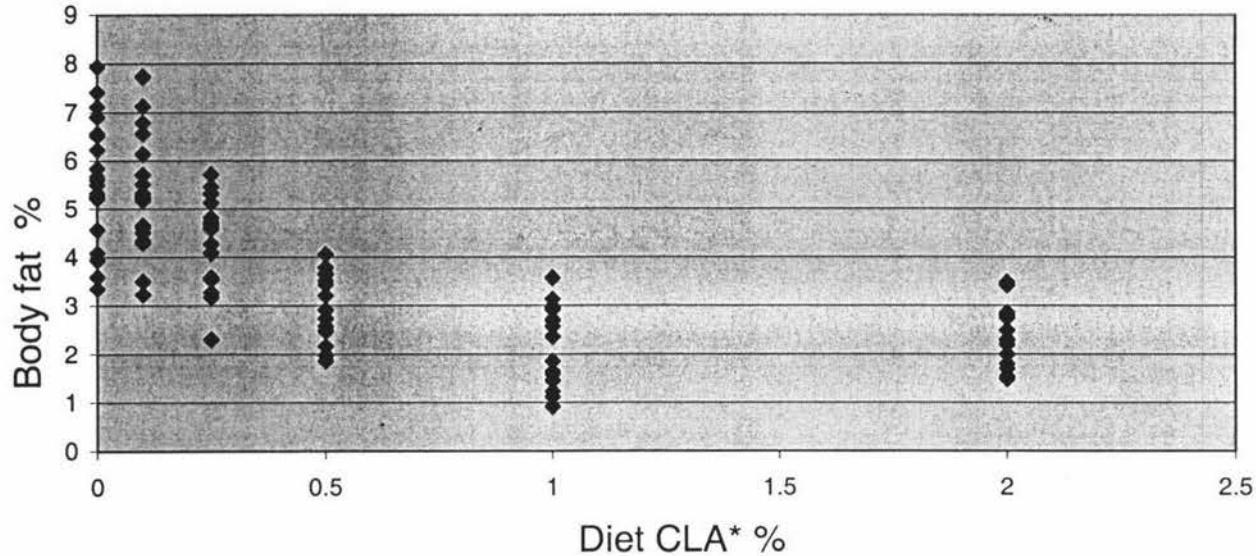
\* &lt; 0.05

\*\* &lt; 0.01

\*\*\* &lt; 0.001

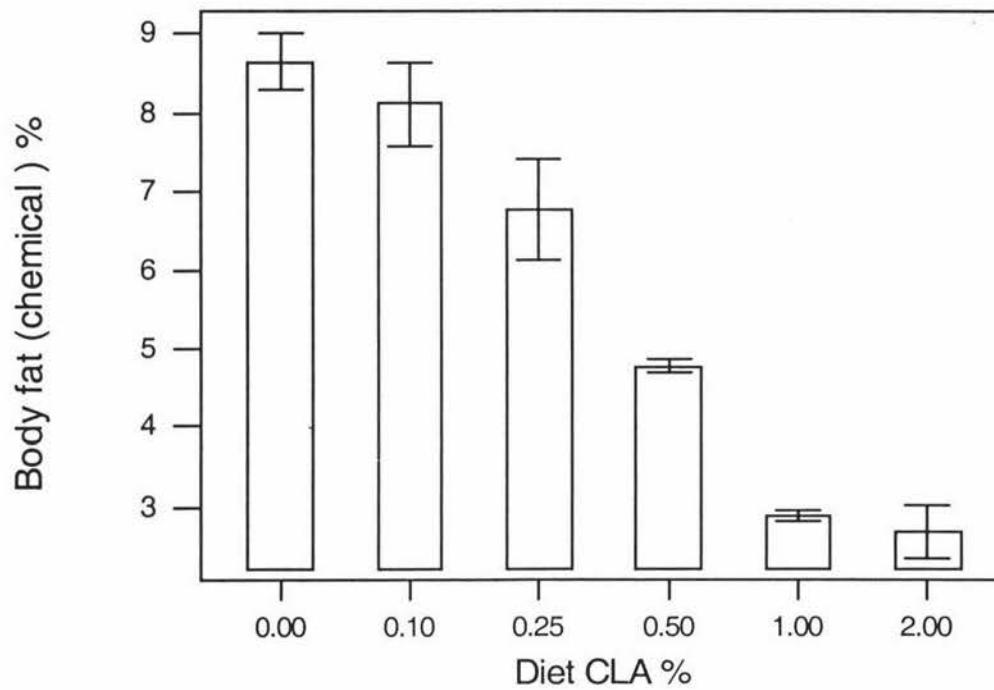
NS Not significant (ie no significant difference between means of the group)

Graph 2.2 Body Fat (dissection) % versus Diet CLA %



\* Mixed CLA isomers containing 37 % 9c11t-CLA and 46 %.10t12c-CLA (refer Table 2.4). CLA % w/w in diet.

Graph 2.3 Body Fat (chemical) versus Diet CLA %

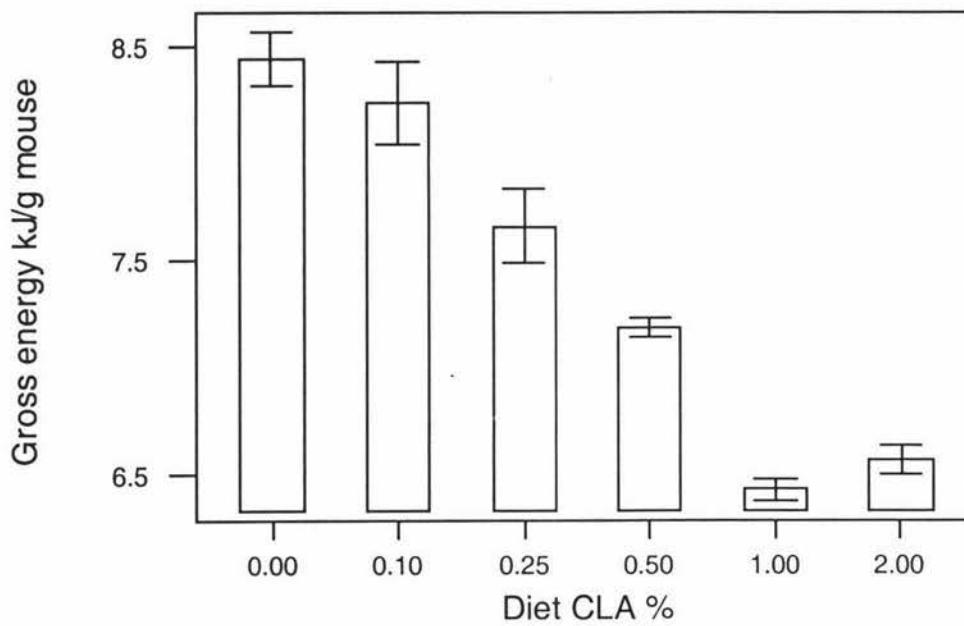


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\* Mixed CLA isomers containing 37 % 9c11t-CLA and 46 % 10t12c-CLA (refer Table 2.4). CLA % w/w in diet

Error bars ± standard deviation.

Graph 2.4 Gross Energy versus Diet CLA %



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\*Mixed CLA isomers containing 37 % 9c11t -CLA and 46 % 10t12c-CLA (refer Table 2.4) . CLA % w/w in diet.

Error bars  $\pm$  standard deviation.

Graph 2.5 Body Fat (dissection) % versus Body Fat (chemical) %

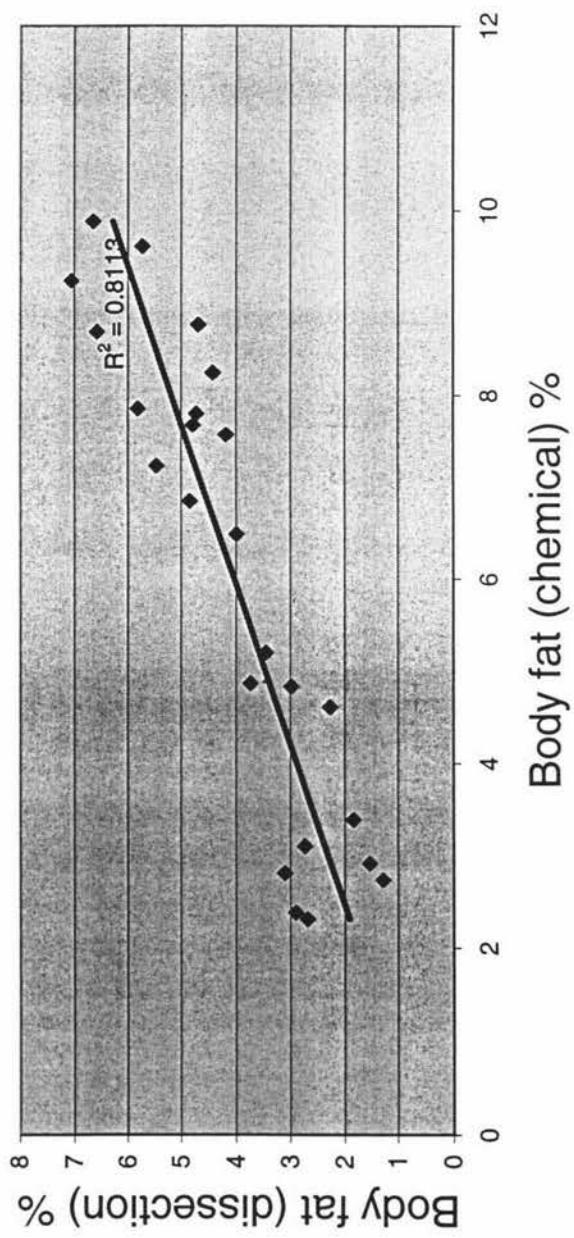


Table 2.12 Lean body mass; moisture, protein and ash and comparison of group means

Diet CLA %	n <sup>1</sup>	Moisture <sup>2</sup>		Protein <sup>2</sup>		Ash <sup>2</sup>		<sup>3</sup>
		%	g/mouse <sup>3</sup>	%	g/mouse <sup>3</sup>	%	g/mouse <sup>3</sup>	
0.0	5	65.87 ± 0.56	12.74 ± 0.43	20.75 ± 0.38	4.01 ± 0.10	3.88 ± 0.21	0.75 ± 0.02	
0.1	5	66.34 ± 0.87	12.59 ± 0.78	20.88 ± 0.74	3.97 ± 0.24	3.79 ± 0.14	0.72 ± 0.05	
0.25	4	67.18 ± 1.22	12.57 ± 0.32	20.92 ± 0.50	3.91 ± 0.10	3.99 ± 0.29	0.75 ± 0.04	
0.5	3	69.01 ± 0.43	13.02 ± 0.45	21.33 ± 0.14	4.02 ± 0.09	4.00 ± 0.26	0.75 ± 0.04	
1.0	4	69.84 ± 0.33	12.34 ± 0.43	22.60 ± 0.25	3.99 ± 0.18	4.19 ± 0.19	0.74 ± 0.05	
2.0	3	69.76 ± 0.34	11.93 ± 0.28	22.91 ± 0.67	3.92 ± 0.06	4.40 ± 0.13	0.75 ± 0.04	
Statistical significance		0.1 0.25 0.5 1.0 2.0	NS	0.1 0.25 0.5 1.0 2.0	NS	0.1 0.25 0.5 1.0 2.0	NS	
0.0		NS NS ** ** **		NS NS NS *** ***		NS NS NS NS *		
0.1		- NS ** ** **		- NS NS ** ***		- NS NS NS **		
0.25		- * ** **		- NS ** ***		- NS NS NS		
0.5		- NS NS		- NS NS		- NS NS		
1.0		- NS		- NS		- NS		
2.0		-		-		-		

± standard deviation

1 Carcasses analysed in groups of four

2 Based on weight of group of four combined homogenised carcasses prior to freeze drying

3 Mean value for each mouse in group of four

4 Significance level

\* &lt; 0.05

\*\* &lt; 0.01

\*\*\* &lt; 0.001

NS Not significant (ie no significant difference between means of the groups)

**Table 2.13 Lean body mass and comparison of group means**

Diet CLA %	n <sup>1</sup>	LBM <sup>2,3</sup>					n <sup>5</sup>	LBM <sup>6</sup>									
		% <sup>4</sup>			g/mouse <sup>7</sup>			% <sup>4</sup>					g/mouse <sup>7</sup>				
0.0	5	90.50 ± 0.87					20	94.40 ± 1.27					19.86 ± 1.02				
0.1	5	91.00 ± 1.00					20	94.56 ± 1.27					19.63 ± 1.44				
0.25	4	92.09 ± 1.26					18	95.62 ± 0.92					19.21 ± 1.27				
0.5	3	94.34 ± 0.14					16	97.05 ± 0.68					20.08 ± 1.39				
1.0	4	96.63 ± 0.26					18	97.77 ± 0.82					18.76 ± 1.07				
2.0	3	97.08 ± 0.36					14	97.63 ± 0.64					18.26 ± 1.43				
Statistical significance <sup>8</sup>		0.1	0.25	0.5	1.0	2.0	NS	0.1	0.25	0.5	1.0	2.0	0.1	0.25	0.5	1.0	2.0
0.0		NS	NS	***	***	***		NS	***	***	***	***	NS	NS	NS	*	**
0.1		-	NS	***	***	***		-	***	***	***	***	-	NS	NS	NS	*
0.25		-	NS	***	***	***		-	***	***	***	***	-	NS	NS	NS	NS
0.50		-	*	**				-	**	NS			-	*	**		
1.0		-	NS						-	NS				-	NS		
2.0		-								-					-		

1 Carcasses analysed in groups of four

1 Based on weight of group of four combined homogenised carcasses prior to freeze drying

2 LBM = moisture % + protein % + ash % (by chemical analysis)

4 Mean value for each mouse in group of four

5 Carcass analysed individually

6 LBM = 100 % - fat % (by dissection)

7 Based on individual carcass weight at start of dissection

8 Significance level

\* &lt; 0.05

\*\* &lt; 0.01

\*\*\* &lt; 0.001

NS Not significant (ie no significant difference between means of the groups)

## **2.4 Discussion**

### **2.5.1 Mouse deaths**

The number of mouse deaths, 5, from 120 mice, or 4 %, was higher than seen in other mouse trials conducted by the Milk and Health Research Centre. While one mouse died following sickness, the remaining four died as a result of aggression from the mouse it was housed with. There were no deaths in the control and 0.1 % dietary CLA groups. Possible explanations for aggression from these male mice include stress from handling and change of environment during energy expenditure measurements, dietary induced stress, physiological stress related to reduction in body fat, or a combination of factors. It is not possible to suggest which, if any, of these factors were important.

### **2.5.2 Food Intake**

Dietary treatments had similar energy content. CLA content, calculated from quantities of ingredients added when the diets were made was 0.0, 0.1, 0.25, 0.5, 1.0, 2.0 % w/w CLA. The proportion of the 9c11t-CLA isomer was assumed to be 37.1% and the 10t12c-CLA isomer 46.3 %. This was on the basis of all CLA originating from Tonalin and the GLC analysis of the isomer content of CLA in the same batch of Tonalin. This is a reasonable assumption as the only other significant fat source in the diets was corn oil, which does not contain CLA. There was no fat from ruminant animals and < 0.1 % milk fat in the skim milk powder portion of diets. Dietary treatments were isoenergetic, similar in gross composition and contained increasing dosages of CLA from a level of 0.1 to 2.0 % w/w. The control (0.0 % CLA) was formulated without the addition of Tonalin.

Feed and consequently energy intake (since diets were isoenergetic), were similar for all six groups of mice except the group receiving the 0.25 % CLA diet. Intakes were significantly higher for this group than both the control and the 1.0 % diet groups ( $p < 0.05$ ). The reason for this is not clear. Body composition and weight measurements for this group fit a CLA dose-dependent trend. This group had significantly less body fat by dissection than the 0.0 % and 0.1 % groups. There was no measurable difference in energy expenditure for this group. There was also no significant difference in the difference between rate of oxygen consumption per gram body weight at two and four weeks in this group compared with other groups. The effect of higher energy intake in

the 0.25 % CLA group was not seen in the body composition or energy expenditure results.

Faecal energy loss was not measured in this trial. A comparison of digestible energy intake between groups of mice has not been investigated. Digestible energy is that portion of energy not excreted in the faeces and therefore assumed to be absorbed by the animal (McDonald, 1996). Digestible energy was measured in the CLA rat feeding trial reported in Chapter 3.

#### 2.5.3 Energy Expenditure

The R values (mean 0.933 at the end of the trial) indicate energy was obtained mainly from metabolism of carbohydrate in these mice, and a lesser amount from fat metabolism.

There was no difference in rate of oxygen consumption, energy metabolism indicated by R, metabolic rate, or activity between mice on dietary treatments of CLA ranging from 0.0 % to 2.0 %. There was also no measurable difference in oxygen consumption rate, oxygen consumption rate per gram of body weight, R or activity between all mice after two weeks on the trial diet and at the end of the trial.

A relationship between activity and metabolic rate was observed, showing that activity was an important determinant of energy expenditure. Increased activity resulted in measurably higher metabolic rate. Dietary treatment, on the other hand, did not measurably effect energy expenditure.

The reason for low serum free fatty acids in the 1.0 % and 2.0 % groups compared with the 0.5 % group (but not the control), is not clear (refer Table 2.8). Repeat measurements are needed to confirm this finding. It is noted that these two groups had significantly less body fat, reduced weight gain, and reduced weight of LBM compared with some groups on lower levels of dietary CLA and/or the control. We did not measure FFA turnover, but if turnover was unchanged by diet, then this lower concentration of FFA would be due to decreased fat oxidation. This may occur when body fat stores are reduced and carbohydrate or protein are the preferential energy source.

#### 2.5.4 Body Composition

Body weight parameters measured were weight gain during the trial, live weight at the end of the trial and carcass weight. These were not affected until a high dosage of CLA was consumed. At the two highest levels of dietary CLA, 1.0 % and 2.0 %, some or all of these parameters were significantly less than the control group, and some dietary CLA groups including the 0.5 % group. In particular, the weight gain of the 2.0 % group during the four week trial was less than the control ( $p < 0.01$ ), the 0.1 % group ( $p < 0.01$ ), the 0.25 % group ( $p < 0.05$ ) and the 0.5 % group ( $p < 0.01$ ). These results suggest high levels of dietary CLA reduce weight in mice. The mice in this trial (6 weeks old at the start) were still growing. Reduced weight gain suggests growth rate has decreased.

Body fat results show a dose response effect of dietary CLA on body fat. At levels of 0.1 % CLA, there is no difference in body fat compared with the control containing no CLA. As dose increases, up to 1.0 % CLA, body fat decreases. At a higher level, 2.0 % CLA, there is no further reduction in body fat.

The high correlation ( $r = 0.90$ ) between total body fat % chemical and total body fat % dissection provides confidence for upper and lower body fat measurements shown in Table 2.10. These were obtained by dissection, without chemical analysis. While both showed significant dose dependent body fat reduction, lower body fat reduction began at 0.25 % dietary CLA, and upper body fat reduction at 0.5 %, compared with controls.

Gross energy of mouse body carcasses showed a highly significant dose response trend, similar to the body fat reduction. These results support the trend of body fat reduction as CLA dosage increases, since body fat has more energy per g than lean body mass. Fat contains approximately 39.8 kJ/g and protein approximately 16.7 kJ/g.

LBM % increased as CLA dosage increased. These results are a consequence of the decrease in body fat. Weight of LBM is maintained up to the 0.5 % CLA group. At high dietary CLA levels, 1.0 % and 2.0 %, the results show a reduction in weight of lean body mass, compared with the control, 0.1 % (2.0 % group but not the 1.0 % group) and

0.5 % group. Body carbohydrate was not analysed separately. It is possible that some of the reduction in LBM was due to reduced carbohydrate stores.

The body fat, carcass gross energy, weight gain and lean body mass results should be considered together. They show a significant reduction in adipose tissue deposits as dietary CLA % group increases. As body fat decreases, relative LBM increases. Weight of LBM in the 1.0 % and 2.0 % groups is reduced. Weight gain of animals consuming 2.0 % CLA was less than the control, and groups of animals (0.1, 0.25, 0.5 % CLA) on lower dietary CLA levels. Reduction in weight gain (2.0 % CLA group) and/or LBM weight reduction (1.0 % and 2.0 % group), in comparison with other groups, occurs when high dosages of CLA are consumed, although body fat reduction begins at a much lower CLA level (0.25 %). The reduction in weight gain is due to both reduced body fat and reduced LBM at high CLA dosages.

Reduction in weight gain and LBM in mice of this age means growth reduction has occurred in the 1.0 and 2.0 % CLA groups. Reduction in body fat is generally considered beneficial, whereas growth reduction is not. Levels of 0.25 % to 0.50 % w/w dietary CLA resulted in reduced body fat without growth reduction. The 1.0 % CLA group had further body fat reduction and reduced growth. Levels of 2.0 % CLA did not further reduce body fat and did reduce growth.

These mice were fed *ad lib* and food intakes were similar for the 0.5 %, 1.0 %, and 2.0 % groups, compared with the control and 0.1 % diets, although intake for the 0.25 % group was higher. With the exception of the 0.25 % group, it is interesting to note that although body fat decreased as CLA dose increased, mice did not compensate by consuming more food. Body fat reduction was first seen in the 0.25 % group (which was an exception and did have a greater food intake). Body fat reduction increased in the 0.5 % and 1.0 % group and food intake did not change. This suggests that the reduction in body fat at higher CLA levels was not associated with a feeling of hunger or starvation, causing the mouse to eat more. This suggests blood glucose was maintained, despite the apparent decrease in carbohydrate stores. This might reflect preservation of liver glycogen at the expense of muscle glycogen since only the liver can contribute glucose to the circulation.

The 0.25 % w/w dietary CLA group was the first to show a reduction in body fat. For comparison with the CLA feeding trials using rats in Chapter 3 it is useful to state dietary CLA on a percent of energy basis. The CLA % energy in the 0.25 % w/w CLA diet group was 0.58 % (refer appendix 2.3 for calculation).

The observed reduction in body seen in this trial as a result of dietary CLA is in agreement with the findings of other researchers (Park *et al.*, 1997; Sugano *et al.*, 1998; West *et al.*, 1998 and DeLany *et al.*, 1999). The reduction in lean body mass at high levels of dietary CLA supports the findings of West *et al.* (1998) and is opposite to the findings of Park *et al.* 1997 and DeLany *et al.* (1999).

#### 2.5.5 Isomers

In this trial, CLA originated from Tonalin. The proportion of the 9c11t-CLA isomer in each diet group was 37.1 % and 10t12c-CLA was 46.3 %. At the time of starting the trial, all the published reports investigating the effect of CLA on health in animals, used synthetic CLA with a similar isomer ratio. These publications assumed 9c11t-CLA was the biologically active form because it was the major isomer found in tissue of animals fed mixed isomer diets (Ha *et al.*, 1990, Ip *et al.*, 1991).

#### 2.5.6 Further Research

A further study will be conducted to investigate the effect of both synthetic CLA and bovine CLA on energy balance. The bovine CLA will contain approximately twice as much of the 9c11t-CLA isomer per unit weight of CLA compared with the synthetic CLA used in this feeding trial. Rats will be used since they are larger and individual measurements can be made throughout. In addition to the components of energy balance measured in this first trial, faecal energy will be measured, digestible energy intake calculated, and diet-induced thermogenesis will be calculated from the difference between fed and fasted energy expenditure.

## CHAPTER 3

### Trial 2; Investigation of the Effect of Bovine Conjugated Linoleic Acid on Energy Balance in Rats

#### 3.1 Abstract

##### Introduction

This study was conducted to investigate the effect of consumption of bovine CLA (86 % 9c11t-CLA and 14 % other CLA isomers) and synthetic CLA (37 % 9c11t-CLA isomer and 46 % 10t12c-CLA isomer) on energy balance in female Sprague-Dawley rats.

##### Methods

Four standard milk powder based diets were formulated with equivalent total fat (14 %) and variable fat composition. The variable in these four diets was fatty acid profile, including CLA content and CLA isomer ratio. Diet 1 was a control diet with fat source cornoil (0.0 % w/w CLA), diet 2, cornoil and synthetic Tonalin (0.21 % w/w CLA, 37 % 9c11t-CLA), diet 3, milk fat fraction containing a high level of CLA (0.29 % w/w CLA, 86 % 9c11t-CLA), diet 4, typical milk fat fraction (0.23 % w/w CLA, 78 % 9c11t-CLA) and diet 5, standard rodent chow diet (0.01 % w/w CLA). The trial feeding period was four weeks.

The measured components of energy balance were;

- (i) Food intake, energy intake, faecal energy and digestible energy.
- (ii) Energy expenditure by indirect calorimetry in fed and fasted rats, diet-induced thermogenesis (DIT) and activity by observation.
- (iii) Stored energy, measured by chemical analysis of body fat, protein, moisture and ash, and weight of dissected adipose tissue deposits.

##### Results

Data was analysed using a one way analysis of variance and Tukey comparison. We found no significant differences in digestible energy intake, energy expenditure, body fat, body moisture, or body protein content between the milk powder based diet groups

and the standard rodent diet control. Feed intake ( $p < 0.01$ ), energy intake ( $p < 0.05$ ) and faecal energy ( $p < 0.01$ ) were significantly less for all of the milk powder based diets compared with the standard rodent diet control. Feed efficiency for all of the milk powder based diets, excluding diet 1 (cornoil), was greater than the standard rodent diet ( $p < 0.01$ ). Body ash % w/w of rat carcasses, was significantly less in the typical milk fat diet group than the high CLA milk fat fraction diet ( $n = 7$ ,  $p < 0.05$ ). Absolute weights of body ash were not different between groups.

### Conclusions

Natural bovine CLA and the synthetic CLA used in the rat diets had no effect on energy balance.

The CLA level used in these diets was the highest level that could be formulated using natural CLA present in milk fat. The effect on energy balance of a higher level of natural CLA in the diet is not known. A milk fat fraction with a higher CLA content is required before this can be examined. Although not currently technologically possible, an alternative to a milk fat fraction containing more CLA may be pure CLA extracted from milk fat.

The CLA in milk fat contains approximately 86 % of 9c11t-CLA and synthetic CLA 37 % 9c11t-CLA. Three recent studies demonstrate the biological activity of 10t12c-CLA in relation to fatty acid metabolism, energy metabolism and body composition. It is important to be aware of the CLA isomer ratio, as well as the total CLA content, in any research investigating the biological activity of dietary CLA.

### 3.2 Experimental materials and procedures

The Massey University Ethics Committee approved all aspects of this study.

#### 3.2.1 Animals and housing.

Forty female Sprague-Dawley rats were supplied by Otago Medical School. They were aged eleven and twelve weeks and were housed in individual hanging cages, at Crop and Food Research Institute, Palmerston North (see Table 3.2 for age at the start of the trial). The cages were 175 mm wide and high, and 260 mm long. Trays covered in sawdust and a sheet of blotting paper, were placed with a 5cm gap beneath each cage to collect spilt food and faeces. The cages were hung on one trolley in a room maintained at 22 ° C (range 21.0 to 23.0 ° C) with constant 12 hours light 7.30am to 7.30pm, and 12 hours dark.

#### 3.2.2 Rat diets

Rats were divided into five groups of eight. They were fed one of four skim milk powder based diets, or a standard ground rodent chow (Reliance Stockfoods Ltd, Dunedin) *ad lib*, during the four-week trial. The milk powder based diets were controlled for composition except fatty acid profile. The total fat and energy content in these four diets was controlled. The fatty acid composition varied due to the following differences in ingredients (refer to Table 3.2);

Diet 1; cornoil (control diet A)

Diet 2; cornoil and Tonalin

Diet 3; a fraction of bovine milk fat containing a higher proportion of long chain fatty acids, including CLA, than typical milk fat

Diet 4; typical milk fat

Diet 5; ground rodent chow (control diet B)

The ingredients used to make the diets are shown in Table 3.1.

**Table 3.1 Ingredient composition of rat diets**

<b>Ingredient g/ 100 g diet</b>	<b>Diet treatments</b>			
<i>Standard milk powder base</i>	Diet 1 Cornoil	Diet 2 Cornoil and Tonalin	Diet 3 High CLA milkfat	Diet 4 Typical CLA milkfat
<i>CLA mg / 100g</i>	0	210	290	230
Skim milk powder (bovine)	54.3	54.3	54.3	54.3
Mineral mix <sup>1</sup>	5.0	5.0	5.0	5.0
Vitamin mix <sup>1</sup>	5.0	5.0	5.0	5.0
Cellulose	5.0	5.0	5.0	5.0
Cornflour	16.7	16.7	16.7	16.7
Cornoil <sup>2</sup>	2.0	2.0	2.0	2.0
<i>Additional fat</i>				
Tonalin <sup>3</sup>	-	<b>0.3125</b>	-	-
Cornoil	<b>12.0</b>	<b>11.7</b>	-	-
Milk fat fraction with high CLA	-	-	<b>12.0</b>	-
Milk fat fraction with typical CLA	-	-	-	<b>12.0</b>
<b>Total</b>	100.0	100.0	100.0	100.0

1 Refer section 2.2.2 Mouse diets.

2 Linoleic acid requirement in diet.

3 Tonalin in triglyceride form.

The ground rodent chow was used by Otago Medical School (Reliance Stockfoods Ltd, Dunedin), to feed the rats since weaning, and was formulated as an optimum diet for growing rats. The rats in this group represented the effect of no dietary change (control diet B). This chow contained the following ingredients; barleymeal, broll (bran etc from wheat flour milling), wheatmeal, fishmeal, meat and bonemeal, milk powder, soybean, soy oil, methionine, salt, vitamins and trace minerals including selenium. These ingredients suggest it is higher in fibre content than the other four skim milk powder based experimental diets.

The level of CLA in experimental diets was determined by consideration of a number of factors.

- As this was an energy balance study, it was important to match energy intake to energy expenditure and the fat level in the experimental diets was limited to 14 % w/w. The fat level in the standard rodent chow (diet 5) was 6.1 %.

- The minimum level of CLA shown to effect body fat in the mouse trial described in Chapter 2 was 0.25 % w/w dietary CLA. This was equivalent to 0.58 % of energy.
- The highest level of CLA in the bovine milkfat fraction available at the time was 2.4 % w/w CLA. The 14 % w/w dietary fat level (2 % from cornoil and 12 % from additional fat), delivered 290 mg CLA / 100 g in the high CLA milkfat diet (0.29 % w/w CLA).
- At the time of starting this study it was generally believed, although not proven, that the biologically active CLA isomer was 9c11t-CLA (refer 1.2.1). The mouse diets contained 37 % 9c11t-CLA. CLA in the high CLA milk fat diet milk fat diet contained 86 % 9c11t-CLA. The rat diets would contain approximately twice as much of the 9c11t isomer in any given amount of total CLA, when compared with the mouse diets. Rat diets were formulated to give similar total % CLA as the 0.25 % mouse diet. Analysis showed they contained 0.21 % CLA (diet 2, cornoil and Tonalin), 0.29 % CLA (diet 3, high CLA milk fat) and 0.23 % CLA (diet 4, typical CLA milk fat) (refer Table 3.4).

Diet 1 (control diet A, cornoil) and diet 2 (cornoil and Tonalin) were used to investigate the effect of synthetic CLA obtained from Tonalin on energy balance in the rat. Diet 1 contained no CLA. Diet 3 (high CLA milk fat) introduced a bovine CLA source, present in a milk fat fraction, which also contained a higher proportion of long chain fatty acids than typical milk fat. We used the milk fat fraction with the highest CLA content available at the time of the experiment.

Diet 2 and diet 3 were formulated to contain similar amounts of total CLA, one from a synthetic source and the other from milk. The CLA isomer ratio was different as described above. These diets were to compare the effect of synthetic and natural CLA on energy balance.

Diet 4 (typical CLA milkfat) used typical milk fat, and was a comparison with the high CLA and long chain fatty acid milk fat fraction in diet 3.

The high CLA milk fat, used in diet 3, was obtained from early season milk (September) from cows grazed on pasture with a supplementary feed. The cow trial work was confidential and details of cow feed manipulation were not available. The

milk from these cows was processed into butter and the milk fat phase was then extracted to obtain anhydrous milk fat. The milk fat was then fractionated in a laboratory scale fractionation system. The soft fraction obtained from the first fractionation was reprocessed and a second soft fraction was collected as the test sample.

The typical milk fat sample was from anhydrous milk fat produced in the New Zealand Dairy Research Institute (NZDRI) pilot plant from cream from late season (May) production.

Nutrient requirements for rat diets were obtained from published data (National Research Council, 1995). Amino acid composition of skim milk and essential fatty acid composition of corn oil was obtained from published data (DSIR, 1989, DSIR, 1991). Corn oil was included in all diets to provide linoleic acid, which is considered to be essential for rats of this age (National Research Council, 1995).

The rat diets were analysed for protein, fat, ash, moisture, and total energy and results are shown in Table 3.3. The fatty acid profile of diets is shown in Table 3.4.

### 3.2.3 Experimental procedure

The experimental protocol for the rat feeding trial is summarised in Table 3.2.

**Table 3.2 Protocol for Trial 2**

Number of female Sprague-Dawley rats	40	
Rat supplier	Otago Medical School, Department of Laboratory Animal Sciences.	
Diet treatments; Diets 1-4; diets each with standard base and experimental fat composition. Diet 5; standard rodent chow	Diet 1 Diet 2 Diet 3 Diet 4 Diet 5	Cornoil Cornoil and Tonalin High CLA milkfat Typical CLA milkfat Ground rodent chow
Number of rats in each treatment diet	8	
Number and content of groups	2 groups of 20 rats, comprised of 4 rats on each of 5 diet treatments.	
Acclimatisation period, before trial start date	Group 1 Group 2	1 week 2 weeks
Age of rats at start of trial	Group 1 Group 2	11 weeks 12 weeks
Trial start dates	Group 1	Monday, 9/11/98

	Group 2	Monday, 16/11/98
Trial period	4 weeks	
Age of rats at end of trial	Group 1 Group 2	15 weeks 16 weeks
<b>Measurements</b>		
Live weight	Weekly	
Food consumed	3 times per week.	
Food spilt	Weekly	
Faecal output	Faeces collected daily and composited, during the fourth week of the trial.	
Calorimetry	Sub-group of 3 animals from diet groups 1,2,3 and 5 selected for fasted and fed calorimetry measurements at the end of the trial.	
Body fat by dissection	Abdominal cavity adipose, pelvic limb adipose and interscapular brown adipose tissue deposits dissected and weighed from all carcasses.	
Body composition	Chemical analysis of fat, dry matter, ash and nitrogen.	

The rats were divided into two groups of twenty for practicality of handling calorimeter measurements at the end of the trial.

During the acclimatisation week, rats were fed the ground rodent chow. They were then ranked according to weight, and weight matched groups were randomly allocated to different diets. The rats were fed *ad libitum*, with feed being placed in a small jar, inside a larger bowl. The feeding jars were cleaned and filled weekly on Monday, and topped up regularly on Wednesday and Friday. Spilt food and faeces was collected on the sheet of absorbent blotting paper, on top of the sawdust, as previously described. The animals had free access to water, which was fed in a vertical dispenser attached to the outside of the cage.

During the last week of the trial the calorimeter cage was taken into the rat housing unit. A subgroup of rats (refer 3.3.2.12) were placed in the calorimeter cage for approximately 30 minutes, three times on separate days, to accustom them to this environment.

On day 28 rats were euthanased by inhaling isoflurane overdose. The carcasses were frozen until subsequent dissection to assess body fat content.

### 3.3 Physical and chemical methods

#### 3.3.1 Intake of food and energy

##### 3.3.1.1 Food intake

Food intake was determined on a weekly basis for individual rats. It was the difference between food offered and food remaining. It was assumed that there was no loss of food, since any spilt food would have been on the spillage tray located under the cages. Food left in the feeder was weighed before washing or topping up the feeder. The absorbent paper on the spillage tray collected spilt food and faeces. It was removed on a Monday and left to dry for one week at room temperature, 22°C and 55 % relative humidity. Each tray was then tipped and scrapped onto a sieve, where powdered food was separated and weighed. The weight was added to the weight of food left in the feeder.

Weekly food intake was the sum of food offered, minus spilt food and food left in the feeder. This was measured for each of four weeks during the trial, summed to obtain the weight of food consumed during the trial, and divided by 28 to obtain feed intake per day.

##### 3.3.1.2 Feed efficiency

Feed efficiency was;  $\frac{\text{weight gain of a rat over four weeks}}{\text{food intake of a rat over four weeks}}$

##### 3.3.1.3 Rat diet composition

###### 3.3.1.3.1 Preparation of rat diet samples for chemical analyses

The powdered or ground rat diet sample was taken at the time of the trial and placed in a sealed plastic container in the freezer. Samples for analysis were weighed directly from these containers.

### 3.3.1.32 Compositional analysis

Fat, nitrogen, dry matter, ash and energy were determined for the five diets by the methods described in sections 3.3.3.22. Analytical results for fat %, ash % and energy (kJ/g) were used directly. Protein % was obtained by multiplying nitrogen % by 6.38<sup>8</sup>. Moisture % was obtained by subtracting dry matter % from 100 %.

### 3.3.1.33 Fatty acid profile, including conjugated linoleic acid

CLA was analysed at NZDRI using accelerated solvent extraction (ASE) prior to fatty acid methyl (FAME) analysis by capillary gas chromatography. Tridecanoic acid internal standard and antioxidant 2,6, *Di tert*-butyl 4 methylphenol (BHT) were also added into the sample before extraction. This antioxidant was added to protect CLA from oxidation.

### 3.3.1.4 Energy Intake

Energy intake was obtained by multiplying food intake (g) by the gross energy content (kJ/g) of each diet. This energy value was obtained for each week of the trial, summed to obtain a value for the whole trial, and divided by 28 to obtain daily energy intake.

### 3.3.1.5 Faecal Gross Energy

Faeces were collected daily during week four, frozen daily and the composite freeze-dried. The gross energy value was obtained by the method described in 2.3.3.22.6. The total faecal gross energy for week four was obtained by multiplying the gross energy value (kJ/g) by the total weight of freeze-dried faeces (g) for the week.

### 3.3.1.6 Digestible Energy

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<sup>8</sup> (McDonald *et al*, 1996) An average conversion factor of 6.38 often used for milk protein has been selected since skim milk powder is the major protein containing component in the diets.

Digestible energy was obtained during week four by subtracting the faecal gross energy from the energy intake for that week.

### 3.3.2 Energy Expenditure

#### 3.3.2.1 Calorimetry

The calorimeter system was the same as described in section 2.3.2.1. It was located in the Physiology Laboratory in the Institute of Food, Nutrition and Human Health at Massey University.

#### 3.3.2.11 *Preliminary studies*

Preliminary studies were carried out to develop a method to measure diet induced thermogenesis (DIT) by indirect calorimetry in rats.

##### (a) Preliminary studies involving two rats.

###### Introduction

The energy expenditure ascribable to food intake is DIT (Linder, 1991). The difference in energy expenditure between a fasted animal, and the post-prandial energy expenditure is assumed to be DIT. Pre and post-prandial energy expenditure rats fed isoenergetic diets with variable fatty acid profile (ie fat component of diet obtained from beef tallow, high oleic acid safflower oil, safflower oil or linseed oil) has been measured by indirect calorimetry (Shimomura *et al*, 1990, Takeuchi *et al*, 1995). We used two Sprague-Dawley rats, not on the feeding trial, to develop a method to measure DIT.

###### Method

A rat was fasted overnight, and then placed in the calorimeter. One of the milk powder based diets, covered by a lid, was also placed in the calorimeter. Respiratory gas exchange was measured for an hour in this fasted state. After one hour, the food lid was removed, without disturbing or effecting the concentrations or flow rate of gas through the calorimeter. A lid, handle and sealed calorimeter roof attachment were built for this purpose. The rat ate food immediately and settled to sleep. The lid was replaced on the food after one hour, again, without effecting the gas concentrations or flow rate. Respiratory gas exchange was measured for the post-prandial period for three to four

hours, until the post-prandial oxygen consumption peak was passed. An individual rat was in the calorimeter for a period of up to six hours.

Both of the rats used in this preliminary study were sexed at the animal housing unit and assumed to be female. However, one was a pregnant female, and one male.

#### Results

The two rats in the preliminary study ate between 2.5 g and 6 g of food during the feeding period. Rates of oxygen consumption and carbon dioxide production and R values increased immediately after eating. R values during the fasted period were in the range 0.70 to 0.75. Post-prandially they peaked, at a value in the range approximately 0.80 to 0.85 before trending downward gradually. The difference between the post-prandial peak and pre-prandial fasted oxygen consumption and R values were used to calculate DIT.

(b) Preliminary results involving trial rats.

#### Purpose

To measure DIT in trial rats by indirect calorimetry.

#### Method

At the end of the feeding trial, individual trial rats were fasted overnight before placing in the calorimeter to follow the procedure described above to measure DIT.

#### Results

When the feed lid was removed, each rat ate a small amount of food, less than 0.5g, and went to sleep. The procedure was repeated on four individual rats and on each occasion insufficient food was eaten to measure appreciable changes in respiratory gases.

#### Discussion

The trial rats did not behave in the same way as the practice rats. There were several possible explanations for the differences in eating patterns between the two rats in the preliminary study and the trial rats. The food intake requirements of the preliminary study rats, a male and a pregnant female, may be more than the female trial rats. The trial rats, accustomed to *ad lib* feeding, and with nocturnal habits, were not interested in more than a small amount of food during daylight hours, even after fasting. Some

training may have been necessary to change the feeding habits established during the four trial weeks.

It was necessary to refine the protocol for measurement of DIT in trial rats. The calorimetry procedure was changed to measure respiratory exchange gases from a fed rat, and the same rat fasted, the next day. The difference between fed and fasted energy expenditure in the same rat was attributed to DIT (refer 3.3.1.12 for calculation), provided activity levels did not affect energy expenditure. Activity was measured by observation and recorded during the fed and fasted calorimetry sessions.

### Conclusion

The modified method measured energy expenditure in fed rats, and the same rats fasted, 24 hours later. There was a measurable positive difference in energy expenditure and it was assumed to be DIT.

#### *3.3.2.12 Calorimetry measurements on trial rats.*

A subgroup of four rats from diet 1, 2, 3 and 5 was selected for calorimetry. The reduced number of rats, from four out of the five diet groups, was necessary to expedite calorimeter measurements with the single calorimeter available. We did not want body composition measurements affected by a wide age range at euthanasia.

Two rats from each of diets 1, 2, 3 and 5 were selected from the four in Group 1 (started trial diet 9/11/98) and another two from the four in Group 2 (started trial diet 16/11/98). The two rats were selected by removing the two rats with the least and most weight gain during the trial, from the same diet treatment, in each of Group 1 and Group 2.

Respiratory exchange gas measurements were initially made on a fed rat. The rat was then fasted overnight, in an elevated cage with a wire mesh floor, the same as used in the housing unit. The second set of measurements, using the same rat fasted, were made the next day.

The gas analyser displayed a signal-drift during the 45 minute measurement period. This drift was measured in the following way. The oxygen consumption and carbon dioxide production measurements of the calibrated calorimeter, before the rat was

introduced, were zero. Running room air through the calorimeter system for five minutes before introducing the rat checked this. At the end of the 45 minute experimental calorimetry period, the calorimeter was flushed with room air for ten minutes, followed by measurement of steady state gas levels for five minutes. An oxygen or carbon dioxide measurement at the end of this period, was attributed to drift. This drift was assumed to be linear and the respiratory gas measurements were corrected accordingly.

The calorimeter respiratory exchange gas measurements were used to calculate the respiratory quotient, energy equivalence of oxygen and metabolic rate using the formula described in 2.3.2.1.

Diet induced thermogenesis was calculated as the difference in metabolic rate between a fed rat and the same rat fasted.

### 3.3.2.13 Activity

Visual observations of rat activity were recorded at five-minute intervals during calorimetry. The rat activity scoring scale is shown in Table 3.3.

**Table 3.3 Rat activity score**

Score	Description of rat activity
1	Sleeping
2	Resting, crouching with eyes open
3	Grooming
4	Slow or moderate movement, feeding
5	Vigorous movement

### 3.3.3 Body Composition

#### 3.3.3.1 Live weight

Individual rats were weighed weekly in the morning on the same day each week. Total weight gain over the four week trial and live weight at the end of the trial were determined.

### 3.3.3.2 Measurement of body composition

#### 3.3.3.2.1 *Body composition by dissection*

The frozen rat carcass was thawed at room temperature before dissection and kept in the refrigerator until dissection on the same day. The carcass was weighed. The gastrointestinal tract was dissected by cutting where the oesophagus entered the stomach and at the anus. The adipose tissue adhering to this part of the gastrointestinal tract was carefully removed and retained. The gastrointestinal tract was weighed, and discarded.

Adipose tissue located in the abdominal cavity, on the pelvic limb and interscapular brown adipose tissue (BAT) deposits were dissected from the carcass and weighed. Abdominal cavity adipose deposits were weighed separately in three groups, according to the location. This was adipose tissue attached to the dissected intestinal tract, adipose tissue attached to the genital organs from the ovaries, along the uterine horns to the uterus, and adipose tissue surrounding the kidneys and lining the anterior of the abdominal cavity.

The carcass was cut into approximately 2.5cm square pieces, combined with the dissected adipose tissue and frozen until sample preparation for chemical analysis.

#### Preliminary work

Dissections were carried out on a different group of rats to improve technique before this trial.

### 3.3.3.22 *Body composition by chemical analysis*

#### 3.3.3.22.1 *Preparation of rat samples for chemical analysis*

Rats were individually prepared for chemical analysis. The frozen chopped carcass was semi-thawed before mincing through 2mm orifices, flattened to less than 1cm and freeze dried (Cuddon, Model 0610 freeze drier). The mincer (Kenwood, Chef model) was washed and dried between each sample preparation. It was not possible to grind the rats in the hammer mill used for the mice in Chapter 2, because of the high fat content, which caused a paste to form in the mill. Instead, the freeze dried material was refrozen and blended frozen (Breville Mini Wizz). Duplicate samples were taken for the fat, dry

matter and ash analysis. The defatted material was ground through a 1 mm screen (Glen Creston cyclic hammer mill) and duplicate samples taken for nitrogen determination.

### **3.3.3.22.2                  Fat Determination**

The fat determination method is described in section 2.3.3.22.2, Fat Determination.

### **3.3.3.22.3                  Nitrogen Determination**

The nitrogen determination method is described in section 2.3.3.22.3, Nitrogen Determination.

### **3.3.3.22.4                  Dry Matter Determination**

The dry matter determination method is described in section 2.3.3.22.4, Dry matter Determination.

### **3.3.3.22.5                  Ash Determination**

The ash determination method is described in section 2.3.3.22.5, Ash Determination.

### **3.3.3.22.6                  Formula used to calculate rat body composition**

The analytical results for fat, nitrogen, moisture and ash content of the freeze-dried sample were used to calculate rat body composition. Body fat, protein, moisture, ash and lean body mass were calculated according to the formula described below. In these formulae, FD is freeze-dried, and weight A is the weight of the minced rat carcass prior to freeze drying.

Worked examples of calculations are shown for the following set of hypothetical results; The hypothetical rat minced carcass weight was 200.0 g. After freeze drying the weight was 76.88 g, fat 24.15 %, nitrogen 9.49 %, dry matter 94.40 %, and ash 9.86 %.

#### **(i)      Fat**

The percent fat in a rat carcass was calculated from the fat in the freeze-dried sample using the following formula;

$$\text{Fat \%} = \frac{\text{fat \% in FD sample} \times \text{weight FD sample}}{\text{weight A}}$$

$$\text{Fat \%} = \frac{24.15 \% \times 76.88 \text{ g}}{200.0 \text{ g}} = 9.28 \% \text{ fat}$$

The weight of fat for a hypothetical rat was  $9.28\% \times 200.0\text{ g} / 100\% = 18.56\text{ g}$

(ii) Protein

The percent protein in a rat carcass was calculated from the nitrogen in the freeze-dried sample using the following formula;

$$\text{Protein \%} = \frac{\text{nitrogen \% in FD sample} \times 6.25^9 \times \text{weight FD sample}}{\text{weight A}}$$

$$\text{Protein \%} = \frac{9.49\% \times 6.25 \times 76.88\text{ g}}{200.0\text{ g}} = 22.80\% \text{ protein}$$

The weight of protein for a hypothetical rat was  $22.80\% \times 200.0\text{ g} / 100\% = 45.60\text{ g}$ .

(iii) Moisture

The percent moisture in a rat carcasses was calculated from the moisture in the freeze-dried sample and the moisture lost during the freeze drying process, using the following formula;

$$\text{Moisture \%} = \frac{\text{weight A g} - (\text{\% dry matter in FD sample}/100 \times \text{weight FD sample})\text{g}}{\text{weight A}} \times 100$$

$$\text{Moisture \%} = \frac{200.0\text{ g} - (94.40\% / 100 \times 76.88\text{ g})}{200.0\text{ g}} \times 100 = 63.71\% \text{ moisture}$$

The weight of water for a hypothetical rat was  $63.71\% \times 200.0\text{ g} / 100\% = 127.44\text{ g}$

(iv) Ash

The percent ash in a rat carcass was calculated from the ash \% in the freeze-dried sample using the following formula;

$$\text{Ash \%} = \frac{\text{ash \% in FD sample} \times \text{weight FD sample}}{\text{weight A}}$$


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<sup>9</sup> (McDonald *et al.*, 1996). An average conversion factor of 6.25 often used for meat protein has been selected. The protein value calculated from nitrogen using this conversion factor will differ from true protein because of the assumptions that all nitrogen is present as protein and all protein contains 160 g nitrogen/kg.

$$\text{Ash \%} = \frac{9.86 \% \times 76.88 \text{ g}}{200.0 \text{ g}} = 3.79 \%$$

The weight of ash for a hypothetical rat was  $3.79 \% \times 200.0 \text{ g} / 100 \% = 7.58 \text{ g}$ .

(v) Lean body Mass

The percent lean body mass (LBM) in a rat carcass was calculated by summing the percent protein, moisture and ash for the same hypothetical rat, using the following formula;

$$\text{LBM \%} = \text{protein \%} + \text{moisture \%} + \text{ash \%}.$$

e.g For a hypothetical rat, the protein was 22.80 %, the moisture was 63.71 % and the ash 3.79 %.

$$\text{LBM \%} = 22.80 \% + 63.71 \% + 3.79 \% = 90.30 \%$$

The weight of LBM in a hypothetical rat  
was  $90.30 \% \times 200.0 \text{ g} / 100 \% = 180.6 \text{ g}$

It is acknowledged that this value for LBM will not include the carbohydrate in the body.

### 3.4 Results

#### 3.4.1 Data analysis

Individual measurements were recorded for food intake, faecal energy, digestible energy, rate of oxygen consumption, rate of carbon dioxide production, live and carcass weights, body fat by dissection, and body fat, protein, ash and moisture by chemical analysis.

Results were analysed using the statistical package MINITAB version 12.1 A one-way analysis of variance (ANOVA) was used to analyse each experimental response for a rat grouped according to diet treatment. Where the F test showed a significant difference between means, a Tukey comparison was used to determine which means differed, together with the level of significance.

### 3.4.1.1 Data excluded from analysis

Two rats failed to thrive, had reduced food intake and lost weight during the four week trial. These rats, from diet treatments 2 and 4, were not included in any of the data analysis.

One set of food intake data was excluded because an error was made in weighing one feed container in week two for a rat in group 3. The container weight was not correctly tared. Food intake over the whole trial and the related value, feed efficiency, was not included for this rat.

### 3.4.2 Food Intake

Trial diet composition, including CLA content is presented in Table 3.4. Fatty acid profile for each of the five diets is shown in Table 3.5.

**Table 3.4 Trial diet composition**

<b>Component % w/w</b>	<i>Trial diet treatments</i>				
	Diet 1 Cornoil	Diet 2 Cornoil and Tonalin	Diet 3 High CLA milkfat	Diet 4 Typical CLA milk fat	Diet 5 Ground rodent chow
Protein %	24.95	24.56	24.88	24.69	25.90
Fat %	14.13	14.18	14.13	14.17	6.05
Ash %	8.30	8.09	8.06	7.92	7.15
Moisture %	5.00	5.34	4.85	4.66	10.76
Total energy kJ/g	19.47	19.33	19.34	19.34	17.10
CLA <sup>1</sup> %	0.0	0.21	0.29	0.23	0.01
9c11t-CLA (% of total CLA)	-	37	86	78	-

1 NZDRI gas chromatography analysis.

**Table 3.5 Diet fatty acid profile<sup>1</sup>**

Fatty acid	Fatty acid (wt % of fat)				
	Diet 1 Cornoil	Diet 2 Cornoil and Tonalin	Diet 3 High CLA milkfat	Diet 4 Typical CLA milk fat	Diet 5 Ground rodent chow
C4:0			2.94	3.26	
C6:0			1.83	2.32	
C8:0			1.00	1.44	
C10:0			1.79	3.13	0.28
C10:1			0.15	0.28	
C12:0			1.81	3.43	0.15
C14:0	0.14	0.17	5.65	8.81	0.70
C14:1			0.53	0.73	
C15:0		0.06	0.65	0.82	0.18
C16:0	10.87	10.71	13.04	17.81	15.16
C16:1	0.16	0.20	1.17	1.95	0.87
C17:0		0.06	0.27	0.39	0.43
C17:1			0.12	0.32	0.25
C18:0	2.13	2.20	10.08	7.21	7.19
C18:1	25.89	25.49	39.53	31.01	24.37
C18:2	58.04	56.62	12.04	9.94	42.01
C18:3	0.71	0.73	1.34	1.22	4.82
C20:0	0.36	0.41	0.19	0.12	0.27
<b>Total CLA isomers</b>	<b>0.00</b>	<b>1.38</b>	<b>1.87</b>	<b>1.53</b>	<b>0.27</b>
Total saturated fatty acids	13.5	13.6	39.3	48.7	24.4
Total monounsaturated fatty acids	26.1	25.7	41.5	34.3	25.5
Total polyfatty acids	58.8	58.8	15.3	12.7	47.2

1 Fatty acid composition analysed by capillary gas chromatography at NZDRI.

Protein, fat and energy values were similar for the four powder based diets. The rodent chow had a lower fat content and energy value. The CLA contents were 0.00, 0.21, 0.29, 0.23 and 0.01 % w/w for diets 1, 2, 3, 4 & 5 respectively. In diets 2, 3 and 4 the percent of the 9c11t-CLA isomer was 37 %, 86 % and 78 % respectively. The CLA in diet 2 originated from Tonalin, in diet 3 from the high CLA milk fat fraction, and in diet 4 from the typical milk fat fraction. The 9c11t-CLA isomer proportion of CLA in these milk fats are comparable with published values for butter of 88 % and 79 % (NZDRI, Fong, 1998; Shantha *et al.*, 1995), and milk, 92 % and 92 % (NZDRI, Fong, 1998; Chin *et al.*, 1992).

Feed intake, weight gain and feed efficiency over the four week trial are shown in Table 3.6. Energy intake, faecal energy and digestible energy in the fourth week of the trial are shown in Table 3.7.

**Table 3.6 Feed intake, weight gain and feed efficiency for whole trial**

Diet	n	Feed intake g / day	Weight gain g	Feed efficiency <sup>2</sup> $\times 10^{-2}$
Diet 1; Cornoil	8	13.98 ± 0.92	30.14 ± 10.20	7.59 ± 2.20
Diet 2; Cornoil and Tonalin	7	13.10 ± 0.90	31.11 ± 6.91	8.46 ± 1.60
Diet 3; High CLA milk fat	8	14.26 ± 0.54 <sup>1</sup>	33.44 ± 4.78	8.51 ± 1.13 <sup>1</sup>
Diet 4; Typical CLA milkfat	7	14.09 ± 1.31	35.73 ± 6.49	9.00 ± 0.86
Diet 5; Ground rodent chow	8	16.88 ± 0.92*	26.48 ± 5.70	5.60 ± 1.15**

± standard deviation

<sup>1</sup> n = 7, due to error made in weighing one food container

<sup>2</sup> feed efficiency = weight gain (g) / food intake (g), both over four weeks

\* diet 5 feed intake > diets 1, 2, 3 & 4, p < 0.01

\*\* diet 5 feed efficiency < diets 2, 3 & 4, p < 0.01

**Table 3.7 Energy intake, faecal energy and digestible energy in week four of trial**

Diet	n	Energy intake kJ/week	Faecal energy kJ/week	Digestible energy kJ/week
Diet 1; Cornoil	8	1988.4 ± 162.5	185.63 ± 20.61	1802.8 ± 144.8
Diet 2; Cornoil and Tonalin	7	1867.3 ± 150.0	173.78 ± 14.46	1693.6 ± 138.6
Diet 3; High CLA milk fat	8	1948.9 ± 163.3	176.63 ± 28.39	1772.3 ± 141.0
Diet 4; Typical CLA milkfat	7	1984.7 ± 150.8	181.95 ± 12.31	1802.7 ± 139.7
Diet 5; Ground rodent chow	8	2260.5 ± 141.8*	374.01 ± 27.11**	1886.5 ± 120.6

± standard deviation

\* diet 5 energy intake > diets 1, & 4, p < 0.05, diet 5 > diets 2 & 3, p < 0.01

\*\* diet 5 faecal energy > diets 1, 2, 3 & 4, p < 0.01

There were no significant differences in digestible energy between the four milk powder based diets and the standard rodent diet control. Rats consuming rodent chow, which was relatively low in energy and high in fibre, had significantly higher feed intake (p < 0.01), energy intake (diet 1 (cornoil) & diet 4 (typical CLA milkfat) p < 0.05, diet 2 (cornoil and Tonalin) & diet 3 (high CLA milkfat) p < 0.01) and faecal energy (p < 0.01) than all four milk based powder diets.

Feed efficiency was significantly less for rats consuming diet 5, the standard rodent chow control, than all of the powdered diets, except diet 1 (cornoil).

### 3.4.3 Energy Expenditure

Energy expenditure results for fasted rats are shown in Table 3.8 and for fed rats are shown in Table 3.9.

**Table 3.8 Rate of oxygen consumption, rate of carbon dioxide production, respiratory quotient, energy equivalence of oxygen and metabolic rate for fasted<sup>1</sup> rats**

Diet	n	$\dot{V}O_2^2$ ml/minute	$\dot{V}CO_2^3$ ml/minute	R	Energy equivalence of oxygen kJ/l	Metabolic rate kJ/minute $\times 10^2$
Diet 1; Cornoil	4	3.43 ± 0.20	2.58 ± 0.20	0.75 ± 0.02	19.84 ± 0.10	6.80 ± 0.41
Diet 2; Cornoil and Tonalin	4	3.50 ± 0.39	2.55 ± 0.29	0.73 ± 0.01	19.73 ± 0.07	6.90 ± 0.77
Diet 3; High CLA milkfat	4	3.34 ± 0.44	2.39 ± 0.28	0.72 ± 0.03	19.67 ± 0.15	6.56 ± 0.84
Diet 5; Ground rodent chow	4	3.78 ± 0.35	2.85 ± 0.26	0.75 ± 0.04	19.85 ± 0.19	7.51 ± 0.67

± standard deviation

1 Rat fasted for 15 to 18 hours

2 Rate of oxygen consumption measured in calorimeter

3 Rate of carbon dioxide production measured in calorimeter

**Table 3.9 Rate of oxygen consumption, rate of carbon dioxide production, respiratory quotient, energy equivalence of oxygen and metabolic rate for fed<sup>1</sup> rats**

Diet	n	$\dot{V}O_2^2$ ml/minute	$\dot{V}CO_2^3$ ml/minute	R	Energy equivalence of oxygen kJ/l	Metabolic rate kJ/minute $\times 10^2$
Diet 1; Cornoil	3	4.26 ± 0.43	3.35 ± 0.42	0.79 ± 0.03	20.03 ± 0.17	8.52 ± 0.90
Diet 2; Cornoil and Tonalin	3	4.26 ± 0.62	3.27 ± 0.51	0.77 ± 0.06	19.94 ± 0.33	8.48 ± 1.23
Diet 3; High CLA milkfat	3	4.13 ± 0.31	3.29 ± 0.34	0.80 ± 0.03	20.08 ± 0.18	8.23 ± 0.66
Diet 5; Ground rodent chow	3	3.99 ± 0.32	3.17 ± 0.33	0.80 ± 0.02	20.07 ± 0.12	8.01 ± 0.69

± standard deviation

1 *Ad lib* feeding

2 Rate of oxygen consumption measured in calorimeter

3 Rate of carbon dioxide production measured in calorimeter

For both fed and fasted rats there were no significant differences in the measured parameters of energy expenditure, oxygen consumption and carbon dioxide production between the four diet groups tested. There were no significant differences between the calculated parameters of energy expenditure, R, energy equivalence of oxygen, and metabolic rate. These diets contained cornoil, corn oil and Tonalin, high CLA milk fat fraction and rodent chow, respectively.

The difference in the respiratory quotient, energy equivalence of oxygen and metabolic rate between fed and fasted rats is shown in Table 3.10.

**Table 3.10 Difference in fed and fasted respiratory quotient, energy equivalence of oxygen and metabolic rate between fed and fasted rats**

Diet	n	R	Energy equivalence of oxygen kJ/l	Metabolic rate kJ/minute $\times 10^2$
Diet 1; Cornoil	3	0.04 ± 0.03	0.21 ± 0.18	1.79 ± 1.02
Diet 2; Cornoil and Tonalin	3	0.04 ± 0.06	0.23 ± 0.30	1.90 ± 1.71
Diet 3; High CLA milkfat	3	0.06 ± 0.03	0.33 ± 0.13	1.83 ± 0.95
Diet 5; Ground rodent chow	3	0.05 ± 0.04	0.24 ± 0.19	0.79 ± 0.82

± standard deviation

All of the fed energy expenditure values were larger than the fasted values. The difference in metabolic rate is energy expenditure due to DIT. There was no significant difference in DIT for any of the diet groups.

#### 3.4.3.1 Diet induced thermogenesis

Energy expenditure data was pooled and analysed to compare all fed animals with all fasted animals. This would show whether a measurable impact of feeding on energy expenditure could be demonstrated with our calorimeter. Mean energy expenditure values for fed and fasted rats are in Table 3.11.

**Table 3.11 Mean oxygen consumption, carbon dioxide, respiratory quotient and metabolic rate for all rats grouped together when fed or fasted**

Group	n	$\dot{V}O_2^1$ ml/minute	$\dot{V}CO_2^2$ ml/minute	R	Metabolic rate kJ/minute x 10 <sup>2</sup>
Fasted	12	3.41 ± 0.33	2.52 ± 0.24	0.74 ± 0.02	6.75 ± 0.64
Fed	12	4.16 ± 0.39*	3.27 ± 0.36*	0.79 ± 0.04*	8.33 ± 0.79*

± standard deviation

1 Rate of oxygen consumption measured in calorimeter

2 Rate of carbon dioxide production measured in calorimeter

\* fed group significantly higher than fasted group  $p < 0.001$

The mean of all fed rat energy expenditure values was compared with all fasted values ( $n = 12$ ). A paired t-test was used since the fed and fasted measurement was made with the same rat. The group of fed rats, on all diets combined, was found to have significantly higher energy expenditure ( $p < 0.001$ ), than the fasted rats.

The data was analysed to find out if the difference in energy expenditure between fed and fasted rats could be attributed to activity. Rat activity was generally scored low, with rats sleeping (score of 1) or crouching with eyes open (score 2) for the majority of the calorimeter session. There was no significant difference in activity between fed and fasted rats (Mann-Whitney statistical test for nonparametric data). Mean group activity scores for rats in the four diet groups, both fed and fasted, ranged from 1.2 to 1.8. Therefore a plot of metabolic rate versus activity did not show correlation as we saw in the mouse trial described in chapter 2 (refer Graph 2.1).

#### 3.4.4 Body Composition

Average live weights of rats in the five diet groups are shown in Table 3.12. The coefficient of variation for rat weight was calculated by repeat weighing three rats each fourteen times. The coefficient of variation (standard deviation / mean), expressed as a percent was very low (0.17 %).

Relative and absolute amounts of body fat dissected from the carcass and results of chemical analysis of body fat are also shown in Table 3.12. Lean body mass is shown in Table 3.13.

There were no significant differences between the five diets for relative and absolute amounts of body fat, moisture, protein, or lean body mass. Ash % in diet group 4 was significantly less than diet group 3 ( $p < 0.05$ ). Diet 4 contained the typical milk fat fraction and diet 3 the high CLA milk fat fraction. Variation in ash % was measured by repeat ash analysis on one rat sample. Six separate subsamples from the same rat were analysed. The mean was 10.10 % ash, standard deviation 1.72 %, and coefficient of variation 17 %. The weight of ash was not significantly different between diet groups.

**Table 3.12 Live weight and body fat, relative and absolute, by dissection and chemical analysis**

Diet	n	Carcass weight g	Live weight g	Body fat by dissection					Body fat chemical	
				Total %	Total <sup>1</sup> g	Abdominal cavity fat <sup>2</sup> g	Interscapular brown adipose tissue g	Subcutaneous pelvic limb deposit g	%	g
Diet 1; Cornoil	8	245.93 ± 18.52	255.61 ± 19.44	7.43 ± 1.76	17.05 ± 5.11	13.40 ± 4.38	0.82 ± 0.09	2.83 ± 0.94	12.35 ± 3.02	28.42 ± 9.10
Diet 2; Cornoil and Tonalin	7	245.22 ± 15.41	256.86 ± 13.80	7.30 ± 1.66	16.43 ± 3.40	13.09 ± 2.94	0.70 ± 0.12	2.63 ± 0.47	12.02 ± 3.08	25.73 ± 7.04
Diet 3; High CLA milk fat	8	245.15 ± 17.98	257.73 ± 17.84	6.88 ± 1.52	15.80 ± 4.76	12.56 ± 4.21	0.71 ± 0.14	2.53 ± 0.59	12.54 ± 2.60	28.78 ± 8.09
Diet 4; Typical CLA milk fat	7	255.02 ± 17.17	262.36 ± 19.32	8.24 ± 1.14	19.60 ± 3.89	15.69 ± 3.36	0.77 ± 0.17	3.14 ± 0.51	14.22 ± 2.06	31.27 ± 9.52
Diet 5; Ground rodent chow	8	240.71 ± 11.14	253.37 ± 11.64	6.14 ± 0.87	13.64 ± 2.29	10.66 ± 2.08	0.72 ± 0.16	2.26 ± 0.48	11.52 ± 1.24	25.57 ± 3.50

± standard deviation

1 Sum of abdominal + interscapular BAT + subcutaneous adipose tissue deposits

2 Sum of adipose tissue deposits attached to intestine + genital organs + kidney region

**Table 3.13 Components of lean body mass; moisture, protein, ash, lean body mass relative and absolute, by chemical analysis**

Diet	n	Moisture		Protein		Ash		LBM	
		%	g	%	g	%	g	%	g
Diet 1; Cornoil	8	61.94 ± 2.40	140.69 ± 8.17	23.31 ± 2.33	56.04 ± 3.62	3.39 ± 0.40	7.73 ± 1.27	88.64 ± 4.08	204.45 ± 9.95
Diet 2; Cornoil and Tonalin	7	62.20 ± 2.27	140.62 ± 12.12	23.48 ± 2.73	56.03 ± 6.94	3.64 ± 0.45	8.20 ± 0.90	89.32 ± 3.82	204.85 ± 16.11
Diet 3; High CLA milkfat	8	62.49 ± 1.73	141.41 ± 7.58	22.70 ± 1.23	53.66 ± 4.05	3.87 ± 0.90	8.72 ± 1.75	89.06 ± 3.24	203.80 ± 11.41
Diet 4; Typical CLA milkfat	7	61.23 ± 1.70	144.50 ± 7.71	23.29 ± 3.04	57.40 ± 6.74	2.99 ± 0.53*	7.08 ± 1.44	87.52 ± 4.33	208.98 ± 12.33
Diet 5; Ground rodent chow	8	63.30 ± 0.97	140.08 ± 6.42	23.86 ± 2.00	55.76 ± 5.36	3.36 ± 0.48	7.42 ± 1.03	90.52 ± 2.35	203.26 ± 10.47

± standard deviation

\* diet 4 ash % &lt; diet 3, p &lt; 0.05

### **3.5              Discussion**

#### **3.5.1          Food Intake**

The protein, fat, moisture and ash analyses of the four powder diets were similar, as were the total energy contents. The fatty acid profiles varied. The diets containing milk fat had higher proportions of saturated and mono-unsaturated fatty acids. The diets containing corn oil had higher proportions of polyunsaturated fatty acids and were high in linoleic acid.

The CLA contents in diets 1, 2, 3, 4 and 5, respectively were 0.00, 0.21, 0.29, 0.23 and 0.01 % w/w. The percent of the 9c11t-CLA isomer in diets 2, 3 and 4 was 37 %, 86 % and 78 % respectively. The percent of 10c12t-CLA in diet 2 was assumed to be 46 %, as the CLA was derived from Tonalin (refer Table 2.4). The amount of 10c12t-CLA in diets 3 and 4 was not measured, and was assumed to be approximately 10 % or less of CLA in diet 3, and 20 % of CLA in diet 4.

The % energy from CLA in diets 2, 3 and 4 was 0.44 %, 0.60 % and 0.47 % respectively (refer appendix 3.1 for calculation).

The difference in CLA content of the two milk fat diets (diet 3 and 4) was obtained by seasonal variation in milk fat CLA level, and processing of the milkfat. CLA in milk fat is higher at the start and end of the dairy season (NZDRI, MacGibbon and Hill, 1997), from September to April. In August, before the main milk flow, and in May, when most cows have stopped producing milk, the values are lower. The high CLA milk fat fraction was obtained from September milk, and the low CLA milkfat fraction from May milk. The September milk fat CLA level was further enhanced by fractionation to obtain a soft milk fat (the fatty acid profile is shown in Table 3.5).

The CLA level used in these diets was the highest level that could be formulated at the time, using natural bovine CLA, present in a milk fat fraction. The total dietary fat was limited to 14 % of energy, to prevent animals either decreasing their food intake or becoming obese. The fat level in the standard rodent chow was 6 %.

Feed intake, weight gain, feed efficiency, energy intake, faecal energy and digestible energy were not significantly different between the four milk powder diets during the trial period. Fatty acid profile, including CLA content, and CLA isomer ratio, were the variables in these diets.

Although not related to dietary CLA content, it is interesting that animals consuming diet 5 (rodent chow) had similar digestible energy and increased feed and energy intake, compared with the other four skim milk diet treatments. This diet contained less than half the fat content of the other diets and 88 % of the total energy. To obtain energy intake equivalent to the four powder diets, rats consuming diet 5 would have to consume 1.14 times the weight of food. These rats actually consumed 1.22 times the weight of the mean of the four powder diets. The high proportion of whole grain cereal components listed in the ingredients used to make this commercial rodent chow suggest it has a high fibre content. Faecal energy was significantly higher in animals consuming this diet. Despite the increased faecal energy, there was no significant difference in the digestible energy between all of the diets. The rats in this trial, fed *ad lib*, were able to self regulate intake of digestible energy to similar intake levels between different diets.

The self regulation involved increased food intake in diet 5 rats for two reasons. Firstly, the feed was less energy dense, requiring consumption of more food to obtain equivalent energy. Additionally, increased faecal energy meant greater loss of energy, which was compensated for by further increased food intake. Rats feeding allowed to fed *ad lib*, consuming highly refined, high fat, energy dense diets (diets 1, 2, 3 and 4) had the same digestible energy intake as rats consuming a whole grain, cereal based, medium fat, reduced energy diet (diet 5). Dietary fat content and fatty acid profile, including CLA content, did not interfere with the self regulation of digestible energy.

Feed efficiency for three of the four milk powder based diets was significantly more than the standard rodent chow, control diet 5. Diet 2 (cornoil and Tonalin) diet 3 (high CLA milkfat), and diet 4 (typical CLA milkfat) had greater feed efficiency ( $p < 0.01$ ). Diet 1 (cornoil) feed efficiency was not significantly different compared with the control diet 5.

### 3.5.2 Energy Expenditure

There were no significant differences in any of the measured and calculated components of energy expenditure between diet groups. Respiratory exchange gases from rats from diets 1,2,3 and 5 were measured in a calorimeter. The calculated components of energy expenditure were respiratory quotient R, energy equivalence of oxygen, metabolic rate and DIT. There were no diet group differences between diet groups for fed rats ( $n = 3$  in each of four groups), fasted rats ( $n=4$  in each of four groups), or the difference between fed and fasted rats ( $n = 3$  ).

The difference in metabolic rate, between fed and fasted animals, was not statistically significant at any level ( $p < 0.50$ ). There was considerable variation in measurements for animals in each group. A larger group may be necessary to obtain meaningful results and determine statistical significance ( $p < 0.05$ ) for this type of energy expenditure work. This would require increased calorimeter resources, or staggering of ages and start time of rats, to ensure similar ages at time of calorimetry and body composition measurement.

Energy expenditure was measured in fasted, and fed animals. In comparison to fasted animals the fed animals consumed more oxygen and produced more carbon dioxide. R values and energy expenditure were greater in fed animals. When data was grouped as all fed rats compared with all fasted rats, energy expenditure for fed animals was significantly ( $p < 0.001$ ) higher. Activity score for fed animals was compared with activity score for fasted animals and found not to be different. This suggested increased activity in fed rats was not the reason for increased metabolic rate. Therefore the increased metabolic rate after eating is probably DIT (the energy cost of digesting and assimilating food).

The lower respiratory quotient found in the group of fasted rats (0.74, refer Table 3.11) showed most metabolism was from oxidation of the energy source fat. The significantly higher post-prandial respiratory quotient (0.79) were from increased carbohydrate metabolism relative to fat metabolism. The increased respiratory quotient suggests a significant movement toward carbohydrate metabolism in fed rats

compared with fasted rats. We did not find a significant difference in respiratory quotient between diet groups.

### 3.5.3 Body Composition

Energy is partitioned into lean body mass, or body fat. Energy in excess of current requirements is stored in adipose tissue. We found no differences in body fat or lean body mass between the diet groups.

There is some evidence that CLA impacts on bone metabolism (Li and Watkins, 1998, Cook *et al*, 1997). Therefore we were interested in assessing body ash since this is an index of bone mineral content. There were no significant differences between all five diets for percent and weights of body fat, moisture, protein, or lean body mass, however the ash % in diet 4, the typical CLA milk fat group, was significantly less than diet 3, the high CLA milk fat ( $p < 0.05$ ). There was a large standard deviation for repeat analysis on the same rat, and the sample size was small ( $n = 7$ ). One possible cause of variation was lack of homogeneity in the freeze-dried whole body samples resulting in sampling error. Although percent ash was different, the weight of ash was not significantly different.

Interscapular fat weights, assumed to be principally BAT, were similar for all groups. It is suggested that BAT in rodents has a metabolic function dissipating surplus energy intake, and is the site of facultative DIT (refer section 1.3.2) (Rothwell and Stock, 1979; Richard *et al.*, 1988; Oudart *et al.*, 1997). It would have been interesting to measure BAT enzyme activity in order to see whether BAT metabolism differed between diet groups. If BAT function is related to tissue size, similar BAT weights indicate no difference in BAT activity between diet groups. This would be expected from the equivalent digestible energy intakes and energy expenditures observed in these animals.

### 3.5.4 CLA Isomers

The biological activities of CLA isomers are an area of active current research (refer section 1.2). As already discussed (refer 1.2.1), the majority of publications, assumed

9c11t-CLA is the biologically active form. Three very recent publications support biological activity from the 10t12c-CLA isomer in relation to atherosclerosis, body composition and energy metabolism. Deckere and Rudrum (1999) were able to demonstrate dietary 10t12c-CLA altered LDL, HDL and VLDL blood cholesterol in hamsters compared to controls, while 9c11t-CLA had no significant effect (refer 1.2.4). Park *et al.* (1999b) found reduced body fat and increased lean body mass in mice related to consumption of 10t12c-CLA, and not 9c11t-CLA (refer 1.2.6.2). Park *et al.* (1999a) concluded preferential metabolism of 10t12c-CLA from the depletion pattern of CLA isomers from skeletal muscle following mixed isomer feeding.

The CLA in milk fat contains approximately 80 % to 90 % of 9c11t-CLA (Chin *et al.*, 1992; Shantha *et al.*, 1995; NZDRI, Fong, 1998). Synthetic CLA generally contains 37% 9c11t-CLA and 46 % 10t12c-CLA. It is important to be aware of the CLA isomer ratio, as well as the total CLA content, in any research investigating the biological activity of dietary CLA.

### 3.5.5 Milk samples

Milk samples used in animal feeding trials where diets are designed to examine an effect of one milk component compared with another milk component must be selected and controlled in the best manner available. It is known that milk composition varies due to a number of factors including breed of cow, time of season, stage of lactation, number of lactations, cow feed and geographic area (refer 1.1.5). Time of season is particularly important in New Zealand, where the majority of cows calve in July and August, and continue to be milked until drying-off around April.

When a milk fraction is used in experimental trial work, careful consideration must be given to selection of appropriate experimental and control milk samples. Ideally samples only vary in the component under investigation, without differences in other milk components. Differences in the factors known to effect milk composition must be eliminated as far as practical, while still producing variation in the component under investigation. When seasonal variations are not under investigation, samples must be obtained on the same date, and from cows in similar geographic locations,

ideally farmed together. For some applications twin herd milk (milk obtained from a herd of identical twin cows, in which milk from one twin is the control milk, and milk from the other twin is the experimental milk) may be ideal, as this will remove variables from genetic variation and farm factors. Twin herd milk may be the best choice when cow feed manipulation is used to alter milk composition. Obviously it would not be suitable when genetic variation is utilised to produce differences in milk composition. Following selection of raw milk samples, processing or laboratory preparation to obtain a suitable milk fraction, must be the same, as far as practical, for control and experimental samples.

The cow feeding trial from which milkfat samples containing high CLA levels were obtained for this trial was confidential. It is possible that alternative manipulation of milk production may further increase CLA content of milkfat.

## CHAPTER 4

### General discussion

In the first feeding trial, described in Chapter 2, synthetic CLA, fed to six week old, male BALB/c mice in a four week trial (0.0, 0.1, 0.25, 0.5, 1.0 and 2.0 % w/w CLA), reduced body fat in a dose response manner. It was effective in reducing body fat over the range 0.25 % to 1.0 % CLA. The observed reduction in body fat in mice has been reported in other studies feeding 0.5 to 1.0 % synthetic CLA. The dose response pattern of body fat reduction seen in this feeding trial showed clearly the effective CLA dose range, and that no further body fat reduction is obtained by feeding above 1.0 % synthetic CLA in the diet.

Compared with controls and some groups fed lower levels of dietary CLA the 2.0 % CLA group had reduced weight gain and the 1.0 % and 2.0 % CLA groups had reduced lean body mass. The data obtained from feeding up to 2.0 % synthetic CLA showed the effect of higher dosages on body weight gain and lean body mass. These results suggest that levels of 1.0 and 2.0 % synthetic CLA fed to growing mice reduce growth.

The observed reduction in body fat in mice fed 0.25 % to 1.0 % w/w synthetic CLA was not balanced by measurable reductions in feed intake or increased energy expenditure. The mechanism by which equivalent energy intakes from diets differing only in a small portion of their total fatty acid profile can result in body fat percent (by chemical analysis) ranging from 8.65 % (control) to 2.90 % (1.0 % CLA), is not known. We were not able to elucidate whether this mechanism was associated with altered feed intake, or altered energy expenditure. Repeat work measuring energy expenditure by doubly labelled water may be necessary to measure small differences in energy expenditure, which are not detectable using indirect calorimetry.

The reduction in body fat seen in mice fed synthetic CLA in the diet confirms earlier research. Park *et al.* (1997) observed reduced fat deposition in weanling male, or seven week old female mice fed 0.5 % w/w CLA for four weeks. Sugano *et al.*

(1998) found perirenal adipose tissue in male rats fed a soybean oil based diet supplemented with 1.0 % w/w CLA to be significantly less than the group supplemented with 1.0 % linoleate. West *et al.* (1998) supplemented low-fat or high-fat male mice diets with CLA (1.0 % and 1.2 % w/w CLA) for a six week feeding trial. CLA significantly reduced carcass lipid content and adipose tissue weights. Recently DeLany *et al.* (1999) found 6 week old male mice, on a high fat diet supplemented with CLA had reduced body fat.

The level of synthetic CLA found to be effective in reducing body fat (0.25 % to 1.0 % w/w CLA) is similar to the dietary level shown to be effective in other studies demonstrating the biological activity of CLA. Although the mechanisms of CLA activity in relation to protection against carcinogens and in relation to energy balance may well be different, it is interesting to note that similar dietary levels are effective. Early anticarcinogen studies, conducted by Ip *et al.*, in 1991, were the first to show dose dependent inhibition of tumour incidence induced by an oral carcinogen at levels of 1 % w/w CLA and below, and as low as 0.1 % w/w CLA (Ip *et al.*, 1994). There was no further protection from higher doses. Repeat experiments by a number of researchers, confirmed consumption of 1 % CLA during the active period of mammary gland development in the rat, inhibited chemically induced mammary carcinogenesis at a later stage. In other feeding trials, investigating food intake and weight gain, suckling and weanling rats had greater weight gain and higher feed efficiency when consuming up to 0.5 % w/w synthetic CLA in the diet.

In the second feeding trial, described in Chapter 3, synthetic CLA at a level of 0.21 % w/w, and bovine CLA at a level of 0.29 % w/w or 0.23 % w/w did not effect energy balance in female Sprague-Dawley rats. There are a number of possible explanations for the reduction in body fat seen in the mouse trial (at 0.25 % synthetic CLA) and not demonstrated in the rat trial (at 0.21 % synthetic CLA).

One possible explanation is the standard deviation in body fat percent seen in corresponding treatments in the two trials. The standard deviation in body fat (by dissection) in rats feed synthetic CLA was 1.66 % body fat (mean %  $\pm$  standard deviation %;  $7.30 \pm 1.66$ , n = 7). In mice feed synthetic CLA at a similar level in the

diet, the standard deviation was 0.92 % body fat (mean %  $\pm$  standard deviation %;  $4.38 \pm 0.92$ , n = 18). These results suggest many more than seven rats were needed to show any significant difference at this low level of dietary CLA.

The two trials used different species and gender, and altered total diet composition. As described above, dietary CLA had been shown to reduce body fat in both male and female mice, and male rats, and it was believed change of species and gender would not be important. Dietary CLA had also been shown to be effective in reducing body fat in both high fat and low fat diets.

A comparison of the percent of energy in the diet from CLA shows similar levels for the two diets. The percent of energy from CLA in the mouse diet (0.25 % w/w synthetic CLA) was 0.58 %. The rat diet 2 (0.21 % w/w synthetic CLA) contained 0.44 % energy from CLA. The rat diet 3 (0.29 % w/w CLA) containing high CLA milkfat had 0.60 % energy from CLA and the rat diet 4 (0.23 % w/w CLA) containing typical milkfat had 0.47 % energy from CLA.

Although the proportion of energy from CLA was similar in these diets, the proportion of the 9c11t-CLA isomer in the CLA was not. The mouse diets (and the synthetic CLA rat diet) contained 37 % 9c11t-CLA (and 46 % 10t12c-CLA). The rat diet 3 contained 86 % 9c11t-CLA (and < approximately 10 % 10t12c-CLA), and diet 4 contained 78 % 9c11t-CLA (and < approximately 20 % 10t12c-CLA). These two rat diets delivered considerably more 9c11t-CLA (and less 10t12c-CLA) than the 0.25 % w/w synthetic CLA mouse diet, the first to show significant reduction in body fat. The level of 9c11t-CLA in the rat diets was closer to the level in the 0.5 % synthetic CLA mouse diet, which showed even greater reduction in body fat in mice. This suggests the biological activity of CLA effective in reducing body fat is not related to the 9c11t isomer. A further study with purified 10t12c CLA and purified 9c11t-CLA is recommended to assess which isomer is active.

The trials were designed to examine the effects of CLA on energy balance. At the same time, other interesting aspects of energy balance were observed. These had a greater influence on energy balance than dietary CLA. In the first trial, mouse activity was highly correlated with energy expenditure, whereas the effect of dietary

supplementation with CLA was not measurable. In the second trial, rat fasting energy expenditure was significantly less than post-prandial energy expenditure. The difference was assumed to be diet induced thermogenesis. In the same trial, the effect of dietary supplementation with CLA on DIT was not measurable.

In the second trial, digestible energy intake for all groups was not significantly different, although rats fed a high fibre, low energy rat chow had significantly higher food intake, energy intake and faecal energy than rats fed energy rich milk powder based diets. Rats were able to self-regulate digestible energy intake to the same level, across diets with different total energy, fat content and fat composition. Dietary fat content and composition (including CLA content) did not interfere with the ability to self-regulate digestible energy. This suggests that mechanisms the rat has to self regulate digestible energy intake are not effected by these levels of dietary CLA.

CLA is common in western diets containing dairy products and meat and meat products from ruminant animals. Daily intakes are suggested in the order of 460 mg / day to 1500 mg / day (refer section 1.1.3). A direct extrapolation of the levels found effective in animal trials to human levels was made by Ip *et al.*, 1994. They calculated the 0.1 % CLA level found effective against chemically induced mammary carcinogenesis in a rat model was equivalent to a 3 g daily CLA dosage in a 70 kg person.

Data extrapolation from feeding trials in one species to make comparisons with another species is not without difficulties and must be treated with caution. Another basis for comparison is CLA as percent of energy in the diet. The 0.58 % of energy from CLA in the mouse diet (0.25 % w/w synthetic CLA) can be extrapolated to a human diet as follows. A person consuming 10,000 kJ / day must have a daily CLA intake of 1450 mg CLA to obtain 0.58 % of energy from CLA (refer Appendix 4.1 for full calculation). Using the same comparison, the 0.5 % dietary CLA level, shown to further reduce body fat, represents consumption of 3 g CLA per day. The 1.0 % dietary CLA level, which demonstrated possible adverse effects on growth associated with reduced weight gain and lean body mass, represents 6 g CLA consumption per day.

The results from these two animal feeding trials support recent research demonstrating that the 10t12c-CLA isomer and not the 9c11t-CLA isomer is biologically active, at least in relation to fatty acid metabolism, energy metabolism and body fat composition (Deckere and Rudrum, 1999; Park *et al.*, 1999a; Park *et al.*, 1999b). It is worth noting that 10t12c-CLA is not present as a natural constituent in the diet except in extremely minor proportions. The quantities used to demonstrate biological activity in animal feeding trials were obtained by chemical synthesis from linoleic acid, a reaction that does not occur in human food sources. The majority of CLA found in food sources is obtained by biohydrogenation of linoleic acid, resulting in the formation of mainly 9c11t-CLA.

Dietary CLA is obtained from mainly ruminant meat and dairy products where the major component is 9c11t-CLA, and 10t12c-CLA is found in only minor proportions. Therefore a comparison of the daily human dietary intake of 10t12c-CLA, compared with that found to be biologically active in feeding trials, may be more relevant than extrapolating total CLA data. A daily human intake of 1450 mg of 9c11t-rich CLA, when extrapolated back to mice, contains considerably less 10t12c-CLA than the 0.25 % w/w synthetic CLA diet found to be biologically active in reducing body fat in the mouse feeding trial. Similarly the 6 g / day of dietary CLA suggested above to possibly cause adverse affects, is a considerable under estimation of the toxic level if 10t12c-CLA alone is responsible for biological activity.

It may be possible to enhance specific CLA isomers during processing of foods (refer section 1.1.6), although more research is required in this area. In addition, as the biological activities of specific isomers become evident, analysis of the CLA content of foods must include isomer proportions as well as total CLA content.

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## APPENDICES

Appendix Table 1.1

### CLA content of food

	NZDRI Fong, 1998	Chin <i>et al.</i> , 1992		Shantha <i>et al.</i> , 1994 & 1995			
	mg CLA/g fat	9c11t- CLA %	Mg CLA/g fat	9c11t- CLA %	mg CLA/g fat	g fat / 100 g food	mg CLA <sup>3</sup> / 100 g food
<b>Country</b>	N.Z.	U.S.A.	U.S.A.	U.S.A.	U.S.A.		
<b>Uncooked meat and seafood</b>							
Beef, ribeye				61	6.4		
Beef, sirloin				57	5.8		
Beef, T-bone				59	6.1		
Fresh ground beef	84	3.8					
Veal	84	2.7					
Lamb	92	5.6					
Pork	82	0.6					
Chicken	84	0.9					
Fresh ground Turkey	85	2.5					
Egg yolk	82	0.6					
Salmon	n.d. <sup>2</sup>	0.3					
Shrimp	n.d. <sup>2</sup>	0.6					
Mussels	n.d. <sup>2</sup>	0.4					
<b>Oils and fats</b>							
Safflower	44	0.7					
Sunflower	38	0.4					
Peanut	46	0.2					
Canola	44	0.5					
Corn	39	0.2					
Coconut	47	0.1					
Olive	47	0.2					
Margarine (2 brands)	0,0					81.8 <sup>1</sup> , 81.8 <sup>1</sup>	0
Pastry fat (3 brands)	0,0,0					85.1 <sup>1</sup> , 83.7 <sup>1</sup> , 81.7 <sup>1</sup>	0
Shortening	5.7					81.6 <sup>1</sup>	460 <sup>1</sup>
Beef tallow		84	2.6				
<b>Processed food</b>							
Beef frank		83	3.3				

Smoked bratwurst		77	2.4				
Smoked bacon ( 3 brands)		75, 78, 76	2.6, 1.7, 0.8				
Corned beef		85	6.6				
Meat patty	6.8				24.6 <sup>1</sup>	170 <sup>1</sup>	
Luncheon	7.5				13.1 <sup>1</sup>	98 <sup>1</sup>	
Mince pie	3.6				13.9 <sup>1</sup>	50 <sup>1</sup>	
Pastry	5.4				23.5 <sup>1</sup>	13 <sup>1</sup>	
Peanut butter		n.d. <sup>2</sup>	0.2				
Muesli bar	0				19.9 <sup>1</sup>	0 <sup>1</sup>	
Chocolate coated biscuit	0				26.3 <sup>1</sup>		
Sweet biscuit	0				15.2 <sup>1</sup>	0 <sup>1</sup>	
Savoury cracker	0				23.8 <sup>1</sup>	0 <sup>1</sup>	
White bread	0				1.4 <sup>1</sup>	0 <sup>1</sup>	

1 Fong

2 n.d.: not detectable

3 Total CLA isomers

Appendix Table 1.2

## **CLA Content of dairy products**

Processed American		91	93	5.0						91 <sup>2</sup>
Processed cheese spread		85, 39								39 <sup>2</sup> - 85 <sup>2</sup>
Cream	94	143	88	3.8						94 - 143 <sup>2</sup>
Roka blue spread	100									100
Cheese Whiz	881									881
<b>Fermented dairy products</b>										
Buttermilk		6								6 <sup>2</sup>
Sour cream		76	90	4.6	78	7.5	140			76 <sup>2</sup> - 140
Yoghurt low fat			83	1.7	71	5.3	0.3			0.3
Yoghurt medium fat		7	86	4.4	82	9.0	9.0			7 <sup>2</sup> - 9.0
Yoghurt high fat			84	4.8	83	5.1	17			17
<b>Milk</b>										
4.0 % fat	70									70
Homogenised milk			92	5.5			9.2	24		24
Reduced fat milk							8.1	11		11
3.2% fat		14								14 <sup>2</sup>
2 % fat		8								8 <sup>2</sup>
Evaporated milk		22, 44								22 <sup>2</sup> - 44 <sup>2</sup>
Whipping Cream		129								129 <sup>2</sup>
Butter			88	4.7	79	8.1	649	12	993	649 - 993
Ice cream			86	3.6	76	5.0	50			50

1 total CLA isomers

2 9c11t-CLA

Appendix Table 1.3

**CLA content of breast milk**

Notes	CLA mg / g fat	9c11t-CLA %	9c11t-CLA mg / 100 g milk
McGuire <i>et al.</i> , 1997. One sample collected from each women (n = 14), between 3 days to 10 months postpartum. Sample collected by complete breast expression from healthy breastfeeding women. Idaho, USA.	3.81 ± 0.92 (mean ± SD) 2.23 to 5.43 (range)	83 to 100 (range) (8 of the 14 samples contained 100 % 9c11t-CLA)	11 ± 8 (mean ± SD) 2 to 30 (range)
Jensen <i>et al.</i> , 1998. 20 samples, collected by complete breast expression, from 5 women taken at 1, 7, 14 and 21 days postpartum. Connecticut, USA.		100	18 ± 2 (mean ± SD) 14 to 28 (range)

Appendix Table 1.4

**CLA content of infant formula**

Infant formula	Chin <i>et al.</i> , 1992	Shantha <i>et al.</i> , 1995	McGuire <i>et al.</i> , 1997	
	9c11t-CLA	mg CLA / g fat	9c11t-CLA %	mg CLA / g fat
Formula (soy protein)	n.d. <sup>1</sup>	0.7		
Formula (milk protein)	n.d. <sup>1</sup>	0.3		
SMA powder			82	2.2
Similac			n.d.	n.d.
Enfamil			n.d.	n.d.
SMA RTF <sup>2</sup>			86	2.2
Similac RTF <sup>2</sup>			n.d.	n.d.
Enfamil RTF <sup>2</sup>			n.d.	n.d.

1 n.d. not detectable

2 RTF; ready to feed

Appendix Table 1.5

## Feeding trials investigating the CLA content of cow's milk

Country	Description	CLA ( mg / g fat)	Conclusions	Reference
New Zealand	Milkfat from NZ butter from 5 regions. Cows pasture fed.	Mean: 11 Range: 7 – 15	Summer values (mid season) tend to be lower than mid-spring and mid-autumn values.	NZDRI, 1997, MacGibbon and Hill
New Zealand	Milk from 35 herds supplying a single factory.	25 % to 75 % quartile ranges;  Friesian: 9 – 19 Jersey: 6 – 13	Milk from Friesian herds significantly higher than milk from Jersey herds.  Summer values tend to be lower in both breeds.  Wide individual cow variation in CLA content of milkfat within each breed.	NZDRI, 1997, MacGibbon and Hill
Ireland	Cows fed high (20 kg / cow / day) or low (16 kg / cow / day) grass allowances for 19 weeks.	Mean, high: 5.9 Mean, low: 3.9	Reduced levels of grass intake caused a reduction in CLA levels.	Stanton <i>et al.</i> , 1997
Ireland	Cows fed high and low full-fat rapeseed – supplemented diets for 8 weeks. Pasture fed control.  9c11t-CLA was the major form in milk fat. Quantities of 10t12c-CLA were too low for accurate quantification.	Mean, high: 7.9 Mean, low: 5.2 Mean, control: 4.8  Variation among individual cows over both trials: 1.5 - 16	Full-fat rapeseed supplements resulted in increases in CLA levels.  Additionally: - Lactation number of cow may contribute. - No effect due to stage of lactation during the two trial periods.	Stanton <i>et al.</i> , 1997
Germany	Black Pied breed cows from three farms; (i) Indoor feeding all year with cereal-rich maize silage ration, (ii) Pasture farming during summer with maize and grass	(i) Mean, indoor farming: 3.4  (ii) Mean pasture	Grass feeding produced higher CLA levels. Difference between the two grass fed groups not understood (suggestion that in ecological dairy farming the rations poorer in energy (starch) and richer in crude fibre, cause a more intense activity of rumen bacteria).	Jahreis <i>et al.</i> , 1997

	silages during other seasons, (iii) "Ecological" farming – grazing during summer with clover/alfalfa, grass silages during other seasons.	farming: 6.1  (iii) Mean ecological farming: 8.0		
Finland / USA	Twelve Holstein cows randomly assigned peanut oil, sunflower oil, and linseed oil supplemented diets in a 3 x 3 Latin square design. Milk samples collected on days 10 – 14 of diet treatment.	Peanut oil supplement: 13  Sunflower oil supplement: 24  Linseed oil supplement: 17	Supplementation of diets containing plant oils high in unsaturated fatty acids, particularly linoleic acid, enhanced the milk fat content of CLA.  Substantial individual variation in CLA content of milk eg individuals consuming the sunflower oil diet ranged from 9.9 to 52.  During all treatments, milk fat content was low compared with pre-treatment levels.	Kelly <i>et al.</i> , 1998
Sweden	28 Swedish Red and White cows, in three groups, one fed a restricted amount of diet containing 50: 50 forage to concentrate ratio (control group), and two trial groups fed a 35: 65 forage to concentrate ration in restricted or unrestricted amount. 9c11t-CLA levels reported.	Mean, control diet, restricted feeding: 5.0  Mean, trial diet, restricted feeding: 11.3  Mean, trial diet, unrestricted feeding: 6.6  Variation among all trial animals: 2.5 to 17.7	The concentration of CLA in milk can be increased through a suitable dietary regimen.	Jiang <i>et al.</i> , 1996

Appendix Table 1.6

**Effect of CLA supplemented diet<sup>1</sup> on body weight, food intake and feed efficiency in animal feeding trials**

Trial notes <sup>2</sup>	CLA isomers	Body weight	Food intake	Feed efficiency <sup>3</sup>	Reference
6 week old male AKR/J mice. High-fat (24 %) or low-fat (6.7 %) diet with or without CLA (2.46 mg / kcal). Six week trial period.	CLA obtained from Nu Chek Prep. Composition ascertained by HPLC 39.1 % 9c11t-CLA, 40.7 % 10t12c-CLA, 4.2 % minor CLA isomers.	Body weight significantly reduced in both CLA groups.	CLA treatment significantly reduced total energy intake independent of diet group. Reduction greatest during the first 3 weeks.	Insufficient data to calculate.	West <i>et al.</i> , 1998
6 week old female SENCAR mice. Levels of CLA (0.5 %, 1.0 %, 1.5 %) added to a 5.0 % corn oil control diet at the expense of dextrose. Six week trial period.	CLA obtained from Nu Chek Prep. Composition reported by supplier 43 % 9c11t-CLA, 45 % 10t12c-CLA, 6 % minor CLA isomers.	Body weights significantly lower in mice fed 1.0 % and 1.5 % CLA diets.	No difference	Decreased in all CLA groups.	Belury and Kempa- Stecko, 1997
7 week old female ICR mice. 0.5 % CLA added to a 5.5 % corn oil control diet at the expense of corn oil. Four week trial period.	Mixture of CLA isomers synthesised from linoleic acid as described (Chin <i>et al.</i> , 1992).	No difference.	No difference	No difference	Park <i>et al.</i> , 1997
Weanling male ICR mice. Diet as above. 32 day trial period.	Mixture of CLA isomers synthesised from linoleic acid as described (Chin <i>et al.</i> , 1992).	No difference.	No difference	No difference	Park <i>et al.</i> , 1997
8 week old female Fisher rats. 0.5 % CLA added to a 5.6 % corn oil diet at the expense of corn oil. Diets fed throughout gestation and lactation.	Mixture of CLA isomers synthesised from linoleic acid as described (Chin <i>et al.</i> , 1992).	No difference in maternal and fetal weights at 20 days gestation. Pups significantly heavier at day 10 of lactation in CLA group.	No difference in maternal food intake.  Milk intake not measured for pups.	No difference for dams.  Pup milk intake not measured	Chin <i>et al.</i> , 1994b

10 week old female Fisher rats. Male and female pups. Control and 0.5 % CLA diet as above. Additionally, 0.25 % CLA diet throughout, and 0 % CLA diet during gestation followed by 0.5 % CLA diet during lactation. Diets fed throughout gestation, lactation and to 8 weeks (male) or 10 weeks (female). Pups fed same diet as mother after weaning.	Mixture of CLA isomers synthesised from linoleic acid as described (Chin <i>et al.</i> , 1992).	Pups significantly heavier at day 10 of lactation in 0.5 % CLA throughout group than control and 0.25 % CLA throughout. 0.5 % lactation only group intermediate in weight. Pup final body weights (8 weeks for males, 10 weeks for females, both weaned at 20 days) for 0.25 % and 0.5 % CLA groups significantly heavier than control.	No difference in food intake for either male (five weeks after weaning) or female pups (seven weeks after weaning).	Feed efficiency for the 0.5 % CLA group significantly higher than the control.	Chin <i>et al.</i> , (1994b)
8 week old male hamsters. Three levels of CLA; 0.06, 0.11, and 1.1 energy % CLA added to hypercholesterolemic control diet. Eleven week trial period.	CLA obtained from Nu Chek Prep. Composition reported by supplier GC analysis showed 9c11t-CLA, 10t12c-CLA accounted for 94 % of total CLA isomers.	No difference at 4 and 8 weeks. At 11 weeks body weights of hamsters on the CLA-supplemented diets significantly less than control.	No difference in food disappearance over the course of the trial.	Reduced.	Nicolosi <i>et al.</i> , 1997
2 groups of 3 male and 3 female New Zealand white rabbits. Control semi-synthetic hypercholesterolemic diet. CLA-supplemented diet 0.5 g CLA per day (0.5 % CLA). Both diets restricted to 100 g per day). Twenty-two week trial period.	Not reported.	CLA-rabbits gained more weight during the first few weeks. At completion of the trial body weights were similar.	No difference in food intake throughout the study.	Increased in CLA fed rabbits during the first few weeks. No difference after 22 weeks.	Lee <i>et al.</i> , 1994
4.5 week old male Sprague-Dawley rats. 1.0 % linoleic acid, 0.5 % each linoleic acid and CLA, 1.0 % CLA, added to a 10.0 % soybean oil diet at the	Mixture of CLA isomers synthesised from linoleic acid as described (Ip <i>et al.</i> , 1991). 29.8 % 9c11t-CLA, 29.6 % 10t12c-CLA, 18.6 % 9t11t-	No difference	No difference	No difference.	Sugano <i>et al.</i> , 1998.

expense of soybean oil. Three week trial period.	CLA, 2.7 % minor CLA isomers.				
3 week old male Sprague-Dawley, rats. 1.0 % CLA added to a 7.0 % soybean oil control diet at the expense of soybean oil.	CLA provided by Kraft General Foods. Isomer composition not reported. However CLA isomer content of rat diets shows majority of CLA isomers were nearly equal proportions of 9c11t-CLA and 10t12c-CLA, and small amounts of minor CLA isomers.	No difference	Not reported, but assumed reduced in CLA fed rats (since feed efficiency improved, and weight was not different).	Significantly improved (p<0.01) in rats fed CLA diet.	Li and Watkins, 1998
8 week old female BALB/c mice. Levels of CLA (0.15 %, 0.3 %, 0.9 %) added to a 4.1% corn oil and 0.9% safflower oil diet, at the expense of safflower oil. Three or six week trial period.	Mixture of CLA isomers synthesised from linoleic acid as described (Chin <i>et al.</i> , 1992). Reported purity 34.5 % 9c11t-CLA, 38.8 % 10t12c-CLA., 6.9 % minor CLA isomers.	No difference	No difference	No difference	Wong <i>et al.</i> , 1997

(summary continued on next page)

Appendix Table 1.6 continued

**Summary data; number of feeding trials showing significant effect or no significant effect when diet supplemented with CLA**

Effect	Body weight	Food intake	Feed efficiency <sup>2</sup>
No significant difference	5	8	5
Reduction	3 total; 1 male mice (6 weeks old and 6 weeks feeding). 1 female mice (6 weeks old and 6 weeks feeding). 1 male hamster (8 weeks old and 11 weeks feeding).	1 total: 1 male mice (6 weeks old and 6 weeks feeding).	2 total: 1 female mice due to reduced body weight (6 weeks old and 6 weeks feeding). 1 male hamsters due to reduced body weight (8 weeks old and 11 weeks feeding).
Increase	2 total; 1 suckling male and female rat pups at day 10 of lactation. 1 weanling rats, males at 8 weeks, females at 10 weeks.	None	2 total; 1 weanling rats males at 8 weeks, females at 10 weeks, females (due to body weight increase). 1 weanling male rats (assumed due to food intake reduction).
Insufficient data provided in reference		1 total; 1 suckling rat pups milk intake not measured	1 total; 6 week old male mice.
Total number of trials	10	10	10

1 Trials described were straight feeding trials. Data from trials where animals are subject to challenge such as carcinogen or endotoxin not included.

2 CLA percentage on weight CLA / weight diet basis (w/w), unless otherwise stated.

3 gram of body weight gain per gram of food consumed

Appendix Table 2.1

**Ingredient composition of pre-acclimatisation and acclimatisation diets**

<b>Component</b>	<b>Pre-acclimatisation diet %</b>	<b>Acclimatisation diet (milk free pellets, ground) %</b>
Meat bone meal	6	6
Lucerne meal	5	5
Fish meal	7	7
Milk powder	1.5	-
Soybean meal	-	1.5
Wheat	43.85	43.85
Barley	30	30
Wheat by-products	5	5
Vitamin mix	0.15	0.15
Mineral mix	0.5	0.5
Soy bean oil	1	1
Total protein content	17	17
Total fat content	7	7

**Appendix 2.2 Conversion of CLA mg / day human diet to % CLA w/w (dry basis)**

Based on total energy consumption of 10,000 kJ / day and no alcohol consumption

And 35 % of energy from fat (3500 kJ)

65 % of energy from carbohydrate and protein (6500 kJ)

And 39.8 kJ / g fat (9.5 kcal / g x 4.186 kJ / kcal) (Linder, 1991)

16.7 kJ / g protein or carbohydrate (Linder, 1991)

Fat consumption / day = 3500 kJ / 39.8 kJ / g = 87.9 g / day

Protein and carbohydrate consumption / day = 6500 kJ / 16.7 / g = 389 g / day

Total consumption of fat, protein and carbohydrate / day = 477 g = approximate dry weight of food

460 mg CLA / day (NZDRI, Fong, 1998) = 0.46 / 477 g x 100 = approximately 0.1 % w/w (dry basis) CLA

1500 mg CLA / day (Parodi, 1994) = 1.5 / 477 g x 100 = approximately 0.3 % w/w (dry basis) CLA

**Appendix 2.3 Conversion of 0.25 % w/w CLA mouse diet to percent of energy from CLA basis**

0.25 % w/w CLA = 0.0025 g CLA / g diet

Assume CLA contains 39.8 kJ / g

0.0025 g CLA contains  $0.0025 \times 39.8 \text{ kJ/g} = 0.0995 \text{ kJ/g}$

The 0.25 % w/w CLA diet contained 17.21 kJ / g (by bomb calorimetry, refer Table 2.5)

Therefore % total energy from CLA =  $0.0995 \text{ kJ} / 17.21 \text{ kJ} \times 100 = 0.578 \text{ % of energy}$

## Appendix 2.4 Calorimeter System and Operating Method

### Principle

Respiratory gas exchange in a group of mice was measured by indirect calorimetry. The inlet air composition was assumed constant and the oxygen and carbon dioxide composition of the calorimeter were measured. The difference between these gas concentrations in ingoing and outgoing air reflects respiratory gas exchange by the animals. From these measurements, respiratory quotient, energy equivalence of oxygen and metabolic rate were calculated.

### Equipment

Calorimeter

Cylinder of oxygen free nitrogen calibration gas

Cylinder of 3.24 % carbon dioxide calibration gas (gases both beta standard gas; BOC Analytical Gases, Wellington)

MACLAB Chart software 3.5.6/s

### Description of calorimeter system

The indirect calorimeter used a perspex box 280 x 350 mm side and 160 mm in height with an open base. This was placed over two mice cages leaving a small gap (< 1 mm) between the base of the box and the laboratory bench through which ingoing air was drawn. Outlet gas from the mice cages was withdrawn from the centre of the two cages using a Jun ACO 5503 aquarium air pump and passed through a Fleisch head temperature regulator (size 00: OEM Medical , Richmond, Virginia), to prevent possible condensation. A validyne differential pressure transducer (DP45-16, VacuMed, Ventura, California) across the Fleisch head measured gas flow through the system. A rotameter was connected in the system after the Fleisch head to provide visual evidence of airflow. Gas passed to a mixing chamber with an internal volume of approximately 200 ml where temperature was measured, using a thermistor, which had been previously calibrated using a mercury in glass thermometer. Gases passed through a 300mm

column of desiccant (Drierite, Hammond Drierite Co., Xenia, Ohio) before analysis by a Servomex OA 137 oxygen analyser (Servomex Controls, Crowborough, England) and a Datex Normocap carbon dioxide analyser (Datex Medical, Helsinki, Finland). These had been modified to give an analogue electrical output of gas composition. These outputs, as well as temperature and airflow were captured using a MacLab 8/s A/D converter (ADI Instruments, Sydney, Australia) at 100 Hz. MacLab Chart software (Version 3.5.6/s; ADI Instruments) was used to calculate  $\text{VO}_2$ ,  $\text{VCO}_2$ , and R.

### Procedure

Mice were studied in groups of four, from both the same diet treatment, and the same group. Mice were grouped in fours because one mouse consumes approximately 1.3 ml / minute of oxygen and the apparatus was not sensitive enough to measure respiratory gas exchange from one mouse.

#### 1. Start up

The Servomex Datex, pump, Fleish head heater, Input converter to voltage rack, MACLAB AS/8 AD instrument and computer were switched on thirty minutes before the first measurement.

The desiccant (anhydrous calcium sulphite) located prior to the gas analysers was replaced when half the tube length was coloured.

Atmospheric pressure was obtained from a recording barograph (GH Zeal), located at Massey University, in the morning and afternoon.

The MACLAB Chart software used the following equations.

Channel	Measurement and units	Equation Setting
1	Inlet airflow ml / minute.	No calculation, direct measurement
2	Temperature air °C	No calculation, direct measurement
3	Inlet airflow corrected for standard temperature and pressure ml / minute.	$(\text{Pressure} \times \text{Channel 1} \times 273^1) / (760^2 \times (\text{Channel 2} + 273))$
4	Fractional concentration of oxygen in expired gas measured by the gas analyser	No calculation, direct measurement
5	Fractional concentration of carbon dioxide in expired gas measured by the gas analyser	No calculation, direct measurement

6	Calculated oxygen consumption ml / minute.	$(0.2094^3 - \text{Channel 4}) \times \text{Channel 3}$
7	Calculated carbon dioxide production ml / minute.	$\text{Channel 3} \times (\text{Channel 5} - 0.0004^4)$
8	R	Channel 7 / Channel 6

- 1 Standard temperature  
 2 Standard pressure  
 3 Concentration of oxygen in air  
 4 Concentration of carbon dioxide in air

## 2. Calibration

A high gas sample frequency (100 measurements / minute) was set during calibration to obtain maximum data for an accurate calibration.

The calorimeter hood was down during calibration to ensure air flow and pressure was the same as in experimental runs. First airflow was calibrated, then carbon dioxide and finally oxygen. The upper and lower end of the range was set during each calibration at the levels shown below. The charts were left recording for a minimum of thirty seconds on each stabilised upper and lower value. The two calibration gases were run at minimum pressure through the gas analyser, just sufficient to obtain a valid reading.

Calibrate	Upper level	Lower level
Airflow rate ml / minute	650	400
	Span	Zero
Carbon dioxide %	3.24 (composition of calibration gas)	0.04 (air)
Oxygen %	20.94 (air)	0.0 (oxygen free nitrogen)

Note; The composition of room air was not measured. The air that was drawn through the calorimeter came from a well ventilated room in which fresh air was available from open windows.

### (a) Airflow

The rotameter was adjusted to 400 ml / minute for the lower flow rate and 650 ml / minute for the upper flow rate. The flow rate was returned to 500 ml / minute, ready for the mice calorimetry measurements. 500ml / minute was sufficient to meet the animal's

oxygen requirements and was chosen to provide maximum sensitivity for analysis of respiratory exchange gases, and least error in flowrate measurement.

(b) Carbon dioxide and oxygen

Air was pumped through the gas analyser for the lower carbon dioxide level (0.04% carbon dioxide) value and upper oxygen level (20.94% oxygen) value.

Carbon dioxide calibration gas (3.24 %) was used for the upper carbon dioxide level.

Oxygen free nitrogen was used for the lower oxygen level.

3. Calorimeter run

The airflow rate through the calorimeter was set at 500 ml / minute. The sample frequency was set at 10 measurements / minute, to obtain sufficient data without overloading storage files.

(a) No mice test

The calorimeter was run for five minutes, with no mice. The oxygen channel (Channel 6), was checked to read in the range – 0.20 to + 0.20 ml / minute oxygen and the carbon dioxide channel (Channel 7), – 0.20 to + 0.20 ml / minute carbon dioxide. If these values were exceeded the calorimeter system was recalibrated.

(b) Calorimeter measurement

Four mice, from two cages of two mice were placed under the calorimeter hood. Ambient temperature, pressure and start time at the beginning of the run was recorded. The calorimeter run period was forty-five minutes. Mice activity score was recorded every five minutes during the run. Chart label details were Group (1 to 5), diet colour, calorimeter status (start, middle, end), and date.

(c) No mice test

Fresh room air was flushed through the system for approximately ten minutes, following removal of the mice cages. The calorimeter was run for five minutes, with the hood down, with no mice. The oxygen channel reading was checked to be in the range – 0.20 to + 0.20 ml / minute oxygen and Channel 7, – 0.20 to + 0.20 ml / minute carbon dioxide. If these values were exceeded, the calorimeter was recalibrated and the run repeated.

### Data Handling

Data was transferred from the MACLAB chart to a spreadsheet using a Macro. This calculated a mean value for each one minute of recorded measurement for inlet airflow rate corrected for standard temperature and pressure, fractional concentration of oxygen in expired gases, fractional concentration of carbon dioxide in expired gases, volume of oxygen consumed, and R.

Appendix 3.1 Conversion of w/w CLA in rat diets to percent of energy from CLA

0.21 % w/w CLA diet

$$0.21 \% \text{ w/w CLA} = 0.0021 \text{ g CLA / g diet}$$

Assume CLA contains 39.8 kJ / g (Linder, 1991)

$$0.0021 \text{ g CLA contains } 0.0021 \times 39.8 \text{ kJ / g} = 0.0836 \text{ kJ / g}$$

The 0.21 % w/w CLA diet contained 19.33 kJ / g (by bomb calorimetry, refer Table 3.4)

Therefore % of total energy from CLA =  $0.0836 \text{ kJ} / 19.33 \text{ kJ} \times 100 = 0.438 \% \text{ of energy}$

0.29 % w/w CLA diet

$$0.29 \% \text{ w/w CLA} = 0.0029 \text{ g CLA / g diet}$$

Assume CLA contains 39.8 kJ / g (Linder, 1991)

$$0.0029 \text{ g CLA contains } 0.0029 \times 39.8 \text{ kJ / g} = 0.115 \text{ kJ / g}$$

The 0.29 % w/w CLA diet contained 19.34 kJ / g (by bomb calorimetry, refer Table 3.4)

Therefore % of total energy from CLA =  $0.115 \text{ kJ} / 19.34 \text{ kJ} \times 100 = 0.595 \% \text{ of energy}$

0.23 % CLA diet

$$0.23 \% \text{ w/w CLA} = 0.0023 \text{ g CLA / g diet}$$

Assume CLA contains 39.8 kJ / g (Linder, 1991)

$$0.0023 \text{ g CLA contains } 0.0023 \times 39.8 \text{ kJ / g} = 0.0915 \text{ kJ / g}$$

The 0.23 % w/w CLA diet contained 19.34 kJ / g (by bomb calorimetry, refer Table 3.4)

Therefore % total energy from CLA =  $0.0915 \text{ kJ} / 19.34 \text{ kJ} \times 100 = 0.473 \% \text{ of energy}$

Appendix 4.1 Conversion of CLA as % energy to mg / day

If % total energy from CLA = 0.578 %

Assume a person consumes 10,000 kJ per day.

$$0.578 \% \times 10,000 \text{ kJ} = 57.8 \text{ kJ.}$$

Assume CLA contains 39.8 kJ / g (Linder, 1991)

$$57.8 \text{ kJ} / 39.8 \text{ kJ / g} = 1.45 \text{ g CLA per day}$$