Dietary fats in the Australian diet and their impact on obesity, appetite and metabolism

A thesis submitted by

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Doctor of Philosophy

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Declaration

I, Shaan Naughton, declare that this PhD thesis entitled ‘Dietary fats in the Australian diet and their impact on obesity, appetite and metabolism’ is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma, except where explicitly stated. Except where otherwise indicated, this thesis is my own work.

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Abstract

Obesity is a worldwide problem, and as such a large body of research has focused on potential treatments to decrease excess body weight and the associated loss of adequate metabolic health. Obesity risk is influenced by more sedentary lifestyles, higher energy intakes and greater consumption of convenience and processed foods. Worldwide evidence indicates that these factors occur as part of nutrition transitions, typified by an increase in energy density and plant derived fats, refined carbohydrates and a reduction in the variety of food consumed. It is believed that this increase in availability of particular plant derived fats can influence health, with the monounsaturated fatty acid (MUFA) oleic acid (OA) appearing to preserve metabolic health, and the polyunsaturated fatty acid (PUFA), linoleic acid (LA), having potential negative impacts, though this has not been fully explored.

The first study of this thesis aimed to investigate changes in the Australian diet from historically available population level food disappearance data and how the availability of common dietary fats, specifically OA and LA changed, and what foods have influenced this. This study found that over the time period 1961-2009 total available energy (TAE) from lipids increased 16.67 %, with a slight reduction in TAE from carbohydrates. Cumulative change in TAE from LA was +120.48 %, found to result from total plant oils having a + 627.19 % cumulative change in LA availability.

As the effect of these fatty acids (FA) on weight gain and metabolic health is yet to be fully elucidated, a rodent study was performed, with 60 Sprague Dawley rats being fed a high fat 'Western' style diet to induce obesity for 9 weeks. The animals were then switched to diets with varying FA compositions, namely a standard 'Western' style diet,

a high OA 'Mediterranean' style diet and a high LA diet for 6 weeks. The impact of these diets on body weight and composition, energy intake, glucose handling and adipose tissue and skeletal muscle metabolism was also investigated. As adiponectin is an insulin sensitising adipokine the ability of this to interact with FA and influence gene expression relating to FA metabolism in skeletal muscle was also investigated through incubation of excised muscles in physiological levels of globular adiponectin. 6 weeks of specific high fat diet feeding in male Sprague Dawley rats resulted in no significant differences between body weights, food intake, blood pressure, percentage body fat, or adipose depot specific change to FA metabolism genes for the 3 high fat fed groups. Despite this, this study does show that there is a potential for the type of dietary fat to modulate food intake and energy efficiency (week 6 high OA vs. high LA, 0.020 ± 0.005 and 0.037 ± 0.003, g gain/mJ, $p=0.033$), with reductions in both of these occurring for the high OA consuming animals towards the end of the study. Additionally, investigation of changes to gene expression in the muscles from these animals found the ability of adiponectin incubation to increase FAT/CD36, β-HAD and CPT1 mRNA expression in both the EDL and soleus from high OA fed animals (all $p \leq 0.05$ compared to tissue incubated without adiponectin), a response which was not seen in any of the other diet groups. This may indicate that high OA feeding preserves adiponectin sensitivity in both of these muscle types and may confer metabolic benefits not seen in the other high fat diets tested here.

Finally, the ability of different FA to modulate acute appetite and hormonal responses following consumption of isoenergetic breakfasts containing elevated contents of carbohydrate, OA and LA respectively, was investigated in eight overweight and obese individuals (age 45.8 ± 3.6 years, BMI 32.0 ± 1.3 kg/m^2) in a 3-way crossover single-blinded study. Consumption of all meals resulted in a significant increase in fullness and a reduction in desire to eat, with the control and high OA meals decreasing prospective

food intake (p= 0.006 and p= 0.049 respectively), though this did not occur following ingestion of the high LA meal (p= 0.183). Additionally, following the consumption of the LA meal there was a significant increase in ghrelin production (compared to the control meal, p= 0.018). This was coupled with a spike in resistin production at the one hour time point, indicating potential impairment of insulin signalling. Taken together this indicates that high LA meals may promote excess energy intake and impair glucose handling, though further investigation is required.

The results of this thesis indicate that LA availability in Australia has increased over the preceding 5 decades as a result of increased plant oil availabilities, as has total fat, possibly contributing to the increasing rates of obesity and obesity associated co-morbidities. Results from the animal and human studies indicate that a higher OA intake as opposed to LA may help to preserve skeletal muscle FA metabolism and potentially limit food intake, indicated by the reduction in food intake and energy efficiency seen in the high OA diet consuming rats in the rodent study and the increase in the appetite and food intake stimulating hormone ghrelin following consumption of the high LA meal in the human study.

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Publications

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Meza, C., Montenegro, C., De La Peña, C., O'Keefe, L., **Naughton, S.**, Simcocks, A., Skelly, D., Mathai, M., McAinch, A. & Bajpeyi, S. (2017). High Fat Diet Induced Obesity Impairs Skeletal Muscle Glycogen and Lipid Preservation After Adiponectin Incubation. In *International Journal of Exercise Science: Conference Proceedings* (Vol. 2, No. 9, p. 28).

Presentations arising from this thesis

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Abbreviations

µg: Microgram

13-HODE: 13-hydroxy-octadecadienoic

20-HETE: 20-Hydroxy-5,8,11,14-eicosatetraenoic acid

2-AG: 2-arachidonyl glycerol

9-HODE: 9-hydroxy-octadecadienoic

AA: Arachidonic acid

ABS: Australian bureau of statistics

Ad: Adiponectin

AE: Available energy

AEA: Anandamide

ALA: α-Linolenic acid

AMP: Adenosine monophosphate

AMPK: Adenosine monophosphate -activated protein kinase

AMPKα2: Adenosine monophosphate -activated protein kinase alpha subunit 2

ANOVA: Analysis of variance

ANZCCART: Australian & New Zealand Council for the care of animals in research and teaching

APPL1: Adaptor protein, phosphotyrosine interacting with pH domain and leucine zipper 1

ATP: Adenosine triphosphate

AU: Arbitrary units

AUC: Area under the curve

β-HAD: Beta-hydroxyacyl-CoA dehydrogenase

BMI: Body mass index (kg/m^2)

BSA: Bovine serum albumin

Ca^{2+}: Calcium

cAMP: Cyclic adenosine monophosphate

CB1: Cannabinoid receptor 1

CB2: Cannabinoid receptor 2

CD 68: Cluster of differentiation 68

cDNA: complementary deoxyribonucleic acid

CEBP β: CCAAT-enhancer-binding protein beta

CEBP δ: CCAAT-enhancer-binding protein delta

CHO: Carbohydrate

COX2: Cyclooxygenase-2

CPT1: Carnitine palmitoyltransferase 1

CRP: C-reactive protein

CVD: Cardiovascular disease

DEPC- Diethylpyrocarbonate

DXA: Dual-energy X-ray absorptiometry

DHA: Docosahexaenoic acid

DHEA: Docosahexaenoylethanolamide

DIO: Diet induced obesity

DNA: Deoxyribonucleic acid

EDL: Extensor digitorum longus

EDTA: Ethylenediaminetetraacetic acid

EI: Energy intake

EPA: Eicosapentaenoic acid

EPEA: Eicosapentaenolyethanolamide

FA: Fatty acid

FAAH: Fatty acid amide hydrolase

FADS1: Fatty acid desaturase 1

FADS2: Fatty acid desaturase 2

FAS: Fatty acid synthase

FAT/CD36: Fatty acid translocase/ cluster of differentiation 36

FAOSTAT: United Nations food and agriculture organisation statistics division

FBG: Fasting blood glucose

FBS: Food balance sheets

FSANZ: Food standards Australia and New Zealand

g: Gram

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GIP: Glucose-dependent insulinotropic peptide

GLP-1: Glucagon-like peptide 1

GLUT4: Glucose transporter type 4

GPCR: G protein coupled receptor

GPR55: G protein-coupled receptor 55

GPR119: G protein-coupled receptor 119

GTT: Glucose tolerance test

H_2O: Water

HO-1: Heme oxygenase 1

HPRT1: Hypoxanthine guanine phosphoribosyl transferase1

IFN-γ: Interferon gamma

IL-10: Interleukin 10

IL-12p70: Interleukin 12 p70

IL-13: Interleukin 13

IL-1β: Interleukin 1β

IL-2: Interleukin 2

IL-3: Interleukin 3

IL-4: Interleukin 4

IL-5: Interleukin 5

IL-6: Interleukin 6

iNOS: Inducible nitric oxide synthase

IST: Insulin sensitivity test

IU: International units

JNK: c-Jun N-terminal kinase

kDa: Kilodalton

kg: Kilogram

kJ: Kilojoule

LA: Linoleic acid

LIP: Lipid

LKB1: Liver kinase beta 1

LPS: Lipopolysaccharide

MAPK: Mitogen-activated protein kinase

MCP-1: Monocyte chemoattractant protein-1

mg: Milligram

MGL: Monoacylglycerol lipase

mJ: Mega joule

mmHg: Millimetres of mercury

mmol/L: Millimoles per litre

MRI: Magnetic resonance imaging

mRNA: Messenger ribonucleic acid

MUFA: Monounsaturated fatty acid

NAD^+: Nicotinamide adenine dinucleotide

NAE: *N*-acylethanolamide

NAPE: *N*-arachidonoyl phosphatidylethanolamine

NAPE-PLD: *N*-acyl phosphatidylethanolamine specific phospholipase D

NF-κβ: Nuclear factor kappa-light-chain-enhancer of activated B cells

NHMRC: National health and medical research council (Australia)

OA: Oleic acid

OXLAMs: Oxidised linoleic acid metabolites

PA: Palmitic acid

PAI-1: Plasminogen activator inhibitor-1

PEA: Palmitoylethanolamide

PGC1α: Peroxisome proliferator-activated receptor gamma co-activator 1 alpha

PKA: Protein kinase A

PPARα: Peroxisome proliferator-activated receptor alpha

PPARβ: Peroxisome proliferator-activated receptor beta

PPARγ: Peroxisome proliferator-activated receptor gamma

PPARδ: Peroxisome proliferator-activated receptor delta

PRO: Protein

PUFA: Polyunsaturated fatty acid

RANTES: Regulated on activation, normal T cell expressed and secreted

RNA: Ribonucleic acid

ROS: Reactive oxygen species

SCD-1: Stearoyl-coenzyme A desaturase 1

SD: Standard deviation

SEM: Standard error of the mean

SFA: Saturated fatty acid

SREBP1c: Sterol regulatory element-binding protein 1c

TAE: Total available energy

TAG: Triacylglycerols

T2DM: Type 2 diabetes mellitus

TNF-α: Tumour necrosis factor α

U: Units

USDA: United States department of agriculture

UTEP: University of Texas at El Paso

VAS: Visual analogue scale

VPR: Volume pressure recording

WHO: World Health Organisation

X g: times gravity

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Chapter 1: Literature Review

Substantial portions of this Chapter have previously been published:

Sections 1.8-1.16 inclusive and 1.25 have been published in the manuscript: Naughton, SS, Mathai, ML, Hryciw, DH, McAinch, AJ, 2016 "Linoleic acid and the pathogenesis of obesity", *Prostaglandins and Other Lipid Mediators,* 125, 90-99.
Please note one section and one figure relating to linoleic acid and the endocannabinoid system have been removed from this Chapter to avoid repetition.

Sections 1.17-1.24 inclusive have been published in the manuscript: Naughton, SS, Mathai, ML, Hryciw, DH, McAinch, AJ, 2013 "Fatty Acid Modulation of the Endocannabinoid System and the Effect on Food Intake and Metabolism," *International Journal of Endocrinology*, vol. 2013, Article ID 361895, 11 pages, 2013. doi:10.1155/2013/361895

1.1 Obesity in Australia and its causes

Obesity is characterised as the deposition of excess body fat at which point an individual's body mass index (BMI) is greater than 30 kg/m^2 or a waist circumference greater than 88 cm for females or 102 cm for males (Cameron et al., 2003) or to a point beyond which an individual's health is negatively impacted (World Health Organisation, 2000). For this excess body weight to develop, a positive energy balance is required, either through insufficient energy expenditure or due to excessive energy intake (Hill, 2006). Additionally, a large body of research has found relationships between obesity and cardiovascular disease (CVD), type II diabetes mellitus (T2DM), osteoarthritis and certain types of cancers (Dixon, 2010). The most recent Australian Health Survey data (2014-2015) found that 27.9 % of the Australian population over the age of 18 were obese, with an additional 35.5 % of the adult population being overweight, with a higher prevalence found in disadvantaged and remote areas (Australian Bureau of Statistics, 2015). These rates are often described as an epidemic and at

an individual level, result in loss of years of lifespan, increased morbidity and mortality and a decrease in quality of life (Dixon, 2010). At a societal level these rates of obesity and related comorbidities increase health expenditure, and contribute to indirect costs as a result of disability, workplace accidents, loss of productivity and carer costs (Dixon, 2010). This financial burden is currently estimated to be $58.2 billion annually, a sizeable proportion of which accounts for loss of wellbeing and the treatment associated with obesity related non-communicable diseases (Access Economics, 2008). Importantly, this strain is felt not only by the federal and state governments, it is also borne by the individual, their families and the rest of society (Obesity Australia, 2014).

The factors influencing the development of obesity are wide ranging, with high birth weights and parent weight status being a predictor of adult adiposity in children (Parsons et al., 1999). In addition genetic factors have been found which can promote the pathogenesis of obesity (Farooqi & O'Rahilly, 2007) and also interact with intestinal microbiota to influence the development of obesity (Ussar et al., 2015). The environment in which we live can also influence adiposity, with lower socio-economic areas having higher rates of obesity; similarly, increased access to supermarkets and decreased access to takeaway/fast food businesses is associated with lower rates of obesity (Giskes et al., 2002). The main influencing lifestyle factors are believed to be a trend towards sedentary activity levels, larger portion sizes (Ledikwe et al., 2005) and increased intake of highly palatable, fat and sugar rich foods (Erlanson-Albertsson, 2005). This change in food intake is part of what is considered to be a nutrition transition, a shift in dietary environment which has been documented in many 'Western' and 'Westernised' countries (Popkin, 2001). Nutrition transitions are typified by an increase in urban area size, advances in food supply chains and the presence of multinational food corporations and usually occur with a concomitant decrease in communicable diseases and an increase in the prevalence of lifestyle related diseases such as obesity and T2DM, due

to an increase in energy availability (Drewnowski & Popkin, 1997). At a food intake level there is a decrease in the consumption of traditional diet components and an increase in the intake of high fat and refined carbohydrate processed and convenience foods, instigated by an increase in availability and a decrease in cost (Kearney, 2010).

1.2 Type II Diabetes Mellitus

T2DM is typified by decreased insulin stimulated glucose transport and metabolism in adipocytes and skeletal muscle and by impaired suppression of hepatic glucose output, resulting in increased levels of blood glucose (Kahn & Flier, 2000). This may result from the inability of insulin to bind to the insulin receptor or due to impaired translocation of the insulin responsive Glucose Transporter Type 4 (GLUT4) to the cell membrane (Kahn & Flier, 2000). A current theory relating to the development of insulin resistance proposes that it is not the inability of insulin to bind to the insulin receptor that is the major issue *per se*, it is more so that an intracellular accumulation of triacylglycerols (TAG) or ceramides in lipid rafts interferes with the translocation of the GLUT4 transporter to the cell membrane (Muoio & Neufer, 2012). This is also supported by research utilising primary cultured myocytes from morbidly obese and lean individuals, with the cells from morbidly obese individuals having a significantly reduced rate of complete fatty acid (FA) β-oxidation and a significantly increased ratio of incomplete to complete oxidation products when compared to cells from lean subjects (Bell et al., 2010). Adding to these contributing factors to insulin resistance there is also a reduction in insulin secretion, possibly due to a decrease in β-cell numbers (Butler et al., 2003) and a systemic increase in inflammation, as well as the effects of lipid deposition in tissues such as the liver (Olokoba et al., 2012).

Results from the 2014-2015 Australian Health survey show that 1 million (4.4 % of the adult population) Australians have T2DM, an increase of almost 200,000 since the 2011-2012 survey (Australian Bureau of Statistics, 2015). As with the prevalence of obesity, incidence is higher

in low socio-economic and remote areas (Australian Bureau of Statistics, 2015). T2DM has numerous comorbidities including dyslipidaemia, hypertension and depression (Caughey et al., 2008). Moreover, common T2DM complications included chronic kidney disease, retinopathy, lower limb amputation and stroke, due to damage to micro and macro vasculature structures and increased risk of infection associated with chronically elevated blood glucose levels (Forbes & Cooper, 2013). This has resulted in Australian people with T2DM having a higher rate of mortality compared to those without T2DM (Harding et al., 2014), with approximately 75–80 % of people with T2DM dying of CVD worldwide (Alberti et al., 2007). Importantly, many of the factors that influence the development of T2DM are modifiable lifestyle factors such as obesity, physical inactivity, excessive energy intake and a low dietary fibre intake (Alberti et al., 2007).

1.3 Skeletal muscle and its role in metabolic health

Skeletal muscle is one of the highest contributors to basal metabolic rate (BMR), accounting for between 20- 30 % of BMR (Zurlo et al., 1990), making it a primary site of glucose and fat utilisation for energy. As such, adequate skeletal muscle metabolism is a predictor of total BMR and influences whole body energy expenditure and balance (Zurlo et al., 1990). Moreover, a reduced BMR is a predictor of the development of excess body weight (Zurlo et al., 1990). Compounding this is the issue that the rate of FA oxidation in skeletal muscle from obese and diabetic individuals is decreased (He et al., 2001), creating a potential cycle of fat deposition.

Skeletal muscle fibres can be categorised based on their vascularity, mitochondrial content and preferred substrate, these categories are slow-twitch oxidative fibres (type I fibres), fast-twitch glycolytic fibres (type IIX fibres) and combined fibre types, type IIa which are predominately oxidative, type IIb which are predominately glycolytic and a mixed type IId (Bonen, et al., 1981; Hämäläinen & Pette, 1993). In humans, the distribution of these fibre types within

specific muscles appears to not follow a specific pattern (Johnson. et al., 1973), though some rodent skeletal muscles are almost entirely of the one type, making them an excellent model in which to investigate changes to metabolism relating to the utilisation of lipids or glucose for energy (Bonen et al., 1981). For example, in rodents the soleus muscle is primarily made up of the type I slow-twitch oxidative type, whilst the extensor digitorum longus muscle is made of a combination of fast-twitch glycolytic and the type IIa fast-twitch oxidative glycolytic (Bonen, et al., 1981).

1.4 Factors regulating skeletal muscle substrate availability and metabolism

Several key components regulate the provision of lipids into muscle fibres for metabolism, one of which being fatty acid translocase/ cluster of differentiation 36 (FAT/CD36) a 78- 88 kilodalton (kDa) cell membrane glycoprotein which allows the movement of long chain FA into tissues, including skeletal muscle and adipose tissue (Bonen, Arend, Campbell, et al., 2004; Vallvé et al., 2002). In skeletal muscle it is found at the sarcolemma and also within intracellular vesicles, with the protein being able to be translocated from inside the cell to the outer membrane to allow for FA influx (Luiken et al., 2002) which can also be triggered by insulin binding (Bonen et al., 1981). There is also evidence that it may physically pair with carnitine palmitoyltransferase 1 (CPT1) at the mitochondrial membrane, facilitating a higher rate of FA transport through the mitochondrial membrane (Campbell et al., 2004). Other research has found that FAT/CD36 increases FA metabolism more so through increasing cellular esterification into triglycerides and lipid droplets than through an increased rate of uptake (Xu et al., 2013). Due to the intrinsic role of FAT/CD36 in the uptake of circulating long chain FA it is, as would be expected, expressed more in oxidative muscle types compared to glycolytic muscle types (Bonen et al., 1999). FAT/CD36 transcription may be Peroxisome Proliferator-activated Receptor Gamma (PPARγ) dependant (Vallvé et al., 2002) and expression is modulated in the presence of insulin resistance and high fat feeding (Greenwalt

et al., 1995), with a deficiency of FAT/CD36 being implicated in the development of insulin resistance (Miyaoka et al., 2001) and associated with CVD (Kashiwagi et al., 1996). Moreover, in an obese resistant rat model fed a 45 % fat diet, silencing of hypothalamic FAT/CD36 led to the development of an obese phenotype without hyperphagia or a change in body weight, suggesting a decrease in both lean mass and energy expenditure (Le Foll et al., 2015).

Once FA have entered the cytoplasm, CPT1 is required to *trans*-esterify fatty acids to acyl-carnitine before they can cross the inner mitochondrial membrane, after which the acyl-carnitine is *trans*-esterified back to free carnitine and long-chain acyl-CoA by carnitine palmitoyltransferase 2 (Holloway et al., 2008; Stephens et al., 2007). This is considered an essential step in shifting the pathway of FA metabolism to β-oxidation, as opposed to forming lipid intermediates such as diacylglycerides and ceramides in the cytosol (Koves et al., 2008). In skeletal muscle, insulin can inhibit CPT1 (Vallvé et al., 2002) and over expression of CPT1 has been found to restore muscle insulin sensitivity caused by high fat feeding induced intramuscular triglyceride accumulation in the EDL (Bruce et al., 2009). Oleic acid (OA) has been found to increase both CPT1 and AMP-activated protein kinase (AMPK) in the liver of rats with experimental sepsis and their control OA fed litter mates (Gonçalves-de-Albuquerque et al., 2016).

AMPK is a heterotrimer complex containing 3 subunits, a catalytic α unit, a β unit containing a glycogen-sensing domain, and a γ subunit that contains 2 regulatory sites that bind adenosine monophosphate (AMP) and adenosine triphosphate (ATP) (Towler & Hardie, 2007). AMPK is a key regulator of energy metabolism, with the ability to increase the uptake and metabolism of both FA and glucose in muscle, with long term activation of AMPK leading to increased expression of genes involved in mitochondrial biogenesis and oxidative metabolism (Turner et al., 2014). Activation of AMPK occurs through phosphorylation of the α-subunit at Thr172 by an upstream AMPK kinase such as liver kinase beta 1 (LKB 1) or calcium (Ca^{2+}) -calmodulin-

dependent protein kinase. Furthermore, it is a sensor of intracellular energy state, with activation occurring when there is an increase in the AMP to ATP ratio, with its activation also leading to increased CPT1 activation (Yoon et al., 2006). Coupled with this switch towards energy production, AMPK has energy preservation actions at a cellular level, with a stalling of the cell cycle and apoptosis occurring during prolonged activation (Towler & Hardie, 2007). Activation of AMPK occurs naturally via exercise (depleting the amount of cellular ATP) and also through hypoxia, though more recent research has found that adipokines, such as leptin and adiponectin exert much of their action through AMPK, specifically the α2 isoform (AMPKα2), in skeletal muscle (Towler & Hardie, 2007). At a whole organism level AMPK has been found to increase food intake in mammals, with leptin inhibiting AMPK in the hypothalamus yet activating it in skeletal muscle, possibly indicating further roles yet to be fully elucidated (Hardie, 2004). Furthermore, the previously unknown cellular mechanism of Metformin in lowering circulating glucose levels has since been attributed to AMPK activation (Zhou et al. (2001). Peroxisome proliferator activated receptor γ coactivator- 1 alpha (PGC-1α), is expressed predominately in metabolic tissues such as skeletal muscle, and activates mitochondrial biogenesis and oxidative metabolism, with higher expression in type I fibres (Lin et al., 2002). Human skeletal PGC-1α expression is down regulated in T2DM (Patti et al., 2003). PGC-1α expression is capable of triggering a transition to a more oxidative fibre type, mitochondrial biogenesis and an increase in oxidation (Lin et al., 2002). Additionally PGC-1α may also help prevent against inflammation within cells, with PGC-1α knockout mice showing increased expression of interleukin 6 (IL-6), tumour necrosis factor alpha (TNF-α) and other proinflammatory mediators (Handschin & Spiegelman, 2008). A study utilising lean, young men has found that short term high fat feeding with an increase in energy from fat in isoenergetic meals down regulated vastus lateralis PGC-1α mRNA (Sparks et al., 2005). An animal study performed by the same researchers utilising C57BL/6J mice fed a high fat diet

for 3 weeks found the same reduction in gastrocnemius muscle (Sparks et al., 2005). Interestingly, a study performed in male Wistar rats found that high fat feeding did not alter PGC-1α mRNA expression, though increased PGC1-α protein levels, when compared to chow fed control animals, indicating post-transcriptional regulation (Hancock et al., 2008). Unfortunately, though the study used two differing high fat diets (a flax/olive oil diet and lard/corn oil diet), changes in expression between the two high fat meals was not evaluated (Hancock et al., 2008). Though the strain of rodent used may be capable of influencing the effects of high fat feeding on PGC1-α mRNA and protein expression, a study performed by Turner et al. (2007), utilising muscle samples from high fat fed male C57BL/6J mice and Wistar rats, *db/db* mice and obese Zucker rats found that when compared to their respective chow fed controls, all animals had an increase in PGC1-α protein expression. This increased expression, and the concomitant increase in the expression of multiple subunits of the mitochondrial respiration chain indicate that there is an increase in mitochondrial oxidative capacity as a result of high fat feeding. Similar results to this study were found by Hoeks et al. (2008) in male Wistar rats fed a high fat diet, which also found that there was intramyocellular lipid accumulation despite upregulation of PGC1-α.

The beta-hydroxyacyl-CoA dehydrogenase (β-HAD) enzyme is part of the trifunctional protein subunit that catalyses the last three steps of mitochondrial long-chain fatty acid β-oxidation, thus providing a significant source of cellular energy (Lu & Claypool, 2015). Additionally, it is a rate limiting step of mitochondrial β-oxidation due to its requirement of nicotinamide adenine dinucleotide (NAD^+). If reoxidisation of the reduced NAD does not occur then β-HAD activity is impaired, potentially leading to a build-up of metabolic intermediates (Bartlett & Eaton, 2004). In female Sprague Dawley rats, high fat feeding results in increased β-HAD activity in both the soleus and EDL when compared to high carbohydrate fed animals (McAinch et al., 2003). In addition, as little as 5 days of high fat feeding has been found to

increase *vastus lateralis* β-HAD mRNA expression in well trained men when compared to both baseline expression and that after 5 days of high carbohydrate feeding (Cameron-Smith et al., 2003).

Adiponectin is an insulin sensitising adipokine with anti-inflammatory roles (Turer & Scherer, 2012), released predominately from adipose tissue, the rate of which is inversely correlated to fat mass, influencing glucose handling. Adiponectin circulates in the plasma at relatively high levels in comparison to other adipokines and is found in three main isomeric forms, the trimeric low molecular weight form (which can bind to albumin), the hexameric medium molecular weight form, and the oligomeric high molecular weight form (Liu & Sweeney, 2014). The mixture of these is referred to as full length adiponectin though it is believed that globular adiponectin, which only contains the C-terminal globular domain (which can be enzymatically cleaved from full length adiponectin) is the most metabolically active (Liu & Sweeney, 2014). Adiponectin may have protective mechanisms in the early stages of insulin resistance by stimulating FA oxidation by increasing FA transport into the mitochondria secondary to stimulation of AMPK and inhibition of CPTI in the soleus (Ritchie & Dyck, 2012). However, this ability is quickly downgraded after commencement of high fat feeding in the short term (several days) to protect the mitochondria from excessive fatty acid content and the generation of reactive oxygen species (ROS), leading to adiponectin resistance (Ritchie & Dyck, 2012). Activation of AMPK by adiponectin occurs through Adaptor Protein, Phosphotyrosine interacting with pH domain and Leucine zipper 1 (APPL1) dependent LKB1 translocation to the cytosol (Zhou. et al., 2009).

Adiponectin receptors 1 and 2 have been found to be expressed in human skeletal muscle (Punyadeera et al., 2005) and in this tissue type adiponectin activates and phosphorylates AMPKα (the α2 isoform more so over the α1), p38 Mitogen-Activated Protein Kinase (MAPK), Peroxisome Proliferator-activated Receptor Alpha (PPARα) and CPT1 (Yoon et al.,

2006). A negative correlation has been found between plasma globular adiponectin concentrations and insulin resistance and obesity in rodents, non-human primates (Hotta et al., 2001) and humans (Arita et al., 1999). Administration of low doses of globular adiponectin (2.5 μg/kg body weight) have been found to increase skeletal muscle FAT/CD36 expression in chow fed C57BL/6J mice (Yamauchi et al., 2001). Incubation of EDL muscle with globular adiponectin increases AMPK expression, which does not occur when full length adiponectin is used (Tomas et al., 2002), indicating the requirement of proteolytic cleavage to a functional form (Yamauchi et al., 2001). The systemic ability of thiazolidinedione's to increase insulin sensitivity has been found to be reliant on the ability of adiponectin to activate AMPK through research using mice with disruptions to the adiponectin coding genetic locus (Nawrocki et al., 2006). Research using cultured C_2C_{12} myotubes has found that incubation of cells with globular adiponectin leads to an increase in CPT1 expression resulting from AMPK activation, demonstrated by an absence of change when challenged by the overexpression of a dominant-negative form of AMPK (Yoon et al., 2006). Taken together this indicates the importance of the interaction between adiponectin and cellular metabolic influencers to promote adequate glucose and FA metabolism in skeletal muscle**.**

1.5 Adipose tissue and its role in metabolic health

Adipose tissue was long considered to be primarily a metabolically inert store of excess energy, though the discovery of the adipokine class of metabolic mediators led to the realisation that it has the ability to effect systemic metabolism and influence appetite and other processes, such as insulin sensitivity. Broadly, adipose tissue has two distinct cell types, unilocular white adipocytes and multilocular brown adipocytes, so named due to the darker colour resulting from an increased mitochondrial content. Additionally it has been found that from a developmental point of view, brown adipocytes are more similar to skeletal muscle (and can form from the same precursor cells) with a gene profile and mitochondrial proteomic signature

closer to that of skeletal muscle than white adipocytes (Kajimura et al., 2010). The other main difference between the two cell types is the ability of brown adipocytes to contribute to non-shivering thermogenesis when adrenergically stimulated (Cannon & Nedergaard, 2004). This is possibly due to the presence of uncoupling proteins in the electron transport chain, thus generating heat as opposed to energy (Cannon & Nedergaard, 2004). There is also a cell type referred to as 'beige' or 'brite', having a mixed phenotype and capable of thermogenic activity (Tiraby et al., 2003). In humans, brown adipose tissue mass (when compared to total mass) is greatest in the neonatal and infant stages of life, with the overall mass decreasing to low levels in adults. In contrast, rodents have significant brown adipose tissue mass throughout their lifespan, most likely due to their small size and the requirement to maintain an adequate body temperature, making them an ideal model in which to investigate this type of tissue (Cypess et al., 2009). Brown adipose tissue mass and its thermogenic capacity has been a focus of research due to the theory that if either of these can be increased then basal metabolism will be increased, potentially a mechanism which could aid in weight loss, especially as energy lost via thermogenesis is not fully compensated for by an increase in appetite or food intake (Cannon & Nedergaard, 2009). Entry of FA to adipocytes can occur through several different mechanisms, through passive diffusion down the concentration gradient, as a result of insulin stimulation or through facilitated transport, most commonly mediated by the previously discussed FAT/CD36, with the suppression of hormone sensitive lipase also playing a role (Lewis et al., 2002). In both white and brown adipocytes the main influencer of adipocyte differentiation is PPAR-γ, increased expression of which is also associated with increased lipid deposition within the cells (Rosen & Spiegelman, 2006). In contrast, PPAR-α is involved in the transcription of genes which generally relate to FA catabolism, namely cellular uptake and β-oxidation and the synthesis of lipoproteins (Kersten et al., 2000). Obesity is typified by an increase in both the number of adipocytes and the volume of lipid within the cells (Nishimura

et al., 2007), with a higher number of large adipocytes being associated with the development of T2DM (Skurk et al., 2007) and with large adipocytes producing greater amounts of proinflammatory cytokines and leptin, (Skurk et al., 2007) though less adiponectin than small adipocytes (Meyer et al., 2013).

1.6 Regulation of appetite and food intake

Food intake is influenced greatly by appetite, with homeostatic food intake being in response to an increase in appetite triggered by a decrease in energy availability; conversely, hedonic food intake is triggered by appetite in response to endogenous and exogenous stimuli and often occurs in satiated or postprandial states (Mela, 2006). Both homeostatic and hedonic ingestion results in an increase in circulating neurotransmitters, hormones and glucocorticoids which have the potential to regulate the activity of a number of G-protein coupled receptors (GPCR), including the cannabinoid receptors (Devane et al., 1988; Howlett, 1985). Additionally appetite regulation can be through an endocrine manner or through neuronal pathways and can be dysregulated in obesity, and is also likely influenced by gut microbiota (Bauer et al., 2016). Moreover, ingested nutrients are also capable of modulating appetite, adding to a complicated regulatory system, as demonstrated in Figure 1.1.

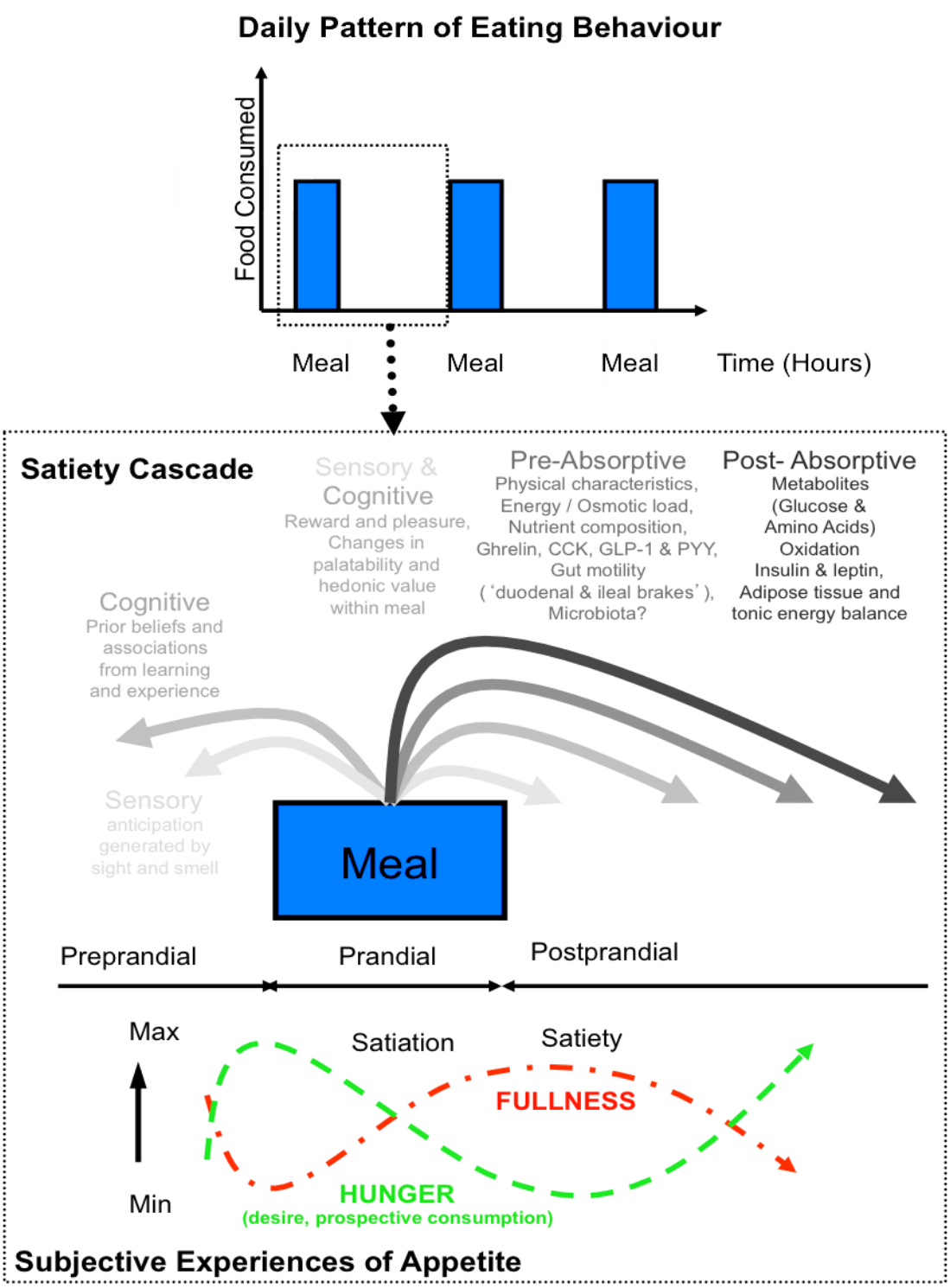


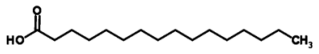
Figure 1.1: Major factors regulating appetite and satiety. CCK, cholecystokinin; GLP-1, glucagon-like peptide-1; PYY, peptide YY. Figure originally published in Halford and Harrold (2012).

For brevity, only the appetite modulating factors discussed in this thesis will be detailed here. Glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) (originally known as Gastric-inhibitory polypeptide) both belong to the incretin class of peptides involved in appetite regulation and postprandial nutrient metabolism. They are both secreted into the small intestine by enteroendocrine cells in the acute phase following food ingestion, and both act on the β cells of the pancreas and other tissues. Despite this they have different roles, GIP promotes adipose tissue energy storage and promotes insulin secretion, with GLP-1 slowing gastric emptying and promoting postprandial satiety (Baggio & Drucker, 2007). In contrast, levels of leptin increase in obesity, potentially leading to leptin insensitivity, an important pathology due to its primary role being in limiting energy intake and promoting satiety (Fantuzzi, 2005). Resistin is one of the more poorly understood adipokines, with current evidence suggesting a role in decreasing insulin sensitivity and in perpetuating inflammation, with circulating levels increased in obese individuals (Al-Suhaimi & Shehzad, 2013).

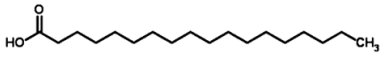
1.7 Dietary fatty acids

The majority of lipids consumed in the human diet are in the form of triglycerides, which are digested to yield 3 FA and a glycerol back bone (Hulbert et al., 2005). The number of carbon atoms and the degree of saturation with hydrogen of these FA greatly influence their roles and uses in the body, and also determine the classes into which they are categorised. FA which have hydrogen atoms bonded to all carbon atoms are considered saturated fats (SFA), with FA which have one double bond between carbon atoms (i.e. not a hydrogen atom bonded at that point) are considered to be mono-unsaturated fatty acids (MUFA), whilst the FA which have multiple double bonds between the carbon atoms being considered poly-unsaturated fatty acids (PUFA).

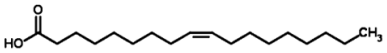
The points at which the unsaturation occur also influences how these are used in the body, with the nomenclature being that the carbon which has the first double bond from the methyl end of the FA being designated the ω or n- carbon, from which FA can further be grouped. Within the MUFA class predominately n-9 FA are found, the most common of which being the 18 carbon FA OA which is the primary FA found in olive oil (Diep et al., 2011). The PUFA group contains two main distinct and often functionally opposite unsaturation types, being n-3 and n-6 FA, though both are essential to the human diet (Simopoulos, 2006). This thesis will focus on some of the those most commonly found PUFA in the human diet, namely the 18-carbon n-6 FA linoleic acid (LA), the 20-carbon n-6 FA arachidonic acid (AA) as well as the 18-carbon n-3 FA α-Linolenic acid (ALA), and the two n-3 FA, the 20-carbon eicosapentaenoic acid (EPA) and 22 carbons docosahexaenoic acid (DHA). The structure of the main FA discussed in this thesis are shown in Figure 1.2.



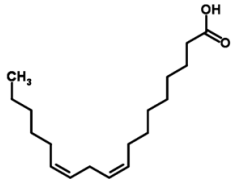
Palmitic acid 16:0



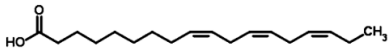
Stearic acid 18:0



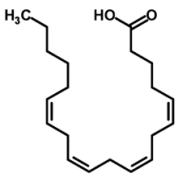
Oleic acid 18:1 n- 9



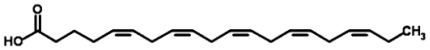
Linoleic acid 18:2 n- 6



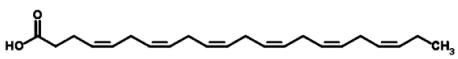
α-linolenic acid 18:3 n- 3



Arachidonic acid 20:4 n- 6



Eicosapentaenoic acid 20:5 n- 3



Docosahexaenoic acid 22:6 n- 3

Figure 1.2: Chemical structure of common fatty acids. The chemical structure of common dietary fatty acids. Carbons have not been labelled for clarity. Additional lines show the location of a double bond. The common fatty acid name is follow by the number of carbons, then the number of double bonds and the location of the first double bond from the methyl end of the carbon chain (n-) where applicable.

In Australia, dietary guidelines are developed and reviewed by the National Health and Medical Research Council (NHMRC) in conjunction with other government departments (National Health and Medical Research Council, 2013b). Historically, one key recommendation of these guidelines was to reduce SFA intake in favour of MUFA and PUFA, with an emphasis placed on plant based lipid sources (National Health and Medical Research Council, 1992), with the current American Heart Association recommendations (Harris et al., 2009) and Australian dietary guidelines continuing to reflect this view (National Health and Medical Research Council, 2013a). As dietary guidelines are produced for the general public, differentiations between particular fatty acids and even between MUFA and PUFA in regard to their physiological roles are not made. This may be an important omission as not all fatty acids in the same class have similar physiological roles (Grande et al., 1970; Mattson & Grundy, 1985). Additionally, as shown in Figure 1.3, the composition of different plant based oils varies widely, making them not nutritionally equal substitutions.

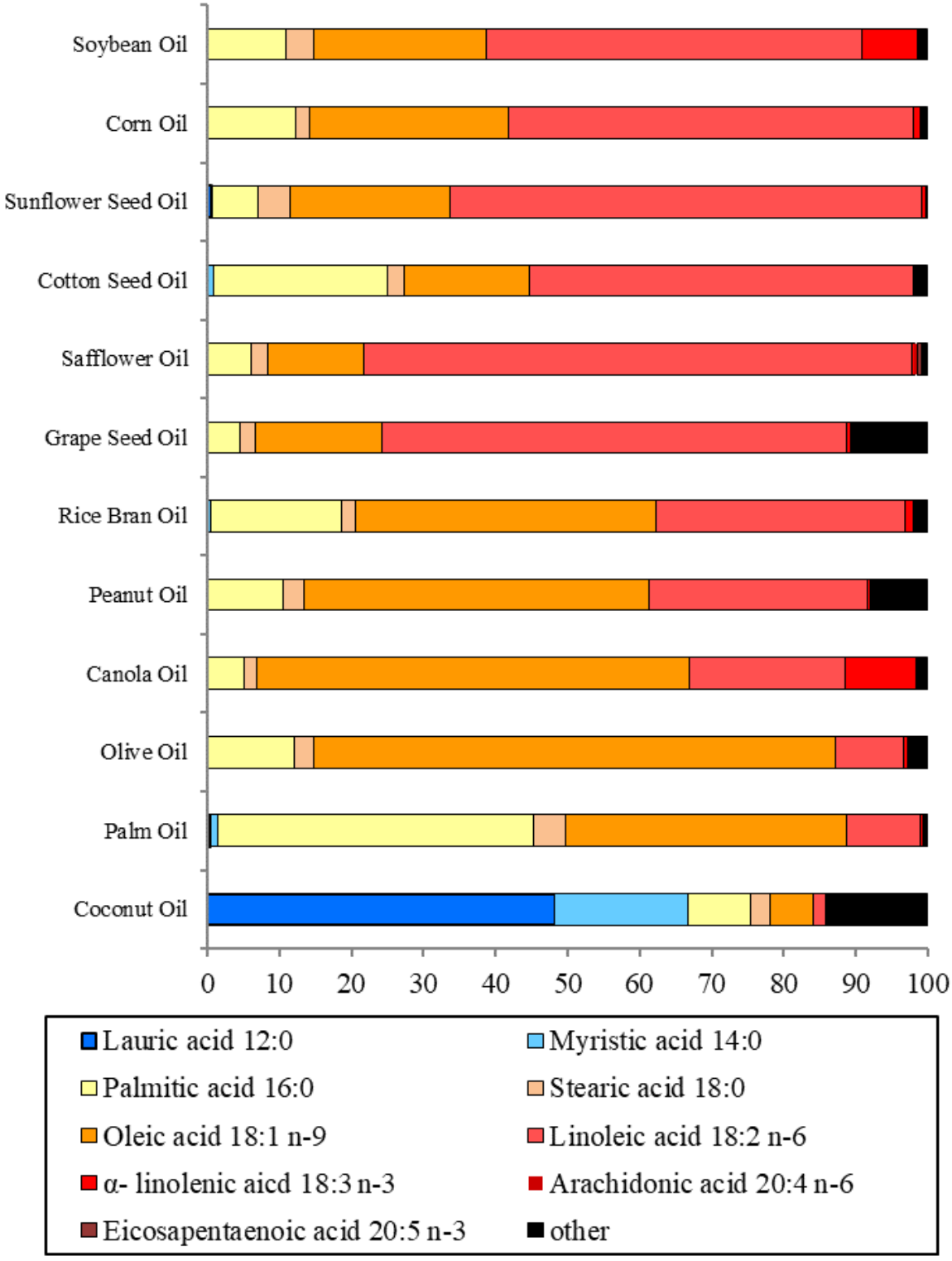


Figure 1.3: The Fatty Acid composition of commonly consumed Plant based oils. The percentage composition of the major fatty acids in commonly consumed plant oils, showing the variety and widely varying compositions. Adapted from Dubois et al., 2007.

1.8 Fatty Acid Essentiality

For the last half century, worldwide dietary guidelines and recommendations by peak public health bodies have emphasised the intake of plant based PUFA, the most abundant of which is LA. Despite this, recent research, and reanalysis of existing data, has questioned the purported beneficial effects of LA and its protective health properties (Ramsden et al., 2013) including requirements to meet physiological needs, with the current estimated requirements of 0.2-0.4 % of total energy intake (EI) being approximately 5-10 times less than what has previously been accepted (Choque et al., 2015; Cunnane & Guesnet, 2011). This is due to the unintentional exclusion of ALA from the diets used in the original research assessing LA adequacy, the addition of which prevents deficiency symptoms at lower LA intakes (Choque et al., 2015). Similarly, a major flaw in the early research investigating replacing SFA with PUFA to decrease CVD risk is the absence of consideration of the n-3 PUFA family (Choque et al., 2014; Ramsden et al., 2010; Ramsden et al., 2013). It has recently been determined that the increase in LA in the original research assessing CVD prevention resulted in a concomitant increase in n-3 PUFA, due to changes to dietary composition, conferring the observed cardiovascular benefits (Ramsden et al., 2010). In addition, there is still a lack of consensus on whether the ratio of FA, the proportions of particular FA (e.g. n-6 PUFA: n-3 PUFA ratio) in the diet, or the specific load of FA is more important in its impact on health and disease, making definitive conclusions difficult to draw.

1.9 Fatty Acid Intake Recommendations in Australia

Current Australian dietary recommendations state that 10 % of energy from LA is an acceptable level, based off levels of intake which appear not to have negative effects on health (National Health and Medical Research Council et al., 2006). US recommendations only state that an intake level of LA is required to prevent deficiency (U.S. Department of Health and Human Services and U.S. Department of Agriculture 2015) with evidence that long term/large scale human research is lacking (Food and Agriculture Organization of the United Nations, 2010).

The current intake of 6.0 % of total energy in in Australian diets and 7.2 % in the United States are however between 14 and 18 times what is required to prevent deficiency (Blasbalg et al., 2011; Naughton et al., 2015).

1.10 Nutrition Transitions and changes in Linoleic Acid Intake

Worldwide, many studies have shown an increase in dietary LA, regardless of the methodologies used to collect the food intake data (e.g. self-reporting recall, food disappearance data/balance sheets, adipose and erythrocyte FA composition) (Blasbalg et al., 2011; Hibbeln et al., 2004; Sanders, 2000). This increase in LA is believed to have resulted from a series of country or region-specific nutrition transitions (Popkin, 2001). Nutrition transitions involve changes to agricultural practice, strengthened transport and trade networks, increased urbanisation of populations and globalisation of food systems (Simopoulos, 2006). These nutrition transitions have resulted in a shift towards a more homogenous diet worldwide, with many traditional foods (e.g. wild yams and cowpea in East Africa) all but disappearing from diets (Raschke & Cheema, 2008), replaced by a higher proportion of processed and ultra-processed snack foods (Kearney, 2010; Khoury et al., 2014; Monteiro et al., 2013). During almost all of the nutrition transitions worldwide (with South Korea being an exception (Kim et al., 2000)) an increase in energy from plant based fats has occurred, resulting in an increase in dietary LA (Popkin, 2001). Furthermore, due to changes in animal feed practices resulting in an increase in grain feeding, animal products now also have an increased LA content than their traditional counterparts (Butler, 2014; Weill et al., 2002). A key follow-on from these nutrition transitions has been an increase in obesity rates and the prevalence of non-communicable diseases such as CVD and T2DM (Popkin et al., 2012).

1.11 Linoleic Acid and the Pathogenesis of Obesity

Due to changes in dietary patterns in developing, African or Eastern countries occurring over a relatively short time span, it is possible to hypothesise that there is a link between the elevated consumption of LA in these populations and an increased risk of developing obesity. Currently

African countries including Botswana, Lesotho, Namibia, Swaziland and South Africa are undergoing nutrition transitions, with the higher socio-economic countries beginning to notice the negative effects associated with dietary change, in the form of increased obesity and non-communicable disease rates (Nnyepi et al., 2015). In southern African countries, the World Health Organisation (WHO) estimates that 40-60 % of adults aged 25-64 are overweight, with urban southern Africans having a higher fat intake than their rural counterparts (due to an increase of all fat classes) (MacIntyre et al., 2002; Steyn & Mchiza, 2014). This increased fat intake is possibly influenced by the increase in vegetable oil consumption due to its lower purchase price, with an intake of 11.5 % of energy from LA being found in a Zambian population subset (Nyirenda et al., 2015). Additionally, Inuit populations of the Canadian Arctic are also currently undergoing a nutrition transition, demonstrated by a decrease in the intake of traditional foods (including marine products) and an increase in dietary and erythrocyte LA levels, with a concomitant decrease in all major n-3 FA (Proust et al., 2014).

1.12 Linoleic Acid Induced Inflammation: Is Adiposity a Factor?

The role of LA in inflammation has been extensively debated, due in part to the majority of dietary guidelines worldwide recommending people replace saturated FA (generally from animal products) with plant oils to decrease CVD risk (National Health and Medical Research Council 2013b). AA, generated from LA or from dietary AA intake is capable of being converted into numerous inflammatory metabolites by cytochrome P450, cyclooxygenase and lipoxygenase pathways (Kuehl & Egan, 1980). The AA metabolite 20-Hydroxy-5,8,11,14-eicosatetraenoic acid (20-HETE) is capable of inducing oxidative stress and is associated with increased adiposity, due to its ability to upregulate adipocyte differentiation (Kim, D. H. et al., 2013). In addition the oxidised LA metabolites 9-hydroxy-octadecadienoic (9-HODE) and 13-hydroxy-octadecadienoic (13-HODE) are able to increase PPARγ (Peroxisome Proliferator-activated Receptor Gamma) expression (Nagy et al., 1998), possibly triggering adipocyte

differentiation (Piscitelli, Fabiana et al., 2011), macrophage activation (Heemskerk et al., 2015) and increased inflammation (Alhouayek & Muccioli, 2014).

Complicating this though, it appears that the ability of dietary LA to increase levels of inflammatory markers is influenced to some degree by the level of adiposity of the individual (Teng et al., 2014). Demonstrating this, after ingestion of a high fat meal (30 % EI) obese subjects had a greater and more prolonged production of reactive oxygen species and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ) than lean individuals (Patel et al., 2007), possibly demonstrating differential modulation of post prandial inflammation in people with obesity. Additionally, obesity is associated with a reduction in the levels of the antioxidant enzyme Heme Oxygenase-1 (HO-1), which stimulates the production of the anti-inflammatory AA metabolite family of epoxyeicosatrienoic acids (Li et al., 2008; Sodhi et al., 2012). Importantly increased HO-1 levels are also associated with increased levels of adiponectin (Abraham et al., 2016). The ability of HO-1 to elevate adiponectin levels is pertinent as adiponectin has a key role in the reduction of lipogenic enzymes and promoting insulin sensitivity (Hu et al., 1996; Maeda et al., 2002)

A meta-analysis completed by Johnson and Fritsche (2012) concluded that LA is not associated with increased inflammation in healthy individuals, though exclusion criteria included obesity (excluding 13 % of the worldwide population (World Health Organization, 2015)) and people who had had a previous cardiovascular event. While Johnson and Fritsche (2012) concluded that LA did not increase inflammation and was still a suitable replacement for SFA in cardioprotective diets, they also stated that there was a lack of long term studies, with many of the studies included in the meta-analysis not accounting for non-steroidal anti-inflammatory use, ALA, EPA or DHA intakes, and at least one study not having matched fat intakes from the control and high LA diet (Johnson & Fritsche, 2012). Directly opposing this is the work of Ramsden and co-authors (2010) whose own meta-analysis found LA intake was correlated with

increased risk of cardiovascular events and all-cause mortality (Ramsden, Christopher et al., 2010) when the presence of obesity and previous cardiovascular events was included. Similarly, another review has found the possibility of increased inflammation resulting from LA, though it seems more likely that the n-6:n-3 ratio of a diet is a more potent regulator than the load of LA (Teng et al., 2014). The differing conclusions of these studies may point towards a maximal amount of LA intake or level of adiposity beyond which chronic inflammation occurs, or a pro-inflammatory macrophage phenotype (Lumeng et al., 2007).

This increase in LA intake is an important factor in health and disease development due to its ability to decrease the incorporation of n-3 PUFA into phospholipid membranes (Blank et al., 2002). In addition LA and ALA compete for the same enzyme (Δ-6 desaturase) for their conversion to AA and DHA and EPA, respectively (Salem et al., 1999) (Figure 1.4 summarises the two conversion pathways). Importantly, high LA levels decrease the conversion of ALA to DHA and EPA (Salem et al., 1999). Though both n-6 and n-3 FA are capable of being converted into inflammatory mediators the n-3 products have lower inflammatory properties and in some instances (e.g. the series 3 prostaglandins and thromboxanes) are anti- inflammatory (Kim et al., 2013).

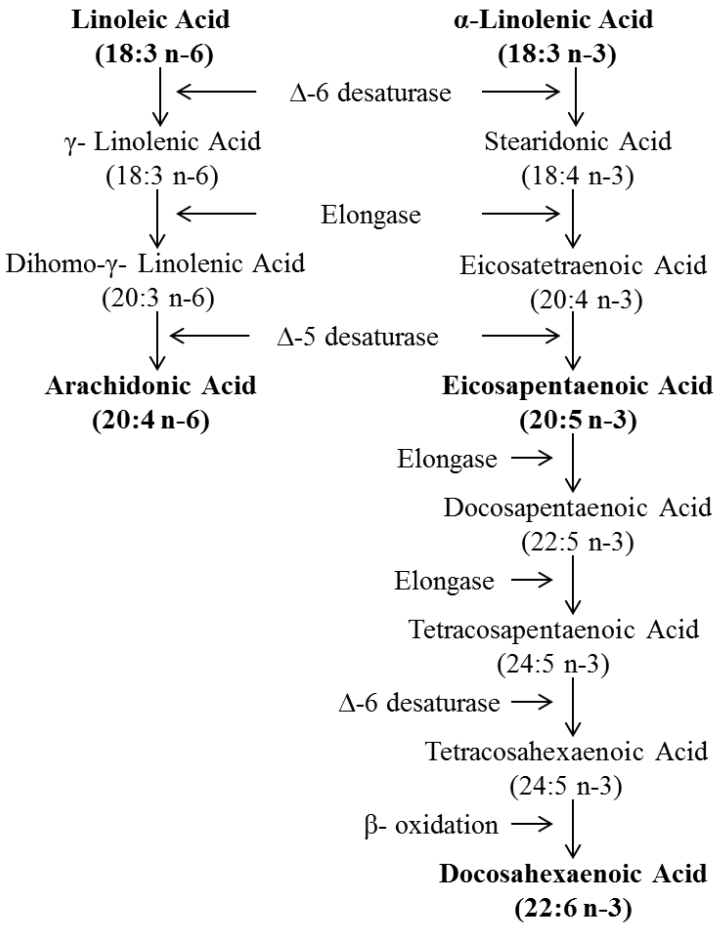


Figure. 1.4: Metabolism of the essential fatty acids linoleic and α-linolenic acid to longer chain fatty acids. This figure shows the common enzymes required for these conversions. Adapted from Salem et al. (1999).

Adding further confusion to the matter, a large study conducted in people over the age of 55 in the Netherlands found that there was an association between n-6 PUFA intake and decreased levels of C-reactive protein (CRP), a marker of systemic inflammation associated with CVD risk (Berg & Scherer, 2005). This prospective study however used dietary intake assessed at baseline for comparison to all subsequent plasma CRP measurements, with the time range of this being between 4 and 10 years (Berg & Scherer, 2005). This presumes that the subjects' dietary intake did not change over this time period, which is unlikely, especially as the composition of foods would change even if the types of foods consumed did not. Additionally, the use of anti-inflammatory or lipid lowering medications was also only assessed at baseline

(Muka et al., 2015), a variable which could reduce the strength of this studies outcomes, especially as there was a significant increase in the use of statins in the Dutch population over the data collection period (Geleedst-De Vooght et al., 2010).

A recent study aimed to address the question of whether the absolute amount of LA or the n-6:n-3 ratio is more important in inducing inflammation. This study tested 9 diets ranging in PUFA % EI from 2.5-10 % with the n-6:n-3 ratio ranging from 1:1 to 20:1 (diets had matched EI from fat with adjustments made by MUFA content) in male C57BL/6J mice and measured the inflammatory response to a non-lethal injection of lipopolysaccharide (Hintze et al., 2016). This study showed that interferon γ and Interleukins 1β, 5, 6 (IL-6) , 10 and 12p70 were increased by the amount of LA, though Interleukins 1β (IL-1β), 17 and 3, as well as macrophage inflammatory protein α, TNF-α, RANTES (regulated on activation, normal T cell expressed and secreted) and monocyte chemoattractant protein-1 (MCP-1) are mainly influenced by the n-6:n-3 ratio of the diet (Hintze et al., 2016). These inflammatory mediators are also involved in the pathogenesis of obesity with IL-6 interfering with rodent adipocyte development, resulting in a pro-inflammatory phenotype (Zeyda & Stulnig, 2007). Additionally, high levels of IL-6 in cultured human adipocytes results in a down regulation of adiponectin expression (Sopasakis et al., 2004). Chronic exposure to IL-1β results in decreased cultured human subcutaneous adipocyte insulin stimulated insulin receptor β subunit phosphorylation and supressed insulin mediated glucose uptake, as well as causing a decrease in adiponectin concentrations (Lagathu et al., 2006). Additionally, TNF-α is capable of inducing adipocyte lipolysis in both rodents and humans (Grant & Stephens, 2015), resulting in an increase in circulating free FA, which can lead to insulin resistance and T2DM development (Bergman & Ader, 2000).This gives rise to the importance of both the reduction of absolute LA and the n-6:n-3 ratio in managing chronic inflammation.

1.13 Linoleic Acid's Obesogenic Properties

There are numerous hypothesised mechanisms through which LA can stimulate weight gain, leading to obesity. The expression of the fatty acid desaturase 1 and 2 genes (FADS1 and FADS2 respectively) code for the Δ-5 and Δ-6 desaturases respectively, which play a key role in the levels of LA and AA available in the body, therefore influencing the production of downstream products which can influence metabolism and inflammation (Zietemann et al., 2010). The expression of different rs174546 single nucleotide polymorphisms in FADS1 appear to modulate the production of these desaturases, resulting in lower LA and higher erythrocyte AA content, showing increased desaturase activity, which has been associated with T2DM and cardiovascular disease mortality (Krachler et al., 2008; Warensjö et al., 2008; Zietemann et al., 2010). A large prospective case controlled study found that dietary LA (and not plasma phospholipid LA) correlated with the risk of T2DM development over a 4 year period (Hodge et al., 2007), possibly providing more evidence for increased Δ-6 desaturase activity (due to polymorphisms of the FADS2 gene) in particular individuals as the association was not significant after adjusting for weight. Supporting this, prospective cohort studies have found Δ-6 desaturase activity a predictor of metabolic syndrome (Steffen et al., 2008; Warensjö et al., 2009) and diabetes (Kröger et al., 2010; Mayneris-Perxachs et al., 2014). Other large scale human studies have found a positive correlation between Δ-6 desaturase activity and obesity (Steffen et al., 2008; Warensjö et al., 2005), and increased CRP (Martinelli et al., 2008). Additionally, there may be gender differences in n-3 and n-6 metabolism, with findings suggesting that females oxidise lower levels of ALA, though men have lower rates of ALA conversion to EPA and DHA, effecting the ability of DHA and EPA to compete for cellular membrane inclusion (Burdge, G., 2004).

Adding further complexity to this area of research, there appear to be numerous genetic factors that influence the associations between different fatty acids and adiposity (Chilton et al., 2014),

with one study finding that an increase in the n-6:n-3 ratio in white South African women resulted in variable increases in adiposity, depending on IL-6 gene polymorphisms −174 G>C, IVS3 +281 G>T and IVS4 +869 A>G) (Joffe et al., 2014). In comparison, the expression of the IVS4 +869 G allele was associated with total fat and adiposity independent of lipid type in native black South African women (Joffe et al., 2014). Therefore, the particular genetic profiles of populations which are currently going through nutrition transitions has the ability to modulate the response to an increase in dietary LA (Chilton et al., 2014), which is also of considerable further interest. It is also possible that the adipogenic properties of LA may be influenced by the macronutrient composition of a diet, with a study finding a high LA intake induced obesity in mice when incorporated into a predominately carbohydrate (sucrose) diet (Madsen et al., 2008). In contrast to these results, when the same %LA EI was combined into an isoenergetic high protein diet, there was insignificant weight gain, with the differences found to be dependent on cyclic adenosine monophosphate (cAMP) activation (Madsen et al., 2008). Oral sensing may also play a role in LA induced adipogenesis, with obese mice having been shown to have a preference for higher concentrations of oral LA, found to occur due to increased lingual expression of FAT/CD36, which has been shown to have a role in fat sensing and tolerance (Chen et al., 2013). Additionally, in this study FAT/CD36 knock-out obese mice consumed less of an LA solution than their wild type controls (Chen et al., 2013). In humans this has been found to result from the rs1761667-AA allele which decreases FAT/CD36 expression, with those who are unable to orally sense LA having a higher fat intake and total EI than those who can (Chevrot et al., 2014).

AA converted from dietary LA is capable of stimulating weight gain and adipogenesis through the prostacyclin pathway, ameliorated via addition of ALA to a high LA diet in mice (Massiera et al., 2003). Through the prostacyclin pathway, AA is capable of stimulating adipogenesis via PPARγ upregulation, either through PPARβ/PPARδ (Peroxisome Proliferator-activated

Receptor Beta/Peroxisome Proliferator-activated Receptor Delta) activation or through the upregulation of both CCAAT/enhancer binding protein β and CCAT/enhancer binding protein δ, with the latter considered to be the most common route (Ailhaud et al., 2006). This ability of AA to stimulate adipogenesis is central to the role of LA in the development and perpetuation of obesity (Gaillard et al., 1989), as adiposity is highly influenced by the number of adipocytes present in an organism (Spalding et al., 2008).

Research using cultured human enterocytes exposed to high levels of LA in combination with TNF-α and IL-6, (both of which are elevated in obesity (Rummel et al., 2016)) resulted in a decrease in the expression of apolipoprotein A-IV, a satiety inducing protein released in response to an increase in high triglyceride chylomicrons postprandially, negating this protein's ability to limit food intake (Li et al., 2015).

This is further supported by a study investigating the effect of AA on preadipocytes, which found that AA increased differentiation through early activation of PPARγ (Massiera et al., 2003). Moreover, this differentiation was impaired by the addition of a COX inhibitor (aspirin) (Massiera et al., 2003). While AA did not have a stronger affinity for PPARγ than other FA, it induced a 15 fold increase in cAMP production and a 3 fold increase in Protein kinase A (PKA) activity (Massiera et al., 2003), a property which is unique among natural FA (Ailhaud et al., 2006). Treatment of these cells with n-3 FA resulted in a decrease in cAMP production, though not to that of control cells (Massiera et al., 2003) (These pathways are summarised in Figure 1.5).

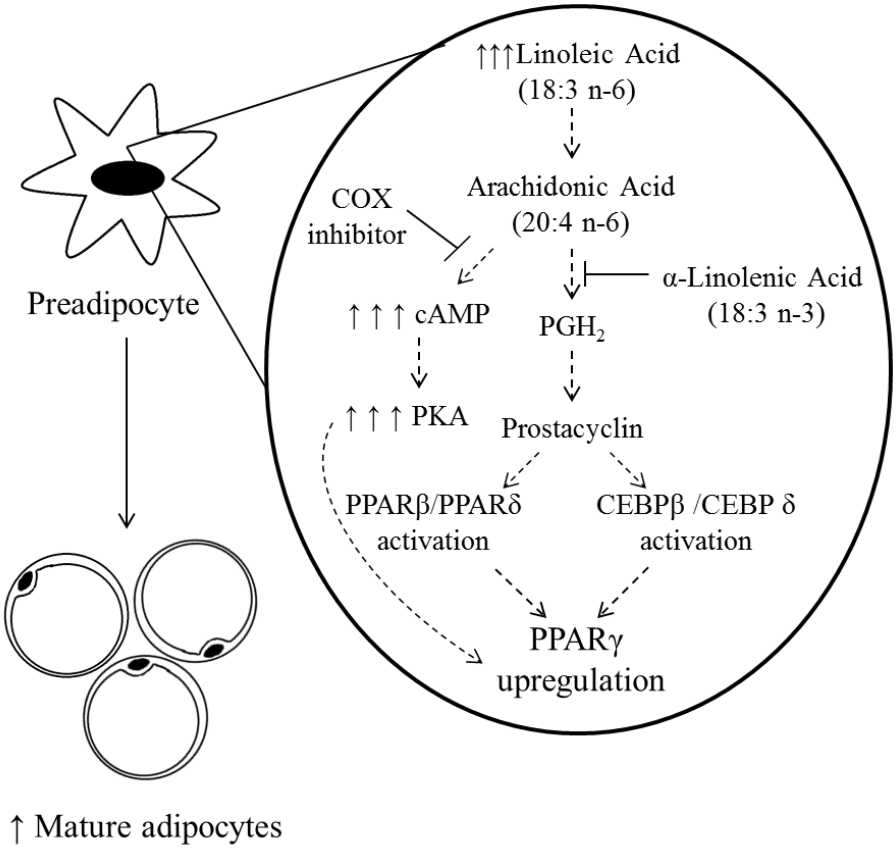


Figure:1.5. The role of linoleic acid in adipocyte differentiation. Excessive cellular arachidonic acid (from dietary linoleic acid intake) is capable of stimulating adipocyte differentiation through several interconnected pathways. Arachidonic acid can be converted to prostacyclin via prostaglandin H2 (PGH2), though this reaction is decreased in the presence of cellular α-linolenic acid. From this point, Prostacyclin is capable of stimulating the peroxisome proliferator-activated receptor (PPAR) family, leading to PPARγ activation, which stimulates adipocyte differentiation. Prostacyclin is also capable of doing this through the CCAAT-enhancer binding protein family (CEBPβ and CEBPδ). Additionally, arachidonic acid is capable of causing PPARγ activation through an increase in cyclic adenosine monophosphate (cAMP) dependent protein kinase A (PKA) activity, which can be disrupted via the addition of a cyclooxygenase (COX) inhibitor. Adapted from Ailhaud et al. (2006); Gaillard et al. (1989); Massiera et al. (2003). Dashed lines indicate simplified pathways shown. Blunt arrows show factors which can decrease arachidonic acid stimulated adipogenesis.

In addition, a study using cultured rat adipocytes found that in the presence of insulin, LA significantly decreased both leptin and adiponectin secretion (Pérez-Matute et al., 2007). Though the adiponectin decreases only occurred at the highest LA concentration, the effect of insulin concentration on this was not investigated, leaving the possibility that lower LA concentrations could elicit the same effect in the presence of elevated insulin. Furthermore, the

decrease in leptin secretion in the presence of LA and insulin may result in a decrease in satiety as this is regulated by leptin through the hypothalamus, leading to excessive energy consumption (Pérez-Matute et al., 2007). In obesity prone C57BL/6J female mice a high LA corn oil diet was associated with reductions in respiratory exchange ratio, spontaneous locomotor activity, whole body glucose uptake and the expression of all of the PPAR subspecies in skeletal muscle (therefore reducing its ability to oxidise FA) in comparison to high OA (from olive oil) fed animals (Wong et al., 2015).

A study using 6-week-old female rats, maintaining LA intake at 2.1 % EI (12 % total energy from fat) for all experimental groups, with an incremental increase in energy from ALA ranging from 0.095 % to 6.3 % (with the energy balance being made up primarily from SFA) investigated the effect of this range of n-6:n-3 ratio on adipocyte gene expression. Over a 3 week feeding period, there was a reduction in the omental expression of fatty acid synthase (FAS), as well as sterol regulatory element-binding protein 1c (SREBP-1c), a major regulator of lipogenesis, which was also correlated with erythrocyte total PUFA content (Muhlhausler et al., 2010). However, this study did not find a difference in the body weights of the animals or the expression of PPARγ (Muhlhausler et al., 2010), which may have been influenced by the relatively short duration of the dietary intervention or the gender of the animals.

1.14 Linoleic Acid and Early Life Obesity Development

The role of maternal diet in early development has been a focus of a large body of recent research due to its ability to impact a child's body composition and metabolism (Moon et al., 2013). In a large prospective study performed in the UK it was found that the mothers plasma n-6 PUFA concentration was a predictor of their child's fat mass at both 4 and 6 years of age (Moon et al., 2013). Enzyme activity has also been linked to the role of LA in inducing obesity during development stages, with a study assessing the plasma phospholipid FA content in obese teenagers with or without metabolic syndrome, finding that obese children with metabolic

syndrome had reduced LA, though increased levels of dihomo-γ-linolenic acid, indicating an increase in Δ-6 desaturase activity (Decsi et al., 2000). This study found no difference in phospholipid AA content between the groups, indicating that obese children with metabolic syndrome may have higher levels of AA metabolites (such as inflammatory mediators) which may be perpetuated in metabolic syndrome. In addition a study investigating obesity in Mexican children found a correlation between obesity and increased plasma Δ-6 desaturase activity (Elizondo-Montemayor et al., 2010), with a study conducted in Korean boys also finding a correlation between obesity and increased plasma Δ-6 desaturase activity, though this was also correlated with insulin resistance, waist circumference and adiposity at follow-up 2 years later (Choi et al., 2014). Moreover a study conducted in Japanese children found that those with abdominal obesity had elevated Δ-6 desaturase activity (Saito et al., 2014). A study performed by the same research group, again in a cohort of Japanese children, found an association between increased Δ-6 desaturase and abdominal obesity and plasma triglycerides (Saito et al., 2013). A study assessing the associations between plasma PUFA and BMI of obese children and aged matched lean controls found that although LA did not correlate with BMI, both dihomo-γ-linolenic acid and AA, did correlate with BMI, leading the authors to speculate that the obese children may have increased Δ-6 desaturase activity (Decsi et al., 1996). Similarly a study assessing the relationship of adipose tissue FA in children in Crete and Cyprus found that increased subcutaneous AA and dihomo-γ-linolenic acid were associated with an increase in BMI, and that all obese children in the study were in the highest quartile of subcutaneous AA levels, with 89 % of the overweight children being in the 3rd or highest quartile (Savva et al., 2004).

The role of excessive LA in the development of obesity and metabolic syndrome in rats and how this is perpetuated over generations shows that subsequent generational exposure to a ‘Western’ style diet with a LA:ALA ratio of 28, results in increasing magnitudes of adiposity

(without a change to EI), inflammatory cytokines, and insulin resistance (Massiera et al., 2010). A similar study performed in wild type B6C3FE mice found a diet with a LA:ALA ratio of 77 resulted in an increase in adiposity, heart hypertrophy, hepatic lipid droplet deposition and a 6-fold upregulation of stearoyl-coenzyme A desaturase 1 (SCD-1) when compared to an isoenergetic diet with a LA:ALA ratio of 9.5 in third generation offspring fed the diets (Hanbauer et al., 2009). It is hypothesised that this generational increase in adiposity is what we are currently experiencing in Western and Westernised populations. A study investigating the impact of differing ALA contents in neonatal guinea pigs using LA to primarily offset energy contribution of ALA found that 3 weeks of low ALA feeding (0.8 % total fat) (high LA feeding) followed by a chow diet for 16 weeks resulted in a 15 % increase in fat mass at 19 weeks of age. In addition these animals had higher adipose tissue proliferation rates than those fed an isoenergetic (with matched fat content) 10 % ALA diet (Pouteau et al., 2010). Though the animals in this study were not fed by their mothers, it is possible to infer the effect of feeding a low ALA/high LA diet in the early postnatal period on subsequent body composition and adipocyte proliferation. A study using C57BL/6J mice tested the ability of high LA diets to promote adiposity in male offspring of dams fed either a high LA diet or a LA and ALA diet. The offspring of the LA fed dams had a greater body weight, fat mass and a 1.9 fold increase in epididymal adipocyte cell size, accompanied by a decrease in adipocyte cell number compared to the LA and ALA fed dam offspring and control animals, demonstrating the ability of a high LA diet to influence adiposity (Massiera et al., 2003). Another study that investigated the ability of a low LA diet (post-weaning) to modify the response to a high fat 'Western' style diet challenge found that results differed between mice and rats. C57BL/6J mice fed a low LA diet had decreased fat accumulation in response to the diet change, when compared to control animals (Oosting et al., 2015). Using the same study design and diets, Wistar rats showed no difference in body composition, though low-LA fed animals had a reduction in retroperitoneal

adipocyte numbers (Oosting et al., 2015). Despite the differing results between species (which may be a result of the strains of the animals used) this study demonstrated that a low LA intake early in life can favourably affect body composition later in life when a higher EI is consumed.

A study performed in Sprague Dawley dams and their offspring showed that the n-6:n-3 ratio can have great effects on offspring development. This study used 3 diets, one of which being an extremely high n-3 diet, almost devoid of LA with an n-6:n-3 ratio of 0.4, a diet with an n-6:n-3 ratio of 9 and an extremely high n-6 diet almost devoid of n-3 with a n-6:n-3 ratio of 216, which were introduced from 10 days prior to delivery (Korotkova et al., 2002). This study found differing body weights across the three diets with the n-6:n-3 ratio of 9 diet animals being heavier, and having higher levels of white adipose tissue (both absolutely and mg/g of body weight) than the other two diets at 3 weeks of age. This is most likely a result of growth restriction of the animals in the n-6:n-3 ratio of 0.4 and n-6:n-3 ratio of 216 groups due to these both containing essential FA levels approaching deficiency. This shows that though it is believed that the n-6:n-3 ratio may be the most important determinant in growth and metabolic adaptations to dietary FA, the presence of essential FA is required for growth and development.

1.15 Linoleic Acid and Obesity: Population Evidence

At a population level, the role of LA in the development of obesity is currently contentious. Due to the plethora of potential lifestyle, dietary and genetic factors which influence obesity development, the singling out of a single nutrient at a population level to have a significant effect on obesity is difficult. Despite this, a prospective study conducted in over 500 American women aged in their 50's found that higher baseline erythrocyte LA levels were correlated with weight gain resulting in overweight or obesity at follow-up (average time of follow-up 10.4 years) (Wang et al., 2015). Though habitual dietary intake was assessed (via food frequency questionnaires) this study removes the variable of relying on self-reported FA intake, as it uses erythrocyte FA content to quantify this, which is an objective measure that reflects habitual

dietary FA intake patterns (Hodson et al., 2008). Similarly, a prospective cohort study performed in Germany found that habitual LA intake correlated with weight gain in women, with a 100 mg increase in LA intake (at the expense of carbohydrate) equating to a 0.42 % increase in body weight over 5 years (Nimptsch et al., 2010). This was found to have occurred mostly in women who were within the healthy weight range (Nimptsch et al., 2010), possibly showing a propagation of weight gain which can lead to obesity and increased Δ-6 desaturase activity.

Moreover, a study assessing the relationship between AA and the presence of metabolic syndrome risk factors in a cohort of more than 480 normal and overweight Costa Rican adults found that adipose tissue AA content was associated with the presence of metabolic syndrome and was also independently associated with abdominal obesity, hypertriglyceridemia, and elevated fasting glucose when adjusted for BMI (Williams et al., 2007). Similarly, a study conduct in a Spanish population found a correlation between the adipose tissue LA content (which also correlated with dietary LA intake), degree of obesity and central adiposity (Garaulet, Marta et al., 2001). Using subcutaneous adipose tissue collected during bariatric surgery in morbidly obese women with T2DM or normal glycaemic control, a correlation has been found between the subcutaneous adipose AA content and expression of the macrophage marker cluster of differentiation 68 (CD68) in the diabetic patients, with increased expression of genes involved in the leukotriene biosynthesis pathway and more crown like structures indicating increased adipocyte death, adipose tissue inflammation and macrophage infiltration (Heemskerk et al., 2015).

1.16 The Effects of Lowering Linoleic Acid Intakes

A high n-6:n-3 ratio and a high LA intake are both thought to influence obesity development, though it has long been considered that at a population level, increasing n-3 FA intake from marine sources (e.g. fish) is unsustainable due to already declining fish populations and

increased demand by populations with an increase in socio-economic status (Jenkins et al., 2009). Though fish can be farmed, other fish are required to feed them. The alternate feeding with plant-based diets results in a reduction in tissue EPA and DHA content and an increase in the n-6:n-3 ratio (Alvheim, Torstensen, et al., 2012). The natural solution to this would be to reduce dietary LA intakes and replace them with monounsaturated fatty acids (MUFA) such as OA (MacIntosh et al., 2013; Wood et al., 2014; Wood et al., 2013). A high OA diet, most commonly defined as being a Mediterranean style diet, with the primary fat source being olive oil, is believed to be cardio-protective and prevent the development of metabolic syndrome and overweight/ obesity (Obesity Australia 2014; Parsons et al., 1999). These effects are believed to be due to the n-9 series of FA (of which OA is the most abundant) not being involved in inflammatory mediator synthesis, as well as promoting a favourable blood lipid composition (Access Economics, 2008). Support for substituting LA intakes with MUFA may be gained from a worldwide study comparing the correlation of female obesity with dietary fat types, with countries that have lower female obesity rates being found to have higher MUFA intakes (Moussavi et al., 2008). In addition, a weight loss diet in 26 overweight individuals, both with low n-6 PUFA contents (4.9 % EI vs. 4.8 % EI), with one being high in n-3 PUFA (3.6 % EI, 725 mg of fish oil) and one being high in MUFA (from high OA sunflower oil) found that both diets resulted in the same amount of weight loss (Kratz et al., 2008). Moreover, both resulted in an increase in plasma adiponectin, though again there wasn't a significant difference between the two groups (Kratz et al., 2008). Interestingly, this study used diets with approximately 2/3rd the amount of energy from n-6 PUFA compared to what is generally found in the American diet (Blasbalg et al., 2011). Another study has found that a 12 week diet with lowered levels of LA (and the equivalent of the average American diet n-3 composition (Hanbauer et al., 2009)) resulted in increased plasma esterified n-3 levels, which was further increased with an increase in dietary n-3 (Taha et al., 2014). Moreover, a 2-week crossover

study in diabetic men has found that a high MUFA OA diet resulted in lower blood glucose and insulin levels than a high LA diet (Madigan et al., 2000). As MUFA are not considered to be involved in the biosynthesis of inflammatory prostanoids, the substitution of dietary LA with MUFA may help to decrease inflammatory states associated with obesity, excessive LA intake, or conversion to AA (Popkin et al., 2013). In addressing CVD risk, a 12 week low-LA diet (2/3 of meals provided) decreased plasma LA levels, which subsequently decreased 4 oxidised LA metabolites (OXLAMs), which are involved in vascular foam cell formation (Ramsden et al., 2012). This change was independent of n-3 intake, with two low-LA diets (both 2.4 % of EI from LA) with differing n-3 contents (1 supplemented with 1500 mg of EPA and DHA) producing the same reduction in plasma LA and OXLAMs, with neither eliciting a change to total plasma phospholipids, triglycerides, cholesterol esters or free FA (Ramsden et al., 2012).

1.17 The Endocannabinoid System

The endocannabinoid system is implicated in both homeostatic and hedonic food intake (Monteleone et al., 2012), with activation of the system resulting in an increase in hunger (Kirkham et al., 2002; Williams & Kirkham, 1999). Specifically, anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), which are derivatives of AA (Devane et al., 1988; Mechoulam et al., 1995; Munro et al., 1993), bind to the main two receptors, cannabinoid receptor 1 (CB_1) and cannabinoid receptor 2 (CB_2), leading to activation of pathways to initiate food intake in the limbic system (Berrendero et al., 1999), hypothalamus (Edwards et al., 2006; Soria-Gomez et al., 2007) and hindbrain (Miller et al., 2004). CB_1 and CB_2 belong to the GPCR class of receptors, generally signalling through $G_{i/o}$ proteins, though chronic low level stimulation triggers a shift to signalling through G_s proteins (Paquette et al., 2007). AEA is a ligand for CB_1 (Devane et al., 1992), with a reduced affinity for CB_2 (Mechoulam et al., 1995), whereas 2-AG binds to both receptors (Hillard et al., 1999; Sugiura et al., 1999; Sugiura et al., 2000).

Though there are structural differences between the glycerol-based and the *N*-acylethanolamide (NAE)-based endocannabinoids, they share common receptor pathways and functions, with all compounds involved in appetite and modulation of metabolism signalling through GPCR or altering GPCR signalling (Oh et al., 2010; Overton et al., 2006; Sugiura et al., 1999).

Endocannabinoids are products of dietary FA and were originally thought to be generated on demand (Alvheim, Malde, et al., 2012; Berger et al., 2001; Petersen et al., 2006), though it is now known that AEA can be stored in intracellular lipid droplets (Oddi et al., 2008). As such, modulation of cannabinoid receptor function can occur via modification of dietary FA intake. Current dietary guidelines recommend a shift away from animal derived fats in favour of plant fats, in an effort to reduce saturated fat intake and cardiovascular disease risk, which has resulted in an increased intake of PUFA, especially that of LA (Blasbalg et al., 2011; Harris & Klurfeld, 2011). LA is easily converted by the human body to AA via γ-linolenic acid and dihomo-γ-linolenic acid, a pathway dependent on the actions of two desaturases and one elongase (Salem et al., 1999). AA can then be converted to AEA via several pathways as shown in Figure 1.6, including the condensation of AA and ethanolamide due to the reverse activity of fatty acid amide hydrolase (FAAH), as reviewed by Sugiura (2008). As FAAH is also the main enzyme responsible for AEA breakdown, its action is also capable of decreasing cannabinoid receptor activation through a reduction in the availability of agonists (Cravatt et al., 2001). Another anabolic pathway involves the biosynthesis of *N*-arachidonoyl phosphatidylethanolamine (NAPE) resulting from the transfer of *sn*-1 position AA from phospholipids to phosphatidylethanolamine by a Ca^{2+} dependent *N*-acyltransferase.

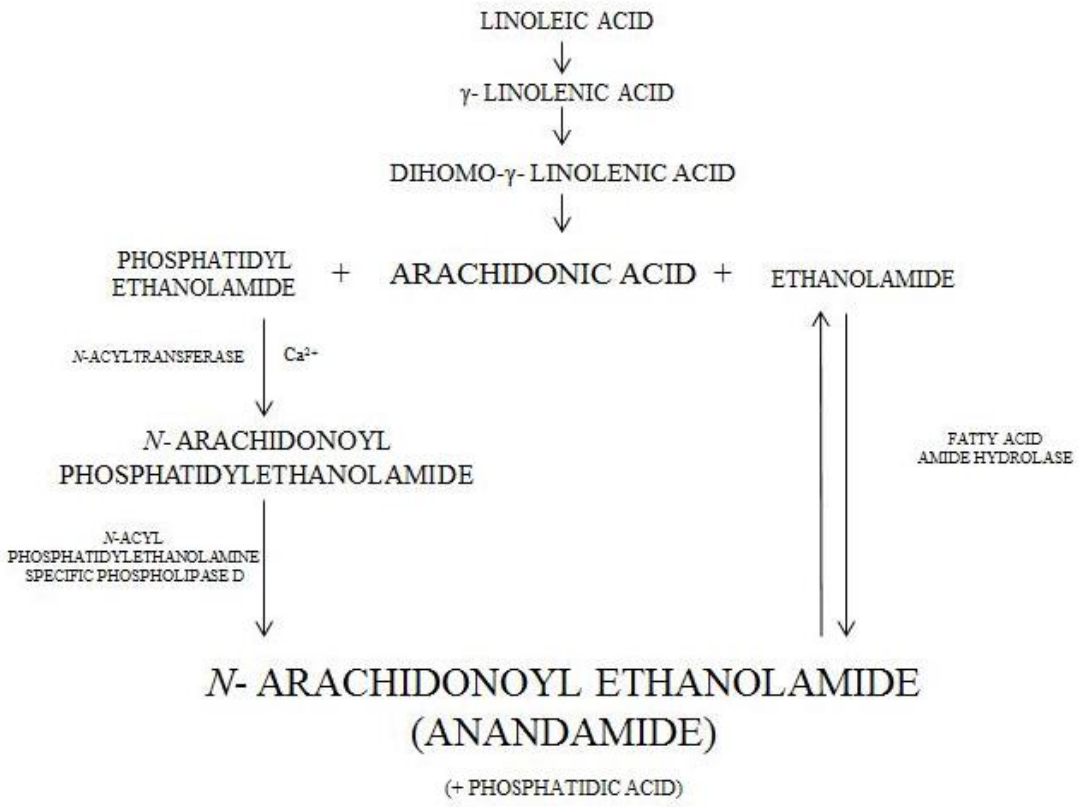


Figure 1.6: Anandamide Synthesis Pathway. Pathways involved in the synthesis of anandamide from dietary linoleic acid and arachidonic acid, via the addition of either phosphatidyl ethanolamide or ethanolamide, the latter also resulting in phosphatidic acid production. Adapted from the works of Salem et al. (1999), Sugiura (2008), Cravatt et al. (2001), Cadas et al. (1997) and Okamoto et al. (2004).

NAPE can then be converted to AEA and phosphatidic acid by *N*-acyl phosphatidylethanolamine specific phospholipase D (NAPE-PLD) and is believed to be the major source of AEA (Cadas et al., 1997; Okamoto et al., 2004). Similarly, there are several pathways through which 2-AG can be synthesised, as shown in Figure 1.7. One of these pathways involves the conversion of diacylglycerol to 2-AG via diacylglycerol lipase, with diacylglycerol being produced from phosphatidylinositol, phosphatidylcholine or phosphatidic acid, the latter two being synthesised by phospholipase C and phosphatases respectively (Kondo et al., 1998; Venance et al., 1997). Phospholipase C is also capable of converting phosphatidylinositol bisphosphate to diacylglycerol and lysophosphatidylinositol to 2-AG,

though this requires a specific phospholipase C isoform (Tsutsumi et al., 1994). As phospholipase C (which is a key enzyme in 2-AG synthesis) is part of downstream GPCR signalling, producing diacylglycerol, it has been found that other GPCRs, including the Angiotensin AT_1 receptor are capable of paracrine transactivation of CB_1 (Turu et al., 2007; Turu et al., 2009), which may indicate that 2-AG synthesis can be influenced by activation of co-expressed GPCRs.

With dietary fats being the only source of FA required for synthesis of endocannabinoids it is possible that what is being consumed is capable of modulating circulating endocannabinoid levels, thus influencing GPCR signalling in an acute timeframe and affecting appetite and subsequent food intake. Also, specific FA, such as AA, are favourably incorporated into phospholipids as opposed to triglycerides (Calder et al., 1994), further affecting their fate in regard to endocannabinoid synthesis due to their cellular location, however the role of storage in the acute effects of dietary fats and later endocannabinoid synthesis requires further investigation.

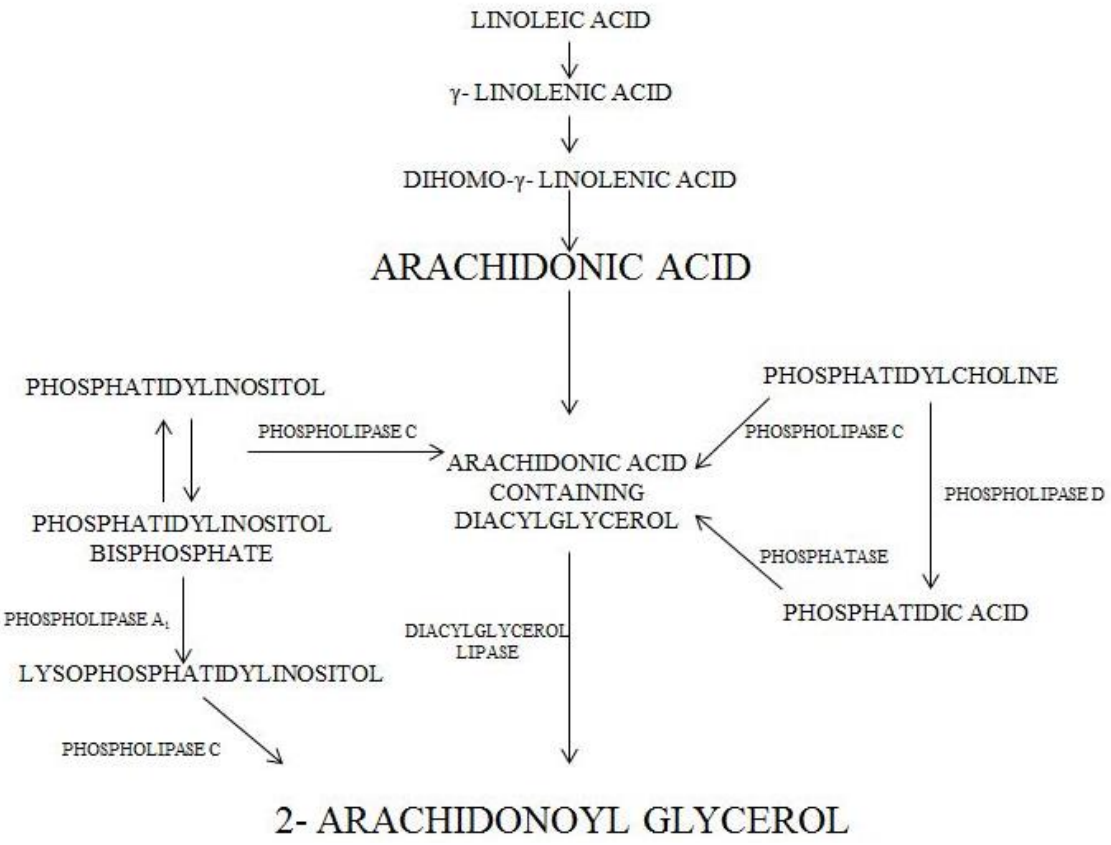


Figure 1.7: 2-Arachidonoyl Glycerol Synthesis Pathway. Pathways involved in the synthesis of 2-Arachidonoyl glycerol from dietary linoleic acid and arachidonic acid, as well as from phosphatidylinositol, phosphatidylinositol bisphosphate, phosphatidylcholine and phosphatidic acid. Adapted from the works of Salem et al. (1999), Venance et al. (1997), Kondo et al. (1998), Tsutsumi et al. (1994).

1.18 Overweight, Obesity and the Endocannabinoid System

Clear associations between body weight and modulation of the endocannabinoid system have been found. The most common of these is that circulating 2-AG levels are significantly increased in obese individuals compared to lean controls (Cote et al., 2007). Furthermore, there are positive correlations between 2-AG and BMI, waist circumference and intra-abdominal adiposity (Cable et al., 2011; Cote et al., 2007). This may be due to the activity of monoacylglycerol lipase (MGL), which primarily degrades 2-AG, not increasing with BMI (Cable et al., 2011; Cote et al., 2007), though expression of FAAH, which is also capable of breaking down 2-AG (Zhang et al., 2009), does increase with BMI (Murdolo et al., 2007). This

increase in 2-AG may also be a result of increased diacylglycerol lipase in obesity, which has been demonstrated in both animal (D'Eon et al., 2008; Massa et al., 2010) and human adipocytes (Karvela et al., 2010; Pagano et al., 2007), though this may be site specific (Pagano et al., 2007) and influenced by dietary composition (Rivera et al., 2013). An increase in FAAH has been found to result in decreased subcutaneous adipose tissue 2-AG levels in obese subjects when compared to lean controls (Bennetzen et al., 2011), with 2-AG also being positively correlated with visceral CB_1 gene expression (Bluher et al., 2006). Body weight has also been found to influence cannabinoid receptor expression, with significant correlations found between CB_1 expression and BMI (Sarzani et al., 2009), percentage body fat (Bluher et al., 2006) and the presence of the metabolic syndrome (independent of BMI) (Sarzani et al., 2009). A correlation in obese individuals has also been found between circulating insulin and increased visceral adipose tissue CB_1 expression, compounded by the presence of the metabolic syndrome, perpetuating visceral lipogenesis due to the role of CB_1 in promoting energy storage in adipose tissue (Sarzani et al., 2009).

Overweight and obese individuals often have a dysregulation of the endocannabinoid system in peripheral tissues, affecting glucose and lipid metabolism (Izzo et al., 2009; Pagano et al., 2007; You et al., 2011). Demonstrating this, a study using paired adipose tissue samples found greater CB_1 mRNA expression in visceral adipose tissue than subcutaneous, with a negative correlation between visceral fat mass and FAAH mRNA expression (Bluher et al., 2006). Similarly, other studies have found that genes involved in 2-AG, CB_1, and MGL synthesis are downregulated in gluteal and up regulated in abdominal adipose tissue of obese individuals (Pagano et al., 2007; You et al., 2011). As activation of CB_1 results in increased glucose uptake (Di Marzo et al., 2009) this may indicate preferential energy storage in abdominal adipose tissue. Supporting this, glucose uptake in adipocytes is increased by treatment with 2-AG (Motaghedi & McGraw, 2008) or AEA (which increased glucose uptake 2-fold) (Gasperi et

al., 2007) with insulin resistant adipocytes from obese mice showing increased expression of endocannabinoid synthesising enzymes and decreased degrading enzymes (D'Eon et al., 2008). Moreover, CB_1 expression is increased in adipocytes during differentiation, as is PPARγ expression which promotes lipid uptake and adipogenesis (Pagano et al., 2007; Tontonoz et al., 1994), both of which are perpetuated by hyperglycaemic conditions (Matias et al., 2006). Also, agonism of CB_1 with either WIN 55212–2 (Perwitz et al., 2006) or HU-210 (Matias et al., 2006) in cultured adipocytes increases PPARγ expression, lipid droplet formation and adipocyte differentiation (Matias et al., 2006; Perwitz et al., 2006). PPARγ activity increases adipocyte differentiation, though during differentiation, CB_1 expression and activity is increased, which further increases PPARγ expression. Thus, chronic stimulation of CB_1 may lead to a cycle of increased adipocyte differentiation and thus further CB_1 expression. In contrast, in normal weight wild-type mice, agonism of CB_1 with HU-210 significantly reduces glucose uptake from skeletal muscle fibres (due to decreased serine/threonine-specific protein kinase (Akt) phosphorylation) curtailing whole body uptake (Song et al., 2011). Similarly CB_1 agonism with arachidonoyl-2-chloroethylamide in lean rat muscle significantly reduces both basal and insulin stimulated glucose uptake (Lindborg et al., 2010).

1.19 Acute Modulation of the Endocannabinoid System

Research on acute modulation of the endocannabinoid system by dietary intake in humans is extremely limited, and has generally focused on macronutrient ratios rather than specific FA intakes. Both Gatta-Cherifi et al. (2011) and Matias et al. (2006) have assessed the effect of food intake on acute concentrations of endocannabinoids (Gatta-Cherifi et al., 2011; Matias et al., 2006). Gatta-Cherifi et al. (2011) compared non-diabetic insulin resistant obese subjects to healthy normal weight participants (though not age- or gender-matched (Gatta-Cherifi et al., 2011)). Whereas Matias et al. (2006) compared healthy participants (though the average BMI was 28.6 ± 1.9 kg/m^2, classifying them as overweight according to the World Health

Organisation (de Onis & Habicht, 1996)), to obese diabetic hyperglycaemic subjects. These studies tested different meal compositions with Gatta-Cherifi et al. (2011) using a meal comprising 35 % of energy from lipids, 45 % carbohydrate and 20 % protein (Gatta-Cherifi et al., 2011), while Matias et al. (2006) utilised a high fat meal (44.15 % of energy from lipids, 39.25 % carbohydrate and 16.6 % protein (Matias et al., 2006)). Both studies showed obese subjects to have increased fasting plasma AEA and 2-AG concentrations, indicating potential chronic cannabinoid receptor overstimulation (Gatta-Cherifi et al., 2011; Matias et al., 2006), with Gatta-Cherifi et al. (2011) finding positive correlations between AEA/2-AG levels and both BMI and waist circumference (Gatta-Cherifi et al., 2011). A positive correlation was also found between AEA and insulin levels in the obese group (Gatta-Cherifi et al., 2011), demonstrating CB_1 overactivity in insulin resistant individuals. This study also found that in the hour after meal consumption AEA levels decreased only in lean subjects, indicating greater orexigenic stimulus in the obese individuals (Gatta-Cherifi et al., 2011), possibly leading to short term hedonic food intake and therefore excess energy intake. Meal consumption by normoglycaemic participants in the Matias et al. (2006) study resulted in transient hyperglycaemia, triggering significant insulin level increases, and a concomitant reduction in AEA levels (Matias et al., 2006) with results from the same study (assessing saliva as opposed to plasma) finding significant reductions in OEA (Matias et al., 2012).

A study by Montelone et al. (2012) investigated the acute (2 hour) influence of hedonic eating in healthy weight satiated individuals with two different meals, one which subjects found extremely palatable and one with the same energy density and nutrient composition which was not considered palatable (Monteleone et al., 2012). One major strength of this study was that participants consumed as much of the palatable food as they wanted in a 10-minute period, and were then given the same volume of the non-palatable meal to eat in the same time frame during a second session, removing the variables of time taken to eat and amounts of ingested nutrients.

In the 120 minutes post consumption, there were no significant differences between the two meals in appetite or satiety scores. Both meals triggered significant AEA and OEA decreases, though the palatable meal resulted in significantly increased plasma 2-AG 2 hours postprandially, accompanied by a significant rise in ghrelin (Monteleone et al., 2012). Supporting this finding, a study assessing 2-AG changes in mice in response to a palatable high fat diet found that levels were increased when compared to control fed animals, which further induced a preference for the high fat diet (Higuchi et al., 2012). This may demonstrate the cyclic nature between hedonic eating, or the intake of pleasurable foods, and increases in 2-AG and orexigenic cannabinoid receptor stimulation.

One study investigating the effect of ethanol on endocannabinoid levels involved the consumption of a test meal (21 % of energy from lipids, 62.9 % carbohydrates and 16.1 % protein) in a group of 19 lean premenopausal women (Joosten et al., 2010). This is the only research thus far, to the author's knowledge, to demonstrate a correlation between serum FA and their respective endocannabinoids (2-AG was not measured in this study) (Joosten et al., 2010), though this study was performed in a non-fasting cohort. This study found the strongest correlation between OEA and its precursor, OA, though a correlation was also found between AA and AEA (Joosten et al., 2010). Furthermore a correlation was found between circulating AEA levels and serum total free FA and BMI over the three hour monitoring period, and no effect of ethanol consumption (Joosten et al., 2010), though unfortunately relationships between consumed FA, serum FA and circulating endocannabinoids were not investigated. With the subjects in this study being lean and having normal blood lipid profiles this demonstrates that without the modulation of the endocannabinoid system by obesity, a high fat meal may still be capable of increasing acute circulating AEA and therefore CB_1 stimulation, possibly perpetuating further food intake, preferential adipose tissue energy storage and adipogenesis.

1.20 Influence of High Fat Diets on Endocannabinoid Synthesis

Worldwide, high fat diets (~40 % of energy) are increasing in prevalence due to the low cost of fats and also due to their palatability (Drewnowski & Popkin, 1997). High fat diets are capable of modulating levels of endocannabinoids regardless of their FA composition (Di Marzo et al., 2008; Diep et al., 2011; DiPatrizio et al., 2011). In animals, high fat diets trigger binge eating patterns (Higuchi et al., 2012) and result in significantly increased intestinal motility (Izzo et al., 2009), and AEA and 2-AG levels (Osei-Hyiaman et al., 2005; Piscitelli et al., 2011) possibly increasing cannabinoid receptor stimulation. High fat diets also result in increased FA synthesis which is in part due to chronic CB_1 activation increasing expression of the lipogenic transcription factor SREBP-1c, triggering greater production of acetyl coenzyme-A carboxylase-1 and fatty acid synthase (Osei-Hyiaman et al., 2005). Increased levels of AEA and 2-AG in response to high fat diets in animals have been found to occur due to decreased MGL and FAAH activity and increased NAPE-PLD action (Aviello et al., 2008), which occurs irrespective of ingestion, as demonstrated by sham feeding studies (DiPatrizio et al., 2011; Dipatrizio et al., 2013). Compounding this, a high fat diet when part of both hypercaloric and isocaloric diets, has been found to decrease OEA levels independent of NAPE-PLD activity, further promoting food intake (Diep et al., 2011).

1.21 The Effect of Dietary Saturated Fat Intake on Endocannabinoid Production

Research into the effect of saturated fats on the endocannabinoid system is extremely limited with the exception of palmitic and stearic acids. One study however, using a pharmacological dose of stearoylethanolamide, has demonstrated a reduction in food intake in starved mice when administered intravenously (Terrazzino et al., 2004). The levels of the palmitic acid based palmitoylethanolamide (PEA) are however not believed to be affected by starvation/refeeding or greatly affected by the intake of any specific nutrients (Fu et al., 2007). Although, levels of PEA have been found to be reduced in rat brain, liver and small intestine when EPA and DHA are administered orally in pharmacological quantities (Artmann et al., 2008). To the authors'

knowledge only one study has found PEA to modulate appetite (Gómez et al., 2002), though it has been demonstrated to be capable of activating PPARα (LoVerme et al., 2006) and is also able to bind to G protein-coupled receptor 55 (GPR55) (Ryberg et al., 2007), however further research is required to confirm these observations.

One study has investigated lauroylethanolamide, from the precursor lauric acid, finding it capable of stopping AEA synthesis in cultured rat basophilic leukaemia and glioma cells (Jonsson et al., 2001). This is supported by human studies which have found that intraduodenal infusion of lauric acid decreases appetite and energy intake (Feltrin et al., 2004; Little et al., 2005) with it having a greater effect on appetite and subsequent energy intake than an OA infusion of the same load (Feltrin et al., 2008), although these studies did not investigate the involvement of the endocannabinoid system.

1.22 The Effect of Dietary Oleic Acid on Endocannabinoid Production

The main MUFA to be investigated in relation to endocannabinoid synthesis has been OA, the primary FA in olive oil (Diep et al., 2011). This is due to OA being the precursor for OEA, with synthesis being dependent on FAT/CD36 (Schwartz et al., 2008). OEA has been found to reduce levels of ghrelin and neuropeptide YY (Serrano et al., 2011) and subsequently food intake (Nielsen et al., 2004) in starved rats when administered intravenously (Gaetani et al., 2003; Soria-Gómez et al., 2010). Oral administration as part of a high fat diet in mice results in increased FAAH and adiponectin gene expression, resulting in decreased food intake and adipose tissue mass indicative of a reduction in CB_1 agonism (Petersen et al., 2006; Thabuis et al., 2010). Oral administration also decreases hepatocyte lipid content, serum triglycerides and cholesterol (Fu et al., 2005), gastric emptying and intestinal motility (Aviello et al., 2008). Furthermore, OEA increases satiety through activation of PPARα (Cluny et al., 2009), as well as increasing PPARα regulated gene expression, including that of PPARα, fatty acid translocase, fatty acid transport protein 1 (Fu et al., 2003) liver fatty-acid binding protein, and

uncoupling protein-2 (Fu et al., 2005). This therefore increases β-oxidation capacity and decreases circulating FA (Dreyer et al., 1992; Motojima et al., 1998) which may be precursors for endocannabinoid synthesis or contribute to decreased glucose uptake as a result of lipotoxicity (Schoonjans et al., 1996). OEA's hyperphagic actions are mediated by G protein-coupled receptor 119 (GPR119), resulting in an increase in cyclic adenosine monophosphate and adenylate cyclase, which is believed to occur through $G_{\alpha s}$ coupling (Overton et al., 2006). Furthermore, activation of PPARα by OEA is believed to reduce inducible nitric oxide synthase (iNOS) gene expression, triggering a decrease in nitric oxide, which reduces vagal afferent stimulation and therefore appetite (Overton et al., 2006). Both OA and oleamide have been found to have similar actions in cultured microglial cells, through inhibition of lipopolysaccharide (LPS) induced iNOS activation, decreasing nitric oxide production as well as phosphorylation of Akt and p38 MAPK (Oh et al., 2010; Oh et al., 2009), which are both also GPCR signalling cascade components.

It has also been found that OEA increases FA release from adipocytes in a dose dependent manner, and skeletal muscle FA uptake and oxidation without affecting glucose utilisation (Guzmán et al., 2004; Yang et al., 2007). Furthermore, OEA can reduce adipose tissue glucose uptake, mediated through the MAPK p38 and c-Jun N-terminal kinase (JNK) pathways (González-Yanes et al., 2005), which inhibits the actions of AEA and 2-AG in adipose tissue, and AEA-induced hyperphagia when both are administered intravenously (Gomez et al., 2002). This may explain the finding of an inverse correlation between adipose tissue MUFA content and degree of obesity (based on BMI and percentage body fat) and central adipose tissue distribution (Garaulet et al., 2001).

1.23 The Effect of Dietary Eicosapentaenoic and Docosahexaenoic Acid on Endocannabinoid Production

The role of dietary EPA and DHA in modulation of endocannabinoid synthesis has been extensively researched due to their ability to displace AA from phospholipid membranes and

reduce its synthesis (Hutchins et al., 2011; Song et al., 2000; Wood et al., 2010), resulting in greater production of eicosapentaenolyethanolamide (EPEA) and docosahexaenoylethanolamide (DHEA) (from the precursors EPA and DHA) (Wood et al., 2010). While EPEA and DHEA do not appear to directly affect appetite, they have been demonstrated to decrease mouse adipocyte IL-6 and MCP-1 production, indicating anti-inflammatory properties (Balvers et al., 2010).

Treatment of cultured mouse adipocytes with EPA/DHA in combination with different free FA found that DHA was able to counteract the conversion of AA to AEA, and importantly was also able to stop the transfer of AA to the sn-1 position of phospholipids, from which AA can be converted to AEA (Matias, Carta, et al., 2008). Supplementation studies in both humans and animals have found that EPA/DHA decrease 2-AG (Banni et al., 2011; Batetta et al., 2009) and AEA (Balvers et al., 2010; Batetta et al., 2009; Wood et al., 2010) levels in obese subjects with a reduction in plasma n-3:n-6 (Balvers et al., 2010; Banni et al., 2011) and a decrease in NAPE-PLD, FAAH and CB_2 mRNA expression (Hutchins et al., 2011), contributing to decreased receptor stimulation. Animal and human studies have also found that DHA/EPA supplementation results in a decrease in brain 2-AG levels (Di Marzo et al., 2010), body mass (Thorsdottir et al., 2007) and prevents the development of obesity (Ruzickova et al., 2004) and further weight gain in mouse models (Rossmeisl et al., 2012). This may be due to an increase in β-oxidation (Froyland et al., 1997) and a decrease in SREBP-1c (Yoshikawa et al., 2002), as well as the reduction in AEA and 2-AG production decreasing cannabinoid receptor stimulation and therefore appetite and food intake. Also, possibly contributing to this is DHA/EPA increasing whole body insulin sensitivity by inhibiting LPS induced phosphorylation of JNK and NF-κβ degradation, and increasing Akt phosphorylation and GLUT4 translocation, via a GPR120 dependent pathway (Oh et al., 2010).

1.24 The Effect of Dietary Linoleic Acid on Endocannabinoid Production

LA has been found to modulate endocannabinoid synthesis due to its ability to be converted to AA by the human body (Salem et al., 1999), although the effect of dietary LA on human endocannabinoid synthesis has not been investigated. This is a pertinent area of research due to the rapid increase in LA content in the Western diet as a result of a shift to plant-derived fats and the greater use of soy and corn oils in food production and manufacturing (Blasbalg et al., 2011; Harris & Klurfeld, 2011). These dietary changes have resulted in a shift in the n-6:n-3, as reviewed Simopoulos (2006), with more than 84 % of PUFA fats consumed in the USA being in the form of the AEA precursor LA (Kris-Etherton et al., 2000). High LA diets promote obesity in both animals and humans (Massiera et al., 2010; Massiera et al., 2003) and are correlated with increased fasting blood glucose, fasting insulin (Madigan et al., 2000) and insulin resistance (Simopoulos, 1994) in humans, making this an important area of further research.

A study by Alvheim et al. (2012) replicated the Western diet LA increase in mouse feed, showing that increasing energy from LA from 1 % to 8 % in a diet with 60 % of energy from lipids caused an increase in AA in the liver and red blood cells. This resulted in a subsequent 3-fold increase in both 2-AG and AEA and increased food intake, plasma leptin and adiposity, possibly as a result of increased cannabinoid receptor activation, however receptor expression and activation were not investigated in this study (Alvheim, Malde, et al., 2012). These changes were abolished with the addition of 1 % n-3 PUFA's to the 8 % diet (resulting in levels comparable to that of the 1% LA diet), again demonstrating the ability of n-3 PUFA's to displace AA and decrease endocannabinoid production (Alvheim, Malde, et al., 2012). A Further study by the same researchers found that substituting fish oil with soy oil in salmon feed increased LA, AA, AEA and 2-AG and decreased DHA and EPA in the salmon flesh and increased fat accumulation in the liver (Alvheim, Torstensen, et al., 2012). These fish were

then fed to mice which resulted in an increased liver content of LA, AA, AEA and 2-AG and decreased DHA and EPA, accompanied by weight gain and adipose tissue inflammation when compared to control fed animals (Alvheim, Torstensen, et al., 2012). This effectively demonstrated how changes in the LA content of animals produced for consumption can negatively affect the end consumer. Similarly, a study by Matias et al. (2008) using mice fed high MUFA and high PUFA diets for a 14-week period found that the high linoleic PUFA diet increased muscle 2-AG levels and induced obesity and hyperglycaemia (with significantly greater blood glucose concentrations than the MUFA diet) (Matias, Petrosino, et al., 2008) indicating endocannabinoid system overactivity. Recently DiPatrizio et al. (2013) found that 30 minutes of oral exposure (through sham feeding) to LA resulted in an increase in both 2-AG and AEA in rat jejunums, which also triggered the rats to develop a preference towards fats with a high LA content, which did not occur when the animals were pre-treated with the CB_1 agonists AM6546 and URB447 (Dipatrizio et al., 2013).

1.25 Conclusion

The role of LA in the development of weight gain leading to obesity is still contentious, though as shown here, there are numerous interacting systems and mechanisms which may promote EI, inflammation and metabolic dysregulation. It is possible that the opposing conclusions of the ability of dietary LA to induce inflammation may be dependent on excessive adiposity to begin with, beyond which dietary LA tips the scale in the favour of a pro-inflammatory pathway, leading to the development of metabolic disturbances. There is also the possibility that genetics or adiposity lead to an increase in Δ-6 desaturase activity, resulting in decreased erythrocyte and adipose tissue levels of LA, and an increase in AA which can then be converted into the pro-adipogenic endocannabinoids or to inflammatory mediators. Although the role of LA in obesity development is not absolute, the data that will come from countries undergoing nutrition transitions currently, especially the rates of obesity related diseases, will help us to

further gauge the negative effects of increasing dietary LA to a level far above what is currently considered to be essential for healthy development. In the meantime, it may be prudent to promote within countries consuming a Westernised diet, and particularly within countries undergoing a nutrition transition towards this dietary style, a more Mediterranean style diet, replacing high LA fats with higher MUFA alternatives to lower the n-6:n-3 ratio. It may also be beneficial to promote health and farming policies that make the availability of this Westernised dietary pattern less prevalent than a more Mediterranean style diet with a higher MUFA intake to displace dietary LA and lower the n-6:n-3.

1.26 Aims

The studies contained within this thesis each have a distinct aim:

Chapter 3: To investigate the changes in diet composition and FA content of Australian diets at a population level for the period of 1961-2009 and identify specific food commodities and food types which have contributed to this.

Chapters 4 and 5: Chapter 4 aimed to determine the influence of differing dietary FA compositions on body weight and composition, glucose handling and adipose tissue gene expression in a rodent model of diet induced obesity. Chapter 5 continues on from this by aiming to determine the effect of the differing dietary fats in fibre type specific skeletal muscle depots on metabolic gene regulation and how this is affected by globular adiponectin.

Chapter 6: This study aimed to investigate acute changes to appetite parameters and appetite and metabolism related markers in the postprandial period after ingestion of specific FA containing meals in overweight and obese individuals.

1.27 Hypothesis

Chapter 3: It is hypothesised that at a population level, the change in total energy availability from fat, LA and LA from plant derived sources will increase over the time period 1961-2009, as has been found in the United States (Blasbalg et al., 2011), the United Kingdom, Ireland and France (Schmidhuber, 2007) over a similar timeframe.

Chapter 4: It is hypothesised that after 6 weeks on the specific high fat diets there will be a reduction in body weight, percentage fat mass and weight of fat pads for the high OA consuming animals when compared to the other high fat fed animals, with the high LA fed animals having a higher body fat percentage (Ikemoto et al., 1996; Javadi et al., 2004). It is hypothesised that the animals fed the high OA diet will have improvements in glucose handling compared to both the 'Western' diet consuming animals and the high LA diet consuming animals, who are hypothesised to have further impairment of glucose handling (Masi et al., 2012). The animals who change from the 'Western' diet to a chow diet at the beginning of the experimental period are expected to have a reduction in percentage body fat and fat pad mass to be comparable with the chow consuming animals and improvement in glucose handling from week 9 measurements. In relation to adipose tissue gene expression it is hypothesised that the high LA consuming animals will have higher white adipose tissue PPARγ mRNA expression compared to the high OA consuming animals (Ailhaud et al., 2006).

Chapter 5: In the skeletal muscle from the animals discussed in Chapter 4 it is hypothesised that the high OA fed animals will have increased expression of FAT/CD36 in both depots compared to the other high fat fed animals. Additionally, it is hypothesised that in the chow, 'Western' diet then chow and high OA consuming animals that adiponectin signalling will be preserved.

Chapter 6: Following consumption of the test meals in this study it is hypothesised that consumption of the high OA meal will result in increased satiety levels postprandially

compared to the high LA and control meals. It is hypothesised that consumption of the high OA meal will result in lower postprandial insulin levels than the LA meal as found in similar research performed by Shah et al. (2007). As LA is precursor for inflammatory cytokines it is anticipated that consumption of the high LA meal will result in higher levels of these in the postprandial period when compared to the other test meals.

Chapter 2: Methods

2.1 Nutrition Transition Dietary Change determination

This study, comprising Chapter 3 of this thesis, aimed to measure the change in lipid availability, including type and major sources over the time-period 1961-2009, during which the Australian diet, at a population level, underwent a significant shift towards a more homogenous, 'Western' style diet.

2.1.1 Data Sources

Annual food supply data in the form of food balance sheets (FBS) for the time-period 1961-2009 (all available data at time of analysis) for Australia was obtained from the United Nations Food and Agriculture Organisation Statistics division (FAOSTAT) (http://faostat.fao.org/). Analytical methods used were based on those published by Blasbalg et al. (2011) and Carden and Carr (2013). The Australian food balance sheets were compiled utilising Australian Bureau of Statistics (ABS) population figures for each corresponding year to determine per capita intake (including infants). FBS show trends in intake at a population level, and though primarily produced to monitor food availability they are also useful and appropriate data sources for monitoring trends in intake over time (Vandevijvere et al., 2013). Data was expressed as grams per day per capita (g/day/capita), and showed the supply of 75 base foods standardised to raw unprocessed commodities/foods (e.g. 'bananas with skin'). These quantities were adjusted by FAOSTAT to reflect importation and exportation and to account for amounts used for seed; fed to livestock or used for non-food products; losses during storage and transportation; farm waste and post-harvest losses and technical losses occurring during the production of processed foods. FAOSTAT performed standardisation on the commodities, with similar products such as 'chicken' and 'turkey' being horizontally standardised to 'poultry'. Actual food commodities were extrapolated out from FAO primary equivalents for

livestock, fish and crops, with the primary equivalents' g/d divided equally across the actual commodities which are commonly consumed in Australia. Commodities were also converted back to quantities expressing raw state (vertical standardisation) to avoid inconsistencies due to differing production processes greatly altering yield and wastage (http://faostat.fao.org/). Commodities with a broad definition (e.g. vegetables- other) were further broken down using ABS 'Apparent Consumption of Foodstuffs' publications produced 1961-1999 (http://www.abs.gov.au/) to provide an accurate representation of foods commonly consumed by the Australian population. Seafood commodity groups were populated based on FAOSTAT definitions and comprised foods commonly available in Australia, in both fresh and processed forms. Where categories were broken down using additional information all supply was evenly divided between constituent foods. Adjustment to reflect edible quantities was performed using United States Department of Agriculture (USDA) Food Yields data (Matthews et al., 1975), which removed bone, skin, shell and other inedible components from a commodities g/d/capita quantity. Commodities which were not shown to be consumed in measurable quantities at any of the time points analysed were removed from the analysis (e.g. plantains), as were commodities which made negligible contributions to available energy (e.g. tea).

2.1.2 Nutritional analysis

The nutritional composition of each commodity was determined using Xyris FoodWorks Professional 7 (Xyris Software Pty Ltd, Highgate Hill, QLD, Australia), encompassing carbohydrate (CHO), protein (PRO), lipid (LIP), PUFA, LA, AA, PA and OA. Primary databases used were from The Royal Melbourne Institute of Technology, Australian fatty acid database, the NUTTAB 1995 and 2010 databases produced by Food Safety Australia and New Zealand (FSANZ) (© Food Standards Australia New Zealand Organisation) and the AUS FA database. As the data resulting from this analysis is highly dependent on the integrity of the nutrition databases used, primary databases used were Australian to remove variations as a

result of analytical, geographical, nutrient and temperature variations. If complete datasets for a particular commodity were not available, the New Zealand FoodFiles database (© Copyright the Ministry of Health (New Zealand) and the New Zealand Institute for Plant and Food Research Limited, 2014) or the USDA National Nutrient Database SR24 database (U.S. Department of Agriculture, Agricultural Research Service. 2011. USDA National Nutrient Database for Standard Reference, Release 24. Nutrient Data Laboratory Home Page, http://www.ars.usda.gov/ba/bhnrc/ndl) were utilised. Where possible conjugated linoleic acid was separated from LA content due to its differing structure and physiological roles.

Availability of specific nutrients was calculated by multiplying the per 100 g/ml quantity of each commodity by the average annual g/day/capita availability. Following this, per capita yearly intake of measured nutrients was calculated and expressed as g/day/capita. Average energy availability per capita in kJ per day was calculated by summing the energy contribution from available macronutrients to determine the contribution of the focus macronutrients to total available energy (TAE) as a percentage. The contribution of the remaining fatty acids not measured in this study was calculated by summing the average yearly intake of investigated FA and subtracting this value from the total available lipids g/day/capita, with the % contribution to total available energy then calculated and this labelled as 'other FA'. Data is expressed as percentage of total available energy to take into account the differences between disappearance (which FBS show) and actual consumption (Sasaki & Kesteloot, 1992). This has been done as FBS methodology results in inherent overestimation (Kearney, 2010) when compared to other forms of dietary monitoring (Serra-Majem et al., 2003).

2.1.3 Data Analysis

Data analysis and area under the curve (AUC) calculations were performed using GraphPad Prism (version 6.02, GraphPad Software Inc.). Cumulative change over time was calculated using the formula:

$$\% \text{ Cumulative change} = \frac{(\text{Actual AUC} - \text{Baseline AUC})}{\text{Baseline AUC}} \times 100$$

This method was used as it assesses all changes that occurred 1961-2009, allowing peaks and troughs in availability to be taken into account. In this formula, the 1961 value is used to determine a "baseline" AUC, which is a flat line hypothesising that availability was at a constant rate from 1961-2009. The actual AUC was calculated using data obtained from the analysis, allowing all change throughout the time period to be taken into account (Carden & Carr, 2013). This method is summarised in Figure 2.1. These two AUC measurements were then used in the above formula to determine the differences between the two AUC (and as such change from baseline), expressing percentage of cumulative change, with negative values indicating a decrease. Relative change was also calculated using the formula:

$$\% \text{ Relative change} = \frac{(2009 \text{ value} - 1961 \text{ value})}{1961 \text{ value}} \times 100$$

This was performed so comparison between the two methods of assessment of change could be made.

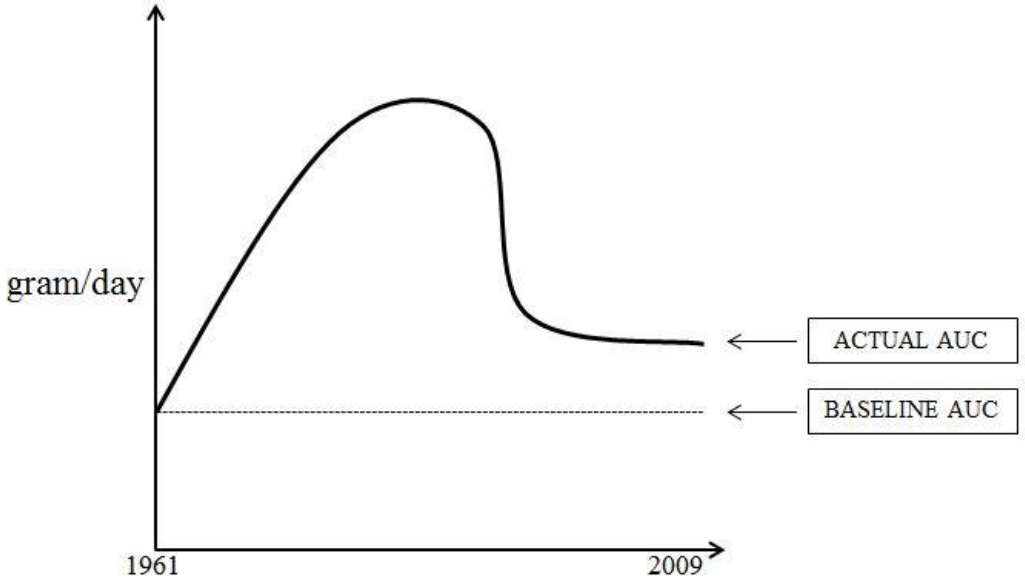


Figure 2.1: Cumulative change representation. This figure is a visual representation of the formula used to calculate the cumulative change of the studied nutrients. Adapted from Carden et al. (2013).

2.2 Experimental Outline of animal study

Animal models of DIO are commonly used due to the ability to sample tissues which are not possible to obtain from humans. Rodent models, including rats, are well accepted models of DIO by the research community world-wide, due in part to their susceptibility to tissue changes as a result of high fat diets and also due to the development of the same co-morbidities found in humans, including heart disease, insulin resistance, systemic inflammation and kidney damage (Abel et al., 2008; Buettner et al., 2007). Sprague Dawley rats were utilised in this study as they are an outbred strain which show either an obese prone or obese resistant phenotype, creating a population which has similar polygenic patterns of response to high fat diets, as seen in humans (Archer et al., 2003).

This study was performed in collaboration with two other PhD candidates; Mrs. Anna Simcocks (shared daily responsibilities) and Ms. Lannie O'Keefe (Euthanasia, anaesthesia, and tissue collection) and comprises of Chapters 4 and 5 of this thesis. The 10 animals per group

was based on a similar study performed within the research group which investigated the influence of pharmacological treatment on skeletal muscle metabolism and glucose handling in DIO Sprague Dawley rats which showed that this number was sufficient for calculation of statistical significance of any differences in the measured parameters.

Ethics approval was granted through the Victoria University Animal Ethics Committee (AEEC 13/005). 60 seven-week-old male Sprague Dawley rats (weight range 200-250 g) were sourced from The Animal Resource Centre (ARC, Perth). Following a seven-day acclimatisation period, the animals were maintained for nine weeks on either a high fat diet (21 % fat, Specialty Feeds, Perth, W.A. SF13-115) to induce obesity or standard chow (4.8 % fat, Meat free Rat & Mouse Feed, Specialty Feeds, Perth, W.A.). The high fat diet used was based off the open source diet D12451, a widely used high fat diet found effective in inducing obesity in rodents, developed in 1996 by Research Diets Incorporated (New Brunswick, New Jersey, U.S.A.) with a slight reduction in digestible energy to meet the suppliers capabilities (Gajda, 2008). Insulin Sensitivity Tests (IST) (beginning of weeks 9 and 14) and Glucose Tolerance Tests (GTT) (end of weeks 9 and 14) were also used to determine effects on whole body glucose homeostasis. Glucose or Insulin were injected peritoneally and blood glucose response was measured from a drop of blood from the end of the animal's tail via the tail snip method.

2.2.1 Experimental diets

Following the nine-week feeding regime, the high fat fed animals were divided into 5 groups of 10 as shown in Table 2.1. The 9 week DIO animals were included to show metabolic and body composition differences between 9-week high fat fed and the 16-week high fat fed animals if required.

Table 2.1: Schedule of Experimental Diets

GROUP	9 WEEKS - PRIOR TO TREATMENT	TREATMENT – 6 WEEKS
CHOW	Standard chow (5 % fat)	Standard chow – lean group (5 % fat)
9 WEEK DIO	High fat 'Western' style diet (21 % fat)	**N/A (**killed at the end of Week 9)
'WESTERN' DIET	High fat 'Western' style diet (21 % fat)	High fat 'Western' style diet (21 % fat)
HIGH OA DIET	High fat 'Western' style diet (21 % fat)	High fat diet predominately oleic acid (OA) (21 % fat)
HIGH LA DIET	High fat 'Western' style diet (21 % fat)	High fat diet predominately linoleic acid (LA) (21 % fat)
'WESTERN' DIET THEN CHOW	High fat 'Western' style diet (21 % fat)	Standard chow (5 % fat)

These diets were chosen to mimic current 'Western' diet patterns consumed in developed countries ('Western' diet), a high PUFA diet comprised predominately of high linoleic plant oils and a 'Mediterranean' style high MUFA diet with the predominant lipid source being high oleic acid olive oil. All diets were considered nutritionally complete. The nutritional composition of the experimental diets, calculated by the manufacturer from previously measured compositions of dietary constituents, is shown in Table 2.2. The standard chow was a fixed formula ration using the following ingredients: wheat, barley, lupins, soya meal, fish meal, mixed vegetable oils, canola oil, salt, calcium carbonate, dicalcium phosphate, magnesium oxide, and a vitamin and trace mineral premix. All diets were from the one manufacturing batch to control for potential seasonal/batch variations of nutritional composition.

The diets were designed to be isoenergetic and have the same proportion of energy from macronutrients, with the exception of the chow diet. Additionally, the diets were designed to have matched energy from the specifically elevated fats, namely OA and LA, as shown in Figure 2.2. The calculated nutritional composition is shown in Tables 2.3 through 2.6. As shown in Table 2.3 the n-6:n-3 of the diets varied, the chow and MUFA diets had the lowest ratios, with the 'Western' diet ratio being slightly below what is currently found in American (Kris-Etherton et al., 2000), with the PUFA diet having 4 fold more LA.

Table 2.2: Composition of experimental diets

	WESTERN	HIGH OA	HIGH LA
Casein (Acid) g/kg	233	233	233
Sucrose g/kg	201	201	201
Linseed (Flax) Oil g/kg	-	4	4.8
Canola Oil g/kg	-	-	2.6
Lard g/kg	210	--	-
Olive Oil g/kg	-	192	-
Safflower Oil (High Linoleic) g/kg	-	-	200
Sunflower Oil g/kg	-	-	2.2
Sunola Oil g/kg	-	14	-
Cellulose g/kg	58	58	58
Wheat Starch g/kg	118	118	118
Dextrinised Starch g/kg	117	117	117
DL Methionine g/kg	3.5	3.5	3.5
Calcium Carbonate g/kg	6.4	6.4	6.4
Sodium Chloride g/kg	2.6	2.6	2.6
AIN93 Trace Minerals g/kg	1.6	1.6	1.6
Potassium Citrate g/kg	19.2	19.2	19.2
Dicalcium Phosphate g/kg	15.1	15.1	15.1
Potassium Sulphate g/kg	1.6	1.6	1.6
Choline Chloride (75%) g/kg	1.3	1.3	1.3
AIN93 Vitamins g/kg	12	12	12

OA; Oleic acid, LA; Linoleic acid

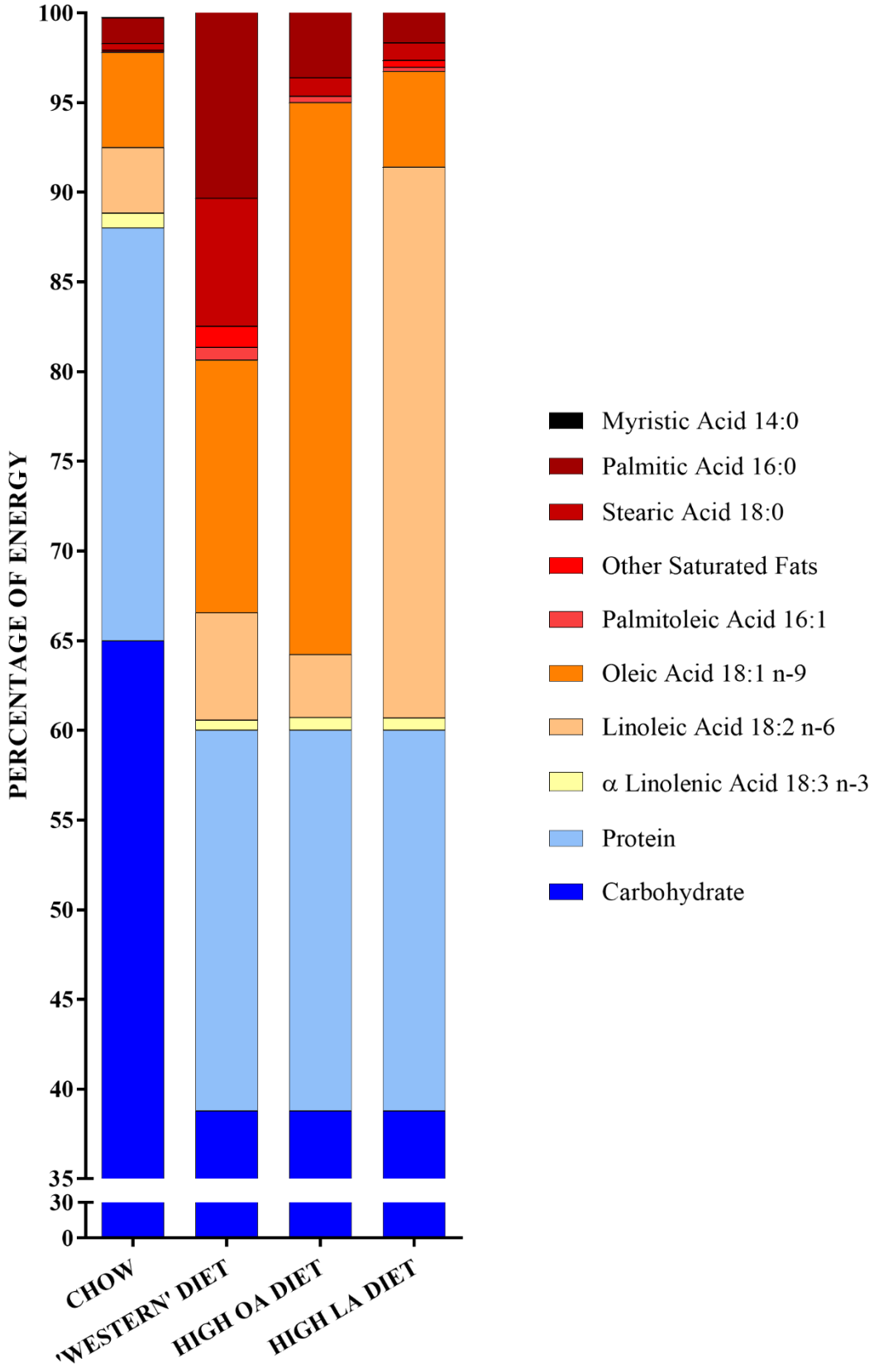


Figure 2.2: Energy ratios of experimental diets. The contribution of carbohydrate, protein and specific fatty acids are shown as a percentage of total digestible energy.

Table 2.3: Calculated Fatty Acid Composition of Experimental diets

	CHOW	WESTERN	HIGH OA	HIGH LA
Saturated Fats ≤ C12:0	-	0.07 %	TRACE	TRACE
Myristic Acid 14:0	0.03 %	0.32 %	TRACE	0.03 %
Palmitic Acid 16:0	0.50 %	5.57 %	2.29 %	1.31 %
Stearic Acid 18:0	0.14 %	3.61 %	0.53 %	0.49 %
Other Saturated Fats	-	0.20 %	TRACE	0.17 %
Palmitoleic Acid 16:1	0.01 %	0.36 %	0.17 %	0.11 %
Oleic Acid 18:1 n-9	1.90 %	7.10 %	15.76 %	2.72 %
Gadoleic Acid 20:1	0.03 %	0.15 %	0.08 %	0.06 %
Linoleic Acid 18:2 n-6	1.30 %	3.02 %	1.80 %	15.65 %
α-Linolenic Acid 18:3 n-3	0.30 %	0.29 %	0.36 %	0.35 %
Arachidonic Acid 20:4 n-6	0.01 %	-	-	-
EPA 20:5 n-3	0.02 %	-	-	-
DHA 22:6 n-3	0.05 %	-	-	-
Total n-3	0.37 %	0.33 %	0.37 %	0.35 %
Total n-6	1.31 %	3.05 %	1.82 %	15.65 %
n-6:n-3	3.54	9.24	4.92	44.7
Total MUFA	2.00 %	7.68 %	16.01 %	2.97 %
Total PUFA	1.77 %	3.48 %	2.18 %	16.00 %
Total SFA	0.74 %	9.76 %	2.82 %	2.00 %
Protein	20.00 %	22.60 %	22.60 %	22.60 %
Total Fat	4.80 %	21.00 %	21.00 %	21.00 %
Crude Fibre	4.80 %	5.40 %	5.40 %	5.40 %
AD Fibre	7.60 %	5.40 %	5.40 %	5.40 %
Digestible Energy mJ/kg	14.0	18.8	18.8	18.8
Total energy from lipids	12.00 %	41.00 %	41.00 %	41.00 %
Total energy from protein	23.00 %	21.20 %	21.20 %	21.20 %
Total digestible energy from carbohydrate	65.00 %	37.8 %	37.8 %	37.8 %

OA; Oleic acid, LA; Linoleic acid, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid.

Table 2.4: Calculated Mineral Composition of experimental diets

	CHOW	WESTERN	HIGH OA	HIGH LA
Calcium	0.80 %	0.71 %	0.71 %	0.71 %
Phosphorous	0.70 %	0.47 %	0.47 %	0.47 %
Magnesium	0.20 %	0.07 %	0.07 %	0.07 %
Sodium	0.18 %	0.14 %	0.14 %	0.14 %
Chloride	-	0.16 %	0.16 %	0.16 %
Potassium	0.82 %	0.83 %	0.83 %	0.83 %
Sulphur	0.20 %	0.25 %	0.25 %	0.25 %
Iron mg/kg	200	65	65	65
Copper mg/kg	23	9.3	9.3	9.3
Iodine mg/kg	0.5	0.23	0.23	0.23
Manganese mg/kg	104	21	21	21
Cobalt mg/kg	0.7	-	-	-
Zinc mg/kg	90	55	55	55
Molybdenum mg/kg	1.2	0.18	0.18	0.18
Selenium mg/kg	0.4	0.4	0.4	0.4
Cadmium mg/kg	0.05	-	-	-
Chromium mg/kg	-	1.2	1.2	1.2
Fluoride mg/kg	-	1.2	1.2	1.2
Lithium mg/kg	-	0.1	0.1	0.1
Boron mg/kg	-	2.3	2.3	2.3
Nickel mg/kg	-	0.6	0.6	0.6
Vanadium mg/kg	-	0.1	0.1	0.1

OA; Oleic acid, LA; Linoleic acid

Table 2.5: Calculated Amino Acid Composition of experimental diets

	CHOW	WESTERN	HIGH OA	HIGH LA
Valine	0.87 %	1.50 %	1.50 %	1.50 %
Leucine	1.40 %	2.10 %	2.10 %	2.10 %
Isoleucine	0.80 %	1.00 %	1.00 %	1.00 %
Threonine	0.70 %	0.90 %	0.90 %	0.90 %
Methionine	0.30 %	1.00 %	1.00 %	1.00 %
Cystine	0.30 %	0.07 %	0.07 %	0.07 %
Lysine	0.90 %	1.70 %	1.70 %	1.70 %
Phenylalanine	0.90 %	1.20 %	1.20 %	1.20 %
Tyrosine	0.50 %	1.20 %	1.20 %	1.20 %
Tryptophan	0.20 %	0.30 %	0.30 %	0.30 %
Histidine	0.53 %	0.70 %	0.70 %	0.70 %

OA; Oleic acid, LA; Linoleic acid

Table 2.6: Calculated Vitamin Composition of experimental diets

	CHOW	WESTERN	HIGH OA	HIGH LA
Vitamin A (Retinol) IU/Kg	10950	4660	4660	4660
Vitamin D (Cholecalciferol) IU/Kg	2000	1170	1170	1170
Vitamin E (Tocopherol acetate) mg/Kg	110	87	90	96
Vitamin K (Menadione) mg/Kg	20	1.2	1.2	1.2
Vitamin C (Ascorbic acid)	-	None added	None added	None added
Vitamin B1 (Thiamine) mg/Kg	80	7.1	7.1	7.1
Vitamin B2 (Riboflavin) mg/Kg	30	7.3	7.3	7.3
Niacin (Nicotinic acid) mg/Kg	145	35	35	35
Vitamin B6 (Pyridoxine) mg/Kg	28	8	8	8
Pantothenic Acid mg/Kg	60	19	19	19
Biotin µg/Kg	410	233	233	233
Folic Acid mg/Kg	5	2.4	2.4	2.4
Inositol	-	None added	None added	None added
Vitamin B12 (Cyanocobalamin) µg/Kg	150	120	120	120
Choline mg/Kg	1640	790	790	790

OA; Oleic acid, LA; Linoleic acid

2.2.2 Animal Housing and Monitoring

Animals were kept at a Victoria University Animal Research Facility and were maintained on a 12-hour light/dark cycle in controlled temperature (18-24 °C) and humidity (40-70 %) conditions. The animals were housed 3 per cage for the first 3 days of acclimatisation, following which they were housed individually for the remainder of the study. Animals were provided with short PVC tubes, shredded newspaper and cardboard for environmental enrichment. Animals were randomly assigned to colour groups during the initial 9 weeks, following which they were ranked and distributed to treatment groups based off body weight,

percentage body fat (determined via Echo MRI), blood pressure and glucose tolerance and insulin sensitivity tests. Animal health (behaviour, coat appearance, alertness etc.) was monitored daily during weighing. Food intake was also measured daily.

2.2.3 EchoMRI body composition assessment

EchoMRI (Echo-MRI™ 900, Houston, Texas, U.S.A.) was performed according to the manufacturer's instructions on the animals at week 5 for acclimatisation and then at weeks 9, 12 and 15. EchoMRI is a quantitative magnetic resonance imaging system that measures whole body fat mass, lean tissue mass, free water, and total body water in live animals up to 1100 grams. This is an easy method of accurately quantifying animal fat mass and lean mass without overly stressing the animals or requiring anaesthesia or sedation (Nixon et al., 2010). Each scan is performed in less than 1 minute, with automatic data analysis based off inputted animal weight.

The system has different sized clear acrylic tubes to accommodate a range of body weight and animal sizes, with air holes for non-restricted breathing. Animals are encouraged to run into the tube, after which they are held securely with a second narrower acrylic tube which attaches loosely with Velcro. During measurement animals still have limited movement capabilities. If at any times animals showed distress during the procedure they were removed from the tube and returned to their home cage.

2.2.4 Glucose Tolerance testing

Glucose tolerance testing was performed at the beginning of weeks 9 and 14 using the protocol developed by Jayasooriya et al. (2008) and modified by Jenkin et al. (2016). Animals were fasted overnight for approximately 16 hours with access to drinking water maintained. A 0.5 g/ml solution of D-Glucose was prepared the night before (to allow for isomerisation to the D configuration) using a 0.9 % saline solution (Mcfarlane Medical, Surry Hills, Victoria, Australia). Animals were weighed and a 3 ml syringe fitted with a 27 G needle was prepared

to contain 2 g glucose per kg of body weight. A fasting blood glucose measurement was taken using a sterile scalpel blade to snip the very end of the tail, with a drop of blood being placed on a glucometer strip inserted into a glucometer (Optimum, Xceed, Abbott, USA). Following this the animals were manually restrained in a 'Duffy Roll' using a small domestic flannel bath towel folded into a triangular shape with each corner wrapped over the animal, swaddling the animal so it can be calmly placed on its back. The glucose solution was administered via intraperitoneal injection, with the animal then removed from the 'Duffy Roll' and returned to its home cage. Blood glucose concentrations were determined at 15, 30, 60, 90 and 120 minutes after administration of glucose by gently removing the scab from the tip of the tail and milking a drop of blood, in some instances the tail was required to be snipped again to enable the collection of adequate blood samples. Once 120 minutes had elapsed the animal's food was returned to the cage.

2.2.5 Insulin Sensitivity testing

Insulin sensitivity testing was performed at the end of weeks 9 and 14 following the protocol of Stengel et al. (2013). Animals were fasted for 2 hours with access to drinking water maintained. A sterile stock solution of 1.0 U/ml Insulin (Humalog, Eli Lilly, Indianapolis, Indiana, United States of America) was prepared using a 0.9 % saline solution. Animals were weighed and an insulin syringe with attached needle was prepared to contain 1.0 U/kg of body weight. Following this the protocol proceeded as per glucose tolerance testing (section 2.2.5).

2.2.6 Blood pressure measurement

Blood pressure was measured in week 5 for acclimatisation to the process then in weeks 9 and 14 using a CODA (Kent Scientific) mouse and rat tail-cuff system. This method was selected due to being non-invasive and providing accurate data using a validated technique (Daugherty et al., 2009). Animals were placed in an appropriate size holder for their body weight, with their head pointing towards the nose cone. The rear hatch was secured to the holder by tightening the screw, ensuring the animals tail was in correct position and no body parts were

pinched. The nose cone was slid toward the rear hatch limiting the movement of the animal though allowing free breathing. Animals were placed in their holders on a towel in a pre-warmed incubator set to 32 °C and allowed to acclimatise. Animals were kept in the incubator for at least 5 minutes to thermoregulate, with their temperature measured frequently with an infrared thermometer. Animals were held in this manner until their temperature reached between 32 °C-35 °C. During this period, the software was set up and calibrated. Animals were carefully moved onto the warming platform of the blood pressure machine and the occlusion tail cuff was fitted by threading the tail through the occlusion cuff, with the cuff being placed as close to the base of the tail as possible without force. Following this the volume pressure recording (VPR) sensor cuff was placed within 2 mm of the occlusion cuff and the tube was secured in the notch on the rear of the holder. Blood pressure was measured in 30 second intervals by inflation of the occlusion tail cuff to impede the blood flow to the tail, the occlusion cuff is slowly deflated and the VPR tail cuff measures the physiological characteristics of the returning blood flow- as the blood returns to the tail the VPR sensor cuff measures the tails swelling because of the arterial pulsations from the blood flow. Systolic blood pressure is automatically measured at the first appearance of tail swelling. Diastolic blood pressure is automatically calculated when the increasing rate of swelling cesses in the tail. Automatic analysis determines successful measurement. After 8 cycles, the cuff and sensor were removed from the tail and the rear hatch and nose cone removed to allow the animal to leave the holder, with data exported in Excel format for analysis. Following this the animal was returned to its home cage.

2.2.7 Euthanasia and Tissue Collection

Two animals were euthanised (n= 1 chow group, n= 1 ‘Western’ diet then chow group) via CO_2 asphyxiation (as per ethical guidelines) during the study due to pathologies not related to experimental conditions/procedures, with cause of illness determined by necropsy performed

by a qualified veterinarian. At the end of week 9 (9 week DIO) and week 15 (all other groups) animals were deeply anaesthetised with Isoflurane (Isoflo, Abbott Laboratories, Animal Health Division, Illinois, USA) using an Australian & New Zealand Council for the Care of Animals in Research and Teaching (ANZCCART) approved method. Anaesthesia was performed by Ms. Lannie O'Keefe. Briefly, animals were placed in an airtight chamber connected to rodent gas anaesthesia instrument equipped with a vaporizer. The vaporizer machine was filled with isoflurane, connected O_2 tank and turned on. The vaporizer was set to 4 % and the O_2 set at a flow of 4 litre per minute to help in circulating the isoflurane. Once the animals were sedated they were removed from the induction chamber and fitted with an individual nose cone, secured with surgical tape. Depending on the animals' size Isoflurane and O_2 were adjusted to an optimal rate to ensure no response to pain stimuli whilst maintaining breathing rate. Response to pain stimuli was assessed throughout the tissue collection process until cardiac puncture was performed and the heart eviscerated. The first tissues collected were the left hind limb extensor digitorum longus (EDL) muscle, followed by the soleus muscle, with each muscle removed from tendon to tendon. The procedure was then repeated on the right hind limb. Immediately following this the muscles were incubated in an organ bath (see 2.2.9). Following this procedure, the diaphragm was lacerated and separation of the lung from the chest wall was performed by opening the ribcage. Cardiac puncture and removal of the heart was performed as a secondary measure of death. Once dead perirenal, epididymal white adipose tissue samples and subscapular brown adipose tissue samples were collected into cryotubes and rapidly frozen in liquid nitrogen. Other organs and tissues were also collected for future analysis outside of the present study.

2.2.8 Muscle organ bath protocol

Immediately following removal, muscles were placed in individual chambers of a specially designed 20 chamber organ bath (Zultek Engineering, Melbourne, Australia). Left and right

leg muscles were alternated for treatment with adiponectin between animals to remove bias. The chambers were filled with Krebs-Henseleit Buffer (Sigma-Aldrich #K3753) to allow for metabolism to continue *ex vivo* and kept at a constant 37 °C. The chambers were also gassed with carbogen (95 % O_2 -5 % CO_2) to help maintain physiological functioning. The protocol for the adiponectin treatment was adapted from Mullenet al. (2010). Briefly, a 0.1 mg/ml stock solution was prepared by diluting 0.01 g of FA free Bovine Serum Albumin (BSA) in 10 ml of sterile phosphate buffered saline (PBS), which was then used to dissolve 1mg of powdered globular adiponectin (Sapphire Bioscience Pty. Ltd., Redfern, NSW, Australia). The amount of adiponectin solution added to each treatment well was calculated based off the amount of Krebs- Henseleit buffer required to submerge the muscle, resulting in a final concentration of 2.5 µg/ml. Following the 30-minute incubation period the muscles were immediately removed from the chambers, blotted on paper towel to remove excess liquid and flash frozen in liquid nitrogen.

2.2.9 Adipose tissue RNA extraction

Rat adipose tissue (100 mg epididymal and perirenal, 35 mg subscapular brown adipose tissue) was homogenised in 1 ml TRIzol (Invitrogen, Carlsbad, CA) with ceramic beads using a Fast Prep tissue homogeniser (FP120 cell disruptor, Electron Corporation, Milford, United States of America) using a protocol developed by Chomczynski and Sacchi (2006) and modified by Cavuoto et al. (2007). After being centrifuged at 12,000 X g for 15 minutes at 4 °C, the supernatant was recovered into a sterile Eppendorf tube, leaving the upper fat layer, cell debris and beads. This was then centrifuged at 12,000 X g for 10 minutes at 4 °C to separate out the fat layer. The lysate was recovered from under the fat layer and this process was repeated twice to ensure the removal of all fat from the sample as per the protocol developed by Cirera (2013). Following this 250 µl of chloroform (Sigma Aldrich, St Louis, MO) was added and briefly vortexed to mix, and then the sample was left on ice for 5 minutes, before being centrifuged at

12,000 X g for 15 minutes at 4 °C to separate out the phases. The clear upper layer was removed (avoiding the interphase), placed in a new sterile Eppendorf tube and 500 µL of 2-isopropanol (Sigma Aldrich, St Louis, MO) and 10 µl of 5 M NaCl in DEPC treated water (H_2O) was added. The lower TRIzol phase and interphase were discarded. Samples were stored overnight at -20 °C to precipitate the ribonucleic acid (RNA). The following day samples were centrifuged at 12000 X g for 10 minutes at 4 °C, resulting in the RNA forming a small white pellet. The supernatant was then removed and the RNA pellet was carefully washed with 400 µL of 75 % (vol/ vol) ethanol made with diethyl pyrocarbonate (DEPC) treated water (Invitrogen Life Sciences), which inactivates any RNAses which may degrade the sample and is free of foreign RNA and DNA. Samples were then centrifuged at 8000 X g for 8 minutes at 4 °C. The ethanol was then aspirated off and the RNA pellet was left to air dry for 10 -15 minutes at room temperature. The pellet resuspended in 10 µL of warm DEPC treated H_2O. 1 µl of each sample was diluted further into 19 µl of DEPC treated H_2O for RNA yield determination.

2.2.10 Skeletal Muscle RNA extraction

Rat skeletal muscle from the organ bath treatment (see section 2.2.8) was cut to 30 mg weights on dry ice and then homogenised in TRIzol (Invitrogen, Carlsbad, CA) with ceramic beads using a Fast Prep tissue homogeniser. After being centrifuged at 12, 000 X g for 15 minutes at 4 °C, the supernatant was recovered into a sterile Eppendorf tube, leaving the cell debris and beads. Following this 250 µl of chloroform (Sigma Aldrich, St Louis, MO) was added and briefly vortexed to mix, the sample was left on ice for 5 minutes, then was centrifuged at 12000 X g for 15 minutes at 4 °C to separate out the phases. The clear upper layer was removed (avoiding the interphase), placed in a new sterile Eppendorf tube and 500 µL of 2-isopropanol (Sigma Aldrich, St Louis, MO) and 10 µl of 5 M NaCl in DEPC treated water was added. The lower TRIzol phase and interphase were discarded. Samples were stored overnight at -20 °C to precipitate RNA. The following day samples were centrifuged at 12000 X g for 10 minutes

at 4 °C, resulting in the RNA forming a small white pellet. The supernatant was then removed and the RNA pellet was carefully washed with 400 µL of 75 % (vol/ vol) ethanol made with DEPC treated water (Invitrogen Life Sciences). Samples were then centrifuged at 8000 X g for 8 minutes at 4 °C. The ethanol was then aspirated off and the RNA pellet was left to air dry for 10 - 15 minutes at room temperature. The pellet resuspended in 5 µL of warm DEPC treated H_2O. 1 µl of each sample was diluted in a further 19 µl of DEPC treated H_2O for RNA yield determination.

2.2.11 RNA yield quantification

Following extraction, RNA content was quantified using nanodrop spectrometry (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE), with the absorbance measured at 260 nm. Samples were considered free of contamination when the 260 nm: 280 nm was 1.8-2.0. Following this samples were diluted to contain 0.5 µg of RNA per 7.5 µl.

2.2.12 cDNA synthesis

0.5 µg of RNA in 7.5 µl DEPC H_2O from each sample was reverse transcribed to yield complementary DNA (cDNA) using an iScript cDNA synthesis kit (BioRad Laboratories Inc. Hercules, California, USA) according to manufacturer's instructions. Briefly, samples were mixed with 2 µl of the 5X Reaction mix and 0.5 µl of the reverse transcriptase and placed in a thermocycler (BioRad Laboratories, Hercules, California, United States of America) with a protocol of priming at 25 °C for 5 minutes, followed by reverse transcription at 46 °C for 20 minutes, with the enzyme then being inactivated by 1 minute at 95 °C, with the samples then being cooled and held at 4 °C. Following this cDNA samples were diluted in 120 µl of DEPC H_2O for 'Real Time' Polymerase Chain Reaction (PCR) quantification.

2.2.13 Oligonucleotide Primer Design Process

Oligonucleotide primers are nucleic acid sequences of approx. 20 base pairs long which bind to specific areas of the DNA sequence and replicate the complementary strand. Primers for genes of interest were designed using Thermo Fisher Scientifics' (Waltham, Massachusetts,

USA) OligoPerfect™ Designer, with the Gene transcript being retrieved from Ensmbl database (release 87) and the U.S.A. National Library of Medicine: National Centre for Biotechnology Information Nucleotide Database. Primer sequences were chosen based on the length of the product strand and primer pairs branching across and exon. Following this the U.S.A. National Library of Medicine: National Centre for Biotechnology Information Basic Local Alignment Search Tool was used to ensure that primers were specific to the gene of interest. After this, primers were ordered from GeneWorks Pty. Ltd. (Thebarton, South Australia). Primers used in Chapters 4 and 5 are shown in Table 2.7.

Table 2.7: Primer Sequences for Genes of Interest

GENE	ACCESSION NUMBER	SEQUENCE
β-Actin	NM_031144	Forward (5'-3') CTAAGGCCAACCGTGAAATGA Reverse (5'-3') CCAGAGGCATACAGGGACAAC
GAPDH	XM_017593963.1	Forward (5'-3') AGTTCAACGGCACAGTCAAG Reverse (5'-3') GTGGTGAAGACGCCAGTAGA
HPRT1	NM_012583.2	Forward (5'-3') GCAGACTTTGCTTTCCTTGG Reverse (5'-3') AGAGGTCCTTTTCACCAGCA
CPT1	XM_006242180.3	Forward (5'-3') TTTGAGATGCACGGCAAGAC Reverse (5'-3') CTGGACAAGAGGCGAACACA
Cyclophilin	NM_017101.1	Forward (5'-3') CTGATGGCGAGCCCTTG Reverse (5'-3') TCTGCTGTCTTTGGAACTTTGTC
FAT/CD36	NM_031561.2	Forward (5'-3') GACCATCGGCGATGAGAAA Reverse (5'-3') CCAGGCCCAGGAGCTTTATT
PGC1α	NM_031347.1	Forward (5'-3') ACCCACAGGATCAGAACAACC Reverse (5'-3') GACAAATGCTCTTTGCTTTATTGC
AMPKα2	NM_023991.1	Forward (5'-3') ACTCTGCTGATGCACATGCT Reverse (5'-3') AGGGGTCTTCAGGAAAGAGG
β-**HAD**	NM_133618.3	Forward (5'-3') TCGTGACCAGGCAATTCGT Reverse (5'-3') CCGATGACCGTCACATGCT
PPARα	NM_013196.1	Forward (5'-3') TGTCGAATATGTGGGGACAA Reverse (5'-3') ACTTGTCGTACGCCAGCTTT
PPARγ	NM_013124.3	Forward (5'-3') TTCAGAAGTGCCTTGCTGTG Reverse (5'-3') CCAACAGCTTCTCCTTCTCG

βActin; beta actin, GAPDH; Glyceraldehyde 3-phosphate dehydrogenase, HPRT1; Hypoxanthine Phosphoribosyltransferase 1, CPT1; Carnitine palmitoyltransferase I, FAT/CD36; Fatty Acid Translocase/ Cluster of Differentiation 36, PGC1α; Peroxisome proliferator-activated receptor gamma co-activator 1 alpha, AMPKα2; AMP-activated protein kinase alpha subunit 2, β-HAD; beta-hydroxyacyl-CoA dehydrogenase, PPARα; Peroxisome proliferator-activated receptor alpha, PPARγ ; Peroxisome proliferator-activated receptor gamma

2.2.14 Primer Optimisation

Primers were diluted out to 1 µmol and the optimal amount of primers determined by running PCRs in the combinations of 3 µM forward + 3 µM reverse, 3 µM forward + 9 µM reverse, 9 µM forward + 3 µM reverse and 9 µM forward + 9 µM reverse for 50 cycles. Following this accuracy of primer binding was determined by measuring the threshold cycle of a set of serial dilutions, linearity of plotted dilutions and threshold cycle values was considered accurate when R= <0.9.

2.2.15 PCR Gene expression quantification

'Real Time' PCR is an analytical process that allows for the quantification of gene expression by measuring the expression of a particular mRNA sequence. This process uses IQ SYBR Green (#1708885, BioRad Laboratories, Hercules, California, USA), a fluorescent dye that binds to double stranded cDNA, with the amount of fluorescence used to quantify mRNA expression. Briefly, a master mix was prepared containing for each required sample 6 µl of sterile DEPC treated water, 2 µl each optimised concentration of forward and reverse primers and 8 µl of the SYBR green was prepared. 18 µl of the master mix was added to the wells of an optically clear 96 well plate (BioRad Laboratories, Hercules, California, USA), following this 2 µL of cDNA was added to each specified well with each sample analysed in triplicate. The plate was covered and sealed using Microseal 'B' Optically clear Film (BioRad Laboratories, Hercules, California, USA) and briefly centrifuged to remove bubbles using a Rotina refrigerated benchtop Centrifuge (Andreas Hettich GmbH & Co.KG Tuttlingen, Germany, model 46 R) until a speed of 430 X g was reached. The plate was then transferred into a BioRad MY iQ® Real-Time PCR detection system. This system is comprised of a camera for measuring the amount of fluorescence emitted from individual wells in real time and a thermocycler which cycles the plate between 95 °C for 15 s and 60 °C for 60 seconds for a set number of cycles (40 for abundant genes, 50 for less abundant). This creates a denaturation process which separates the cDNA into single strands to which the primers can then anneal to

the target sequence. Due to the SYBR green mix containing a DNA polymerase new DNA is synthesised and bound by the SYBR green dye, creating more fluorescence (Bustin, 2000). Finally, this is followed by a process of slowly increasing the temperature of the plate to create a melt curve of denaturation during which the level of fluorescence emitted drops as the product is destroyed to confirm the specificity of the primers and the absence of primer-dimers.

2.2.16 PCR Gene expression analysis

Analysis of gene expression was performed using the Biorad IQ5 Gene analysis software (BioRad Laboratories, Hercules, California, USA 2009, version 2.1.97.1001) and Microsoft Excel (Microsoft, Redmond, Virginia, USA). The threshold cycles of gene expression for individual samples were compared to the average of 3 housekeeping genes for that sample. An average of 3 genes was used as this helps to remove analysis bias due to unexpected alterations in housekeeping gene expression (Vandesompele et al., 2002). Housekeeping genes used for adipose tissue analysis were β-Actin, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Hypoxanthine guanine phosphoribosyl transferase1, (HPRT1). For skeletal muscle analysis β-Actin, GAPDH and Cyclophilin were used. These genes were chosen due to the stability of their expression levels in this tissue type (Svingen et al., 2015; Thellin et al., 1999). Gene expression relative to that of the average housekeeping gene expression was performed using the $2^{-\Delta\Delta C_T}$ method which has been validated and is a widely used and acknowledged method of data analysis (Schmittgen & Livak, 2008).

2.3. Acute fatty acid intake study design

This study was designed as a single blinded, 3-way cross over study. To remove interpersonal variables each participant consumed each test meal after an overnight fast, with one meal consumed each week in a random order with a minimum 5-day period between meals.

2.3.1 Ethical approval and Subject recruitment

Ethical approval was obtained from Victoria University human research ethics committee (HRETH 12/87). Inclusion criteria for the study was a BMI >25 kg/m^2 or waist circumference

>94 cm (male)/ >80 cm (female), weight stable and aged 18-60 yrs. Exclusion criteria were the presence of Type I or II diabetes mellitus, insulin resistance, heart, liver or kidney disease, pregnant (or planning to become pregnant during the course of the study) breastfeeding, and the use of weight loss medication/supplementation or FA supplementation. Potential participants were provided with a plain language document outlining the study (see Appendix, Chapter 9, Figure 1) and informed consent was obtained (see Appendix, Chapter 9, Figure 2). Eligibility status was determined during an initial screening consultation.

The study aimed to recruit 10 subjects, a sample size based on previously observed results by Thomsen et al. (1999) and Robertson et al. (2002) both who investigated acute appetite hormone changes in response to differing fatty acid containing test meals.

2.3.2 Meal challenge sessions

Meal challenge sessions took place with participants commencing at approximately 7:30am, with participants being in an overnight fasted state (post absorptive) approximately 10-14 hours after their last meal. A timeline of the meal sessions is shown in Figure 2.3. Participants were instructed to consume a nutritionally balanced meal the night before testing and refrain from alcohol intake. Upon arrival participants had a cannula inserted their antecubital vein. All meals were consumed at the same time of day (between 9 am and 10 am) to control for diurnal hormonal variation with a maximum of 15 minutes provided for consumption. Participants were encouraged to consume water during the sessions to maintain fluid levels and assist in the collection of blood and instructed to refrain from exercise immediately before and during the test period.

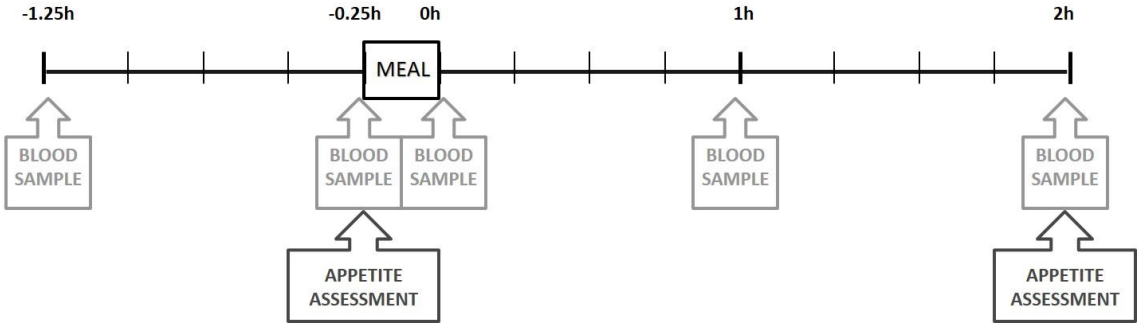


Figure 2.3: Meal Challenge Session Timeline. Participants were required to consume the test meal in a 15-min time frame. Blood sampling occurred on arrival, 1 hour later (immediately before consumption), immediately after consumption and then 1 and 2 hours post consumption. Participants completed VAS questionnaires immediately prior to consumption and 2 hours after.

2.3.3 Test meal compositions

The test meals were comprised of toasted bread, reduced sugar jam, icing sugar and differing oil compositions (extra virgin olive oil, safflower oil and coconut oil), resulting in control, high oleic acid (high monounsaturated), and high linoleic acid (high polyunsaturated) meals, with all ingredients used commonly found in Western diets (for exact composition see Appendix, Table 9.1). The meal compositions took into account palatability and the ability of participants to be blinded, with prior testing indicating that these criteria were met. Meal composition was chosen so as to replicate a prepared mixed composition meal that people would normally consume, as opposed to a liquid formula. As such fibre enriched bread was used, which also had a higher protein content than conventional white breads. Icing sugar was used to increase the energy density of the control meal to match those of the elevated fat meals due to it homogenising easily with the jam and also due to it not adding any further nutrients to the meal which would be absent in the elevated fat meals.

Hot water and lemon was provided with the meals to increase participant compliance and palatability, caffeinated beverages were not allowed while fasting or during the meal sessions due to caffeine's influence on blood glucose levels (Beaudoin et al., 2011). The high fat meals had comparable ratios of all other fats than that which had been elevated, with the amount of

energy from the elevated fats in each high fat meal also being matched (for further details see Table 2.8). The two high fat meals were isoenergetic and had matched contributions from macronutrients to energy content. Each test meal contained 13.74 ± 0.658 kJ/g.

The meals were weighed to the nearest 0.1 gram and provided 30 % of estimated daily energy intake, a figure based on the findings of large scale studies indicating that breakfasts generally comprise 20-25 % of daily energy intake (Preziosi et al., 1999; Purslow et al., 2008; Winkler et al., 1999). This was increased by 5 % to account for the sessions running through to late morning, removing energy intake from foods generally consumed during this time-period. Estimated energy intake was calculated using the Mifflin St Jour equation which has been found to be the most accurate for overweight and obese individuals (Frankenfield et al., 2005; Mifflin et al., 1990; Weijs, 2008). This equation took into account age, gender, height and weight and was adjusted using appropriate activity factors based off participant reported usual activity levels (Volpe et al., 2007).

Table 2.8: Nutritional Composition of Experimental Meals

	CONTROL	HIGH OA	HIGH LA
Carbohydrate (%E)	73.46	39.74	39.74
Protein (%E)	6.91	6.84	6.84
Fat (%E)	23.12	55.13	55.13
- SFA (% total fat)	34.03	14.41	14.54
- MUFA (% total fat)	34.63	70.65	14.41
- oleic acid (% of MUFA)	95.05	97.88	94.94
- PUFA (% total fat)	31.32	14.93	70.74
- linoleic acid (% of PUFA)	94.97	94.08	98.68
n-6:n-3	16.7	13.09	77.20

%E = Percentage of total energy

2.3.4 Body composition Assessment and Anthropometrical Measurements

Body composition (fat mass % and lean mass %) was measured by a licensed professional at Footscray Park Campus using dual energy x-ray absorptiometry (DXA) (Hologic Discovery QDR, Hologic (Australia) Pty Ltd, North Ryde, NSW, Australia) in the first week of the study. DXA was used as it is the most accurate form of indirect assessment of body composition (Haarbo et al., 1991) giving total body and specific region data. This was performed before 9 am in well hydrated, fasting participants. Hydration status was determined from the specific gravity of the first micturition of the day. Participants were instructed to avoid consuming fluids in the time between urine collection and scanning.

Anthropometric measurements were taken twice during the study to assess weight stability. All measurements except weight were taken twice to increase accuracy. Measurements included an initial height measurement using a stadiometer, weight (Tanita HD-351 Digital Weight Scale, Tanita Pty. Ltd. Tokyo, Japan), waist circumference (measured at the narrowest point between the lower costal border and the iliac crest), hip circumference (taken at the level of the

greatest posterior protuberance of the buttocks), blood pressure and heart rate (Omron 5 Series, Omron Healthcare, Inc., Palatine, IL, USA).

2.3.5 Blood collection

Blood samples were taken during each meal challenge session. These occurred on insertion of the cannula (one hour before eating), immediately prior to the meal, immediately after consumption and one and two hours after consumption, with a total of five over each 3 ¼ hour meal session (see Figure 2.3). Samples were collected into plain tubes to yield serum (and stored at room temperature for 30-45 minutes to clot) and EDTA containing tubes to yield plasma, then centrifuged for 11 minutes at 1300 g (Sigma 3-18K Centrifuge, Sigma, Germany), with serum and plasma being aliquoted into sterile Eppendorf tubes coded for participant confidentiality and stored at -80 °C until analysis.

2.3.6 Appetite assessment

Participants completed an appetite questionnaire immediately before consumption of the meal and two hours after. The appetite questionnaire (see Appendix Chapter 9, Figure 3) utilised a visual analogue scale (VAS) which has been found to be a reliable and valid tool for appetite assessment (Flint et al., 2000). This comprised 8 100-mm horizontal lines, where 0 mm represented "sensation not felt at all" and 100 mm represented "sensation felt the greatest" with subjects asked to mark the line at the point which corresponded to how they were feeling at that particular time. The questionnaire developed by Parker et al. (2004) has been used in similar studies assessing the effect of meal composition on appetite and gastrointestinal hormones in humans (Brennan et al., 2012; Little et al., 2005; Parker, Ludher, et al., 2004; Parker, Sturm, et al., 2004; Stewart et al., 2011). The distance from the beginning of the line to the participants' mark was measured from the left-hand side to the nearest 0.5 mm.

2.3.7 Blood glucose analysis

Blood glucose levels in millimoles per litre (mmol/L) were measured in serum samples using an auto-calibrating automated sampler (YSI 2300 Stat Plus Glucose L-Lactate analyser, YSI

Inc. Life Sciences, Yellow Springs, OH, USA) which is precise to ±2 % or 0.2 mmol/L of reading (the greatest of the two).

2.3.8 Human Cytokine determination

Blood samples collected at baseline, 1 hour post and 2 hours post consumption of test meals were analysed. The other 2 timepoints (immediately prior to and immediately following meal consumption) were excluded due to the cost of analysis.

Cytokines and Diabetes related markers (see Table 2.9) were measured in human serum (10-30 µl) based off fluorescent bead region using a Bioplex © 200 multiplex suspension array system (Biorad, California, USA) coupled to a high-throughput fluidics system. The analytes measured were used the pre-configured 10-plex Cytokine plate, the Diabetes Markers plate and a single-plex Adiponectin plate with the manufacturers protocols followed. This system is based on the measurement of fluorescence emitted by small magnetic beads which bond to the analytes in solution, allowing the quantification of multiple analytes on one 96 well plate in a short period of time. Background fluorescence measured from a blank was subtracted and the observed fluorescence from 8 serially diluted standards was automatically analysed to yield a standard curve from which concentrations of analytes in participant samples were then extrapolated. Analytes that did not have a full set of data inside the reporting range (set at 80-120 % of the standard curve range) were excluded from analysis.

Table 2.9: Target Analytes for Human Cytokine and Diabetes Marker Determination

ANALYTE	BEAD REGION
Interleukin 1β	58
Interleukin 2	38
Interleukin 4	52
Interleukin 5	73
Interleukin 6	19
Interleukin 10	56
Interleukin 12 p70	75
Interleukin 13	50
Interferon γ	21
Tumour Necrosis factor α	36
Adiponectin	64
C-peptide	72
Ghrelin	26
Gastric inhibitory polypeptide	14
Glucagon-like peptide-1	27
Glucagon	15
Insulin	12
Leptin	78
Plasminogen activator inhibitor-1	61
Resistin	65
Visfatin	22

2.4 Statistical Analysis

All statistical analysis was performed using Prism GraphPad (Version 7, GraphPad Software, San Diego, California, USA). For Chapter 3 linear regression was performed and Pearson's Correlation determined for change over time. Linearity and significance was set at $p \leq 0.0001$. All other results were considered significant when $p < 0.05$. Dependant on data type, results are reported as mean ± standard deviation (SD) or standard error of the mean (SEM). For Chapters

4 and 5 the 9 week DIO animals are not included in tissue analysis. All data is presented as mean ± standard error of the mean (SEM). Changes in body weight and energy intake over the experimental period were determined using a 2-way ANOVA with Tukey's test of multiple comparisons. Animal energy efficiency (g gained/mJ consumed) over the experimental period was calculated using energy intake (mJ/week) (calculated based on suppliers nutritional composition information and weighed food disappearance measured daily) and weekly weight gain (measured daily), using the formula (adapted from Bellinger et al. (2004)):

$$\text{Energy efficiency} = \frac{\text{g weight gained}}{\text{mJ consumed}}$$

One way ANOVA was used to determine differences between body composition, fat pad/organ weights, blood pressure, fasting blood glucose and AUC responses for GTT and IST. For gene expression samples were considered outliers if the average of the triplicates was greater than 2 standard deviations away from the average for that diet group. One way ANOVA was used to compare between diet groups within fat depots and treated/untreated muscles. Paired two-tailed t-tests were used to compare between muscles (i.e. soleus and EDL) for the untreated muscles, and to compare between treated and untreated muscles for each diet group. In the instance of a missing data point the matching data point (e.g. the corresponding treated or untreated muscle) was removed from analysis for the t-tests.

Chapter 3: Australia's Nutrition Transition 1961-2009: A Focus on Fats

This Chapter has been published in the manuscript: Naughton, S. S., Mathai, M. L., Hryciw, D. H., & McAinch, A. J. (2015). Australia's nutrition transition 1961–2009: a focus on fats. *British Journal of Nutrition*, *114*(03), 337-346. CORRIGENDUM published: Naughton, S. S., Mathai, M. L., Hryciw, D. H., & McAinch, A. J. (2015). Australia's Nutrition Transition 1961-2009: A Focus on Fats- CORRIGENDUM. *British Journal of Nutrition*, *114*(6), 997-997.

N.B: Corrigendum was issued due to the changing of a colour in the figure legend of Figure 3.4 during typesetting, resulting in two identical symbols.

3.1 Abstract

Background: Since the 1960's, Australian diets have changed considerably, influenced by a burgeoning multicultural cuisine, increased urbanisation, and food technology advances. This has been described as a 'nutrition transition', resulting in the adoption of a Western diet pattern, with a shift away from unrefined foods, towards a diet higher in both plant derived high PUFA and total fats, and refined carbohydrates.

Methods: Utilising 1961-2009 annual food supply data from the United Nations FAO, this study investigated changes in macronutrient and specific fatty acid intake in the Australian population, including that of the PUFA linoleic acid (LA), due to its hypothesised role in inflammation and obesity risk. Cumulative change over time for the contribution of specific nutrients to total available energy (TAE) was calculated, as was linearity of change.

Results and Discussion: Over the time-period analysed cumulative change in TAE from carbohydrate was -9.35 % and +16.67 % from lipid. Cumulative change in TAE from LA was +120.48 %. Moreover, the cumulative change in contribution of LA to total PUFA availability was +7.1 %. Utilising the average g/day/capita of LA from selected dietary sources the change

in the contribution of specific foodstuffs was assessed, with total plant oils having a +627.19 % cumulative change in LA availability, equating to +195.61 % cumulative change in contribution to total LA availability. The results of this study indicate that LA availability in Australia has increased over the preceding 5 decades as a result of increased plant oil availabilities, as has total fat, possibly contributing to the increasing rates of obesity and obesity associated co-morbidities.

3.2 Background

Food intake in Australia has changed greatly over the last half century. This is due to a combination of increased urbanisation and gross domestic profit, more efficient import streams and an increasingly multicultural population. Migrants have brought with them their traditional cuisines and ingredients, which over time have been shared and incorporated into everyday diets, resulting in multicultural food customs and a great increase in dietary diversity in Australia (Finkelstein, 2003). Adding further to this changing food culture is the ever evolving food technology industry, which has introduced genetically modified crops and grains (Uzogara, 2000), processed and ultra-processed foods with significantly extended shelf lives (Monteiro et al., 2013), fortified foods and an array of convenience manufactured foods which require very little further preparation or are ready to eat (Kearney, 2010). These have become favourable commodity traits for the increasing number of dual income families with limited food preparation time (Finkelstein, 2003). Moreover, take away meals and dining out now account for more than 23 % of the average Australian household expenditure (Espinel & Innes-Hughes, 2013), with approximately 1 in 3 meals prepared outside of the home (Finkelstein, 2003).

Australia is believed to have gone through what is described worldwide as a 'nutrition transition', resulting in the adoption of a Western diet pattern, typified by a shift away from diets high in unrefined carbohydrates and fibre, towards a diet higher in total fat and refined carbohydrates (Drewnowski & Popkin, 1997). Furthermore, nutrition transitions have been described as having two distinct phases, the first involving an increase in energy availability from cheaper vegetable based products, and the second comprising a shift away from a high carbohydrate diet to one rich in vegetable oils, animal products and sugars (Kearney, 2010). In Australia this has been evidenced by the embracing of Asian and European cuisines over the traditional British style of food (Finkelstein, 2003). A nutrition transition is also usually

accompanied by an increase in non-communicable diseases such as obesity, type II diabetes mellitus (T2DM), cardiovascular disease (CVD) and some types of cancer (Drewnowski & Popkin, 1997) and is generally preceded by a demographic or epidemiological transition (Kearney, 2010). One major impetus for this change in dietary pattern is believed to be an increase in urbanisation, more efficient food import and distribution systems (Popkin et al., 2012), increasing access to fast food franchises (Kearney, 2010), and a greater reliance on large multinational supermarkets (Kearney, 2010). Moreover, once a nutrition transition has occurred these foods are cheaper to purchase than traditional staple commodities, making diets rich in unrefined grains, fruits and vegetables often more expensive to obtain than high fat and refined sugar diets (Drewnowski & Darmon, 2005; Drewnowski & Popkin, 1997; Kearney, 2010).

In Australia, dietary guidelines are developed and reviewed by the National Health and Medical Research Council (NHMRC) in conjunction with other government departments (National Health and Medical Research Council, 2013b), with the first guidelines being published in 1982. The key recommendation of these guidelines was to reduce SFA intake in favour of MUFA and PUFA, with an emphasis placed on plant based sources (National Health and Medical Research Council, 1992), with the current American Heart Association recommendations (Harris et al., 2009) and Australian dietary guidelines continuing to reflect this view (National Health and Medical Research Council, 2013a). As dietary guidelines are produced for the general public, differentiations between particular fatty acids and even between MUFA and PUFA in regard to their physiological roles are not made. This may be an important omission as not all fatty acids in the same class have similar physiological roles (Grande et al., 1970; Mattson & Grundy, 1985).

The 18 carbon n- 6 PUFA, linoleic acid (LA), the most commonly consumed PUFA in the human diet (Alvheim et al., 2014), is considered an essential fatty acid (Holman, 1977), due to its role in modulating cell signalling, gene expression and inflammation (Das, 2006). Furthermore, LA can be converted by the human body to arachidonic acid (AA) (Salem et al., 1999), though the rate of this conversion may be as low as 1 % (Emken et al., 1994), with the same metabolic pathway used for conversion of α-linolenic acid (ALA) to long chain n-3 FA, though this is inhibited by high LA intakes (Burdge & Calder, 2005). Early research determined an intake of 1-2 % of dietary energy from LA as being adequate to prevent deficiency symptoms (Hansen et al., 1963; Holman, 1971), though recent research has shown that requirements may be as low as 0.3 % of total energy intake (Choque et al., 2014). As LA is a precursor for inflammatory mediators (James et al., 2000; Turpeinen et al., 1998), intakes above what is required by the body can result in a pro-inflammatory state, which is further exacerbated in obesity due to the production of inflammatory substances by adipose tissue (Furukawa et al., 2004). LA is also the precursor of the two main endocannabinoid system mediators, anandamide and 2-arachidonoyl glycerol (Naughton et al., 2013), which regulate appetite and metabolism (Cota et al., 2003), though the system is dysregulated in overweight and obese individuals (Matias et al., 2006). High LA diets have also been found to correlate with obesity prevalence (Moussavi et al., 2008), the promotion of obesity in both animals and humans (Massiera et al., 2010; Massiera et al., 2003) and are correlated with increased fasting blood glucose and insulin (Madigan et al., 2000) and insulin resistance (Simopoulos, 1994). Recently, reanalysis of data from the Sydney Diet Heart Study was performed after the identification of missing data points and mortality data. This re-analysis identified that in men with a history of a recent coronary event, replacing dietary SFA with LA resulted in greater CVD events and mortality rates than the control group (no dietary change) (Ramsden et al., 2013). This is supported by a recent meta-analysis finding that, in studies which elevated

dietary LA only, there was no evidence of a decrease in cardiovascular risk (Ramsden et al., 2013).

As the majority of plant based fats are rich in LA (sunflower and safflower >65 %, cottonseed, corn and soy >50 % of total weight (Ramsden et al., 2013)) it has been hypothesised that LA intake has increased at a population level, though recent research investigating this in Australia is lacking. Therefore, the aim of this study is to assess the changes in intake of macronutrients, PUFA and specific fatty acids: LA, AA, the most commonly consumed MUFA oleic acid (OA) and the most commonly consumed SFA, palmitic acid (PA). In addition, food sources which have contributed to LA availability in the Australian diet will also be determined.

3.3 Methods

3.3.1 Data Sources

Further detailed methodology can be found in Chapter 2. Annual food supply data in the form of food balance sheets (FBS) for the time-period 1961-2009 (all available data at time of analysis) for Australia was obtained from the United Nations Food and Agriculture Organisation Statistics division (FAOSTAT) (http://faostat.fao.org/) utilising Australian Bureau of Statistics (ABS) population data (including infants). FBS show trends in intake at a population level, and though primarily produced to monitor food availability they are also useful in monitoring trends in intake over time (Vandevijvere et al., 2013). Data was expressed as grams per day per capita (g/day/capita), and showed the supply of 75 base foods standardised to raw unprocessed commodities/foods (e.g. 'bananas with skin'). These quantities were adjusted by FAOSTAT to reflect importation and exportation and to account for amounts used for seed; fed to livestock or used for non-food products; losses during storage and transportation; farm waste and post-harvest losses and technical losses occurring during the production of processed foods. Adjustment to reflect edible quantities was performed using United States Department of Agriculture (USDA) Food Yields data (Matthews et al., 1975). Commodities which were not shown to be consumed in measurable quantities at any of the time points analysed were removed from the analysis, as were commodities which made negligible contributions to available energy (e.g. tea).

3.3.2 Nutritional analysis

The nutritional composition of each commodity was determined using Xyris FoodWorks Professional 7 (Xyris Software Pty Ltd, Highgate Hill, QLD, Australia), encompassing carbohydrate (CHO), protein (PRO), lipid (LIP), PUFA, LA, AA, PA and OA. Primary databases used were from The Royal Melbourne Institute of Technology, Australian fatty acid database, the NUTTAB 1995 and 2010 databases produced by Food Safety Australia and New Zealand (FSANZ) (© Food Standards Australia New Zealand Organisation) and the AUS FA

database. As the data resulting from this analysis is highly dependent on the integrity of the nutrition databases used, primary databases used were Australian to remove variations as a result of analytical, geographical, nutrient and temperature variations.

Availability of specific nutrients was calculated by multiplying the per 100 g/ml quantity of each commodity by the average annual g/day/capita availability. Following this, per capita yearly intake of measured nutrients was calculated and expressed as g/day/capita. Average energy availability per capita in kJ per day was calculated by summing the energy contribution from available macronutrients to determine the contribution of the focus macronutrients to total available energy (TAE) as a percentage. The contribution of the remaining fatty acids not measured in this study was calculated by summing the average yearly intake of investigated FA and subtracting this value from the total available lipids g/day/capita, with the % contribution to total available energy then calculated and this labelled as 'other FA'. Data is expressed as percentage of total available energy to take into account the differences between disappearance (which FBS show) and actual consumption (Sasaki & Kesteloot, 1992). This has been done as FBS methodology results in inherent overestimation (Kearney, 2010) when compared to other forms of dietary monitoring (Serra-Majem et al., 2003).

3.3.3 Data Analysis and Statistics

Statistical analysis and area under the curve (AUC) calculations were performed using GraphPad Prism (version 6.02, GraphPad Software Inc.). Linear regression was performed and Pearson's Correlation determined for change over time. Linearity and significance was set at $p \leq 0.0001$. Cumulative change over time was calculated using the formula:

$$\% \text{ Cumulative change} = \frac{(\text{Actual AUC} - \text{Baseline AUC})}{\text{Baseline AUC}} \times 100$$

This method was used as it assesses all changes that occurred 1961-2009, allowing peaks and troughs in availability to be taken into account. In this formula, the 1961 value is used to

determine a “baseline” AUC, which is a flat line hypothesising that availability was at a constant rate from 1961-2009. The actual AUC was calculated using data obtained from the analysis, allowing all change throughout the time period to be taken into account (Carden & Carr, 2013). These two AUC measurements were then used in the above formula to determine the differences between the two AUC (and as such change from baseline), expressing percentage of cumulative change, with negative values indicating a decrease. Relative change was also calculated using the formula:

$$\text{Relative change \%} = \frac{(\text{2009 value} - \text{1961 value})}{\text{1961 value}} \times 100$$

This was performed so comparison between the two methods of assessment of change could be made.

3.4 Results

3.4.1 Macronutrient contribution to total available energy

All macronutrients showed a significantly linear relationship to time over the period 1961-2009 (see Figure 3.1), with the slope of linear regression being -0.19 for CHO, -0.02 for PRO and +0.2 for LIP. Cumulative changes during the period 1961-2009 were -9.3 % for energy from carbohydrate, -2.8 % from protein and +16.7 % from lipid (see Table 3.1). Relative change over the period 1961-2009 for carbohydrate, protein and lipid was -15.7 %, -9.8 % and +30.5 % respectively.

Table 3.1: Cumulative change of selected macronutrients and fatty acids. Cumulative change over time was calculated using the formula:

$$\% \text{ Cumulative change} = \frac{(\text{AUC} - \text{Baseline AUC})}{\text{Baseline AUC}} \times 100$$

	1961 availability (%AE*)	**2009 availability (%AE*)**	**cumulative change (%)**
carbohydrate	53.6	45.0	-9.3
protein	14.1	12.7	-2.8
lipid	32.5	42.3	16.7
palmitic acid	7.5	8.7	7.45
oleic acid	9.4	14.8	29.0
linoleic acid	2.2	6.0	120.5
arachidonic acid	0.06	0.05	-3.6
other FA	13.1	12.5	-4.1
total PUFA†	2.9	7.7	104.7
contribution of LA‡ to total PUFA†	75.3	78.6	7.1

* AE; available energy, † PUFA, polyunsaturated fatty acid, ǂ LA**,** linoleic acid

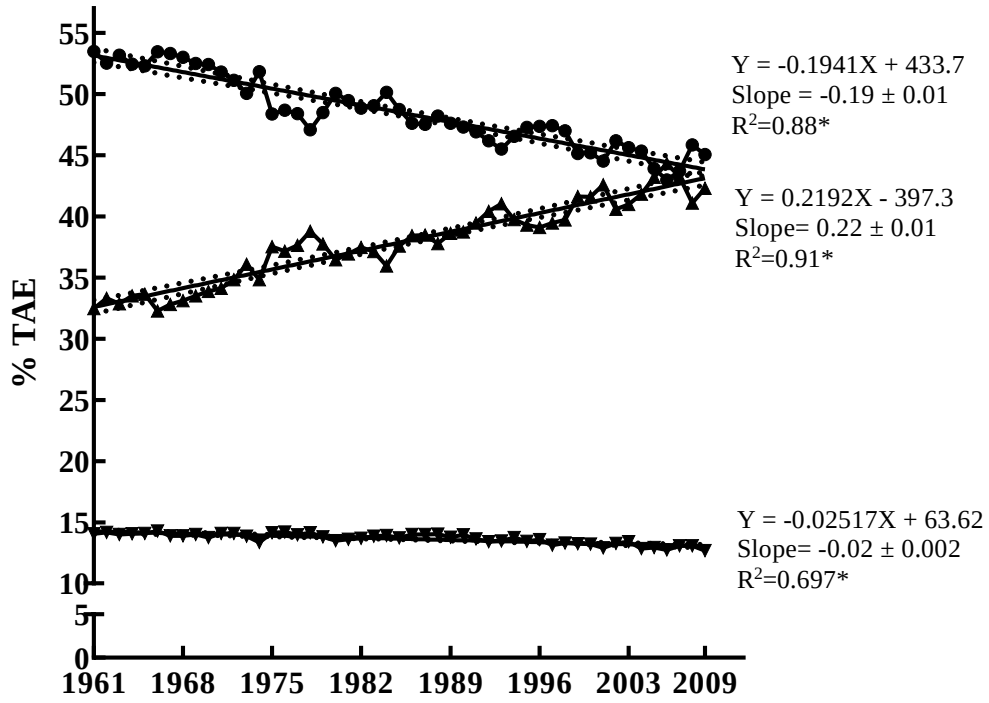


Figure 3.1: Macronutrient contribution to total available energy. The contribution of carbohydrate (●), lipid (▲) and protein (▼) to total available energy (TAE) expressed as average annual percentage for 1961-2009 with linear regression showing 95 % confidence interval bands. *p≤ 0.0001. X= calendar year.

3.4.2 Specific fatty acid contribution to total available energy

PA, OA, AA and LA all showed significantly linear relationships to time over the period 1961-2009 (see Figure 3.2, AA data not shown), with the slope of linear regression being 0.03 for PA, 0.1 for OA, 0.08 for LA and -0.0002 for AA (AA data not shown). The slope of linear regression for the remaining FA not specifically measured in this study was -0.02. As shown in Table 3.1, cumulative changes in energy from specific FA during this period were 7.4 % from PA, 29.1 % for energy from OA, -3.6 % from AA, 120.5 % from LA, and -4.1 % from the remaining unmeasured FA. The relative change of these FA for 1961-2009 were 16.5 % available energy from PA, 58 % from OA, 176.8 % from LA and -20.5 % from AA, showing similar trends to the cumulative change data.

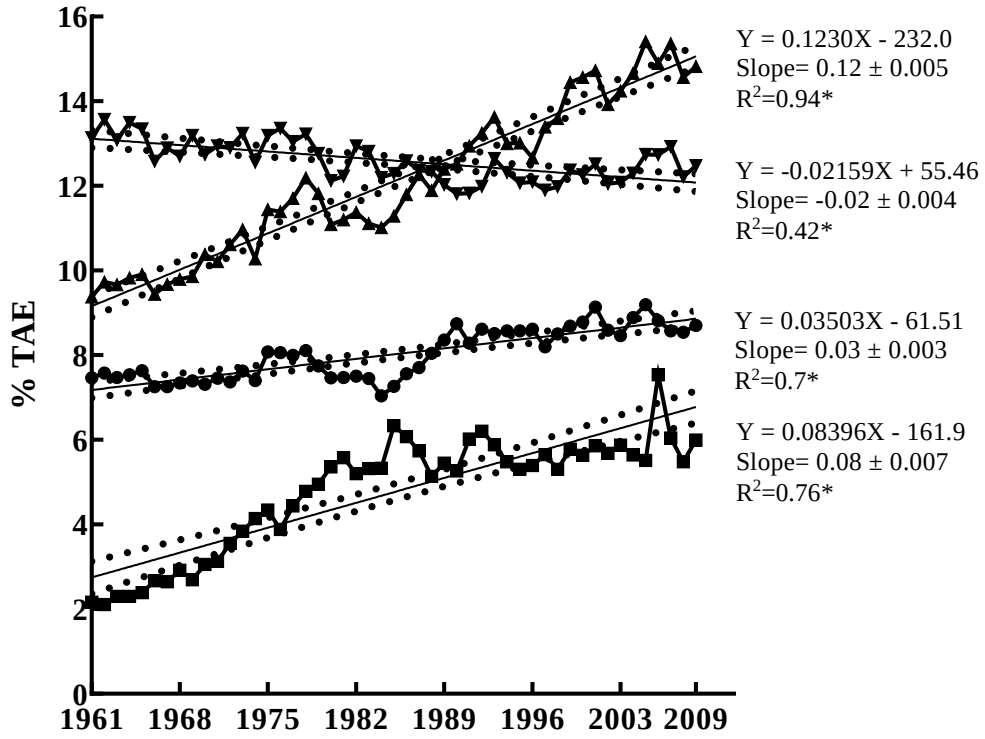


Figure 3.2: Specific fatty acid contribution to total available energy. Contribution of oleic (▲), palmitic (●), linoleic (■) and other (▼) fatty acids to total available energy (TAE) expressed as average annual percentage for 1961-2009 with linear regression showing 95 % confidence interval bands. *p≤ 0.0001. X= calendar year.

3.4.3 Linoleic acid contribution to total PUFA availability

The cumulative change in contribution of LA to total PUFA availability over the time analysed was 7.1 % (see Table 3.2) and the relative change 4.4 %. This low cumulative change is most likely due to the contribution of LA to total available PUFA rising sharply from 73.4 % in 1961 to 85.7 % in 1985, following which it dropped back to 78.6 % by 2009 (see Figure 3.3), though the slope of the regression varied between 0.003 and 0.12.

3.4.4 Linoleic acid availability from specific dietary sources

The change in contribution of specific foodstuffs to LA availability in the Australian diet was determined utilising the FBS commodity lists and divided into plant and animal sources as shown in Figures 3.4 and 3.5. Cumulative change data (Table 3.2) shows that the change in LA availability (gram/day/capita) from the plant based cottonseed, palm, sunflower and soy oils were 1470.9 %, 2510.5 %, 611.9 % and 2016.7 % respectively. Relative change for these

commodities was 2820 % for cottonseed, 6105 % for palm, 265 % for sunflower, and 995 % for soy oils. The cumulative change of contribution from peanut oil was -70.1 % and wheat was -17.7 %. Relative change calculations show that peanut oil decreased 100 % as it was not available at a measurable level in 2009, with wheat having a relative change of -74.25 %. As Rapeseed oil was not available at a measurable level in 1961 cumulative change could not be calculated, nor could relative change. Despite this, as shown in Table 3.2, by 2009 it provided the highest amount of LA from any single food source. The availability of LA from animal sources analysed showed a cumulative change of 345.2 % from poultry, 16.3 % from beef, 58.7 % from pork, -34.0 % from lamb and -19.8 % from eggs. Relative change of these sources found that poultry increased 707.7 %, and pork 110.7 %, though lamb decreased 61.56 % and eggs decreased 50 %. Relative change for beef was a decrease of 6.2 %, in disagreement with cumulative change data. This is most likely due to beef derived LA availability, as shown in Figure 3.4, peaking in 1978 then subsiding back towards baseline by 2009, showing the limitation of using relative change to determine trends in nutrient availability over time.

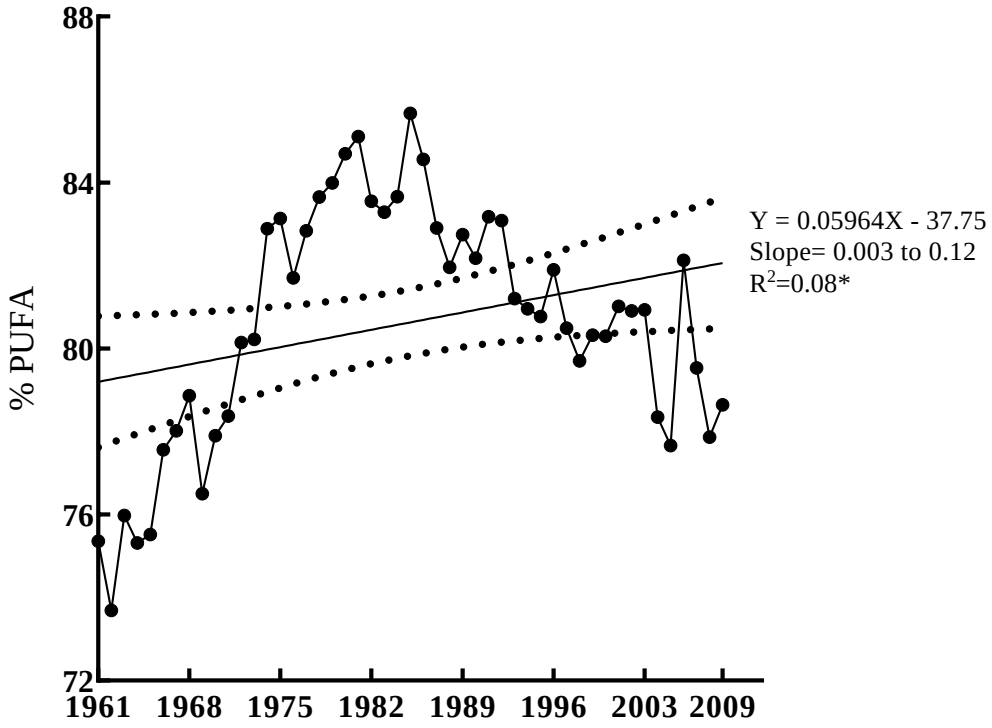


Figure 3.3: Linoleic acid contribution to total available PUFA. Contribution of linoleic acid to the total available PUFA (polyunsaturated fatty acid) expressed as an average annual percentage for 1961-2009 with linear regression showing 95 % confidence interval bands. *p≤ 0.05. X= calendar year.

3.4.5 Linoleic acid availability from total plant derived oils and major animal sources and their contributions to the total dietary linoleic acid availability

Total plant oil LA availability (average annual gram/day/capita) was calculated (encompassing coconut, cottonseed, groundnut, maize, palm, rapeseed, sesame seed, soy, safflower, olive and sunflower seed oils) and showed significant linearity (see Figure 3.6) with a slope of 0.29. As shown in Table 3.2, the cumulative change in plant oil derived LA (gram/day/capita) over the time analysed was 627.1 %, relative change in plant oil derived LA showed an increase of 992.9 %. Analysis of the major animal sources (beef, pork, poultry, lamb, eggs and milk) of LA found that the cumulative change of their total LA contribution (gram/day/capita) during the time-period analysed was 11.3 %, with the relative change being an increase of 13.67 %. As seen in Figure 3.6 combined animal sources provided a relatively stable amount of available

LA over the time-period analysed, having an R^2= 0.05 and a non-significant slope of 0.002. Analysis of the contribution of total plant derived oils to total dietary LA availability showed a cumulative change of 195.6 % and major animal sources -46.0 % over the time-period analysed (see Table 3.3). Relative change analysis showed a 248.8 % increase from plant sources to total LA availability and a 63.7 % decrease from animal sources. As shown in Figure 3.7, the annual average contribution of total plant oils to total LA showed a significantly linear relationship over time with a slope of 0.86, with the contribution of major animal sources having a slope of -0.34.

Table 3.2: Cumulative change of selected linoleic acid sources. Cumulative change over time was calculated using the formula:

$$\% \text{ Cumulative change} = \frac{(\text{AUC} - \text{Baseline AUC})}{\text{Baseline AUC}} \times 100$$

	1961 availability (gram LA*/day/capita)	**2009 availability (gram LA*/day/capita)**	**cumulative change (%)**
cottonseed oil	0.1	4.1	1470.9
peanut oil	0.7	0	-70.1
palm oil	0.02	1.5	2510.5
rapeseed oil	0	4.4	n/a
soy oil	0.3	3.3	611.9
sunflower oil	0.2	0.6	2016.7
total plant oils†	1.4	15.7	627.1
wheat	2.0	1.6	-17.7
poultry	0.1	1.1	345.2
eggs	0.3	0.2	-19.8
pork	0.06	0.1	58.7
lamb	0.7	0.3	-34.0
milk	0.3	0.2	-18.3
beef	0.6	0.5	16.3
major animal sources ǂ	2.2	2.4	11.3

* LA, linoleic acid. †- Total plant oils comprise coconut, cottonseed, groundnut, maize, palm, rapeseed, sesame seed, soy, safflower, sunflower seed and olive oils. ǂ- Major animal sources comprise beef, lamb, pork, poultry, eggs and milk

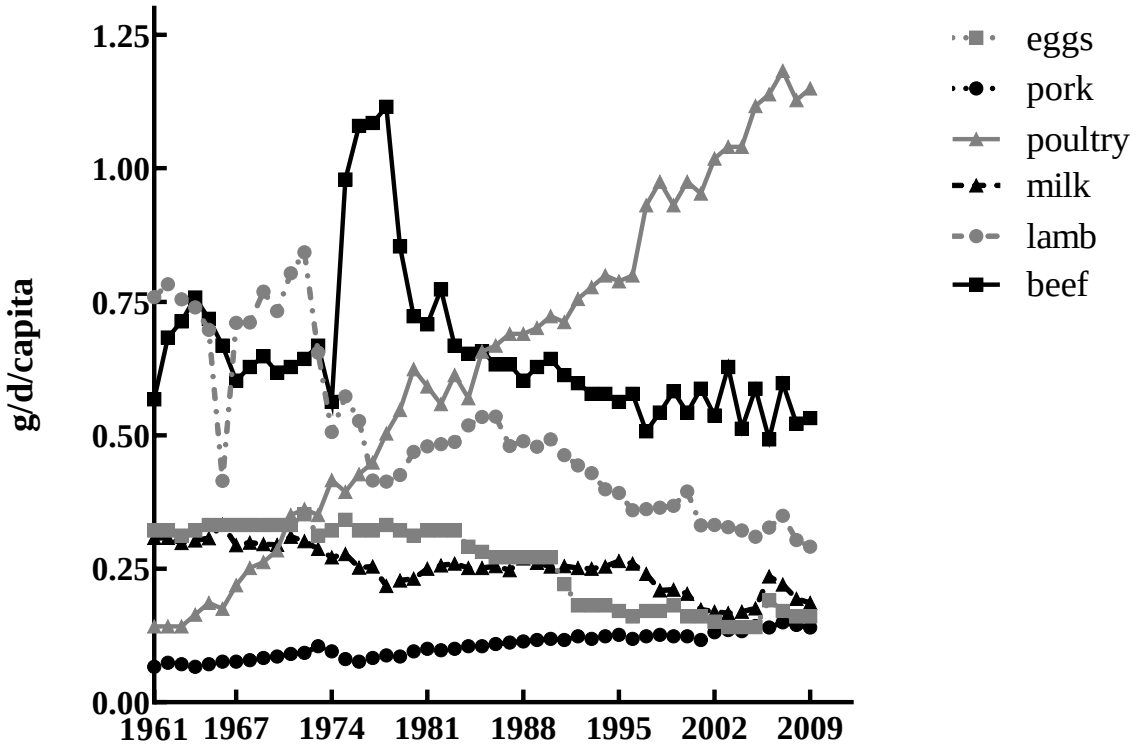


Figure 3.4: Animal product linoleic acid content. Major animal source linoleic acid availability expressed as average annual grams per day per capita of linoleic acid for 1961-2009.

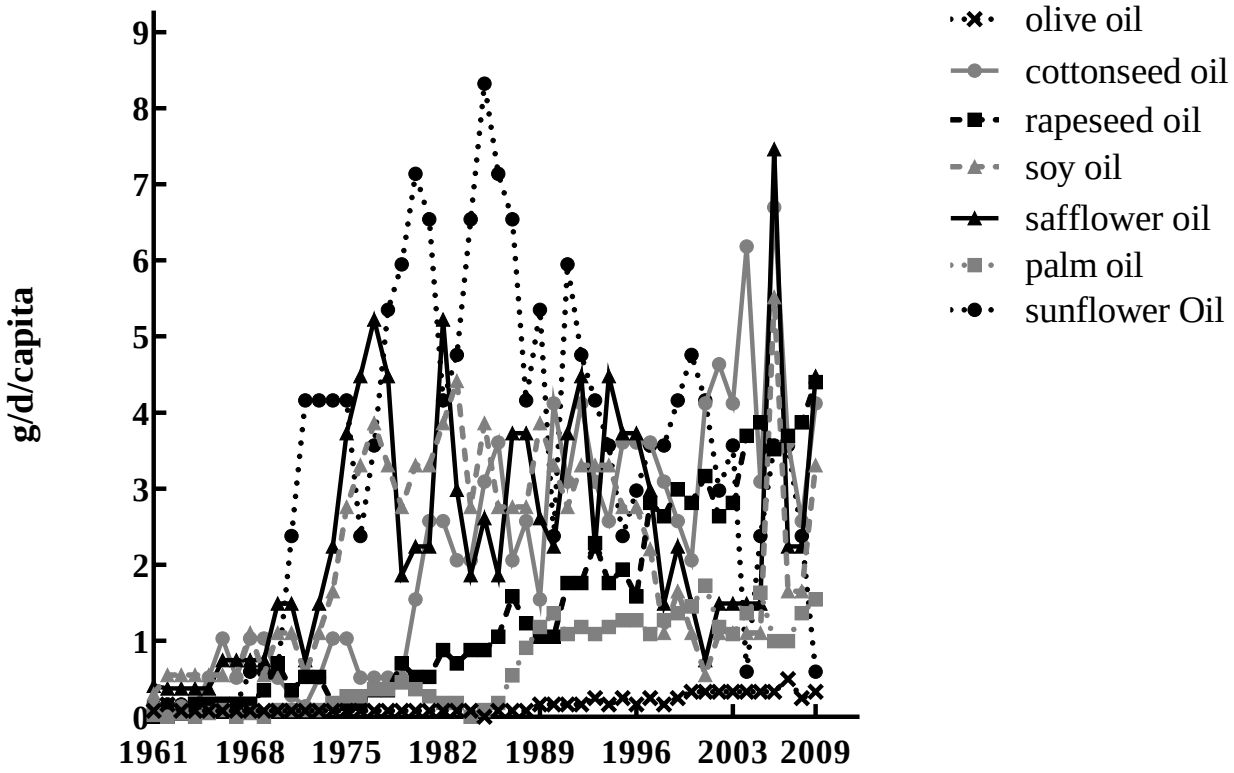


Figure 3.5: Plant derived linoleic acid intake. Major plant oil linoleic acid availability expressed as average annual grams per day per capita of linoleic acid for 1961-2009.

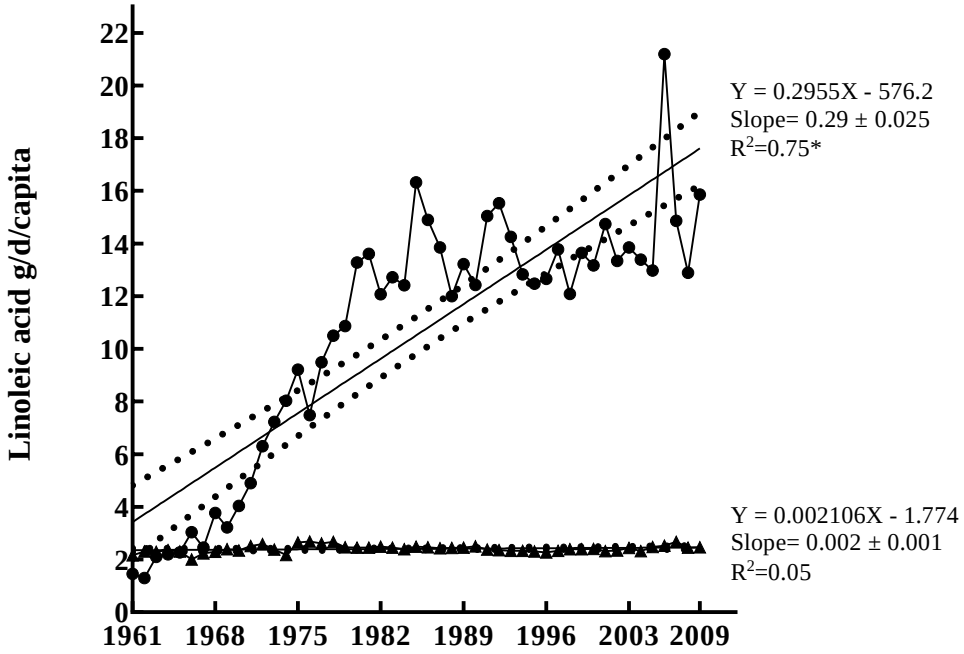


Figure 3.6: Combined animal and plant oil contribution to grams of available linoleic acid. Cumulative total of major plant oil (coconut, cottonseed, groundnut, maize, palm, rapeseed, sesame seed, soy, sunflower seed, olive and safflower oils) (●) and major animal source (milk, poultry, pork, beef, lamb and eggs) (▲) linoleic acid availability expressed as average annual grams per day per capita for 1961-2009 with linear regression showing 95 % confidence interval bands. *p≤ 0.0001. X= calendar year.

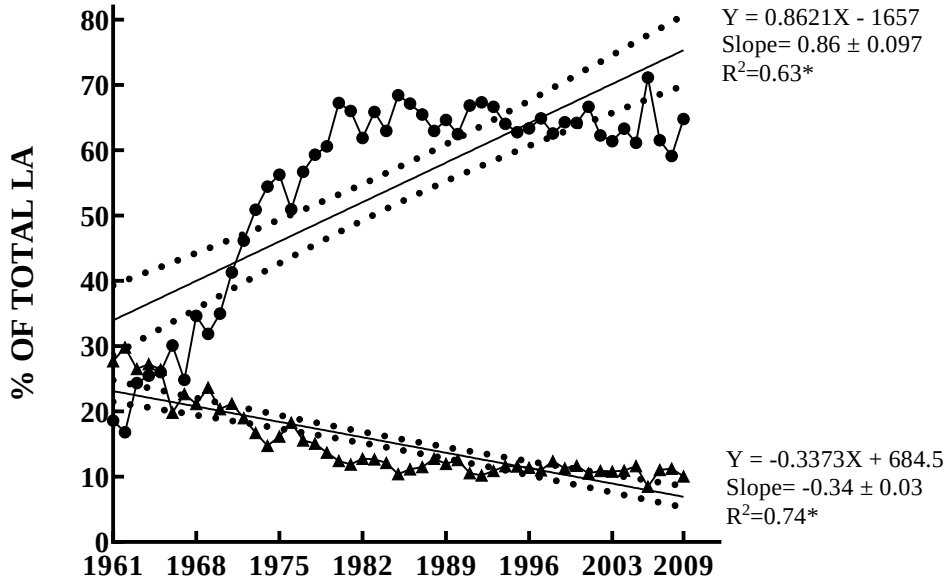


Figure 3.7: Combined animal and plant oil contribution to total linoleic acid. Cumulative plant oil (coconut, cottonseed, groundnut, maize, palm, rapeseed, sesame seed, soy, safflower, sunflower seed and olive oils) (●) and major animal source (milk, poultry, beef, pork, lamb and egg) (◆) contribution to total LA (linoleic acid) expressed as average annual percentage of total available linoleic acid for 1961-2009 with linear regression showing 95 % confidence interval bands. *p≤ 0.0001. X= calendar year.

Table 3.3: Cumulative change of linoleic acid from plant oils and major animal products contribution to total linoleic acid availability Cumulative change over time was calculated using the formula:

$$\% \text{ Cumulative change} = \frac{(\text{AUC} - \text{Baseline AUC})}{\text{Baseline AUC}} \times 100$$

	1961 contribution to total LA* (%)	2009 contribution to total LA* (%)	cumulative change (%)
total plant oils†	18.6	64.8	195.6
major animal sources ǂ	27.7	10.0	-46.0

* LA, linoleic acid. †Total plant oils comprise coconut, cottonseed, groundnut, maize, palm, rapeseed, sesame seed, soy, safflower, sunflower seed and olive oils. ǂ Major animal sources comprises beef, lamb, pork, poultry, eggs and milk

3.5 Discussion

Over the time-period 1961-2009, Australia experienced a major dietary change, or nutrition transition, resulting in an increase in the contribution of lipids to total energy availability, and an increase in both LA availability and the contribution of plant based oils to LA availability. This study is the first (to the authors' knowledge) to assess the changes in specific SFA, MUFA and PUFA and their sources over such a large time period.

The 16.7 % increase in energy availability from lipids found in this study shows a similar trend to a study investigating cumulative change of energy from macronutrients in the USA, which found a 14.6 % increase in the contribution from lipids from 1970 through to 2009 (Carden & Carr, 2013). Furthermore, this present study also shows comparable findings to several similar studies conducted in other Westernised countries such as Ireland (Sheehy & Sharma, 2011), Barbados (Sheehy & Sharma, 2010), Switzerland, France (Guerra et al., 2012) and Europe (Balanza et al., 2007), as well as China, which has recently gone through a nutrition transition (Popkin, 2001). Despite this, the percentage of energy available from lipids in Australia identified in this study in 2001 (42.5 %) was higher than in Austria, Belgium, Denmark, Germany, Greece, Spain and Sweden, which were also determined by FAOSTAT FBS analysis (Schmidhuber, 2007). The mechanism behind increased fat intakes worldwide has been extensively debated, with some schools of thought believing that a preference for high fat foods is innate or developed in childhood, though others believe that fat intake is influenced purely by the amount of fat available in the food supply, and that people will consistently consume the same weight of food, regardless of energy density (Blundell & Macdiarmid, 1997; Drewnowski & Popkin, 1997; Popkin, et al., 2001). Despite this, the cause of the increase in availability of fats in the food supply is generally believed to be the natural result of an increase in production, and therefore availability, of plant derived oils (De Haen et al., 2003). Supporting this are findings from the WHO that the increase in energy availability in Australia

is primarily due to an increased availability of fats (Silventoinen et al., 2004), reflecting the results found in this study.

The increase in availability of energy from fat found in the current study is a result of an increase in the availability of the most common FA of the SFA, MUFA and PUFA classes, being PA, OA and LA. Furthermore, the finding of this study that OA is the most highly consumed FA of those measured is similar to American results which found OA to be the most highly consumed FA, providing 12 % of total energy in 2010 (Vannice & Rasmussen, 2014). This is most likely a result of OA being present in the lipid fraction of a wide range of foods, from animal products and dairy through to plant based oils and a range of vegetables, albeit at lower levels in the later. Though OA accounted for the highest proportion of the FA surveyed here, the greatest cumulative change in FA energy availability was from LA, with a cumulative change of 120.5 % and a relative change of 176.8 % over the time-period analysed. A similar trend was found in the US by Blasbalg et al. (2011), who found an increase in LA availability of 158 % over the time period 1909-1999 when assessing relative change. Comparing the average annual contribution of investigated FA from 1995 found here to similar Australian research conducted by Hibbeln et al. (2006) there is comparable energy availability from both LA (4.7 % as compared to the 5.3 % found here) and AA (0.07 % compared to 0.06 %), with differences possibly attributable to the use of different food composition databases in analysis. Interestingly, though we now have a wider range of foods available than in 1960 (Commonwealth of Australia, 1999), which in itself leads to a greater proportion of energy from lipids (Drewnowski & Popkin, 1997), the results of this study show that over the time period analysed the contribution of the 3 main dietary FA to total lipids has increased (with the 'other FA' group showing a reduction), possibly reflecting a decrease in FA diversity.

Australian dietary guidelines recommend less than 10 % of energy intake from LA, which is a figure based on the 90th centile of intake in the population surveyed in the 1995 Australian National Nutrition Survey, as this level of intake did not appear to have negative effects on health (National Health and Medical Research Council et al., 2006). Though the results of the present study indicate current levels are below this, the intake level above which a negative impact occurs is strongly debated (Choque et al., 2014). One population group which demonstrates the effects of excessive LA consumption is Israelis following kosher diets, consuming LA intakes of between 10 and 12 % of total energy (Dubnov-Raz & Berry, 2008). This is due in part to the high reliance on plant oils, especially soy (Dubnov-Raz & Berry, 2008; Guggenheim & Kaufmann, 1976), and the consumption of high LA nuts such as pecans, pistachios and almonds (Dubnov-Raz & Berry, 2008). Interestingly, despite this population having one of the lowest total fat intakes in Westernised countries and a high intake of fruit and vegetables, they have one of the highest rate of diabetes mortality in the world (Dubnov-Raz & Berry, 2008). Moreover, with LA being promoted as being cardio-protective, it has been found that the standardised mortality rate of atherosclerosis is similar to that in America (Dubnov-Raz & Berry, 2008), indicating that increasing LA intake further may not result in improved CVD prevalence.

Further analysis of the commodities listed in the FBS found that the main source of the increased energy availability from LA was primarily plant oils (by both volume and percentage). This also identified cottonseed, palm and sunflower seed oils as having the greatest increases in availability over the time-period analysed, with rapeseed oil contributing the highest amount of LA from any single food source in 2009. This demonstrates the increase in worldwide availability and decreased cost of plant based oils, with the price of an energy dense high fat diet currently being much lower than a less energy dense diet with adequate fresh fruit and vegetables (Drewnowski & Darmon, 2005; Lee et al., 2011). Research into

worldwide plant oil production in 1984 showed plant oils accounted for 63 % of total fat production, with soybean oil being the most produced, providing nearly half of the worldwide intake of LA (Adam, 1989), with more recent research conducted by FAO showing an increase in vegetable oil supply of 121 % in developed countries from 1961- 2003 (Food and Agriculture Organization of the United Nations, 2010). This increase in plant oil production has been facilitated by trade organisation and government support programs (Morgan, 1993; Popkin, et al., 2012), provision of food aid and credit guarantees (Drewnowski & Popkin, 1997) and also due to technological advances, including the development of low erucic acid rapeseed oil (O'Keefe, 2000).

In comparison, the contribution to total LA availability from combined major animal sources decreased as a percentage over the time-period analysed, though its contribution when expressed in volume has remained relatively steady. This, coupled with the somewhat constant level of AA found over the time-period analysed here indicates that meat intake, which primarily supplies dietary AA, has remained relatively steady throughout the nutrition transition. Despite this steadiness, when looking at individual animal sources of LA, the trend of increased poultry intake in Australian diets (Australian Institute of Health and Welfare, 2012) is evident by the 345.2 % cumulative and 707.7 % relative increase in LA from this source. Westernisation and an expanding population has led to an increased demand for meat and animal products and has spurred agricultural change such as the replacement of grass in livestock feed with grains such as maize and soy (Thornton, 2010). This results in higher feed LA levels, leading to an increase in LA in animal tissues and products, which has been found to negatively affect the n-6:n-3 of consumers (Weill et al., 2002). Furthermore, modern farming techniques have resulted in traditionally lean animals, such as chicken, having a higher fat content and a higher n-6:n-3 (Crawford et al., 2010). In addition, farmed fish have a higher fat content, less DHA and EPA and more LA than their wild counterparts (George & Bhopal,

1995; van Vliet & Katan, 1990). Importantly, cellular phospholipid content is strongly determined by dietary FA intake, with LA and DHA/EPA competing for incorporation. In addition, the synthesis of both AA and DHA/EPA (from ALA) requires the same enzyme, which preferentially converts LA to AA, indicating that a diet high in LA is capable of decreasing tissue DHA/EPA levels (Abbott et al., 2012), further increasing the risk of pro-inflammatory states, endocannabinoid system over-activity and the development of obesity (Naughton et al., 2013).

During the time-period 1961-2009 there was a cumulative change of 7.1 % and a relative change of 4.4 % in the contribution of LA to total PUFA availability. Interestingly, Meyer et al. (2003), when investigating PUFA intake using 24-hour recall data found that in 1995, LA contributed to approximately 88 % of total PUFA, around 8 % higher than found at this time point in the current study, possibly due to the intrinsic differences between the 2 dietary analysis methods. The contribution of LA to total PUFA availability found here is slightly less than the approximately 84 % found in studies assessing American diets (Blasbalg et al., 2011; Harris & Klurfeld, 2011; Kris-Etherton et al., 2000). This is most likely due to soy oil being the most commonly consumed oil in the US (Blasbalg et al., 2011) accounting for approximately 20 % of energy intake (Hibbeln et al., 2006), while the results of this study indicate rapeseed, sunflower and cottonseed oils in combination with soy oil comprise the majority of plant oil availability, with soy oil having a higher proportion of PUFA than MUFA when compared to the other commonly used plant oils found here.

As a population, Australians have adopted the dietary guideline recommendations of consuming reduced fat dairy products (Dobson et al., 1997; Dairy Australia, 2013), lean meat and trimming visible fat (Dobson et al., 1997; Williams, P. & Droulez, 2010), however the Western diet pattern is high in invisible fat sources such as baked and fried foods (Popkin et al., 2001) and highly processed foods utilising butter fat as an ingredient. As a result, the intake

of fat from dairy sources has not reduced (Sanders, 2000). Furthermore, consumption of processed products such as sausages and meat patties/burgers still account for almost half of all meat consumed in Western countries (Kearney, 2010), which may show the influence of price as these products are often cheaper than their leaner counterparts. This is supported by a study utilising data obtained from the 1995 Australian National Nutrition Survey, which found a trend towards more frequent intake of luncheon meats and sausages in lower income groups (Worsley et al., 2003). This demonstrates how cost is a core influencing factor in food choice (Brimblecombe & O'Dea, 2009; Lee et al., 2011), with constraint of food expenditure generally leading to an increase in the energy density of food choices (Brimblecombe & O'Dea, 2009; Drewnowski & Darmon, 2005; Lee et al., 2011) and, in turn, a significant increase in Australian dietary energy intake (Beatriz et al., 2011; Brimblecombe & O'Dea, 2009; Lee et al., 2011).

One criticism of using FBS for determining trends in food supply changes is that they do not show differences between population groups (Vandevijvere et al., 2013), though research comparing male Australians who were vegan, ovo-lacto vegetarian or who habitually consumed large amounts of meat found no significant differences in LA and total PUFA intakes (Mann et al., 2006). Alcohol levels were not able to be quantified due to the categorising of beverages incorporating a wide range of alcohol contents and the data being presented as per capita, not just for the adult population. Also, FBS do not take into account household food wastage, which may artificially increase the availability of energy or particular nutrients (Vandevijvere et al., 2013). Though this has not been investigated in Australia, data from the United Kingdom shows that of household waste, fats and oils account for only approximately 1 % of total waste, with the majority of waste comprising fresh fruit and vegetables, and drinks (Quested et al., 2013). A limitation of this study is the use of current nutritional content data, which reflect modern farming and production practices for all time points, due to the absence of appropriate data from the 1960's. As a result, changes to livestock rearing and plant oil

production which occurred over the time-period analysed, specifically those which increased the LA content of a large number of commodities (Blasbalg et al., 2011), have not been able to be shown, indicating the changes in the availability of nutrients, especially LA, may actually be greater than what is found here.

In Australia, an increase in food diversity and multiculturalism coupled with food technology advances triggered a nutrition transition over the time-period 1961-2009 which resulted in changes to dietary intake at a population level. The most important of these changes identified here is an increase in energy availability from fats, due mostly to the greater availability and lower cost of vegetable based oils. This is also evident by looking at the LA contribution to total available fat, which showed a greater change than the other major dietary FA. Analysis of the dietary sources which contributed to this increase in LA identified plant derived oils as having the greatest contribution, helping to confirm our hypothesis. Though as a population the available energy from LA is below that recommended as an upper level by dietary guidelines, the evidence of the role of LA in increased inflammation, obesity development and CVD risk indicates a need for caution in any changes which may lead to further increases in intake.

Chapter 4: The effect of dietary fats or energy restriction on body composition, glucose handling and adipose depot specific fat metabolism genes in a rodent model of diet induced obesity

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Some of the methodology relating to animal treatment and measurements whilst animals were alive, and tissue collection, as well as results relating to food intake, body weight and composition, insulin and glucose testing have been previously presented in the thesis of Mrs. Anna Simcocks, due to the collaborative nature of this study.

4.1. Abstract

Background: The effects of specific dietary fats on obesity development and metabolism is yet to be fully elucidated. Therefore, this study aimed to investigate the two most common dietary fats in the Australian diet, linoleic acid (LA) and oleic acid (OA) in a rodent model of diet induced obesity (DIO) and if the specific type of fat in a diet is capable of influencing adiposity, metabolic health or depot specific adipose tissue gene expression.

Methods: 60 male 7 weeks old Sprague Dawley rats were fed a standard 'Western' style high fat diet (21 % fat w/w) for 9 weeks to induce obesity. One group (9 week DIO) were killed at the end of week 9. Obese animals were then randomly allocated to four treatment groups and fed either the 'Western' diet (n=10), a high OA diet (21 % fat w/w, 16 % OA w/w, n=10), a high LA diet (21 % fat w/w, 16 % LA w/w, n=10) or a standard chow (5 % fat, n=10) for 6 weeks. 10 additional control animals were fed a standard chow throughout the experimental period (15 weeks). Food intake and weight were measured daily. Body composition was assessed using Echo MRI (weeks 9, 12 and 15); 2-hour glucose tolerance and insulin sensitivity (both during weeks 9 and 14) testings were performed and blood pressure was measured (during weeks 8 and 15) using the tail cuff method. At the end of week 15 the animals were

deeply anaesthetised and killed, following which the epidydimal, perirenal and subscapular fat pads were removed. RNA was extracted from portions of fat pads and PCR was performed for quantification of target gene expression relative to that of three housekeeping genes.

Results and Discussion: Expression of FAT/CD36 mRNA in the perirenal white adipose and subscapular brown adipose depots was not influenced by diet type, though in the epididymal white adipose depot the 'Western' diet then chow fed animals had significantly increased expression when compared to the high LA diet fed animals, which may indicate that energy restriction in these animals may be triggering fat mobilisation from tissues such as the liver (which was significantly lighter compared to the high OA fed animals) for storage in adipose tissue or metabolism. Additionally, DIO appears to down regulate epidydimal adipose tissue PPARα mRNA expression regardless of FA type, though no effect of diet was seen in the subscapular brown or the perirenal white depot. This study found no significant differences between body weights, food intake, blood pressure or percentage body fat for the three high fat fed groups. However, this study does show that there is a potential for the type of dietary fat to modulate food intake and energy efficiency, with reductions in both of these parameters occurring for the high OA consuming animals towards the end of the study. This may support the body of research indicating the benefits of consuming a high OA diet, though a longer experimental period may have resulted in stronger results.

4.2 Background

The current food product landscape is rich in dietary fats, many of which are added during processing and preparation, and are considered 'hidden' fats, which have contributed to the rising obesity rates worldwide (Popkin 2001). Though the consequences of a high dietary fat intake have been thoroughly researched, the role of specific dietary fatty acids (FA) in obesity development and their effect on metabolism, is yet to be elucidated. Therefore, this study aimed to investigate the two most common dietary fats in the Australian diet (as found in Chapter 3), linoleic acid (LA) and oleic acid (OA) in a rodent model of diet induced obesity (DIO) and if the specific type of fat in the diet is capable of influencing adiposity or metabolic health.

Research performed over the last 50 years has shown at a population level that the habitual consumption of Mediterranean style diets can be protective against chronic disease, most notably cardiovascular disease (Estruch et al., 2006; Keys, 1995) and diabetes (Esposito, Maiorino, et al., 2010; Martinez-Gonzalez et al., 2008; Mozaffarian et al., 2007; Paniagua et al., 2007). Though Mediterranean style diets are typified by a high olive oil consumption, there are additional dietary components such as phytochemicals which may also confer health benefits (Visioli et al., 2000), making the role of particular fatty acids less clear. Despite this, studies replacing LA with OA have found an increase in insulin stimulated glucose uptake (Ryan et al., 2000) and a positive association has been found between LA intake and central adiposity, and the reverse for OA intake (Garaulet et al., 2001). To add further to the body of evidence relating to the roles of dietary fats in body weight and metabolic regulation, the current study utilised a Mediterranean style diet, a 'Western' style diet (with a similar fat profile to those found in American diets) and an elevated LA diet to see if increasing the LA composition of the diet would have any negative effects on body weight or metabolism in a rodent model of obesity. The ability of dietary FA to modulate storage of adipose tissue in specific depots has shown some promise, with high OA 'Mediterranean' style diets being

associated with a reduction in visceral adiposity in humans (Garaulet et al., 2001; Paniagua et al., 2007) and a high LA intake being associated with increased visceral fat deposition (Garaulet et al., 2001).

White adipose depots are generally considered storage depots with a low metabolic function, whereas brown adipose tissue is traditionally thermogenic, helping the animal to maintain body temperature (Casteilla et al., 2001). This thermogenic capacity is due to the presence of uncoupling proteins in the electron transport chain, which triggers the production of heat. Rodents have a comparably large amount of brown adipose tissue for their body mass when compared to adult humans. Despite this brown adipose tissue metabolism is of interest in human obesity treatment research due to the theory that if its mass or thermogenic capacity could be increased it would result in an increase in basal metabolic rate, which could decrease excess energy storage within the white adipose tissue mass (Nedergaard & Cannon, 2010).

The aim of this project was to determine the effects of manipulating specific dietary fatty acids in a rodent model of DIO on body composition, glucose metabolism, blood pressure, and adipose tissue energy metabolism. Additionally, the role of weight loss following a high fat diet in DIO and its effects on body composition, glucose metabolism, blood pressure and adipose tissue energy metabolism was also investigated.

4.3 Methods

4.3.1 Experimental Outline

Ethical approval was granted through the Victoria University Animal Ethics Committee (AEEC 13/005). 60 seven-week-old male Sprague Dawley rats (weight range 200-250 g) were sourced from The Animal Resource Centre (ARC, Perth). Following a seven-day acclimatisation period, the animals were maintained for nine weeks on either a high fat diet based on the open source diet D12451 (21 % fat, Specialty Feeds, Perth, W.A. SF13-115) to induce obesity or a standard chow (4.8 % fat, Meat free Rat & Mouse Feed, Specialty Feeds, Perth, W.A.). Animals were kept at a Victoria University Animal Research Facility and were maintained on a 12-hour light/dark cycle in controlled temperature and humidity conditions. Further detailed methodology can be found in Chapter 2.

4.3.2 Experimental diets

Following the nine-week obesity inducing period, the DIO animals were ranked and divided into 5 groups of 10 as shown in Table 4.1. The 9-week DIO animals were included to show metabolic and body composition differences between 9-week-old DIO and the 15-week-old DIO animals. The diets were designed to mimic current 'Western' diet patterns consumed in developed countries ('Western' diet), a high polyunsaturated fat (PUFA) diet comprised predominately of high LA plant oils and a 'Mediterranean' style high monounsaturated fat (MUFA) diet with the predominant lipid source being olive oil which is high in OA.

Table 4.1: Schedule of Experimental Diets

GROUP	9 WEEKS - PRIOR TO TREATMENT	TREATMENT – 6 WEEKS
CHOW	Standard chow (5 % fat)	Standard chow – lean group (5 % fat)
9 WEEK DIO	High fat 'Western' style diet (21 % fat)	**N/A (**killed at the end of Week 9)
'WESTERN' DIET	High fat 'Western' style diet (21 % fat)	High fat 'Western' style diet (21 % fat)
HIGH OA DIET	High fat 'Western' style diet (21 % fat)	High fat diet predominately oleic acid (OA) (21 % fat)
HIGH LA DIET	High fat 'Western' style diet (21 % fat)	High fat diet predominately linoleic acid (LA) (21 % fat)
'WESTERN' DIET THEN CHOW	High fat 'Western' style diet (21 % fat)	Standard chow (5 % fat)

The energy ratios of the diets are shown in Figure 4.1. The diets were designed to be isoenergetic and have the same proportion of energy from macronutrients, with the exception of the chow diet. Additionally, the diets were designed to have matched energy from the specifically elevated fats, namely OA and LA, as shown in Figure 4.1. As shown in Table 4.2 the n-6:n-3 of the diets varied, the chow and MUFA diets had the lowest ratios, with the 'Western' diet ratio being slightly below what is currently found in American diets (Kris-Etherton et al., 2000), with the PUFA diet having four-fold more LA.

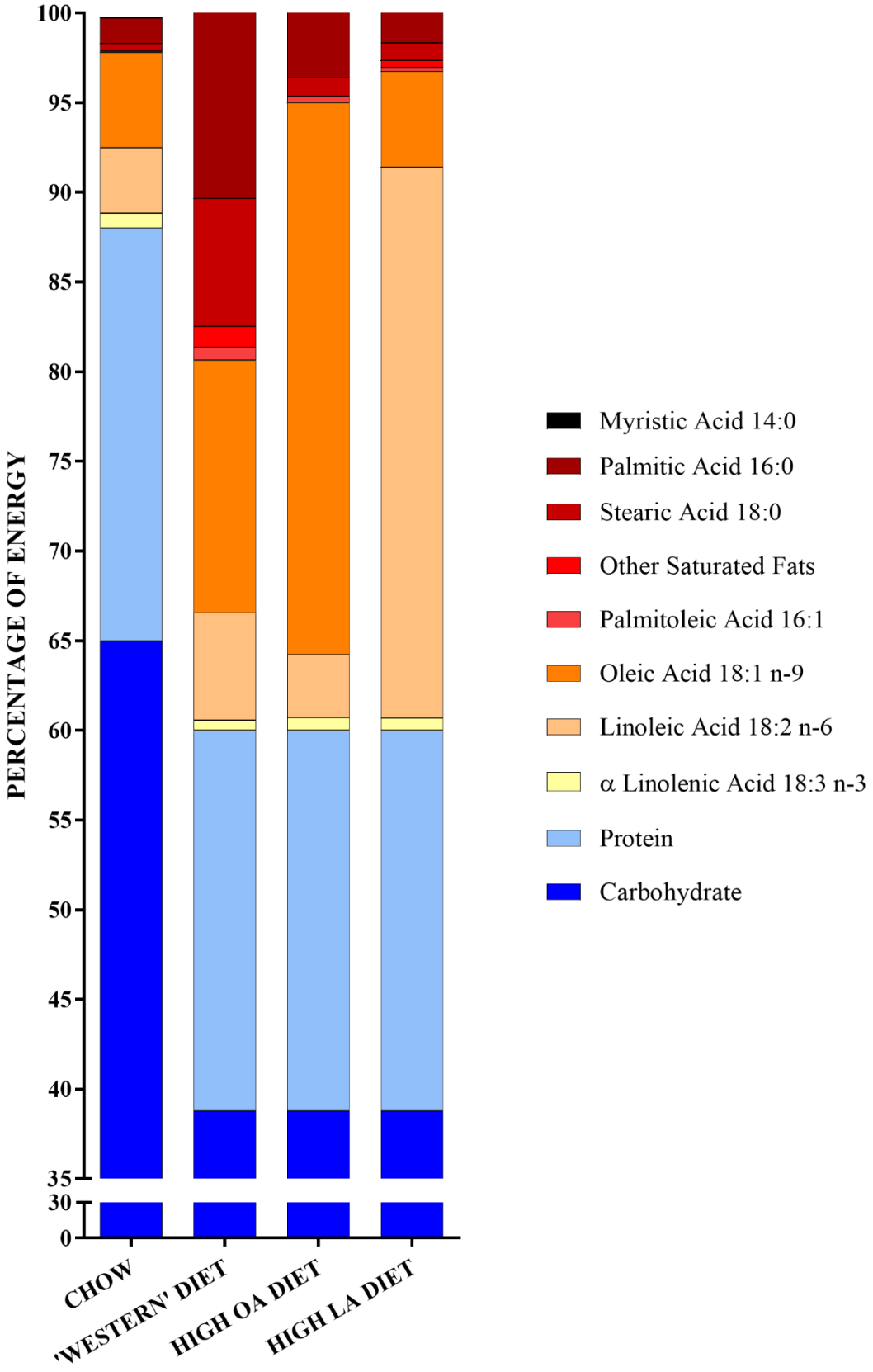

Figure 4.1: Energy ratios of experimental diets. The contribution of carbohydrate, protein and specific fatty acids are shown as percentage of total digestible energy.

Table 4.2: Calculated Fatty Acid Composition

	CHOW	WESTERN	HIGH OA	HIGH LA
Saturated Fats ≤ C12:0	-	0.07 %	TRACE	TRACE
Myristic Acid 14:0	0.03 %	0.32 %	TRACE	0.03 %
Palmitic Acid 16:0	0.50 %	5.57 %	2.29 %	1.31 %
Stearic Acid 18:0	0.14 %	3.61 %	0.53 %	0.49 %
Other Saturated Fats	-	0.20 %	TRACE	0.17 %
Palmitoleic Acid 16:1	0.01 %	0.36 %	0.17 %	0.11 %
Oleic Acid 18:1 n-9	1.90 %	7.10 %	15.76 %	2.72 %
Gadoleic Acid 20:1	0.03 %	0.15 %	0.08 %	0.06 %
Linoleic Acid 18:2 n-6	1.30 %	3.02 %	1.80 %	15.65 %
α-Linolenic Acid 18:3 n-3	0.30 %	0.29 %	0.36 %	0.35 %
Arachidonic Acid 20:4 n-6	0.01 %	-	-	-
EPA 20:5 n-3	0.02 %	-	-	-
DHA 22:6 n-3	0.05 %	-	-	-
Total n-3	0.37 %	0.33 %	0.37 %	0.35 %
Total n-6	1.31 %	3.05 %	1.82 %	15.65 %
n-6:n-3	3.54	9.24	4.92	44.7
Total MUFA	2.00 %	7.68 %	16.01 %	2.97 %
Total PUFA	1.77 %	3.48 %	2.18 %	16.00 %
Total SFA	0.74 %	9.76 %	2.82 %	2.00 %
Protein	20.00 %	22.60 %	22.60 %	22.60 %
Total Fat	4.80 %	21.00 %	21.00 %	21.00 %
Crude Fibre	4.80 %	5.40 %	5.40 %	5.40 %
AD Fibre	7.60 %	5.40 %	5.40 %	5.40 %
Digestible Energy mJ/kg	14.0	18.8	18.8	18.8
Total energy from lipids	12.00 %	41.00 %	41.00 %	41.00 %
Total energy from protein	23.00 %	21.20 %	21.20 %	21.20 %
Total digestible energy from carbohydrate	65.00 %	37.8 %	37.8 %	37.8 %

OA; Oleic acid, LA; Linoleic acid, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid.

4.3.3 Weight and body composition assessment

Animals were weighed daily throughout the study. Body composition (whole body fat mass and lean tissue mass) was measured at weeks 9, 12 and 15 using Echo MRI (Echo-MRI™ 900, Houston, Texas, U.S.A.).

4.3.4 Blood pressure measurement

Blood pressure was measured in weeks 9 and 14 using a CODA (Kent Scientific, Connecticut, USA) mouse and rat tail-cuff system, a non-invasive method which provides accurate data using a validated technique (Daugherty et al., 2009). Briefly, animals were secured in an appropriate size holder with nose cone, and acclimatised in an incubator set to 32 °C to encourage blood flow in the tail. Following this the occlusion tail cuff and the volume pressure recording (VPR) sensor cuff were fitted and blood pressure was measured in 30 second intervals.

4.3.5 Glucose Tolerance testing

Glucose tolerance testing was performed at the beginning of weeks 9 and 14 using the protocol developed by Jayasooriya et al. (2008) and modified by Jenkin et al. (2016). Briefly, animals were fasted overnight for approximately 16 hours, had a fasting blood glucose measurement taken using the tail snip method and administered 2 g/kg body weight of glucose in saline solution via intraperitoneal injection. Blood glucose measurements were taken using a hand-held glucometer (Optimum, Xceed, Abbott, USA) at 15, 30, 60, 90 and 120 minutes after administration of glucose.

4.3.6 Insulin Sensitivity testing

Insulin sensitivity testing was performed at the end of weeks 9 and 14 following the protocol of Stengel et al. (2013). Animals were fasted for 2 hours with access to drinking water maintained, a fasting blood glucose concentration was taken as described in section 4.3.5. and 1.0 U/kg of body weight of Insulin (Humalog, Eli Lilly, Indianapolis, Indiana, United States of America) was administered.

4.3.7 Euthanasia and Tissue Collection

At the end of week 9 (9 week DIO group) and week 15 (all other groups) animals were deeply anaesthetised with Isoflurane (Isoflo, Abbott Laboratories, Animal Health Division, Illinois, USA) and the extensor digitorum longus (EDL) muscles were removed, followed by the soleus muscles, with each muscle removed from tendon to tendon. Following this procedure pneumothorax, cardiac puncture, and removal of the heart was performed. Once dead, perirenal, epididymal white adipose tissue samples and subscapular brown adipose tissue samples were collected into cryotubes and rapidly frozen in liquid nitrogen for subsequent analysis. Other organs and tissues were also collected for future analysis outside of the present study. The 9 week DIO tissues were not analysed further.

4.3.8 Adipose tissue RNA extraction and quantification

Rat adipose tissue was homogenised in TRIzol (Invitrogen, Carlsbad, CA) with ceramic beads using a Fast Prep tissue homogeniser (FP120 cell disruptor, Electron Corporation, Milford, USA). Following this RNA was extracted using a protocol developed by Chomczynski and Sacchi (2006) and modified by Cavuoto et al. (2007) and the RNA content was quantified using nanodrop spectrometry (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE, USA). Samples were diluted with Diethyl pyrocarbonate (DEPC) treated H_2O to contain 0.5 µg of RNA per 7.5 µL, and DNA was reverse transcribed to yield complementary DNA (cDNA) using an iScript cDNA synthesis kit (Biorad Laboratories Inc. Hercules, California, USA) and then further diluted with DEPC treated H_2O for 'Real Time' Polymerase Chain Reaction (PCR) quantification. Further details regarding this process are contained in Chapter 2. PCR was performed using a BioRad MY iQ® Real-Time PCR detection system and analysis of gene expression was performed using BioRad IQ5 Gene analysis software (BioRad Laboratories, Hercules, California, USA 2009, version 2.1.97.1001) and Microsoft Excel (Microsoft, Redmond, Virginia, USA). The threshold cycles of gene expression for individual samples were compared to the average of 3 housekeeping genes for that sample. An average of 3 genes

was used as this helps to remove analysis bias due to unexpected alterations in housekeeping gene expression (Vandesompele et al., 2002). Housekeeping genes used for adipose tissue analysis were β-Actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine guanine phosphoribosyl transferase 1, (HPRT1), with further information shown in Table 4.3. These genes were chosen due to the stability of their expression levels in this tissue type (Svingen et al., 2015; Thellin et al., 1999). Gene expression relative to that of the average of the expression of the housekeeping genes was performed using the $2^{-\Delta\Delta C_T}$ method which has been validated and is a widely used and acknowledged method of data analysis (Schmittgen & Livak, 2008).

Table 4.3: Primer Sequences

GENE	ACCESSION NUMBER	SEQUENCE
β-Actin	NM_031144	Forward (5'-3') CTAAGGCCAACCGTGAAATGA Reverse (5'-3') CCAGAGGCATACAGGGACAAC
GAPDH	XM_017593963.1	Forward (5'-3') AGTTCAACGGCACAGTCAAG Reverse (5'-3') GTGGTGAAGACGCCAGTAGA
HPRT1	NM_012583.2	Forward (5'-3') GCAGACTTTGCTTTCCTTGG Reverse (5'-3') AGAGGTCCTTTTCACCAGCA
PPARγ	NM_013124.3	Forward (5'-3') TTCAGAAGTGCCTTGCTGTG Reverse (5'-3') CCAACAGCTTCTCCTTCTCG
FAT/CD36	NM_031561.2	Forward (5'-3') GACCATCGGCGATGAGAAA Reverse (5'-3') CCAGGCCCAGGAGCTTTATT
PPARα	NM_013196.1	Forward (5'-3') TGTCGAATATGTGGGGACAA Reverse (5'-3') ACTTGTCGTACGCCAGCTTT

βActin; beta actin, GAPDH; Glyceraldehyde 3-phosphate dehydrogenase, HPRT1; Hypoxanthine Phosphoribosyltransferase 1, FAT/CD36; Fatty Acid Translocase/ Cluster of Differentiation 36, PPARα; Peroxisome proliferator-activated receptor alpha, PPARγ; Peroxisome proliferator-activated receptor gamma

4.3.9 Statistical Analysis

All statistical analysis was performed using Prism GraphPad (Version 7, GraphPad Software, San Diego, California, USA). Results were considered significant when $p < 0.05$. All data is presented as ± standard error of the mean (SEM). Changes in body weight and energy intake over the experimental period were determined using a 2-way ANOVA with Tukey's test of multiple comparisons. Energy efficiency (g gained/mJ consumed) over experimental period was calculated using energy intake (mJ/week) (calculated based on suppliers nutritional composition information and weighed food disappearance measured daily) and weekly weight gain (measured daily), using a formula adapted from Bellinger et al. (2004). For gene expression, samples were considered outliers if the average of the triplicates was greater than

2 standard deviations from the average of that diet group. One way ANOVA was used to compare gene expression between diet groups within each fat depot. One way ANOVA was also used to determine differences between body composition, blood pressure, fat pad/organ weights, fasting blood glucose and AUC responses for GTT and IST.

4.4 Results

As shown in Figure 4.2 there were no significant differences in weight gain between the chow and high fat fed animals during the experimental period. The ‘Western’ diet group that were switched to a chow diet at the beginning of the experimental group had a significantly lower rate of weight gain compared to all other diet groups from week 12 onwards. The slight decline in weight gain seen at week 5 can be attributed to the animals being fasted overnight for glucose tolerance testing. When comparing percentage of total weight gain over the experimental period, as shown in Figure 4.3, the ‘Western’ diet then chow group had a significantly lower total weight gain when compared to all other diet groups.

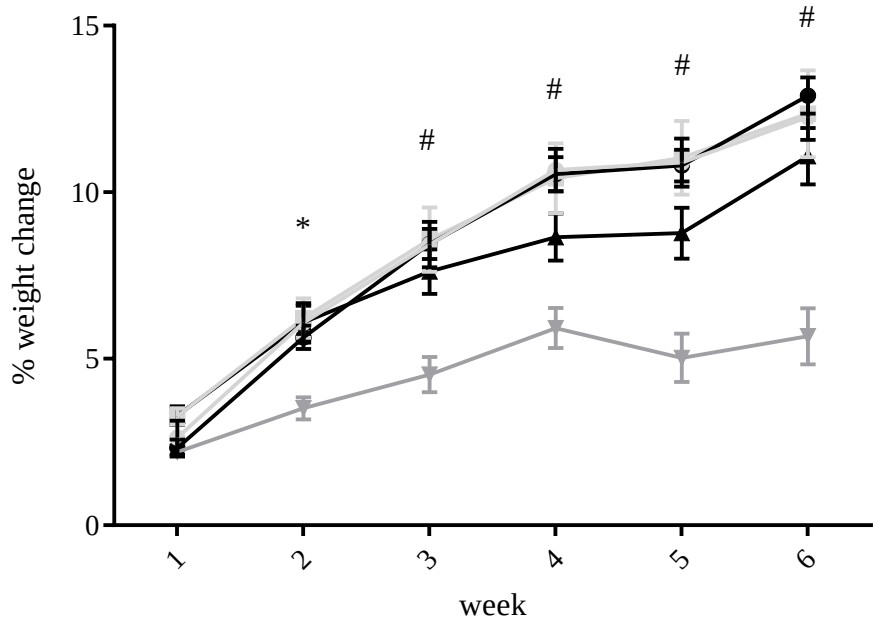

Figure 4.2: Percentage weight change over experimental period. Cumulative percentage weight change is expressed in relation to animal body weight on the first day of the experimental period. ● = chow fed group (n= 9) ● = 'Western' diet group (n= 10), ■ = high OA diet group (n= 10), ▲= high LA group (n= 10), ▼ = 'Western' diet then chow group (n= 9), all data shown as mean ± SEM. * = 'Western' diet then chow significantly different to chow, high OA and high LA diets at this time-point. # = 'Western' diet then chow significantly different to chow, 'Western' diet, high OA and high LA diets at this time-point.

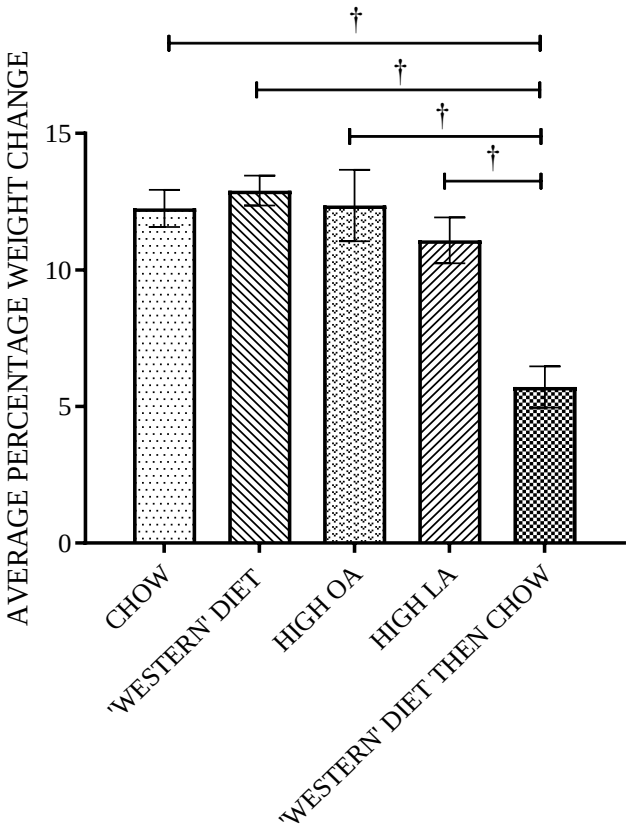

Figure 4.3: Average percentage weight change over experimental period. Average percentage weight change is expressed in relation to animal body weight on the first day of the experimental period. Data shown as mean ± SEM. †= significant difference between diet groups.

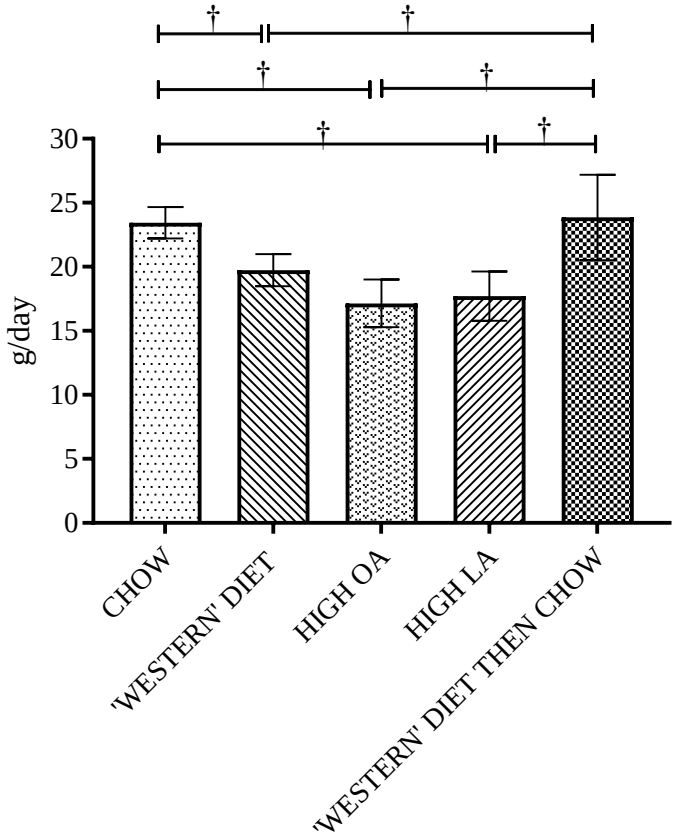

Figure 4.4: Average daily food intake (grams) per day during the experimental period. Food intake was weighed daily. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. † = significant difference between diet groups.

The average daily food intake in grams did not differ between the high fat fed animals. The chow and 'Western' diet then chow animals consumed a significantly higher amount of food when compared to the high fat fed animals, as shown in Figure 4.4.

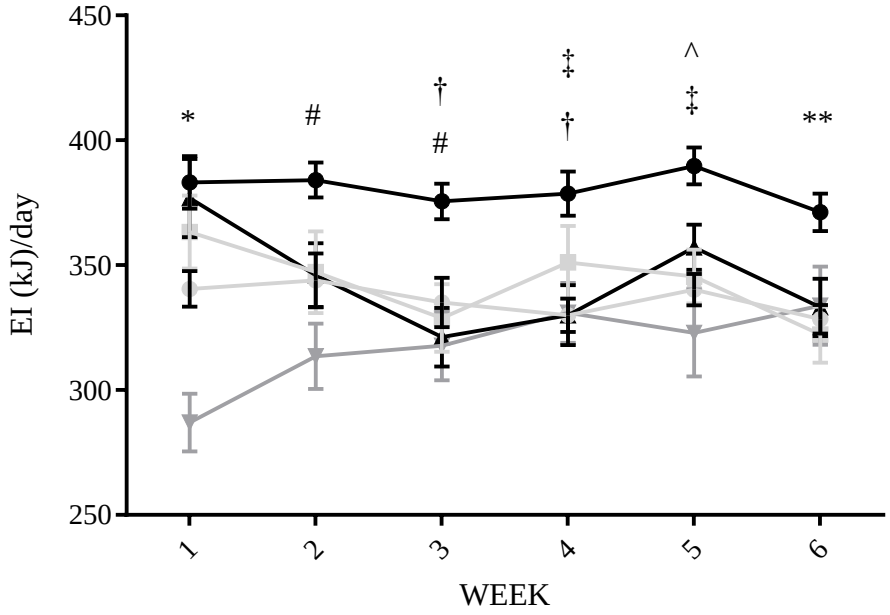

Figure 4.5: Energy Intake (kJ/day) over experimental period. Energy intake (kJ/day) was calculated based on suppliers nutritional composition information and weighed food intake measured daily. ● = chow fed group (n= 9) ● = 'Western' diet group (n= 10), ■ = high OA diet group (n= 10), ▲= high LA diet group (n= 10), ▼ = 'Western' diet then chow group (n= 9), data shown as mean ± SEM. * = 'Western' diet then chow significantly different to chow, 'Western', high OA and high LA diets at this time-point. # = 'Western' diet then chow significantly different to 'Western' diet group at this time-point. †= 'Western' diet then chow and high LA diets significantly different to the 'Western' diet group at this time-point. ‡= chow significantly different to 'Western' diet at this time-point. ^ = 'Western' diet then chow and high OA diet significantly different to 'Western' diet at this time-point. ** = high OA diet significantly different to the 'Western' diet at this time-point.

Throughout the experimental period there were numerous significant differences in energy intake between the dietary groups, as shown in Figure 4.5. Most commonly these were between the group switched to a chow feed from the 'Western' diet at the beginning of the experimental period, with this group having a significantly lower energy intake than all other dietary groups in the first week, and lower energy intakes than the 'Western' diet group for weeks 2- 5. Additionally, the high LA diet group had a significantly lower energy intake compared to the 'Western' diet group during weeks 3 and 4, the chow group having a significantly lower energy intake than the 'Western' diet group during weeks 4 and 5 and the high OA diet group having a significantly lower energy intake than the 'Western' diet group during the 5th and 6th weeks.

Energy efficiency (ratio of weight gained to energy consumed) was calculated for each week of the treatment period. As expected, the 'Western' diet then chow group had a significantly lower energy efficiency than all other diet groups in the 2nd week of treatment, potentially due to the change in diet and texture of diet. This was sustained compared to the chow, 'Western' diet, and high OA diet at the 3-week time-point. At this time-point the high LA diet had a significantly greater energy efficiency when compared to the 'Western' diet. At week 4 the high LA diet was significantly more efficient than the chow diet. The high LA diet had significantly higher energy efficiency at week 6 when compared to the chow and high OA diets. At this time-point the 'Western' diet then chow diet also had a significantly lower energy efficiency when compared to the 'Western' and high LA diets, as shown in Figure 4.6.

At the beginning of the experimental period there were no significant differences in body fat percentages between the high fat fed animals, with the chow group having significantly lower body fat than the animals commencing the high LA diet and the 'Western' diet then chow group (data not shown). As shown in Figure 4.7 at the end of the experimental period the chow group had a significantly lower percentage body fat when compared to the 'Western', high OA and high LA diet groups. There were no significant differences in body fat percentage between the high fat diet groups. The 'Western' diet then chow diet group had a body fat percentage similar to that of the chow group, which was significantly lower than that of the high OA and high LA diet groups.

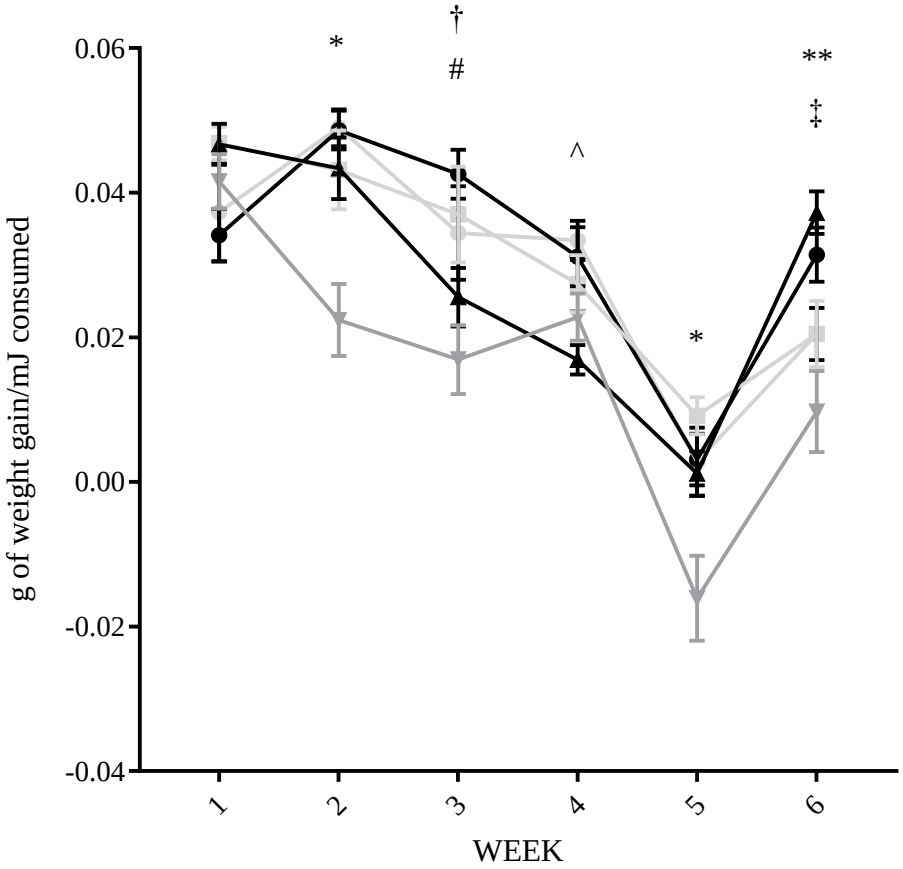

Figure 4.6: Energy efficiency (g gained/mJ consumed) during experimental period. Energy intake (mJ/day) was calculated based on suppliers' nutritional composition information and weighed food intake measured daily, formula adapted from Bellinger et al. (2004). ● = chow fed group (n= 9) ● = 'Western' diet group (n= 10), ■ = high OA diet group (n= 10), ▲= high LA diet group (n= 10), ▼ = 'Western' diet then chow group (n= 9), data shown as mean ± SEM. * = 'Western' diet then chow significantly different to all other diet groups at this time-point. # = 'Western' diet then chow significantly different to chow, 'Western' diet, and high OA diet at this time-point. †= high LA diet significantly different to 'Western' diet at this time-point. ^= chow diet significantly different to high LA diet at this time-point. ‡= high LA diet significantly different to chow and high OA diets at this time-point. **= 'Western' diet then chow diet significantly different to 'Western' diet and high LA diet at this time-point.

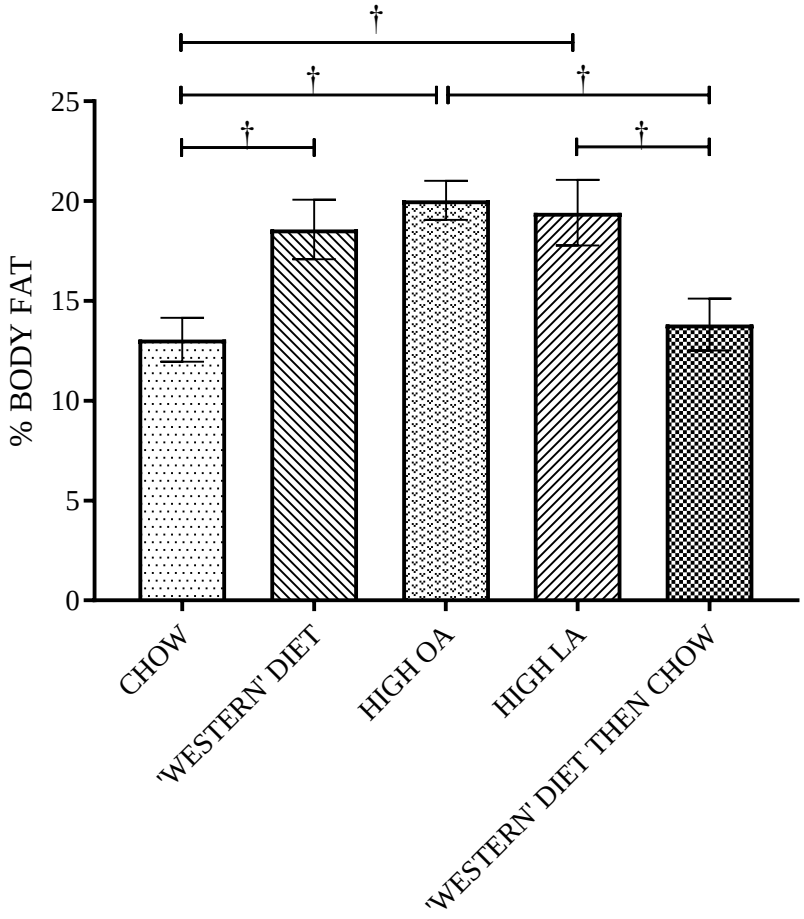

Figure 4.7: Percentage body fat as determined via Echo MRI. Echo MRI was performed in week 15 to determine percentage of body fat compared to lean mass. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. † = significant difference between diet groups.

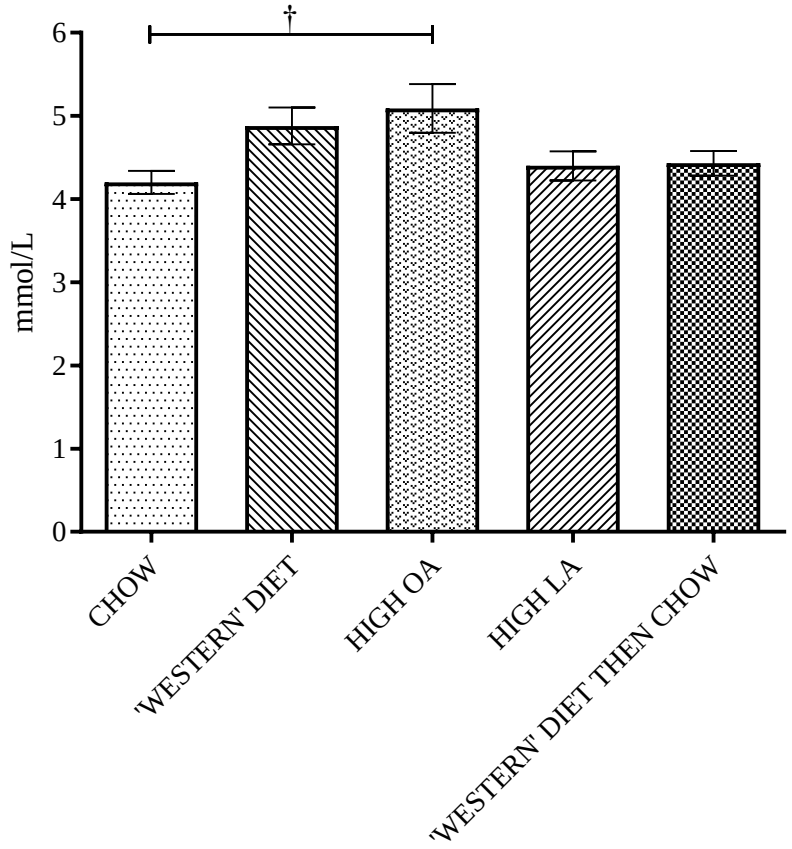

Figure 4.8: Fasting blood glucose levels determined during week 15. Blood glucose levels were measured after a 16 h fast. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. * = significant difference between diet groups.

Fasting blood glucose measurement was performed during week 15 after an approximately 16 hour fast. As shown in Figure 4.8 there were no significant differences between the high fat groups, though the fasting blood glucose levels for the chow animals was significantly lower than the high OA fed animals.

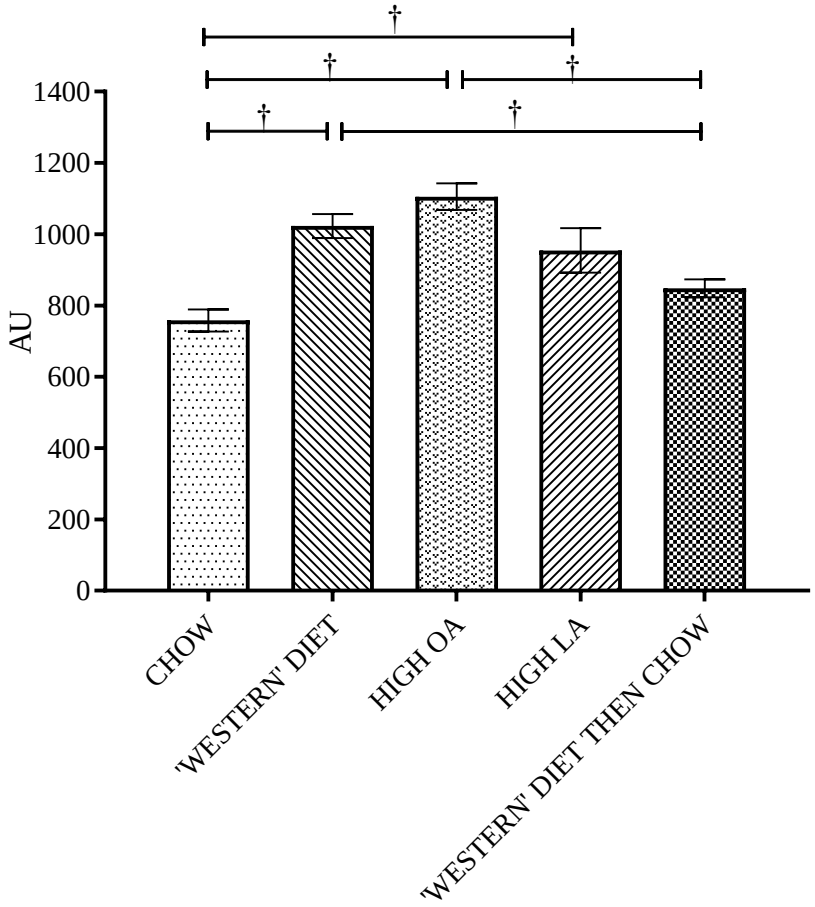

Figure 4.9: Area under the curve (AUC) of blood glucose levels during glucose tolerance testing during week 15. Blood glucose levels were measured every 30 minutes to determine response to intraperitoneal injection of 2 g/kg glucose solution. All data shown as mean ± SEM. Chow n= 8 due to exclusion of one animal due to a prior adverse event; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. AU= arbitrary units. †= significant difference between diet groups.

Blood glucose AUC for glucose tolerance testing completed in week 15 shows that the chow group had a significantly lower AUC when compared to the 3 high fat diet groups, as shown in Figure 4.9. The 'Western' diet then chow group also had a significantly lower AUC when compared to the 'Western' and high OA diet groups.

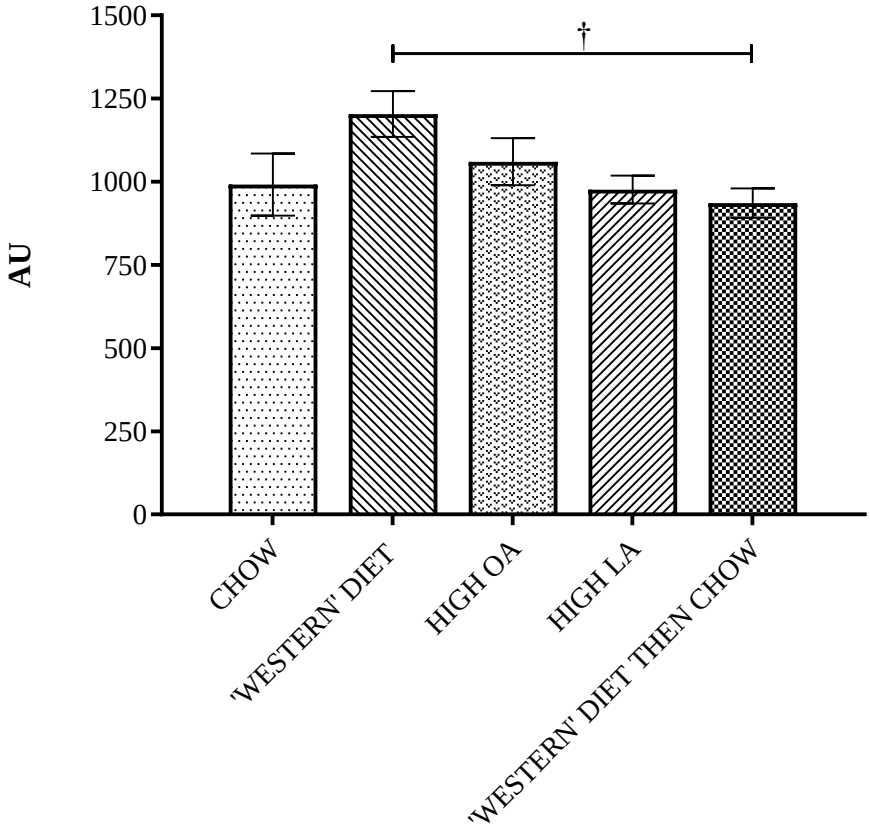

Figure 4.10: Area under the curve (AUC) of blood glucose levels during insulin sensitivity testing during week 15. Blood glucose levels were measured every 30 minutes to determine response to intraperitoneal injection of 1.0 U/kg insulin solution. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. AU= arbitrary units. †= significant difference between diet groups.

Insulin sensitivity testing found no significant differences in AUC of blood glucose responses between the high fat diet groups, as shown in Figure 4.10. The AUC for the 'Western' diet then chow group was significantly lower than the 'Western' diet group. There were no significant differences in AUC of blood glucose responses for insulin sensitivity testing when performed in week 9 (prior to experimental period) (data not shown).

Blood pressure measurement in week 14 showed no significant differences between diet groups for either systolic or diastolic blood pressure, as shown in Figures 4.11 and 4.12. There were also no significant differences when measurements were performed during week 9, nor was there a significant change between the two time-points for either measurement (data not shown).

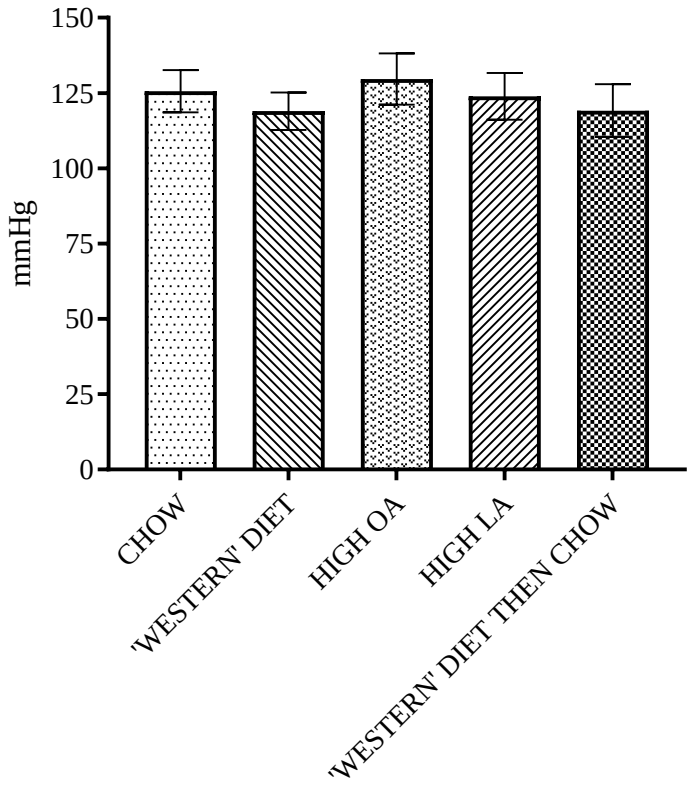

Figure 4.11: Systolic blood pressure measured in week 14. Displayed as millimetres of mercury (mmHg). All data shown as mean ± SEM. Chow n =8; 'Western' diet n= 10; high OA n = 10; high LA n= 9; 'Western' diet then chow n= 9.

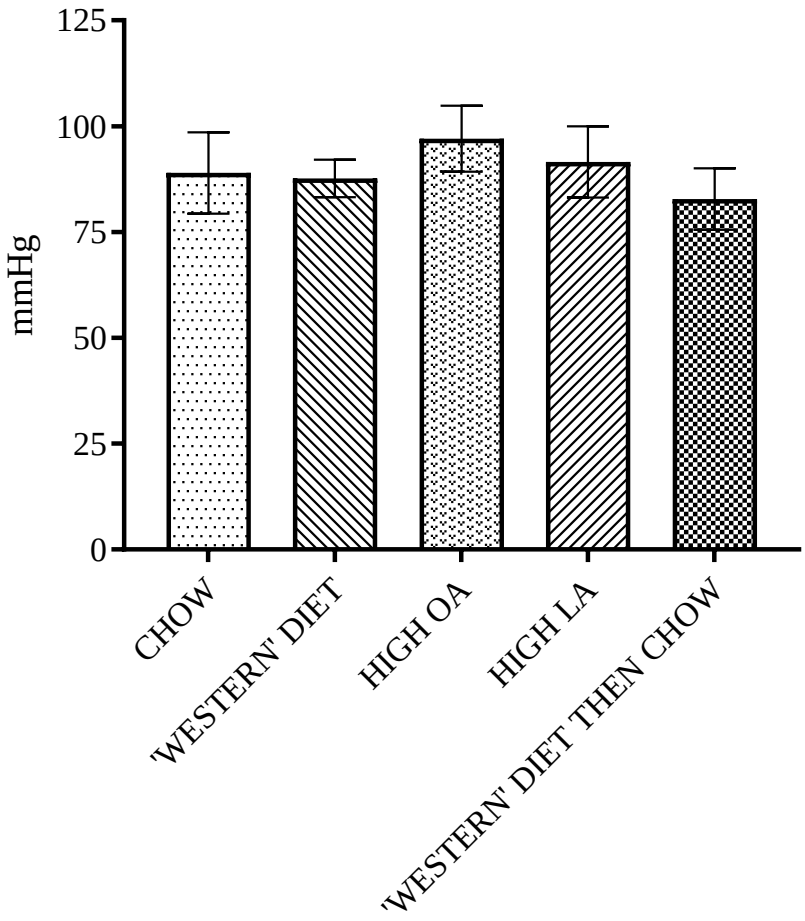

Figure 4.12: Diastolic blood pressure measured in week 14. Displayed as millimetres of mercury (mmHg). All data shown as mean ± SEM. Chow n =8; 'Western' diet n= 10; high OA n = 10; high LA n= 9; 'Western' diet then chow n= 9.

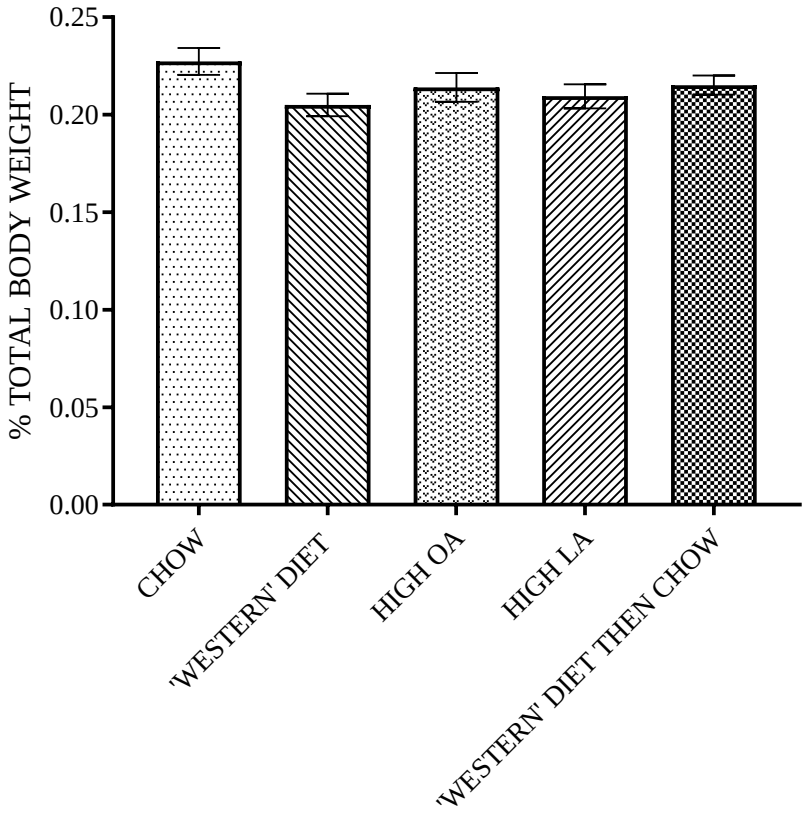

Figure 4.13: Weight of animals' hearts expressed as percentage of total body weight. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9.

As shown in Figure 4.13 there were no significant differences in heart weight when expressed as percentage of total body weight, though as shown in Figure 4.14 the livers of the high OA diet fed animals contributed a significantly greater proportion of total body weight when compared to the 'Western' diet then chow animals.

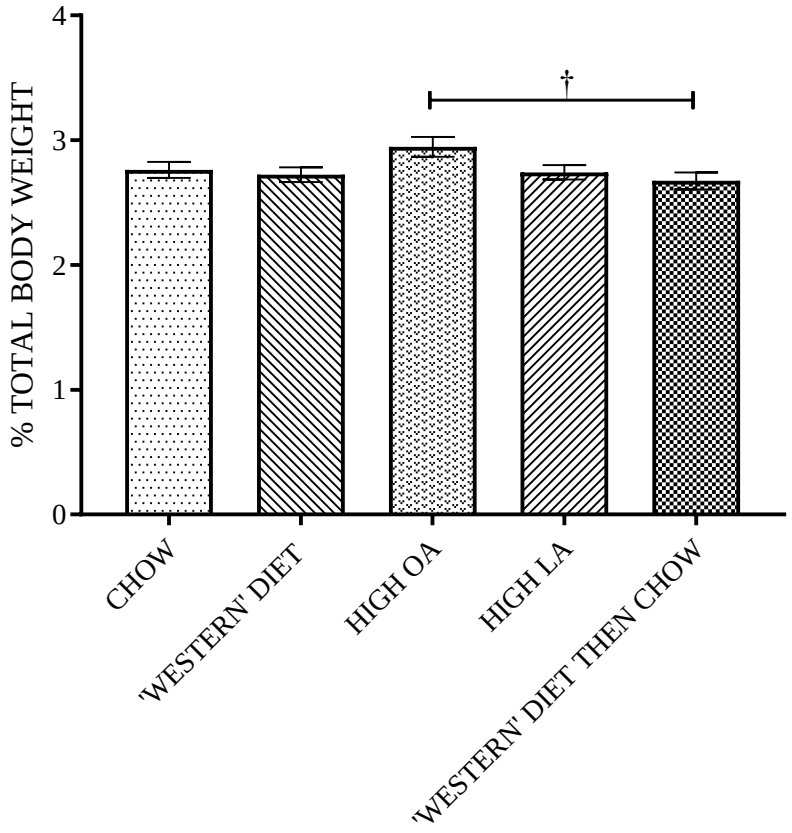

Figure 4.14: Weight of animals' livers expressed as percentage of total body weight. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. † = significant difference between diet groups.

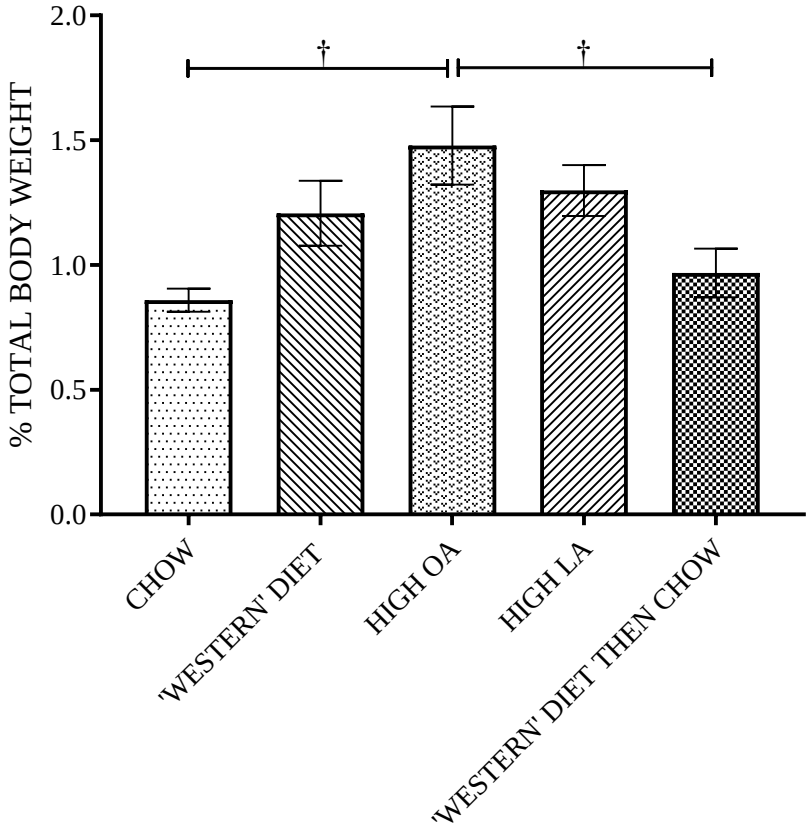

Figure 4.15: Weight of animals' epididymal fat pad expressed as percentage of total body weight. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. †= significant difference between diet groups.

The epididymal fat pad (as percentage of total body weight) was significantly heavier in the high OA diet group compared to the chow and 'Western' diet then chow diet groups (see Figure 4.15).

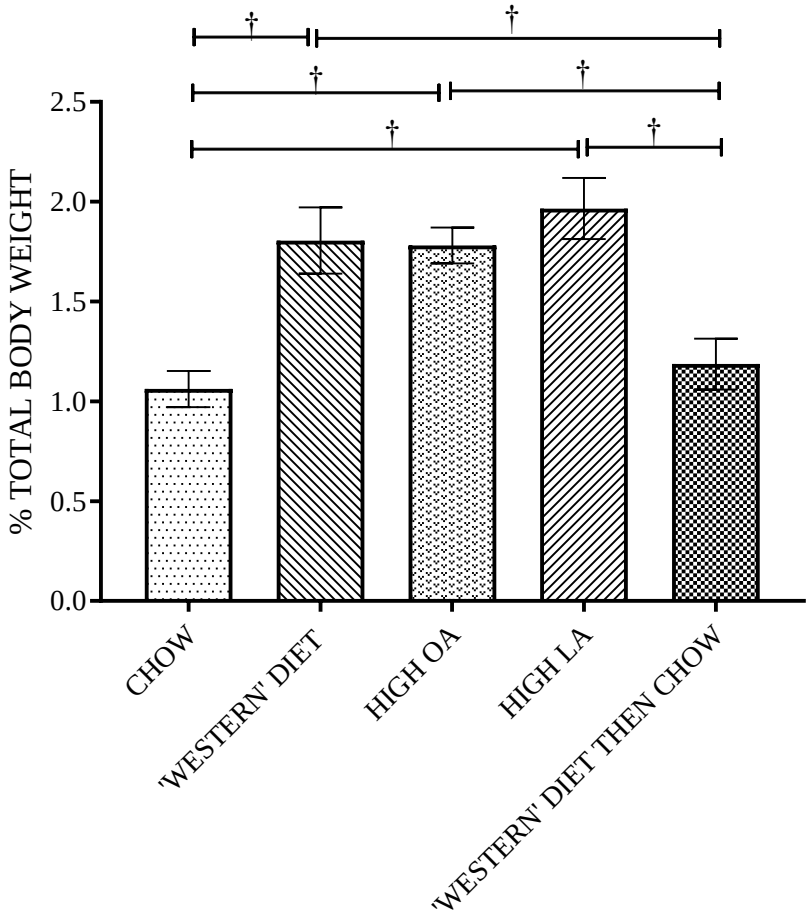

Figure 4.16: Weight of animals' perirenal fat pad expressed as percentage of total body weight. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. † = significant difference between diet groups.

When comparing perirenal fat pad weight as percentage of total body weight there were no significant differences between the high fat diet groups, as shown in Figure 4.16. The chow and 'Western' diet then chow groups had a significantly lower contribution of perirenal fat pad weight to total body weight when compared to all of the high fat fed animals.

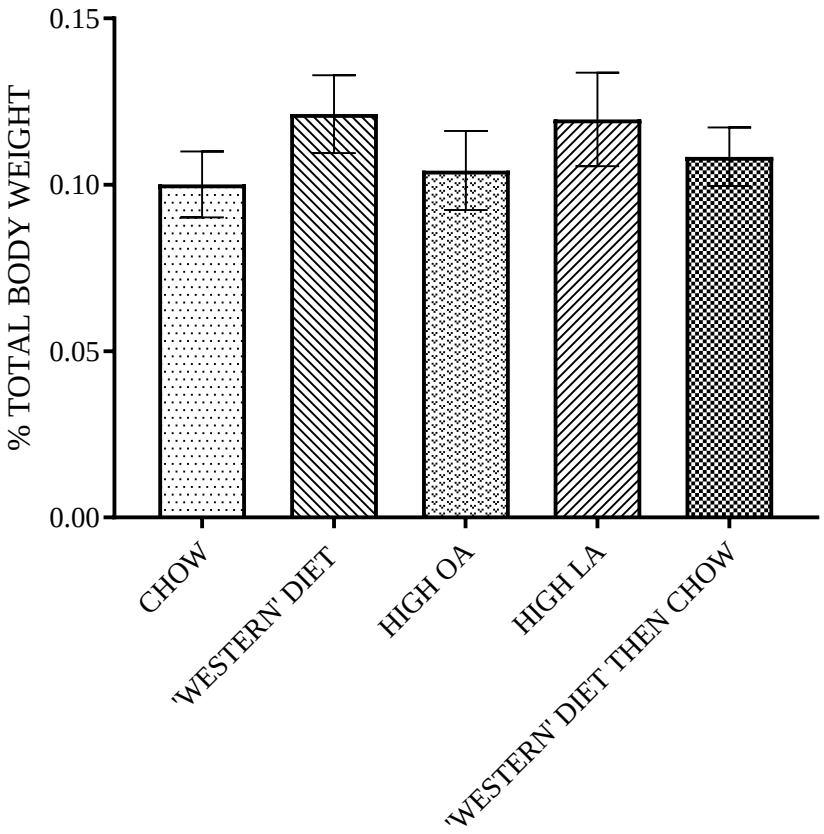

Figure 4.17: Weight of animals' subscapular brown adipose fat pad expressed as percentage of total body weight. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. * = significant difference between diet groups.

When comparing the contribution of subscapular brown fat pads there were no significant differences in contribution to total body weight, as shown in Figure 4.17.

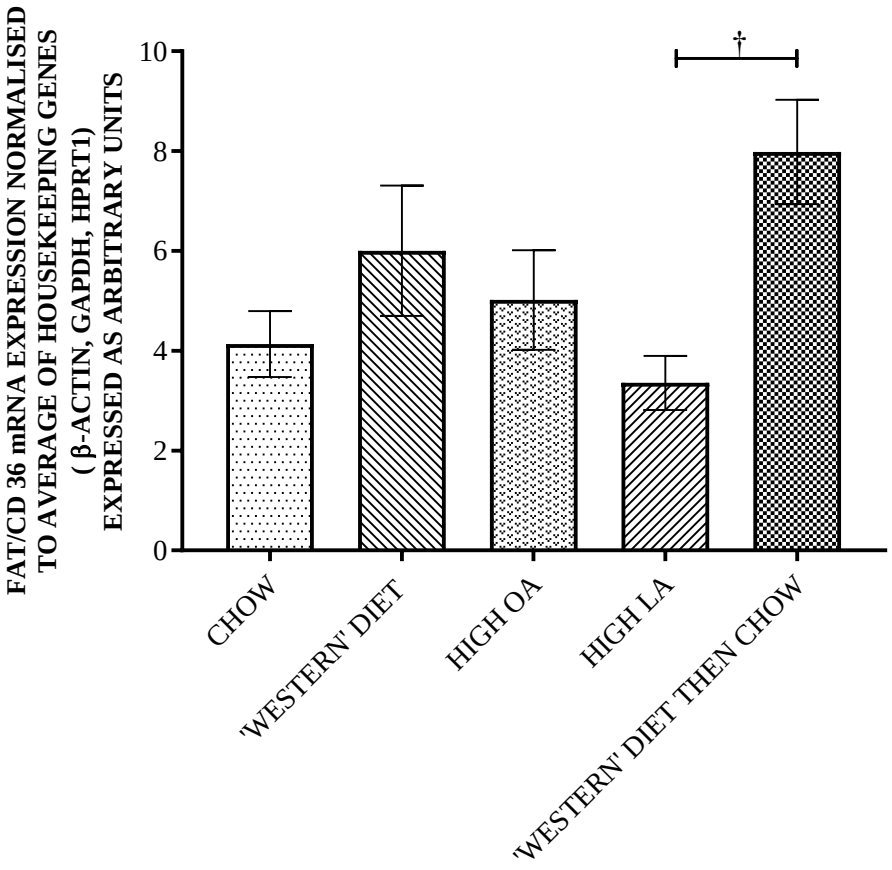

Figure 4.18: **Epididymal fat pad FAT/CD36 mRNA expression normalised to the average of housekeeping genes (β-Actin, GAPDH and HPRT1).** Shown as Arbitrary units. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. † = significant difference between diet groups.

As shown in Figure 4.18 expression of epididymal FAT/CD36 mRNA was significantly lower in the high LA diet group compared to the 'Western' diet then chow group. There was also a non-significant trend ($p = 0.066$) towards higher expression in the 'Western' diet then chow diet group when compared to the chow group.

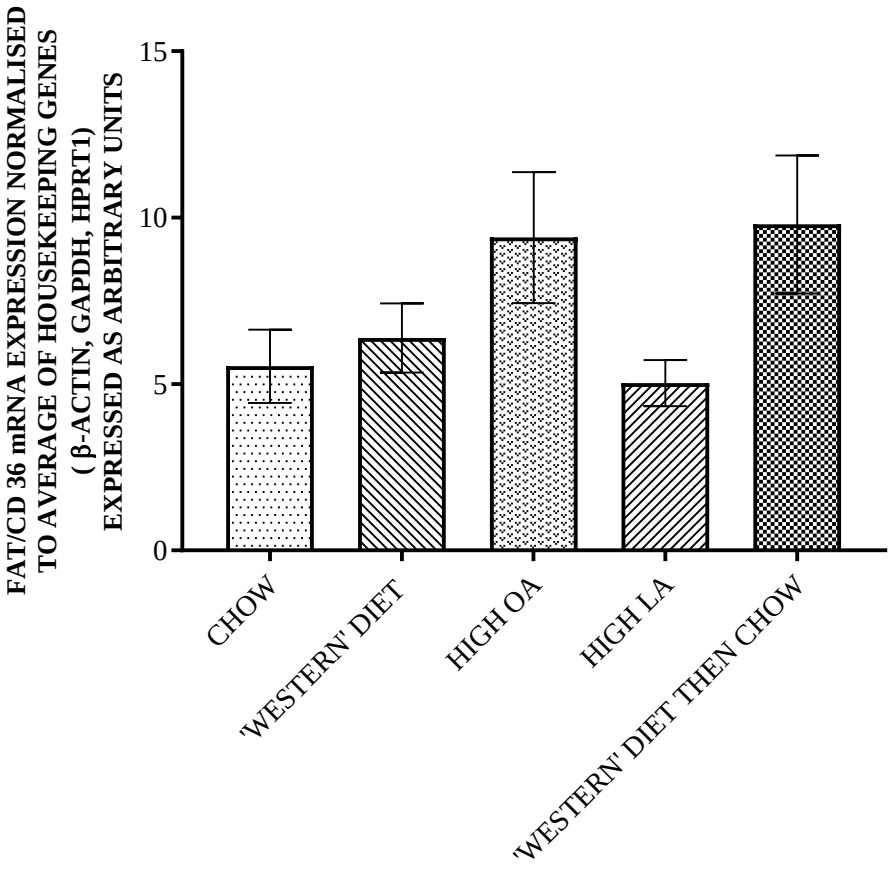

Figure 4.19: Perirenal fat pad FAT/CD36 mRNA expression normalised to the average of housekeeping genes (β-Actin, GAPDH and HPRT1). Shown as Arbitrary units. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9.

As shown in Figures 4.19 and 4.20 there were no significant differences in FAT/CD36 mRNA expression between the diet groups in either the perirenal white or subscapular brown adipose tissue depots.

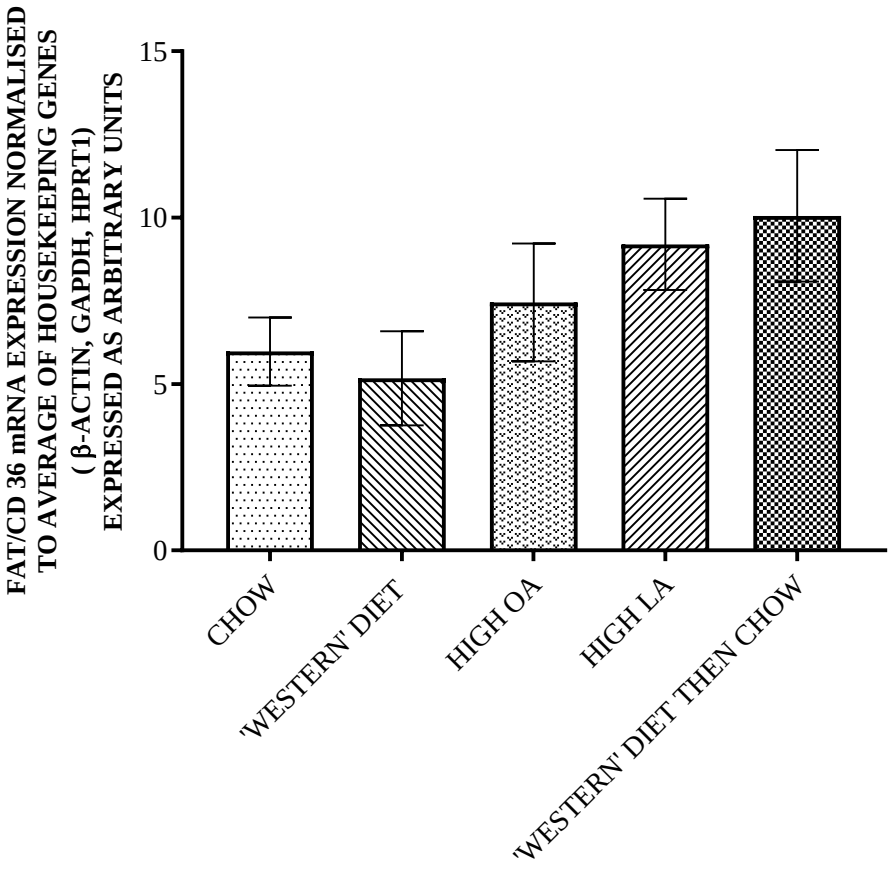

Figure 4.20: Subscapular brown adipose tissue fat pad FAT/CD36 mRNA expression normalised to the average of housekeeping genes (β-Actin, GAPDH and HPRT1). Shown as Arbitrary units. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9.

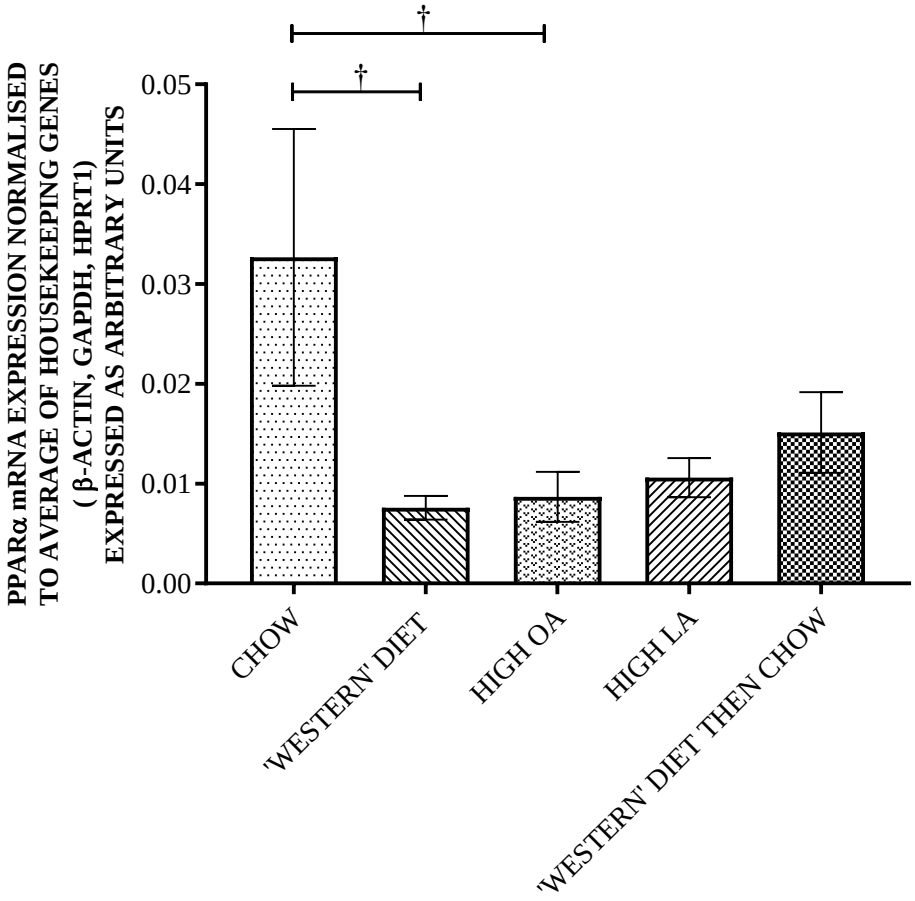

Figure 4.21: Epididymal adipose tissue PPARα mRNA expression normalised to the average of housekeeping genes (β-Actin, GAPDH and HPRT1). Shown as Arbitrary units. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. † = significant difference between diet groups.

Epididymal adipose tissue PPARα mRNA expression was increased in the chow diet compared to both the 'Western' diet group and the high OA diet group. As shown in Figure 4.21, there were no significant differences in expression between the high fat diet groups.

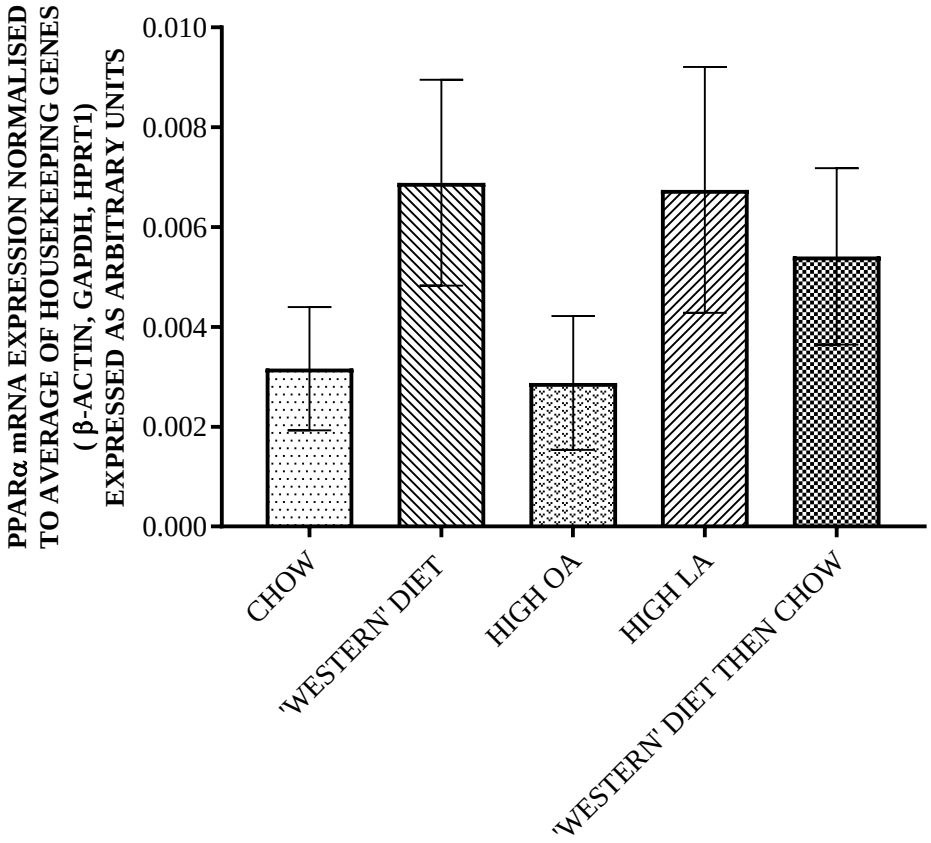

Figure 4.22: Perirenal adipose tissue PPARα mRNA expression normalised to the average of housekeeping genes (β-Actin, GAPDH and HPRT1). Shown as Arbitrary units. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9.

Perirenal white and subscapular brown adipose tissue PPARα mRNA expression was not affected by diet type in either depot, as shown in Figures 4.22 and 4.23.

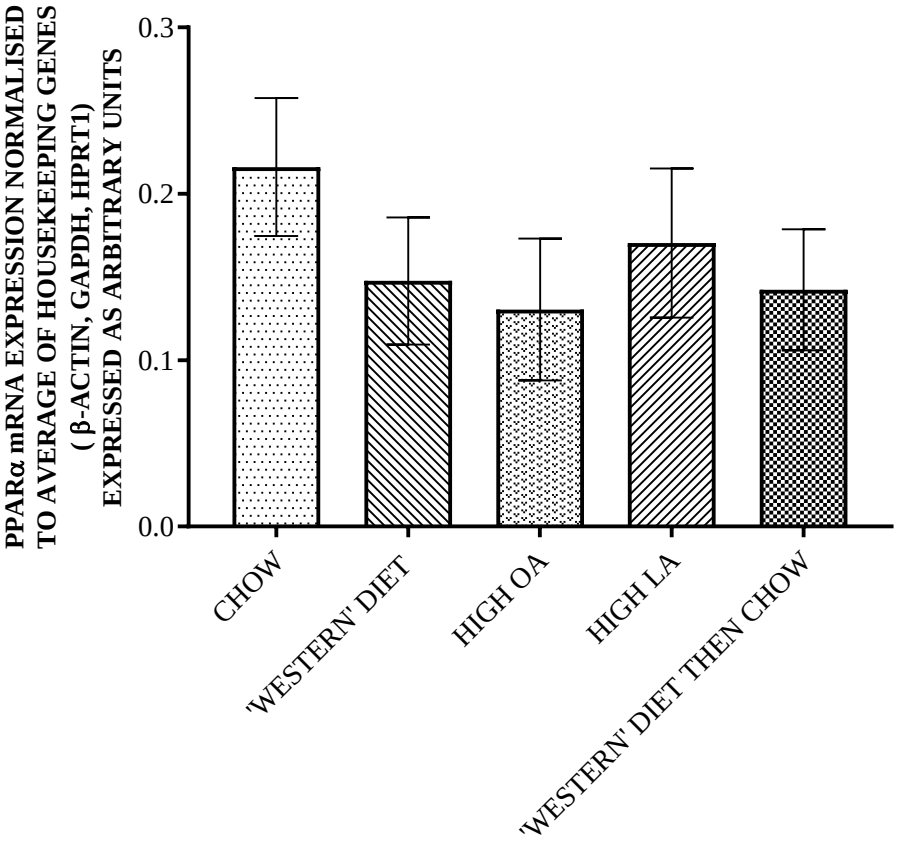

Figure 4.23: Subscapular brown adipose tissue PPARα mRNA expression normalised to the average of housekeeping genes (β-Actin, GAPDH and HPRT1). Shown as Arbitrary units. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9.

As shown in Figures 4.24- 4.26, diet did not alter expression of PPARγ mRNA in any of the adipose depots measured.

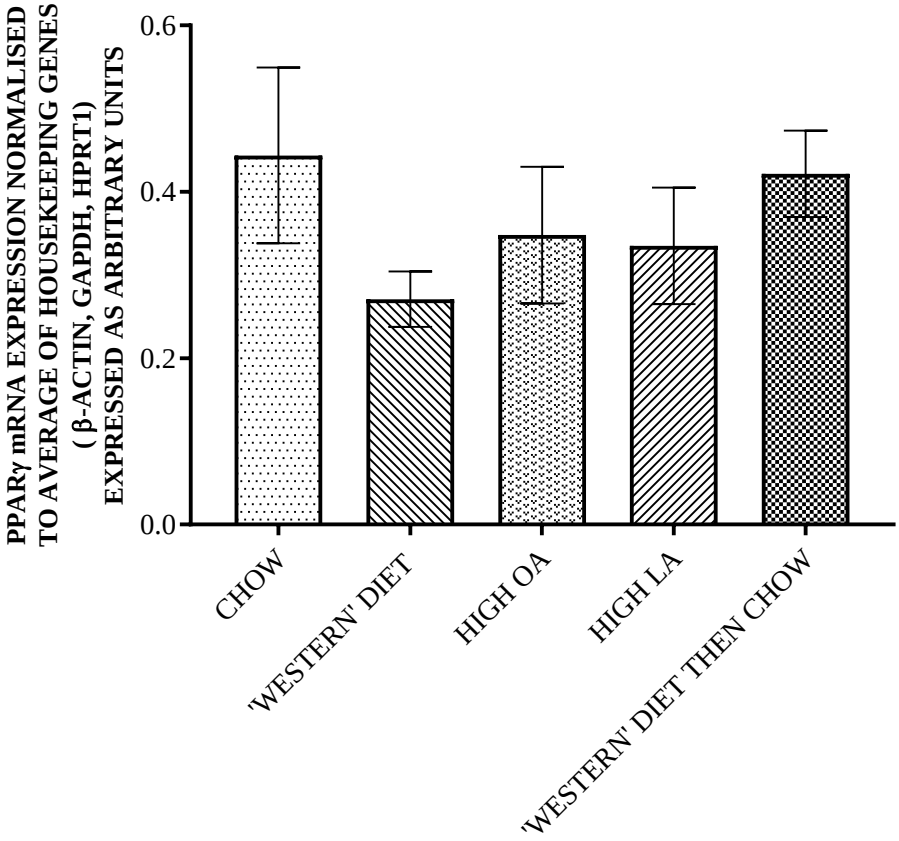

Figure 4.24: Epididymal adipose tissue PPARγ mRNA expression normalised to the average of housekeeping genes (β-Actin, GAPDH and HPRT1). Shown as Arbitrary units. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9.

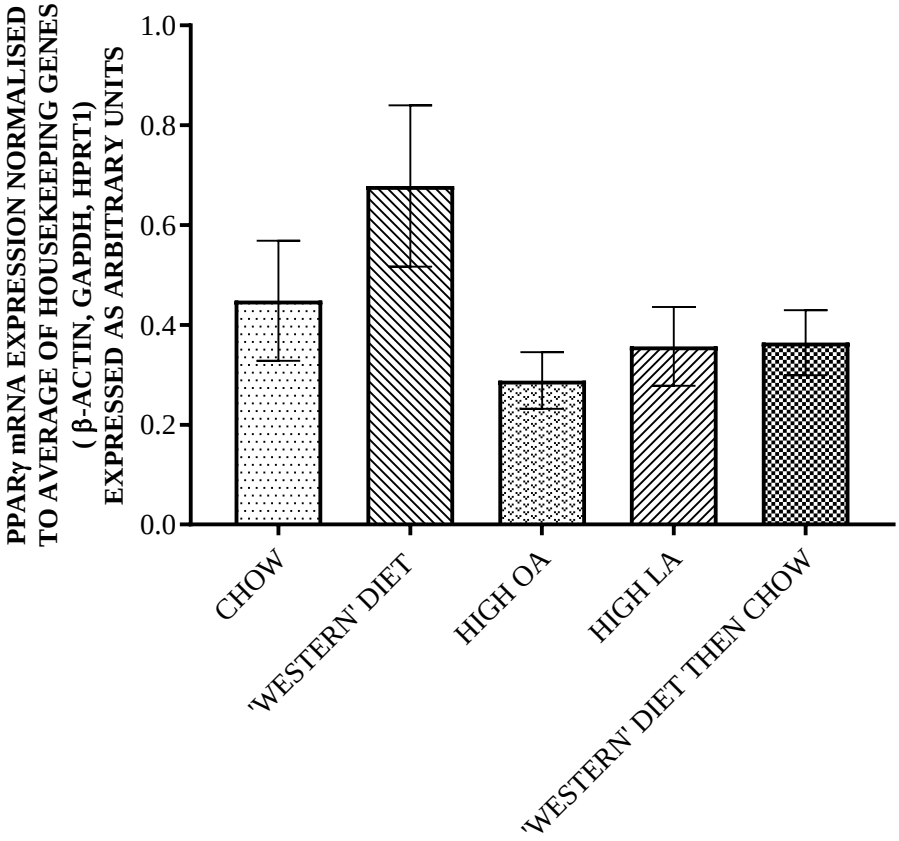

Figure 4.25: Perirenal adipose tissue PPARγ mRNA expression normalised to the average of housekeeping genes (β-Actin, GAPDH and HPRT1). Shown as Arbitrary units. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9.

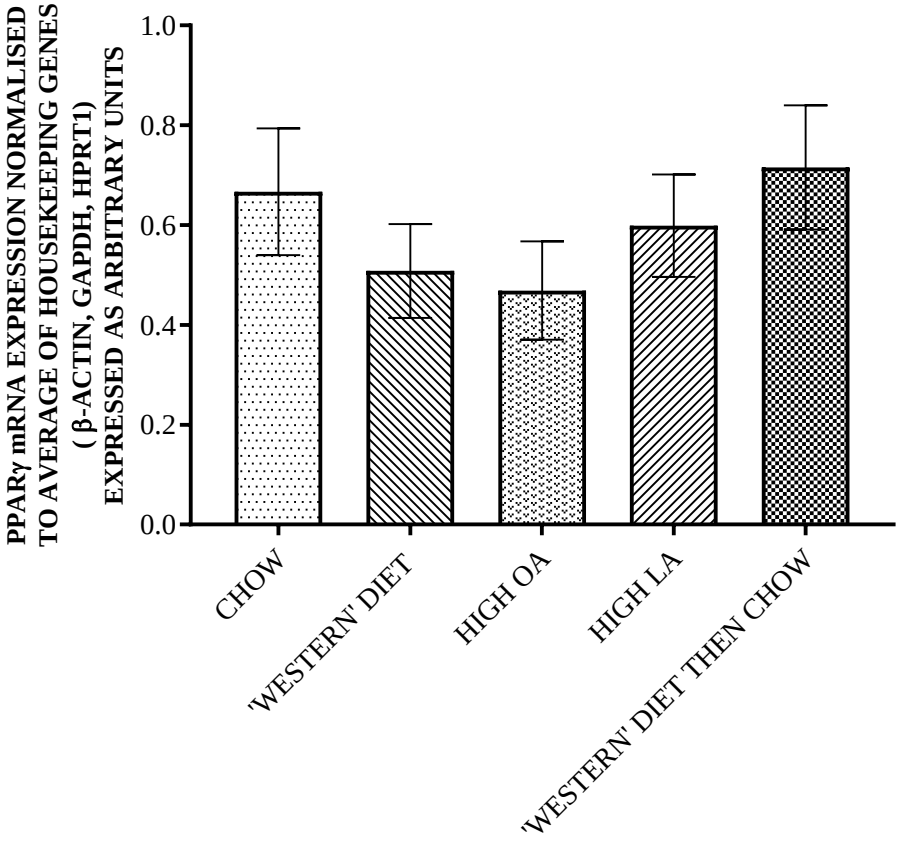

Figure 4.26: Subscapular brown adipose tissue PPARγ mRNA expression normalised to the average of housekeeping genes (β-Actin, GAPDH and HPRT1). Shown as Arbitrary units. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9.

4.5 Discussion

This study investigated the effect of different dietary fats on changes in body composition, metabolic health and adipose depot specific changes to expression of genes involved in adipose tissue metabolism in a rodent model of DIO. Additionally, this study aimed to investigate the changes to these parameters by weight loss in DIO by feeding a lower energy chow diet. Though this study found no significant differences between body weights, food intake, blood pressure or percentage body fat for the 3 high fat fed groups, this study does show that there is a potential for the type of dietary fat to modulate food intake and energy efficiency, with reductions in both of these occurring for the high OA consuming animals towards the end of the study. Additionally, DIO appears to down regulate epididymal adipose tissue PPARα mRNA expression regardless of FA type.

When comparing percentage body weight gain, the 'Western' diet then chow animals had a significantly lower rate of weight gain compared to all other diet groups from week 3 onwards. This group of animals had a reduction in body fat percentage over the experimental period to be on par with that of the chow group, indicating weight loss following DIO, a pattern previously documented in male Sprague Dawley rats which mimics human patterns of dietary restriction (Levin & Dunn-Meynell, 2000). The significantly lower energy intake for the group of animals switched to a chow diet from a 'Western' diet can be attributed to a reduction in the energy density of their feed, requiring a higher intake of food (as seen when comparing food intake in grams), and may also be influenced by the change in texture from a soft processed pellet to a hard grain based pellet, requiring greater energy expenditure to masticate (Oka et al., 2003). Though the 'Western' diet then chow and chow-only animals consumed a significantly greater volume of food than the high fat diet groups, the differing energy density of the chow diet (14 kJ/g compared to 18.8 kJ/g) did not result in significant differences in energy consumption.

At the end of the experimental period, the animals fed the chow diets had significantly less body fat than the high fat fed animals (with no difference between the high fat groups) with the 'Western' diet then chow animals having significantly less body fat than the high OA and LA diet fed animals, indicating obesity had been induced in the high fat fed animals. This lack of difference between diet groups is in contrast to previous research showing animals fed a high LA diet have a significantly higher percentage of body fat than other high diet fat fed animals (Ikemoto et al., 1996; Saidpour et al., 2011; Sospedra et al., 2015), which may have been influenced by the rodent strain or duration of the study.

In the last 2 weeks of the experimental period the high OA fed animals had a significantly lower energy intake compared to the 'Western' diet group which may be representative of a reduction in appetite, though in the final week of the experimental period the high OA diet fed animals had a lower energy efficiency when compared to the high LA diet animals, indicating that they were eating less, and were also gaining weight at a lower rate for the amount of energy consumed. As the intestinal absorption of OA and LA is comparable (99.8 % - 99.9 %) it is unlikely that malabsorption is a contributing factor (Javadi et al., 2004; Jones, Pencharz, & Clandinin, 1985). It is more likely that this may be due to the n-6:n-3 of the high OA diet, which was lower than both the 'Western' diet and the high LA diet, as lowering the n-6:n-3 has been associated with reduced adipose tissue deposition (Hassanali et al., 2010; Ruzickova et al., 2004), energy efficiency (Alvheim, Malde, et al., 2012) and food intake (Buckley & Howe, 2009). Though this only occurred in the final week of the experimental period, this finding is in line with results seen in male BALB/c mice fed a diet with 43 % of energy from fat, which showed a trend towards higher fat deposition in a LA fed group when compared to other 18C FA (including OA) (Javadi et al., 2004). Similarly, a study in male weaning Wistar rats (21 days old, with introduction of a standard chow formula) found that eight weeks consumption of a high soy oil (high LA) diet led to greater energy intake than animals consuming an

isoenergetic olive oil (high OA) diet (Saidpour et al., 2011). In humans a high OA intake has been associated with an increase in postprandial fat oxidation and diet induced thermogenesis (Soares et al., 2004). Interestingly, in a study utilising male Sprague Dawley rats, animals that had the carbohydrate content of their diets decreased by 15 % (without varying FA intake) had a significant reduction in body weight when fed a high OA diet, which did not occur in high LA fed animals, possibly indicating a greater rate of fat oxidation in OA fed animals (Jones et al., 1995).

When comparing the contribution of specific organs and fat depots to total body weight there were no significant differences in heart weight, though the high OA fed animals had significantly larger livers (as percentage of total body weight) when compared to the 'Western' diet then chow animals. This may indicate an increase in liver triglyceride deposition in the high OA fed animals, which has previously been observed in female apolipoprotein E knockout mice (Arbones-Mainar et al., 2007), or a reduction in liver size in the 'Western' diet then chow animals, as a result of energy restriction occurring from a reduction in feed energy density (Browning & Horton, 2004; Omagari et al., 2008). For the adipose depots measured there were no significant differences observed between dietary groups for the subscapular brown adipose depot, with the perirenal white adipose depot being significantly larger in the three high fat groups compared to the chow and 'Western' diet then chow animals, again demonstrating effective inducement of obesity. Greater fat storage in visceral depots, such as the perirenal and epididymal are associated with increased risks of CVD and T2DM (Bjørndal et al., 2011). When comparing epididymal white adipose depots the high OA fed animals had a significantly greater mass than the chow fed and 'Western' diet then chow fed animals. Though an increase in visceral fat deposition is associated with an influx of FA to the liver, the total body fat percentage in the OA group did not differ significantly when compared to the other high fat fed animals, so it is possible that this could be a compensation for a lower level of lipid storage

in another visceral fat depot not measured such as the mesenteric, omental or perivascular, or in subcutaneous deposits or the increased liver weight. Though there is lack of research on the role of OA on fat depot sizes in rodents, a low n-6:n-3, as this diet group had, has been found to reduce both epidydimal and perirenal fat pad weights (Bjørndal et al., 2011; Parrish et al., 1991), though this was not found in the current study, potentially due to the different n-6:n-3 or rodent strain used.

Fasting blood glucose levels measured in the final week of the study show that levels were elevated in the high OA consuming animals compared to the control animals, which may be a reflection of the increased epididymal depot, or liver size, potentially impairing glucose handling (Bjørndal et al., 2011; Samuel et al., 2004). This is in opposition to the findings of other studies that in DIO, a high OA diet preserves adequate glucose handling in male Wistar rats (Alsaif & Duwaihy, 2004). Despite this, high OA fed animals did not display a different response to the other high fat fed animals when administered a glucose challenge. Area under the curve for the 2 hour glucose tolerance test (GTT) found that the chow consuming animals had a significantly faster glucose clearance when compared to all of the high fat fed animals, with the 'Western' diet then chow animals having a significantly lower glucose area under the curve than the 'Western' diet and high OA diet fed animals and a significantly lower insulin sensitivity test (IST) blood glucose AUC when compared to the high fat fed 'Western' diet animals, indicating increased tissue insulin sensitivity. Taken together this indicates a potential improvement in glucose handling conferred by weight loss in this group, as would be expected (Kahn & Flier, 2000), though for the GTT there were no differences between the high fat fed animals, which goes against findings that in female C57BL/6 mice the LA content of the diet is correlated with higher blood glucose levels during GTT (Ikemoto et al., 1996). Similarly, in response to IST there were no significant AUC differences between the high fat fed animals, in contrast to research in male C57BL/6 mice which found that when a high fat (59 % of energy)

diet was supplemented with 2 g/kg body weight, LA insulin resistance was induced, which was not found in animals only consuming the high fat diet (Masi et al., 2012); this again may show the influence of rodent species on results.

Expression of FAT/CD36 mRNA in the perirenal white adipose and subscapular brown adipose depots was not influenced by diet type, though in the epididymal white adipose depot the 'Western' diet then chow fed animals had significantly increased expression when compared to the high LA diet fed animals, with a trend towards increased expression compared to the 'Western' diet fed animals. This may indicate that energy restriction in these animals may be triggering fat mobilisation from tissues such as the liver (which was significantly lighter compared to the high OA fed animals) for storage in adipose tissue as FAT/CD36 is involved in transport of long chain FA into cells (Coburn et al., 2000). Also, as FAT/CD36 is also involved in lipolysis in adipocytes (Wan et al., 2013) this increase in expression could also be mobilising FA for metabolism in other tissues.

Research conducted by Milan et al. (2002) found that in *ob/ob* Zucker rats high fat feeding did not alter PPARγ mRNA expression in subcutaneous or visceral adipose depots, though weight loss in obese animals results in an increase in epididymal PPARγ mRNA expression. This was not seen in this experiment, though the length of the weight loss period before measurement is not indicated by Milan et al. (2002) and changes in PPARγ expression can be transient (Lehrke & Lazar, 2005). Additionally, as FAT/CD36, whose expression was increased in this depot, is capable of modulating PPARγ levels in C57BL/6 mice (Hajri et al., 2007), an increase in PPARγ expression may have been expected. Activation of PPARγ in rat adipocytes also leads to increased high molecular weight adiponectin secretion (Olivares-Garcia et al., 2015), though in line with an absence of effect of diet on PPARγ mRNA expression, there were no significant differences between groups in plasma adiponectin concentrations (Anna Simcocks, unpublished observations).

Though PPARγ is involved in the differentiation of both WAT and BAT, PPARα expression is a distinctive marker of BAT due to the tissues high levels of lipid oxidation (Bonet et al., 2013), which did not seem to be affected by dietary FA source or weight loss in DIO, in the current study. Similar to the patterns of expression of both FAT/CD36 and PPARγ mRNA, the expression of PPARα mRNA in rat fat depots was unaffected by diet or reversal of obesity in both the subscapular brown adipose and perirenal white adipose depots. In the epididymal white adipose depot the chow fed animals had a significantly greater level of PPARα mRNA expression compared to the 'Western' and high OA diet fed animals, indicating obesity may down regulate expression. Interestingly, though the 'Western' diet then chow animals had a reduction in adiposity to be in line with that of the chow fed animals, PPARα mRNA expression was not restored to levels seen in the chow fed animals, indicating expression was not influenced by energy balance in the current study.

Results of this study indicate that 6 weeks of feeding different dietary FA in a high fat diet are capable of maintaining DIO, though not capable of inducing changes to body fat percentage. The animals who were switching from the 'Western' diet to the chow diet at the start of the experimental period showed a reduction in weight gain and body fat percentage and an increase in epididymal FAT/CD36 mRNA expression, which may demonstrate an influence of energy balance on FAT/CD36 mRNA expression. The animals fed a high OA diet had heavier livers compared to the 'Western' diet then chow animals and also higher fasting blood glucose compared to the chow animals, though this did not translate to differences in glucose handling between the high fat fed animals. Despite this, changes to food intake and energy efficiency in the high OA fed animals seen in the final week of the study may indicate beneficial results of higher OA or lower n-6:n-3 content diets. As these results were seen at the end of the study this indicates that a longer experimental period may have resulted in sustained differences between the high fat fed animals which has been observed in other models and in longer term studies.

Chapter 5: The effect of diet on genes regulating skeletal muscle fat metabolism and adiponectin signalling in a rodent model of diet induced obesity

5.1 Abstract

Background: Resting skeletal muscle metabolism has a direct influence on whole body metabolism and energy expenditure, though the rate of fatty acid (FA) oxidation in skeletal muscle from obese individuals is decreased. Moreover, high fat feeding has been found to impair the ability of adiponectin to stimulate lipid oxidation, leading to intramuscular lipid deposition and impairment of insulin stimulated glucose uptake. As such, the role of dietary fats in influencing skeletal muscle metabolism, and preserving adiponectin signalling, is deserving of investigation.

Methods: 60 male 7 weeks old Sprague Dawley rats were fed a 'Western' style high fat diet (21 % fat w/w) for 9 weeks to induce obesity. Obese animals were then randomly allocated to four treatment groups and fed either the 'Western' diet (n=10), a high OA diet (21 % fat w/w, 16 % OA w/w, n=10), a high LA diet (21 % fat w/w, 16 % LA w/w, n=10) or standard chow (5 % fat, n=10) for 6 weeks. 10 additional control animals were fed a standard chow throughout the experimental period. At the end of week 15 the animals were deeply anaesthetised and hind-leg extensor digitorum longus (EDL) and soleus muscles were removed, after which the animals were killed. Immediately following excision, muscles were incubated in Krebs-Henseleit buffer with or without 2.5 µg/ml of globular adiponectin for 30 minutes, alternating left and right muscles to receive adiponectin, then snap frozen for further analysis. RNA was extracted from portions of muscle and real time PCR was performed.

Results and Discussion: Feeding a high OA diet resulted in the maintenance of a lean phenotype of FAT/CD36 expression, with higher expression in the soleus compared to the EDL

which may prevent intramyocellular lipid accumulation in this tissue, which was not seen in the other high fat fed animals. Adiponectin incubation increased FAT/CD36, β-HAD and CPT1 mRNA expression in both the EDL and soleus from high OA fed animals, a response which was not seen in any of the other diet groups. This may indicate that high OA feeding preserves adiponectin sensitivity in both of these muscle types and may confer metabolic benefits not seen in the other high fat diets tested in this study.

5.2 Background

The modern 'Western' diet is typified by a high availability of energy and high levels of plant derived fats (Simopoulos 2006). In Australia, as shown in Chapter 3, changes in plant derived fats in the last 60 years can be attributed to an increase in predominately linoleic acid (LA) containing fats. During this time period we have also seen a dramatic increase in the incidence of overweight and obesity (Haby et al., 2011). As such the type of fats consumed has become a focus of research in finding ways in which metabolic health can be preserved. As skeletal muscle metabolism has been found to heavily influence systemic metabolic disease (Baskin et al., 2015), the role of dietary fats in influencing skeletal muscle glucose and fatty acid (FA) metabolism is deserving of attention.

The rate of FA oxidation in skeletal muscle from obese and diabetic individuals is decreased (He et al., 2001). The ability of specific dietary fats to alter expression of genes related to FA oxidation may provide a potential therapy to restore insulin functioning in skeletal muscle. High fat feeding has been found to impair the ability of adiponectin to stimulate lipid oxidation in the soleus muscle within days (Ritchie & Dyck, 2012). It is currently known in skeletal muscle that adiponectin activates and phosphorylates AMPKα (the α2 isoform more so over the α1), p38 MAPK, PPARα, CPT1 and β-HAD (Coletta et al., 2009; Yoon et al., 2006). Additionally adiponectin is capable of causing microvascular dilatation allowing greater endothelial surface area for insulin delivery and binding (Zhao et al., 2015). A negative correlation has been found between plasma globular adiponectin concentrations and insulin resistance and obesity in rodents, non-human primates (Hotta et al., 2001) and humans (Arita et al., 1999). Additionally, adiponectin resistance has been found to precede intramyocellular lipid accumulation and the loss of insulin stimulated glucose uptake (Mullen, Smith, Junkin, & Dyck, 2007). As such, interactions between adiponectin and particular FA in tissues, which are generally considered to reflect habitual dietary intake (Borkman et al., 1993), could provide

further knowledge regarding which dietary lipids are best for maintaining adiponectin signalling and therefore adequate metabolic health.

This study aimed to investigate the ability of different types of dietary fat to modulate expression of genes related to oxidative capacity in a rodent model of diet induced obesity (DIO). We also aimed to investigate whether specific dietary FA were able to restore adiponectin signalling or alter expression of genes related to oxidation or transport of FA by incubating excised muscles with globular adiponectin at physiologically relevant levels.

5.3 Methods

The experiments conducted in this Chapter are an extension of those performed in Chapter 4. Further detailed information relating to methodology can be found in Chapter 2.

5.3.1 Muscle sample collection

At the end of week 9 (9 week DIO) and week 15 (all other groups) animals were deeply anaesthetised with Isoflurane (Isoflo, Abbott Laboratories, Animal Health Division, Illinois, USA) and the extensor digitorum longus (EDL) muscles were removed, followed by the soleus muscles, with each muscle removed from tendon to tendon. Following this procedure pneumothorax, cardiac puncture, and removal of the heart was performed. After this other tissues and organs were collected, including those discussed in Chapter 4, and for further use in subsequent studies beyond those discussed in this thesis.

5.3.2 Muscle organ bath protocol

Immediately following the removal of soleus and EDL muscles, they were placed in individual chambers of a specially designed 20 chamber organ bath (Zultek Engineering, Melbourne, Australia). Left and right leg muscles were alternated for treatment with adiponectin between animals to remove bias. The protocol for the adiponectin treatment was adapted from Mullen et al., (2010). Briefly, treated muscles were incubated in a solution of Krebs- Henseleit buffer (to allow for metabolism to continue *ex vivo*) containing 2.5 µg/ml of globular adiponectin for 30 minutes. Untreated muscles were incubated in a solution of Krebs- Henseleit buffer without globular adiponectin. The chambers were kept at a constant 37 °C and pre-gassed with carbogen (95 % O_2 -5 % CO_2) to help maintain physiological functioning. Following the incubation period, the muscles were immediately removed from the chambers, blotted on paper towel to remove excess liquid and snap frozen in liquid nitrogen. Muscles were stored at -80 °C for further analysis.

5.3.3 Skeletal Muscle RNA extraction and quantification

The 9 week DIO are not included in this analysis. Rat skeletal muscle from the organ bath treatment were cut to 30 µg weights on dry ice and then homogenised in TRIzol (Invitrogen, Carlsbad, CA) with ceramic beads using a Fast Prep tissue homogeniser (FP120 cell disruptor, Electron Corporation, Milford, United States of America). Following this, RNA was extracted using a protocol developed by Chomczynski and Sacchi (2006) and modified by Cavuoto et al. (2007) and the RNA content was quantified using nanodrop spectrometry (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE). Following this step, samples were diluted with DEPC H_2O to contain 0.5 µg of RNA per 7.5 µL, and DNA was reverse transcribed to yield complementary DNA (cDNA) using an iScript cDNA synthesis kit (Biorad Laboratories Inc. Hercules, California, USA) and then further diluted with DEPC H_2O for 'Real Time' Polymerase Chain Reaction (PCR) quantification. Further details regarding this can be found in Chapter 2. PCR was performed using a BioRad MY iQ® Real-Time PCR detection system and analysis of gene expression was performed using Biorad IQ5 Gene analysis software (BioRad Laboratories, Hercules, California, USA 2009, version 2.1.97.1001) and Microsoft Excel (Microsoft, Redmond, Virginia, USA). The threshold cycles of gene expression for the average of the triplicate for individual samples were compared to the average of 3 housekeeping genes for that sample. Three genes were used as this helps to remove analysis bias due to unexpected alterations in housekeeping gene expression (Vandesompele et al., 2002). For skeletal muscle analysis β-Actin, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Cyclophilin were used. These genes were chosen due to the stability of their expression levels in this tissue type (Svingen et al., 2015; Thellin et al., 1999). Gene expression relative to that of the average expression of the three housekeeping genes was performed using the $2^{-\Delta\Delta C_T}$ method which has been validated and is a widely used and acknowledged method of data analysis (Schmittgen & Livak, 2008). Primers used in this Chapter are shown in Table 5.1.

Table 5.1: Primer Sequences

GENE	ACCESSION NUMBER	SEQUENCE
β-Actin	NM_031144	Forward (5'-3') CTAAGGCCAACCGTGAAATGA Reverse (5'-3') CCAGAGGCATACAGGGACAAC
GAPDH	XM_017593963.1	Forward (5'-3') AGTTCAACGGCACAGTCAAG Reverse (5'-3') GTGGTGAAGACGCCAGTAGA
Cyclophilin	NM_017101.1	Forward (5'-3') CTGATGGCGAGCCCTTG Reverse (5'-3') TCTGCTGTCTTTGGAACTTTGTC
CPT1	XM_006242180.3	Forward (5'-3') TTTGAGATGCACGGCAAGAC Reverse (5'-3') CTGGACAAGAGGCGAACACA
FAT/CD36	NM_031561.2	Forward (5'-3') GACCATCGGCGATGAGAAA Reverse (5'-3') CCAGGCCCAGGAGCTTTATT
PGC1α	NM_031347.1	Forward (5'-3') ACCCACAGGATCAGAACAACC Reverse (5'-3') ACAAATGCTCTTTGCTTTATTGC
AMPKα2	NM_023991.1	Forward (5'-3') ACTCTGCTGATGCACATGCT Reverse (5'-3') AGGGGTCTTCAGGAAAGAGG
β-HAD	NM_133618.3	Forward (5'-3') TCGTGACCAGGCAATTCGT Reverse (5'-3') CCGATGACCGTCACATGCT

β-Actin; beta actin, GAPDH; Glyceraldehyde 3-phosphate dehydrogenase, CPT1; Carnitine palmitoyltransferase 1, FAT/CD36; Fatty Acid Translocase/ Cluster of Differentiation 36, PGC1α; Peroxisome proliferator-activated receptor gamma co-activator 1 alpha, AMPKα2; AMP-activated protein kinase alpha subunit 2, β-HAD; beta-hydroxyacyl-CoA dehydrogenase.

5.3.4 Statistical Analysis

All statistical analysis was performed using Prism GraphPad (Version 7, GraphPad Software, San Diego, California, USA). Results were considered significant when $p < 0.05$. All data is presented as mean ± standard error of the mean (SEM). For gene expression samples were considered outliers if the average of the triplicates was greater than 2 standard deviations away from the average for that diet group. One way ANOVA was used to compare between diet groups for treated/untreated muscles. Paired two-tailed t-tests were used to compare between muscles (i.e. soleus and EDL) for the untreated muscles, and to compare between treated and

untreated muscles for each diet group. In the instance of a missing data point the matching data point (e.g. the corresponding treated or untreated muscle) was removed from analysis for the t-tests.

5.4 Results

As shown in Table 5.2, when comparing between muscle tissues for the untreated groups, FAT/CD36 mRNA expression was significantly greater in the soleus muscle compared to the EDL for the chow (n=9), high OA (n=9) and the 'Western' diet then chow (n=9) groups. When comparing between muscle tissues for the untreated groups, CPT1 mRNA expression was significantly greater in the soleus muscle compared to the EDL for the chow diet (n=8), the high OA (n=9), the high LA (n=10) and the 'Western' diet then chow (n=9) groups. PGC1α and AMPKα2 mRNA expression in the untreated muscles were significantly greater in the EDL compared to the soleus for the 'Western' diet and the high LA groups, with a non-significant trend towards increased PGC1α mRNA expression in the soleus compared to the EDL for the high OA group (n=8). The only diet group to show a difference in β-HAD mRNA expression between untreated muscles was the high LA group which had higher expression in the soleus muscle compared to the EDL (n=8).

Table 5.2: Gene expression differences between EDL and soleus muscles incubated without adiponectin

	CHOW		'WESTERN' DIET		HIGH OA		HIGH LA		'WESTERN' DIET THEN CHOW	
	EDL	**SOLEUS**	**EDL**	**SOLEUS**	**EDL**	**SOLEUS**	**EDL**	**SOLEUS**	**EDL**	**SOLEUS**
FAT/CD36	2.27 ± 0.34	5.77 ± 0.99*	4.02 ± 0.69	4.22 ± 0.46	1.68 ± 0.38	5.83 ± 1.01*	2.53 ± 0.33	2.97 ± 0.53	2.28 ± 0.41	6.03 ± 1.02*
CPT1	0.20 ± 0.02	0.68 ± 0.08*	0.42 ± 0.10	0.57 ± 0.07	0.13 ± 0.02	0.47 ± 0.05*	0.18 ± 0.02	0.31 ± 0.03*	0.15 ± 0.03	0.29 ± 0.02*
PGC1α	0.11 ± 0.02	0.12 ± 0.01	0.15 ± 0.02	0.09 ± 0.01*	0.05 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.03 ± 0.01*	0.06 ± 0.01	0.06 ± 0.01
AMPKα2	0.61 ± 0.08	0.48 ± 0.08	1.04 ± 0.10	0.26 ± 0.03*	0.24 ± 0.03	0.23 ± 0.03	0.47 ± 0.06	0.17 ± 0.04*	0.29 ± 0.05	0.21 ± 0.04
β-HAD	0.12 ± 0.02	0.12 ± 0.02	0.11 ± 0.02	0.06 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.04 ± 0.01	0.10 ± 0.01*	0.06 ± 0.01	0.08 ± 0.01

mRNA expression in arbitrary units normalised to average of housekeeping genes (GAPDH, β-Actin, Cyclophilin). Data shown as mean ± SEM. * = significantly different to EDL. FAT/CD36; Fatty Acid Translocase/ Cluster of Differentiation 36, CPT1; Carnitine palmitoyltransferase 1, PGC1α; Peroxisome proliferator-activated receptor gamma co-activator 1 alpha, AMPKα2; AMP-activated protein kinase alpha subunit 2, β-HAD; beta-hydroxyacyl-CoA dehydrogenase.

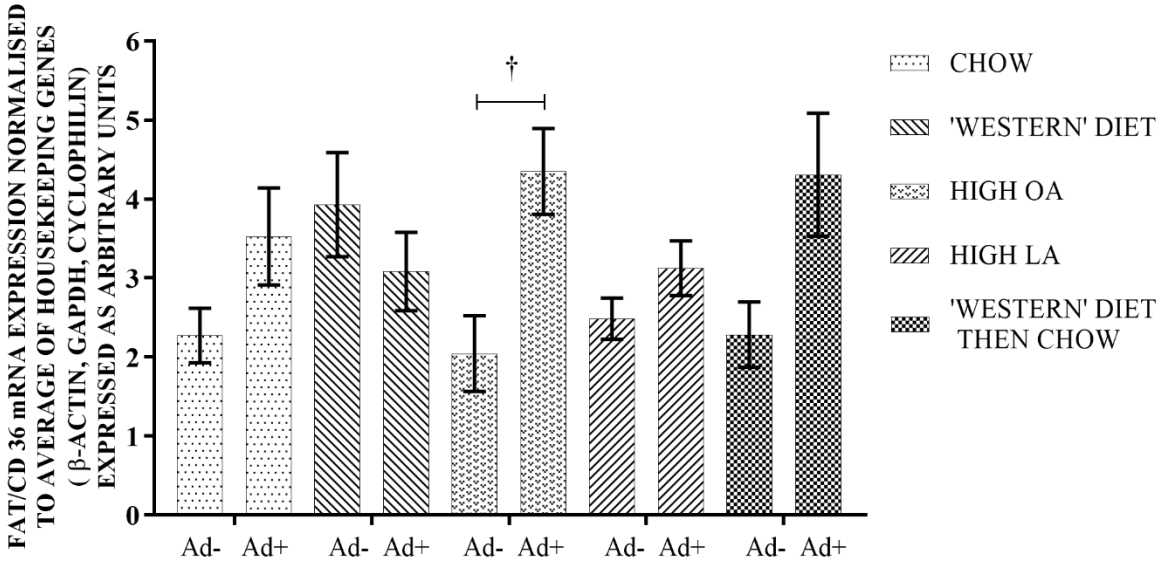

Figure 5.1: Extensor Digitorum Longus muscle FAT/CD36 mRNA expression following 30 min of treatment with or without globular adiponectin normalised to the average of housekeeping genes (β-Actin, GAPDH and Cyclophilin). Shown as Arbitrary units. Ad- = muscle incubated without globular adiponectin, Ad+ = muscle incubated with 2.5 µg/ml of globular adiponectin for 30 min. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. † = significant difference between muscles incubated with or without globular adiponectin.

Treatment of the EDL muscles from the high OA diet group resulted in a significant increase in FAT/CD36 mRNA expression, with a non-significant trend towards an increase (p= 0.056) for the 'Western' diet then chow group, as shown in Figure 5.1.

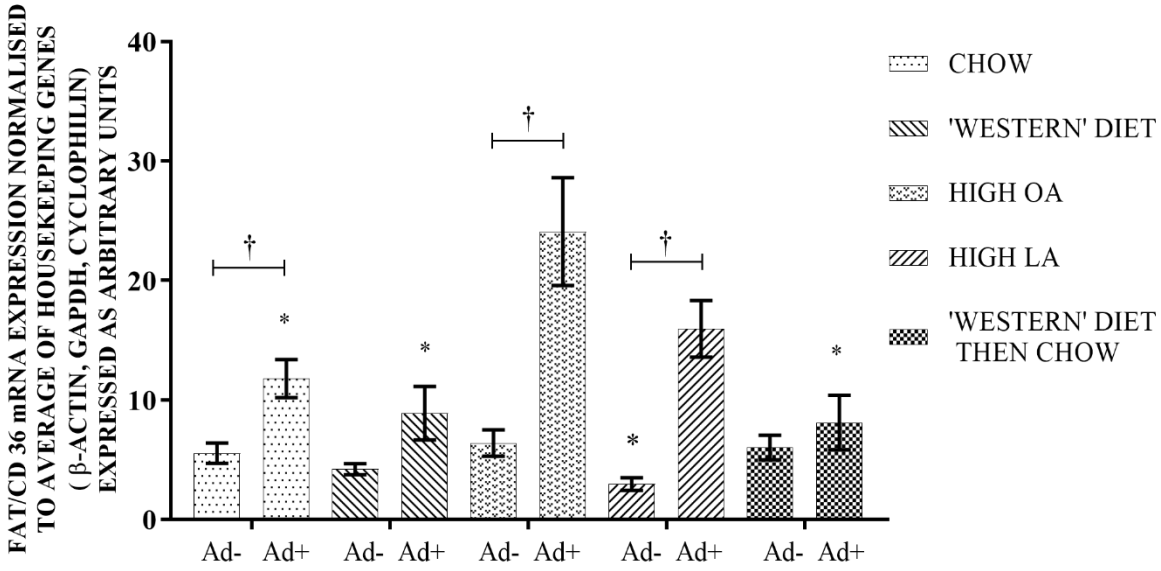

Figure 5.2: Soleus muscle FAT/CD36 mRNA expression following 30 min of treatment with or without globular adiponectin normalised to the average of housekeeping genes (β-Actin, GAPDH and Cyclophilin). Shown as Arbitrary units. Ad- = muscle incubated without globular adiponectin, Ad+ = muscle incubated with 2.5 µg/ml of globular adiponectin for 30 min. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. * = significantly different to high OA diet group for this treatment † = significant difference between muscles incubated with or without globular adiponectin for specific diet group.

The high LA diet group had a significantly lower expression of FAT/CD36 mRNA for the soleus muscles incubated without globular adiponectin compared to the high OA consuming animals, as shown in Figure 5.2. Treatment with adiponectin significantly increased FAT/CD36 mRNA expression in the soleus muscle for the chow, high OA and high LA diet animals. When comparing the adiponectin-treated soleus muscles, the high OA group had significantly increased FAT/CD36 mRNA expression when compared to all other diet groups except for the high LA group.

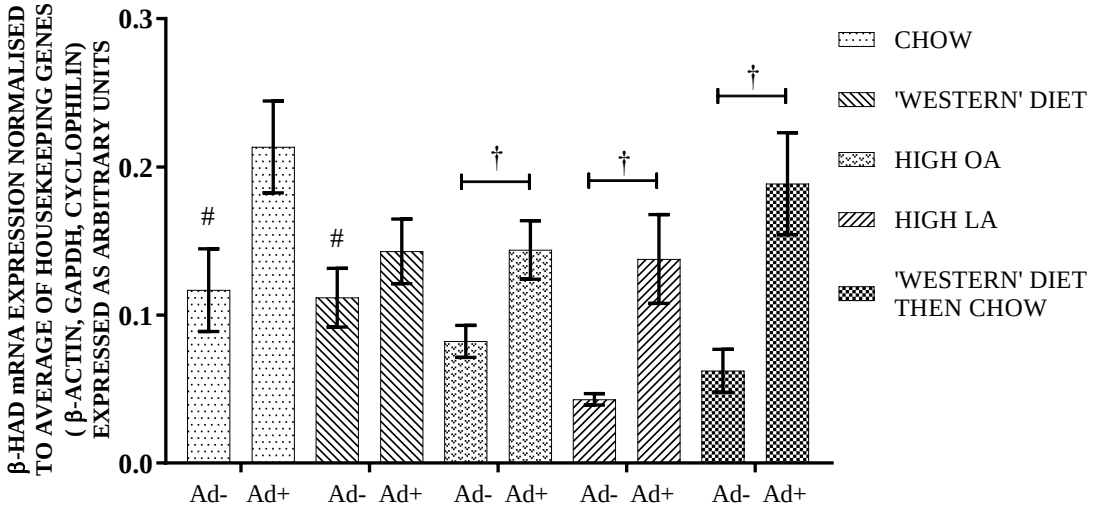

Figure 5.3: Extensor Digitorum Longus muscle β-HAD mRNA expression following 30 min of treatment with or without globular adiponectin normalised to the average of housekeeping genes (β-Actin, GAPDH and Cyclophilin). Shown as Arbitrary units. Ad- = muscle incubated without globular adiponectin, Ad+ = muscle incubated with 2.5 µg/ml of globular adiponectin for 30 min All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. # = significantly different to high LA for this treatment. † = significant difference between muscles incubated with or without globular adiponectin for specific diet group.

Expression of β-HAD mRNA in the untreated muscles was significantly greater in the chow and 'Western' diet groups when compared to the high LA group, as shown in Figure 5.3. Comparing the muscles incubated with and without adiponectin, the expression of β-HAD mRNA was significantly increased with adiponectin incubation for the high OA, high LA and 'Western' diet then chow groups.

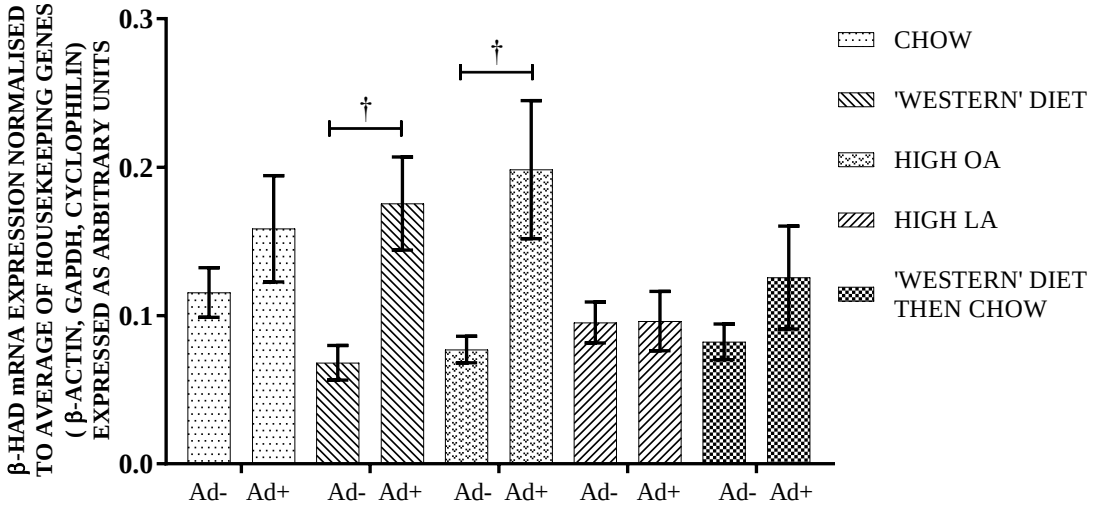

Figure 5.4: Soleus muscle β-HAD mRNA expression following 30 min of treatment with or without globular adiponectin normalised to the average of housekeeping genes (β-Actin, GAPDH and Cyclophilin). Shown as Arbitrary units. Ad- = muscle incubated without globular adiponectin, Ad+ = muscle incubated with 2.5 µg/ml of globular adiponectin for 30 min. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. † = significant difference between muscles incubated with or without globular adiponectin for specific diet group.

As shown in Figure 5.4 there were no significant differences in soleus muscle β-HAD mRNA expression between diet groups for the muscles treated with or without globular adiponectin, though treatment with adiponectin increased β-HAD mRNA expression in the 'Western' and high OA diet groups.

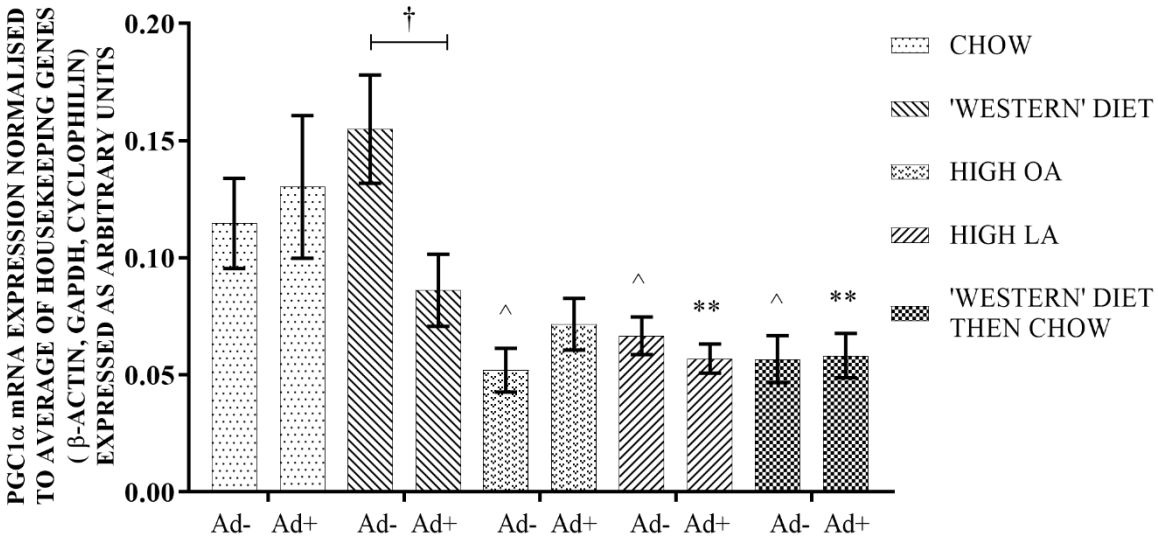

Figure 5.5: Extensor Digitorum Longus muscle PGC1α mRNA expression following 30 min of treatment with or without globular adiponectin normalised to the average of housekeeping genes (β-Actin, GAPDH and Cyclophilin). Shown as Arbitrary units. Ad- = muscle incubated without globular adiponectin, Ad+ = muscle incubated with 2.5 µg/ml of globular adiponectin for 30 min. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. ^ = significantly different to 'Western' diet for this treatment-. **= significantly different to chow for this treatment. † = significant difference between muscles incubated with or without globular adiponectin for specific diet group.

When comparing between EDL muscles incubated without globular adiponectin, the OA, LA and 'Western' diet then chow diet groups had significantly lower PGC1α mRNA expression compared to the 'Western' diet, as shown in Figure 5.5. For the globular adiponectin-treated muscles, the high LA and 'Western' diet then chow diet groups had significantly lower PGC1α mRNA expression when compared to the chow diet. Incubation of the EDL from the 'Western' diet group with adiponectin reduced mRNA expression, which was not seen in other diet groups.

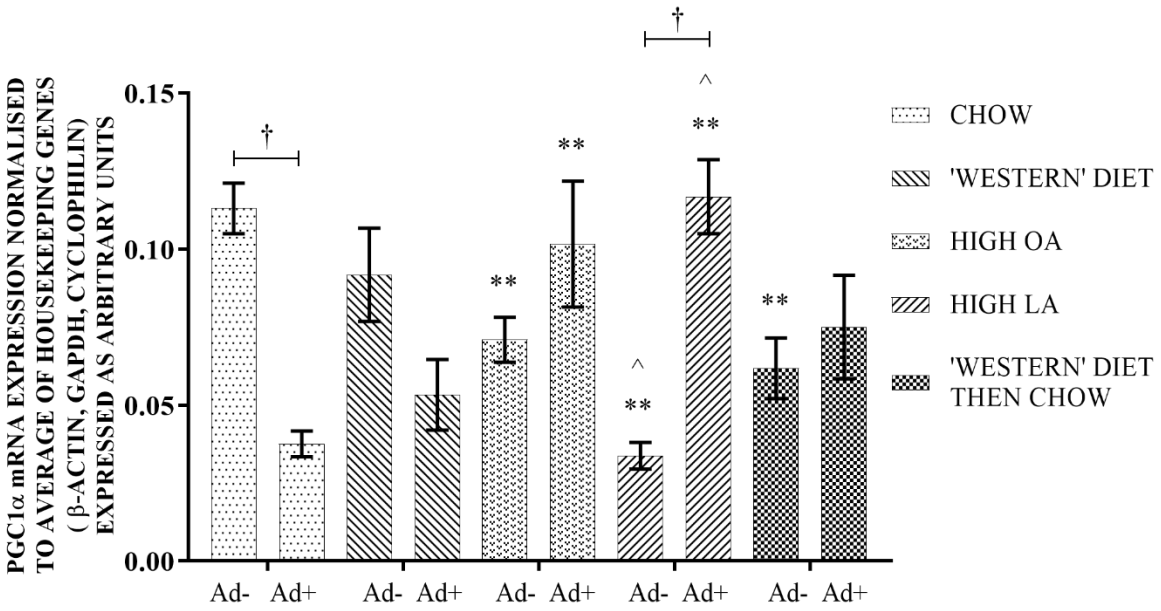

Figure 5.6: Soleus muscle PGC1α mRNA expression following 30 min of treatment with or without globular adiponectin normalised to the average of housekeeping genes (β-Actin, GAPDH and Cyclophilin). Shown as Arbitrary units. Ad- = muscle incubated without globular adiponectin, Ad+ = muscle incubated with 2.5 µg/ml of globular adiponectin for 30 min. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. ** = significantly different to chow for this treatment. ^ = significantly different to 'Western' diet for this treatment. † = significant difference between muscles incubated with or without globular adiponectin for specific diet group.

In the soleus muscles incubated without adiponectin, the high OA, high LA and 'Western' diet then chow diet groups had significantly lower PGC1α mRNA expression when compared to the chow muscles, as shown in Figure 5.6. Additionally, PGC1α mRNA expression was also lower in the high LA diet group when compared to the muscles from the animals fed the 'Western' diet. For the chow group, incubation of the soleus with adiponectin showed a decrease in PGC1α mRNA expression when compared to the muscles incubated without adiponectin. In contrast, the high LA diet group soleus muscles treated with adiponectin showed an increase in PGC1α mRNA expression. When comparing the adiponectin treated muscles, there was significantly greater expression of PGC1α mRNA in the soleus muscle of the high OA and high LA diet animals compared to the chow animals.

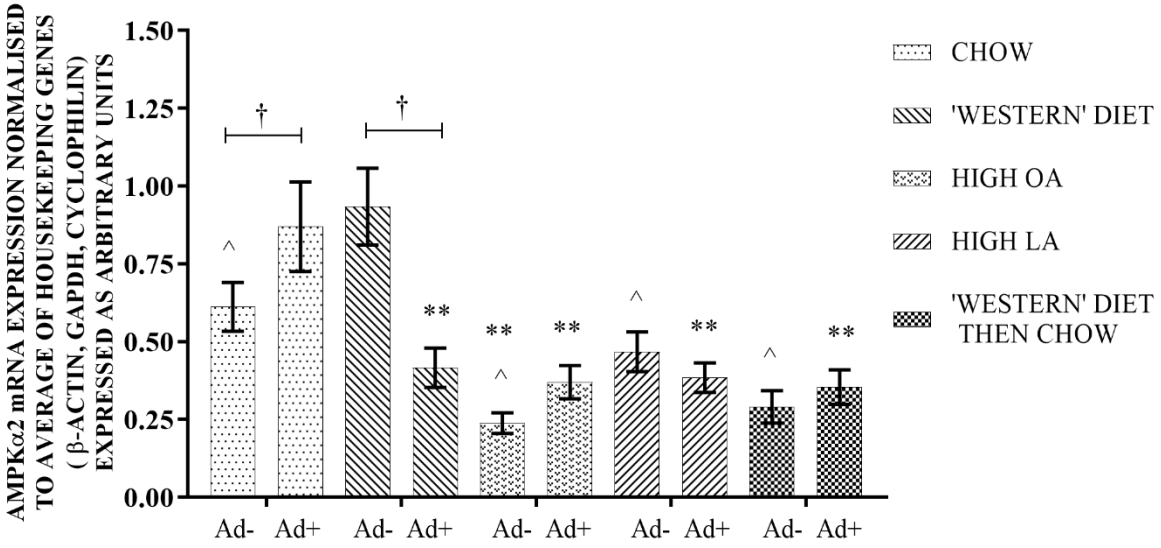

Figure 5.7: Extensor Digitorum Longus muscle AMPKα2 mRNA expression following 30 min of treatment with or without globular adiponectin normalised to the average of housekeeping genes (β-Actin, GAPDH and Cyclophilin). Shown as Arbitrary units. Ad- = muscle incubated without globular adiponectin, Ad+ = muscle incubated with 2.5 µg/ml of globular adiponectin for 30 min. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. ^ = significantly different to 'Western' diet for this treatment. ** = significantly different to chow for this treatment. † = significant difference between muscles incubated with or without globular adiponectin for specific diet group.

In the EDL muscles the 'Western' diet group had higher levels of expression of AMPKα2 mRNA than all other diet groups in the muscles not incubated with adiponectin, whilst the OA group was also significantly lower than the chow group, as shown in Figure 5.7. Treatment of the EDL from 'Western' diet animals with globular adiponectin resulted in a significant reduction in AMPKα2 mRNA expression. In contrast, adiponectin treatment resulted in a significant increase in AMPKα2 mRNA expression in the EDL muscles from the chow animals compared to all other diet groups.

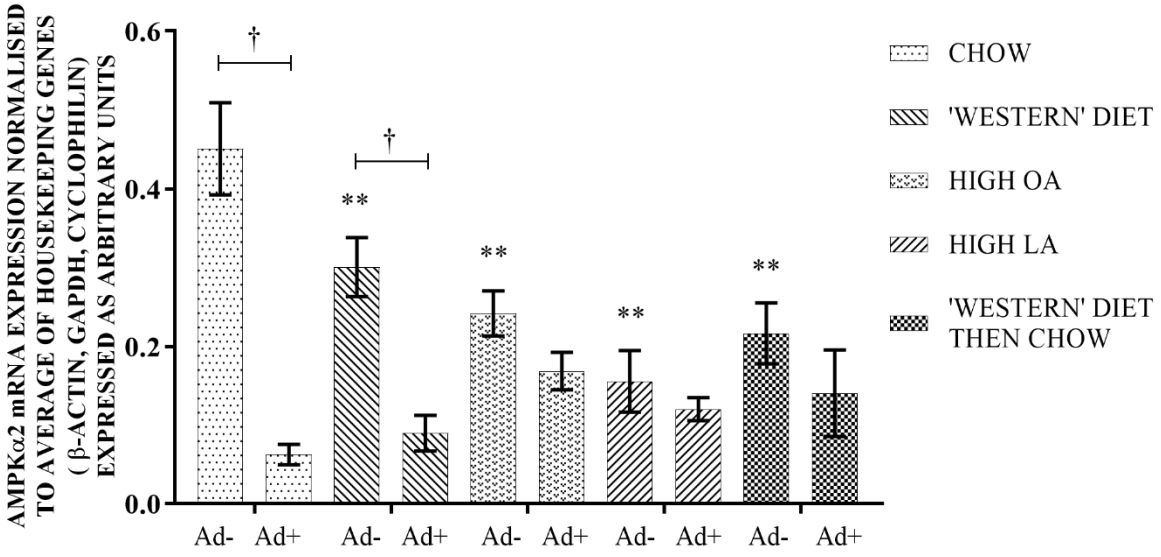

Figure 5.8: Soleus muscle AMPKα2 mRNA expression following 30 min of treatment with or without globular adiponectin normalised to the average of housekeeping genes (β-Actin, GAPDH and Cyclophilin). Shown as Arbitrary units. Ad- = muscle incubated without globular adiponectin, Ad+ = muscle incubated with 2.5 µg/ml of globular adiponectin for 30 min. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. ** = significantly different to chow for this treatment. † = significant difference between muscles incubated with or without globular adiponectin for specific diet group.

As shown in Figure 5.8 the expression of AMPKα2 mRNA was significantly higher in the chow group for the muscles incubated without adiponectin compared to all other diet groups. Incubation with adiponectin resulted in a decrease in AMPKα2 mRNA expression for the chow and 'Western' diet groups, with no significant differences between the diet groups for the adiponectin treated muscles.

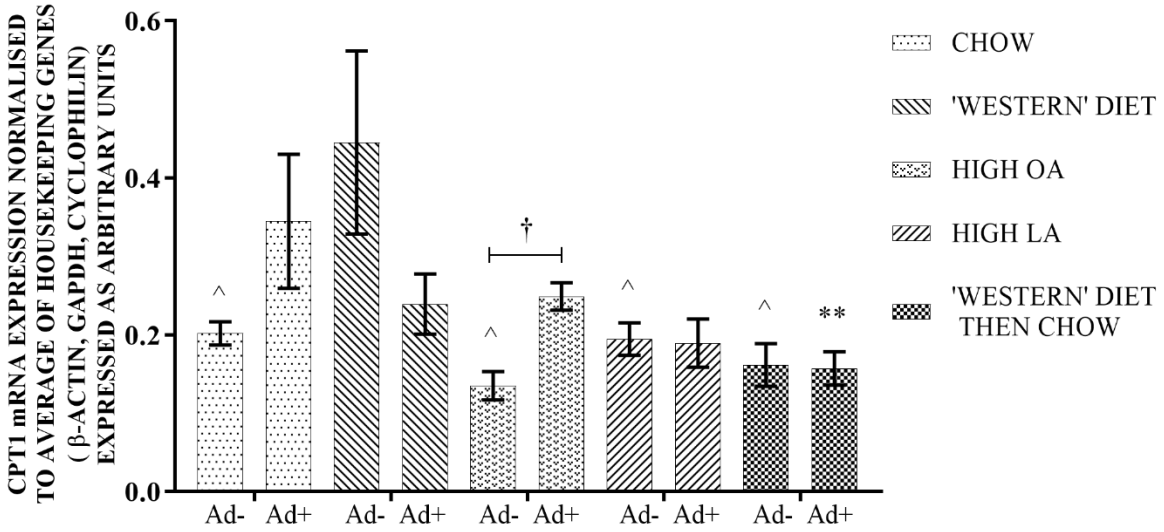

Figure 5.9: Extensor Digitorum Longus muscle CPT1 mRNA expression following 30 min of treatment with or without globular adiponectin normalised to the average of housekeeping genes (β-Actin, GAPDH and Cyclophilin). Shown as Arbitrary units. Ad- = muscle incubated without globular adiponectin, Ad+ = muscle incubated with 2.5 µg/ml of globular adiponectin for 30 min. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. ^ = significantly different to 'Western' diet for this treatment. ** = significantly different to chow diet for this treatment. † = significant difference between muscles incubated with or without globular adiponectin for specific diet group.

CPT1 mRNA expression was significantly greater in the 'Western' diet EDL muscles incubated without adiponectin compared to all other diet groups, as shown in Figure 5.9. Treatment of the muscles with adiponectin resulted in a significant increase in CPT1 mRNA expression in the high OA diet group only. Comparing between the diet groups for the adiponectin treated muscles found that the 'Western' diet then chow animals had significantly lower CPT1 mRNA expression when compared to the chow diet animals.

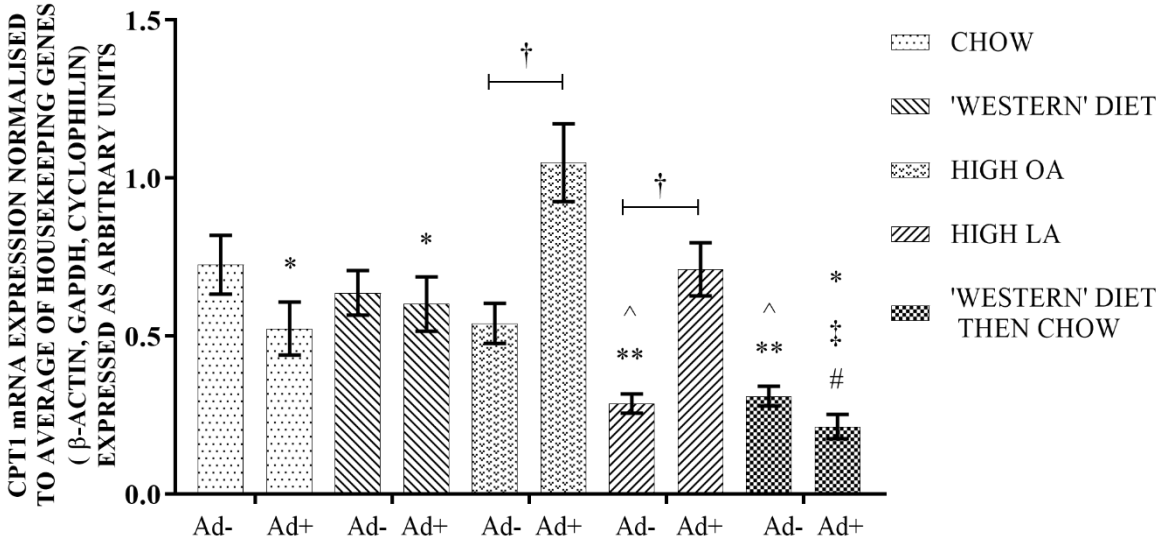

Figure 5.10: Soleus muscle CPT1 mRNA expression following 30 min of treatment with or without globular adiponectin normalised to the average of housekeeping genes (β-Actin, GAPDH and Cyclophilin). Shown as Arbitrary units. Ad- = muscle incubated without globular adiponectin, Ad+ = muscle incubated with 2.5 µg/ml of globular adiponectin for 30 min. All data shown as mean ± SEM. Chow n =9; 'Western' diet n= 10; high OA n = 10; high LA n= 10; 'Western' diet then chow n= 9. ** = significantly different to the chow diet for this treatment. ^ = significantly different to the 'Western' diet for this treatment. * = significantly different to high OA for this treatment # = significantly different to high LA for this treatment. ‡ = significantly different to the 'Western' diet for this treatment. † = significant difference between muscles incubated with or without globular adiponectin for specific diet group.

The soleus muscles incubated without adiponectin from the high LA and the 'Western' then chow diet groups had significantly lower CPT1 mRNA expression than both the chow and 'Western' diet animals, as shown in Figure 5.10. Treatment of soleus muscles from both the high OA and high LA with globular adiponectin resulted in a significant increase in CPT1 mRNA expression. When the soleus muscles were treated with adiponectin, the expression of CPT1 mRNA was significantly greater in the high OA diet animals than the chow, 'Western' and 'Western' diet then chow animals, with a non-significant trend towards an increase also found in the high LA diet group ($p = 0.068$). The expression of CPT1 mRNA in the adiponectin treated muscles from the 'Western' diet then chow animals was significantly lower than the high LA diet group as well.

5.5 Discussion

Skeletal muscle makes a significant contribution to energy expenditure and basal metabolic rate, and as such has a significant influence on metabolic health (Baskin et al., 2015). Obesity is a predictor of metabolic disease and is highly prevalent in our modern society (Australian Bureau of Statistics, 2015) and as such has been a large focus of research into maintaining adequate metabolic health. Despite this, the influence of common fats on skeletal muscle metabolic gene expression and adiponectin signalling has not been fully elucidated. In this study the effects of different common dietary fat compositions, being a predominately 'Western' style, a 'Mediterranean' style high OA and a high LA diet on skeletal muscle gene expression in a rodent model of DIO was investigated. Despite, as found in Chapter 4, the animals having similar body weights and compositions, the consumption of the different dietary compositions for a 6-week period resulted in significant changes to expression of genes involved in FA transport (both into the muscle and the mitochondria) and also to genes involved in FA metabolism. Of note this study demonstrated the ability of adiponectin treatment to increase FAT/CD36, β-HAD and CPT1 mRNA expression in both the EDL and soleus from high OA fed animals, a pattern which was not seen in any of the other diet groups for both muscles investigated. Moreover, chronic dietary intake of specific FA appears to alter metabolic gene mRNA expression in different fibre type muscles.

5.5.1 Dietary influence of gene expression in glycolytic and oxidative muscle types

Expression of FAT/CD36 was higher in the soleus depot compared to EDL for the muscle incubated without adiponectin for the chow, and the 'Western' diet then chow group, a pattern previously found in tissues from lean rodents (Bonen et al., 1999; Bonen et al., 1998). Additionally, in the current study FAT/CD36 mRNA expression was also higher in the soleus muscle compared to EDL in the high OA group. As the 'Western' diet then chow and the chow fed animals had comparable body fat percentages (as shown in

Chapter 4) this may indicate that high fat feeding with the majority of the fat being derived from OA may preserve a lean phenotype of FAT/CD36 mRNA expression, which was not seen in the other high fat fed/DIO animals. As the soleus muscle is primarily oxidative this may indicate that the higher FAT/CD36 mRNA expression in the soleus compared to the EDL from the chow, high OA and 'Western' diet then chow animals is allowing adequate amounts of FA into the cells for oxidation, both at rest and during contraction (Holloway et al., 2008).

Distinct patterns of expression were observed in two of the diets used; the high LA diet fed animals had higher PGC1α, AMPKα2 and CPT1 mRNA expression in the EDL than the soleus muscle, though β-HAD mRNA expression showed the opposite pattern. This may indicate that though there is a capacity for increased mitochondrial density due to increased PGC1α mRNA expression (Choi et al., 2008) and transfer of lipids across the mitochondrial membrane, that the rate of β-oxidation in the EDL is not increased. Similarly, the 'Western' diet animals had higher expression of PGC1α and AMPKα2 mRNA in the EDL compared to the soleus. Comparing between muscle types incubated without adiponectin within the different diet groups, CPT1 mRNA expression was significantly higher in the soleus than the EDL for all diet groups except for the 'Western' diet group, as would be expected in a primarily oxidative muscle type (Stephens et al., 2007).

5.5.2 Effect of diet on gene expression in EDL muscles

Dietary FA composition, FA content or reversal of obesity did not affect expression of FAT/CD36 mRNA expression in the EDL muscle. In contrast, it has been previously found that FAT/CD36 mRNA expression in the EDL muscle from high fat fed female Sprague Dawley rats is increased, which may show the effect of gender (as the current study only included male Sprague Dawley rats) or could be attributed to the difference in

the contribution of fat to the total energy of the diet (41 % here compared to 78 %) (McAinch et al., 2003). Additionally, in male Wistar rats, high fat feeding (30 % of energy) has been found to increase FAT/CD36 expression in the mixed fibre type gastrocnemius muscle independent of fat source (a mixed SFA/MUFA/PUFA, high SFA and mixed with increased n-3 FA) which may indicate rodent strain related differences (Feillet-Coudray et al., 2013).

The EDL from the high LA diet group showed a significantly lower β-HAD mRNA expression compared to the chow and 'Western' diet fed animals, potentially indicating lower levels of fat oxidation. This may possibly be influenced by the high n-6:n-3 of this diet group, as n-3 FA have been found to increase β-HAD activity in cultured myotubes (Wensaas et al., 2009). Overall, in the EDL tissue incubated without adiponectin, expression of PGC1α, AMPKα2 and CPT1 mRNA was higher in the 'Western' diet group compared to all other high fat diet groups, which may indicate greater oxidative capacity in this tissue. This may also represent a shift in fibre type composition as has been previously found in the EDL muscles from male Wistar rats fed a high EPA/DHA diet, which resulted in an increase in the protein levels of PGC1α and the proportion of intermediate-type fibres (determined via myosin heavy chain isoform levels), indicating the ability of FA to alter fibre type in this muscle (Hashimoto et al., 2016; Mizunoya et al., 2013). Additionally, high fat feeding of a 'Western' style diet (40 % TE) increases EDL PGC1α expression in male Long Evans rats compared to chow-fed littermates (Stephenson et al., 2012), though the specific FA composition of the diet is not discussed beyond the amount of saturated and unsaturated fats, making direct comparisons difficult. Despite this, as this diet had the highest proportion of SFA (see Chapter 4) it would be expected that there would be a down regulation of oxidative genes as has been found in similar high fat feeding studies (Martins et al., 2012), though changes to skeletal muscle

gene expression in response to SFA seems to be highly sensitive to the specific chain length of FA's, and the 'Western' diet used in this study, as shown in Chapter 2 (Table 2.3) was a mix of different chain length SFA (Ciapaite et al., 2015).

5.5.3 Effect of diet on gene expression in soleus muscles

In the soleus muscles incubated without adiponectin, expression of FAT/CD36 mRNA was significantly lower in the LA fed animals in comparison to the OA group. As this is primarily an oxidative muscle this may indicate lower substrate availability. Research comparing lean chow fed and diabetic Zucker rats found that the diabetic animals had an increase in FAT/CD36 expression in red muscle fibres when compared to their lean counterparts (Chabowski et al., 2006). This was not found in this study, potentially due to the high fat fed animals still having adequate glucose handling, as shown in Chapter 4, and therefore a supply of glucose into the muscle for metabolism.

From the results of this study, dietary FA composition, obesity or the reversal of obesity do not appear to modulate β-HAD mRNA expression in the muscles incubated without adiponectin. Supporting this is the finding that high fat feeding, varying in contribution of energy (15 % and 30 % of energy) or composition, (a high SFA, mixed SFA/MUFA/PUFA, and mixed with increased n-3 FA) does not modulate β-HAD levels (determined via Western blotting) in gastrocnemius muscle of male Wistar rats (Feillet-Coudray et al., 2013). In opposition, the rate of FA oxidation in skeletal muscle from obese and diabetic individuals is decreased as is the activity of the β-HAD enzyme (He et al., 2001), though mRNA expression and not activity was measured in this study. Furthermore, this finding is in contrast to the findings of increased β-HAD mRNA expression in the soleus muscles from high fat fed female Sprague Dawley rats when compared to high carbohydrate fed animals, which may show an influence of gender or could be attributed to the difference in contribution of fat to the total energy of the diet

(41 % here compared to 78 %) (McAinch et al., 2003). Of note, the high fat diet utilised by McAinch et al., (2003) did not contain any digestible carbohydrate, which may also have influenced the differences seen here.

Soleus muscles (incubated without adiponectin) from the chow fed animals had a significantly higher mRNA expression of AMPKα2 than all other diet groups, indicating that obesity may lead to a decrease in expression regardless of the type of fat consumed and expression may not be recovered with weight loss. As AMPK is involved in maintaining mitochondrial capacity this may indicate that a high fat diet decreases mitochondrial capacity potentially due to lipid deposition in the tissue impairing function (O'Neill et al., 2011), which may not be recovered via returning to a normal diet. Moreover, as AMPK activation has been found to increase FA uptake and metabolism in resting skeletal muscle (soleus) from female Sprague-Dawley rats (Smith et al., 2005) and also in muscle from high fat fed insulin resistant male Wistar rats, this decrease in expression may be perpetuating weight gain through decreasing resting FA oxidation. Finally, as AMPKα2 is a sensor of cellular energy status the positive energy balance found in the high fat fed/DIO animals may be downregulating the mRNA expression due to the energy density of the diet (Hardie et al., 2006).

AMPK is also capable of regulating CPT1, as AMPK is able to phosphorylate and inactivate the enzyme acetyl CoA carboxylase 2 which produces malonyl CoA, an allosteric inhibitor of CPT1 (Turner et al., 2014). In the soleus muscle, CPT1 mRNA expression was significantly lower in the high LA diet and 'Western' diet then chow animals when compared to the chow and 'Western' diet animals for the samples incubated without adiponectin. As CPT1 is required for long chain FA movement across the mitochondrial membrane (Bruce et al., 2009) this may indicate a high LA intake limits substrate availability, also demonstrated by the low FAT/CD36 expression, in this

predominately oxidative muscle type. In male Lewis rats fed a high fat diet there were no significant differences found between CPT1 activity following 10 weeks of consumption of diets rich in OA or LA in the soleus, though in the present study CPT1 expression was measured, not activity (Power & Newsholme, 1997). The reduction in CPT1 mRNA expression found in the animals which had a reduction in fat mass may indicate a down regulation of expression in relation to weight loss/fatty acid availability or potentially to preserve body mass.

PGC1α is a transcriptional co-activator produced by skeletal muscle in response to exercise and is capable of triggering mitochondrial biogenesis and oxidative metabolism in numerous cell types, as well as fibre type switching in skeletal muscle (Handschin & Spiegelman, 2008; Puigserver et al., 1998). Soleus muscles from the high OA and 'Western' diet then chow fed animals all had significantly lower expression of PGC1α mRNA than the chow diet for the muscles incubated without adiponectin, with expression in the high LA fed animals also being lower than the 'Western' diet fed animals. This indicates that high fat feeding, regardless of FA type decreases PGC1α mRNA expression, in line with the findings that in C57BL/6J male mice, 14 weeks of high fat feeding (59 % of energy) down regulated PGC1α mRNA expression in the soleus (Philp et al., 2015) and in the quadriceps after 11 weeks of high fat feeding (42 % of energy) (Crunkhorn et al., 2007). Interestingly, in cultured human vastus lateralis muscles, unsaturated FA (including LA and OA) increased PGC1α expression after overnight incubation (Staiger et al., 2005), which may indicate down regulation of expression with chronic dietary intake.

5.5.4 Effect of adiponectin incubation and diet on gene expression in oxidative soleus muscle

Incubation of the soleus muscles from the chow group with adiponectin resulted in an increase in FAT/CD36 mRNA expression, which was also the case for the high LA fed

animals. Though, when comparing between diet groups, the soleus muscles incubated with adiponectin from the high OA fed animals had a higher FAT/CD36 mRNA expression than all other diet groups aside from the high LA fed animals. In Syrian gold hamsters fed a diet containing 10 % of energy from fat (6 % of energy being either high OA canola oil or high LA corn oil), 29 days of feeding was found to increase hepatic FATCD/36 expression 3-fold over chow animals for the high OA group. This was coupled with a concomitant increase in levels of the OA derived ethanolamide, oleoylethanolamide (OEA) in the liver and small intestine (Lin et al., 2013), though unfortunately skeletal muscle was not analysed. Additionally, the corn oil fed animals were heavier and had a greater body fat percentage than the high oleic canola fed animals (Lin et al., 2013), though as shown in Chapter 4, this was not observed in this study. OEA has become a focus of research investigating body weight and food intake as it has anorectic properties which in the long term can result in reductions in fat mass (Aviello et al., 2008; Lin et al., 2013; Mennella et al., 2014). A relationship between FAT/CD36 and OEA production in the small intestine has been demonstrated by the development of FAT/CD36 knock-out mice (Guijarro et al., 2010), with tissue OEA production increasing circulating OEA levels and increasing hypothalamic mediated satiety (Schwartz et al., 2008). Moreover, FAT/CD36 deletion decreases the uptake of OA across the duodenal and jejunal membrane (Schwartz et al., 2008). This indicates that high OA fed animals in this study may have had an upregulation of FAT/CD36 for production of the OA ethanolamide mediated through adiponectin, though as mentioned previously, despite considerable time investment, ethanolamide levels were unable to be quantified for this thesis.

Incubation with adiponectin of the soleus muscles from the ‘Western’ diet and high OA fed animals increased the mRNA expression of β-HAD, though this did not result in

significant differences between any of the dietary groups. Research conducted by Tishinsky et al. (2012) in the soleus muscles from female Sprague Dawley rats fed diets with differing n-6:n-3 contents found that globular adiponectin incubation (at the concentration used in this study) restored (to that of a lean control group) FA oxidation in the group fed the lowest n-6:n-3 ratio. As β-HAD is integral for mitochondrial FA oxidation (Civitarese et al., 2006), this may show that adiponectin incubation in the soleus muscle of the animals fed high fat diets from the two groups with the lowest n-6:n-3, being the 'Western' diet (n-6:n-3= 9.24) and the high OA group (n-6:n-3= 4.94) were able to have adiponectin restoration of FA oxidation which was not seen in the high LA group which had a higher n-6:n-3 (n-6:n-3= 44.7) (data shown in Chapter 4, Table 4.2). Moreover, OA muscle content is associated with lower levels of muscle FA deposition in unspecified abdominal muscles from male Sprague Dawley rats (Garcia-Escobar et al., 2008), which may indicate greater rates of FA oxidation. As muscle FA composition is influenced by habitual dietary intakes (demonstrated in both humans (Andersson et al., 2002) and rodents (Ayre and Hulbert 1996; Corcoran et al., 2007)) this may indicate that a high OA diet may have a positive influence on lipid deposition in skeletal muscle.

Adiponectin incubation of the soleus muscle from the chow fed animals led to a significant decrease in PGC1α mRNA expression which is in opposition to results found in the soleus muscle of lean mice (Iwabu et al., 2010), which is most likely due to downregulation of PGC1α expression in the present study after 30 minutes of incubation compared to the 5 minutes in the study performed by Iwabu et al. (2010). Though adiponectin decreased PGC1α expression in the lean animals, the opposite was observed in the high LA diet animals. These changes in expression resulted in the high OA and high LA fed animals' muscles having higher PGC1α mRNA expression than the chow fed animals, with the high LA fed animals having higher expression than the muscles

from the ‘Western’ diet fed animals as well. Dietary unsaturated FA have been found to increase PGC1α expression in cultured human primary myotubes (Staiger et al., 2006), and as such this may indicate that adiponectin, may interact with the unsaturated FA of the high OA and LA diets to increase PGC1α expression in DIO rodents and potentially increase FA oxidation. Conversely, a study performed in humans has found that SFA (of which the ‘Western’ diet had the highest content) intake decreases muscle PGC1α expression (Turco et al., 2014), which is consistent with the results found here.

In the soleus muscles from the chow fed animals, incubation with adiponectin decreased AMPKα2 mRNA expression to levels comparable with those from the obese animals, with adiponectin also decreasing expression in the ‘Western’ diet fed animals. As adiponectin incubation generally increases AMPKα2 mRNA expression in tissue from lean animals (Yoon et al., 2006), the reduction in expression observed here may be an indication of downregulation as a negative feedback mechanism, which has been observed in 3T3-L1 adipocytes (Zhang et al., 2015). Comparing the soleus incubated with and without globular adiponectin, the chow and ‘Western’ diet fed animals had a significant reduction in AMPKα2 following incubation. This may show that over the 30-minute incubation period, the expression was down-regulated due to an influx of glucose from the incubation medium into the tissue. Additionally, incubation with globular adiponectin also increases the translocation of GLUT4 to the cell surface (Ceddia et al., 2005). Research conducted by Mullen et al. (2007) has found that high fat feeding impairs adiponectin stimulated FA oxidation, though this pathway is stimulated through an AMPK independent stream (determined by quantification of phosphorylated AMPK) and occurs independently of adiponectin stimulated glucose uptake, which may possibly be seen here. In the soleus muscle of high LA and sucrose fed (30 % energy from fat, 76 % of which being LA) *ob/ob* Zucker rats, there was increased lipotoxicity-induced

endoplasmic reticulum stress and decreased AMPK expression when compared to animals fed an isocaloric matched OA diet (Ohminami, et al., 2014). This is believed to decrease the membrane fluidity and therefore limit the action of insulin (Ohminami et al., 2014). Adiposity and glucose handling may also affect the ability of adiponectin to phosphorylate AMPKα2, with higher doses required in cultured obese human myocytes when compared to lean, with the higher dose not being able to elicit an increase in AMPKα2 phosphorylation in myocytes from obese diabetic subjects (Chen et al., 2005).

Incubation of soleus muscles with adiponectin resulted in an increase in CPT1 mRNA expression in the muscles from the high OA and high LA fed animals, with the high OA diet fed animals having higher expression than the chow and 'Western' diets. Incubation of the 'Western' diet then chow muscles with adiponectin resulted in CPT1 mRNA expression lower than the high LA and OA diets and also the 'Western' diet. As treatment of mouse C_2C_{12} myotubules with globular adiponectin leads to a dose dependant increase in CPT1 mRNA expression (Yoon et al., 2006) and over expression of CPT1 in insulin resistant Male Wistar rats fed a high fat diet resolves lipid-induced skeletal muscle insulin resistance (Bruce et al., 2009), this may indicate that the adiponectin stimulation of CPT1 is leading to clearance of intramyocellular lipid deposits in the high OA and LA fed animals.

5.5.5 Effect of adiponectin incubation and diet on gene expression in EDL muscle

In the EDL there were no significant differences in FAT/CD36 mRNA expression between the diet groups for the muscles incubated with adiponectin, as was found between the muscles incubated without adiponectin, indicating that adiponectin is incapable, or there is adiponectin resistance to modulation of FAT/CD36 mRNA expression in this tissue. This is in line with the finding that high fat feeding, (for as little as 5 days) has been shown to induce inhibition of adiponectin stimulated FA uptake in

the EDL from female Sprague Dawley rats independent of a shift in cellular redox state (Ritchie & Dyck, 2012). Though there were no differences between the diet groups, incubation of the EDL muscle from animals fed a high OA diet with globular adiponectin resulted in an increase in FAT/CD36, β-HAD and CPT1 mRNA expression when compared to the muscles incubated without adiponectin. As the EDL is a mixed fibre type of fast-twitch glycolytic and fast-twitch oxidative glycolytic this may indicate fibre type switching to a more oxidative type. Additionally, over expression of CPT1 has been found to restore muscle insulin sensitivity caused by high fat feeding-induced intramuscular triglyceride accumulation in this muscle type (Bruce et al., 2009). This may indicate that adiponectin upregulation of CPT1 mRNA expression could explain some of the insulin sensitising effects of high OA diets observed in other studies (Madigan et al., 2000; Soriguer et al., 2004; Yoon et al., 2006).

In the EDL muscles of the 'Western' diet group incubated with adiponectin, expression of PGC1α mRNA was decreased compared to the untreated muscle, resulting in levels similar to that of the other diet groups. Comparing between dietary groups for the EDL muscles incubated with adiponectin the 'Western' diet then chow and the high LA diet groups had significantly lower PGC1α mRNA expression than the chow fed animals. Furthermore, studies using transgenic mice over expressing PGC1α have shown that they are more prone to intramyocellular lipid induced insulin resistance due to decreased insulin stimulated glucose uptake, most likely due to a relative increase in fatty acid delivery (Choi et al., 2008). Despite this, studies using transgenic mice only mildly over expressing PGC1α in skeletal muscle have found that they are resistant to diabetes and obesity caused by aging (Wenz et al., 2009). Considered with the results found here, this may indicate that specific FA, or the n-6:n-3 of a diet may affect mitochondrial biogenesis, as the group with the highest n-6:n-3 (the high LA diet) was the only high fat

diet group which had a lower PGC1α expression after adiponectin incubation compared to the chow fed animals. Moreover, though adiponectin incubation of EDL muscles from the LA fed animals resulted in an increase in β-HAD mRNA expression, CPT1 and FAT/CD36 mRNA expression were not upregulated, possibly indicating that though there was an increase in β-oxidative capacity in this muscle type there may not have been an increased supply of substrate into the muscle or mitochondria. A higher LA, or higher n-6:n-3 diet, may impair the ability of adiponectin to increase β-HAD mRNA expression, with the other two high fat, though lower n-6:n-3, diet groups showing an increase in expression in the adiponectin treated muscles.

In the EDL muscles from the animals which were switched from the 'Western' diet to the chow diet at the beginning of the experimental period, adiponectin incubation only increased β-HAD mRNA expression. This possibly indicates that energy restriction, which caused a reduction in body fat percentage on par with the chow fed animals (as shown in Chapter 4), increased the use of FA as an energy source in this usually glycolytic muscle type. As expression of the genes involved in FA movement into the cell and mitochondria were not increased, this may indicate clearing of lipid deposits within the cell by mitochondrial FA oxidation.

Obesity appears to ameliorate the ability of adiponectin to increase AMPKα2 mRNA expression in the EDL muscle, as incubation resulted in a decrease in AMPKα2 mRNA expression in the 'Western' diet group and an increase in the chow group. This increase resulted in the chow group having significantly higher expression than all other groups for the adiponectin treated muscles. Incubation of EDL but not soleus, with the same concentration of globular adiponectin as used in this Chapter, has been found to increase AMPKα2 expression two-fold (Tomas et al., 2002). This results in increased FA oxidation due to AMPK being able to phosphorylate and inhibit malonyl CoA

decarboxylase, which is an allosteric inhibitor of CPT1, allowing CPT1 to transfer adequate FA into the mitochondria for oxidation (Tomas et al., 2002). However, incubation of the EDL muscles with adiponectin only elicited a response in the high OA group (an increase in CPT1 mRNA expression), with the 'Western' diet then chow animals having a lower expression than the chow muscles.

5.5.6 Conclusion

Utilising specially designed diets to mimic dietary FA compositions commonly consumed in humans, this study has shown that FA composition of the diet, or the n-6:n-3, can potentially modulate skeletal muscle metabolic gene expression in Sprague Dawley rats, though further assessment of protein contents and activities would demonstrate if the changes in mRNA expression have downstream affects. Feeding a high OA diet resulted in the maintenance of a lean phenotype of FAT/CD36 expression, with higher expression in the soleus compared to the EDL, which may prevent intramyocellular lipid accumulation in this tissue, which was not seen in the other high fat diets. Moreover, in the high OA diet animals, incubation of both EDL and soleus muscles with globular adiponectin increased expression of FAT/CD36, CPT1 and β-HAD mRNA, the combination of which were not seen in any of the other dietary groups, indicating influx of FA into the muscle and mitochondria and increased FA oxidation capacity and preservation of adiponectin signalling pathways. This may indicate that high OA feeding preserves adiponectin sensitivity in both of these muscle types and may confer metabolic benefits, which was not seen in the other high fat diets tested in this study, and may potentially increase whole body fat oxidation. In contrast, though adiponectin incubation of soleus muscles from high LA fed animals resulted in an increase in FAT/CD36, CPT1 and PGC1α mRNA expression, this did not occur in the EDL. Overall, the results of this study indicate that in DIO, a high OA diet may prevent development of muscle adiponectin resistance in both oxidative and glycolytic fibre type muscles, which has been

found to precede insulin resistance. These findings may add further support to the body of evidence indicating the ability of dietary OA to promote metabolic health.

Chapter 6: The acute effect of mixed composition meals containing different plant fats on appetite parameters and metabolic and inflammatory markers in overweight and obese individuals

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Portions of this Chapter (including parts of the study design, participant characteristics, VAS results and blood glucose responses) were discussed as part of the author's Bachelor of Science (Honours) thesis.

6.1 Abstract

Background: Human appetite and regulatory factors are modulated by a range of factors, though the effects of different plant-derived fats, which are abundant in our diet, have yet to be elucidated. This study aimed to investigate the ability of the two most abundant dietary plant derived fats, oleic (OA) and linoleic (LA) acids to modulate post-prandial appetite perception and levels of circulating appetite and metabolic regulators in overweight and obese individuals.

Methods: This study was designed as a single-blinded 3-way cross over study, with a wash out period between meal challenges. Meals were a predominately carbohydrate control meal, a high OA meal (from extra virgin olive oil) and a high LA meal (from safflower oil) and were comprised of toasted bread, jam and mixed oils, weighed to provide 30 % of participants' estimated energy requirements. Meals were consumed in the morning after an overnight fast, with blood samples collected 1 hour prior to consumption, immediately before and after consumption, and 1 and 2 hours post consumption. Appetite parameters were assessed via a validated visual analogue scale questionnaire immediately before consumption and 2 hours after. Blood glucose levels

were measured using an automated analyser and hormones and other circulating factors were quantified using a Bioplex multiplex system.

Results and Discussion: 8 participants completed the study. For appetite parameters, all meals resulted in a significant increase in fullness and a reduction in desire to eat. The control and high OA meals decreased prospective food intake though this did not occur following ingestion of the high LA meal. Additionally, following the consumption of the LA meal there was a significant increase in ghrelin production, a hormone which encourages hedonic food intake. This was coupled with an acute increase in resistin production at the one hour time point, indicating potential impairment of insulin signalling. Taken together this indicates that high LA meals may promote excess energy intake and impair glucose handling, though further investigation is required.

6.2 Background

Appetite is the main regulator of food intake, and as such is a key component in the regulation of energy intake and body weight. Appetite is modulated by a range of endogenous substances produced by the stomach, small intestine, pancreas, adipose tissue and brain, with many of these operating together, as well as in opposition (Neary et al., 2004). Despite this, it is believed that when high fat foods are available, as are found abundantly in current Western cultures, passive overconsumption occurs due to the body's inability to gauge energy intake accurately, potentially leading to chronic excesses of energy intake, which may lead to weight gain and obesity (Blundell & Macdiarmid, 1997). Additionally, there is the possibility that our hormonal control of appetite, which evolved in conjunction with a low fat diet, may be desensitised to overnutrition by modern high fat dietary patterns (Bray & Popkin, 1998; Duca et al., 2013).

With the exception of breakfast, most meals are consumed by humans during the postprandial state (Lopez-Miranda & Marin, 2010). As such, postprandial regulation of appetite is a key factor in energy intake. More specifically, understanding how postprandial appetite regulation is influenced by our current nutritional environment is important to understand how our food intake affects subsequent intake and ultimately, weight status.

When comparing the satiating effect of the macronutrients, lipids are the least satiating on a kJ basis, possibly leading to excessive energy intake (Rolls, 1995). Despite this, the ability of specific fats to modulate appetite and food intake is yet to be fully elucidated, an important area of research, especially as the dietary availability of linoleic acid (LA) in Australia has increased dramatically in recent times (Naughton et al., 2015). LA is the precursor of the two main endocannabinoids, anandamide and 2-arachidonyl glycerol, both of which promote hedonic food intake and adipose tissue energy storage.

Additionally the system is upregulated in obesity, which may further perpetuate excessive energy intake (Bluher et al., 2006; Engeli et al., 2005). The importance of LA in the postprandial state is not only due to its potential to modulate hunger and satiety, it is also the precursor of arachidonic acid, which can be converted to several different inflammatory mediators (Galli & Calder, 2009). As such there is a possibility that a high intake could perpetuate the low grade systemic inflammation found in overweight and obese individuals.

In comparison, oleic acid (OA) is not involved in the synthesis of inflammatory mediators, with several studies finding that OA and its endocannabinoid derivative oleoylethanolamide (OEA), reduce hunger and subsequent food intake (Diep et al., 2011; Lambert et al., 2007; Schwinkendorf et al., 2011). Despite this, studies comparing OA and LA containing meals in an acute time frame have failed to find a difference in perception of hunger or appetite in the postprandial period (Flint et al., 2003; Strik et al., 2010)

The role of specific fatty acids in modulating satiety regulating hormones and perception of hunger and prospective food intake is yet to be fully elucidated, with no clear associations identified between different fatty acids and appetite modulating hormones (Kaviani & Cooper, 2017). Therefore, this study aimed to assess the acute (2 hour) response of appetite regulating hormones, metabolic markers, and cytokines, as well as self-reported appetite parameters, following the consumption of high LA or OA containing mixed composition meals, in comparison to an energy matched predominately carbohydrate containing meal, in overweight or obese men and women.

6.3 Methods

6.3.1 Study design

This study was designed as a single blinded, 3-way cross over study. To remove interpersonal variables each participant consumed each test meal after an overnight fast, with one meal consumed each week in a random order with a minimum 5-day period between meals.

6.3.2 Ethical approval and subject recruitment

Ethical approval was obtained from the Victoria University human research ethics committee (HRETH 12/87). Inclusion criteria for the study was a BMI >25 kg/m^2 or waist circumference > 94 cm (male)/ > 80 cm (female), weight stable and aged 18-60 yrs. Exclusion criteria were the presence of Type I or II diabetes mellitus, insulin resistance, heart, liver or kidney disease, pregnant (or planning to become pregnant), breastfeeding, and the use of weight loss medication/supplementation or FA supplementation. Further information regarding methodology used throughout the study is contained in Chapter 2.

6.3.3 Meal challenge sessions

Meal challenge sessions took place with participants being in an overnight fasted state. A timeline of the meal sessions is shown in Figure 6.1. Upon arrival participants had a cannula inserted into their antecubital vein. All meals were consumed at the same time of day (between 9 am and 10 am) to control for diurnal hormonal variation, with a maximum of 15 minutes provided for consumption.

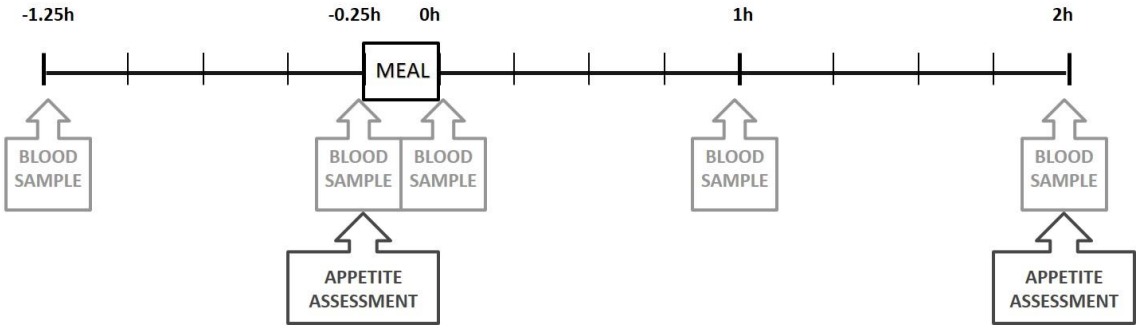

Figure 6.1: Meal Challenge Session Timeline. Participants were required to consume the test meal in a 15-min time frame. Blood sampling occurred on arrival, 1 hour later (immediately before consumption), immediately after consumption and then 1 and 2 hours post consumption. Participants completed appetite assessment via validated VAS questionnaires (developed by Parker, Sturm, et al. (2004)) immediately prior to consumption and 2 hours after.

6.3.4 Test meal compositions

The test meals were comprised of toasted bread, reduced sugar jam, icing sugar and differing oil compositions (extra virgin olive oil, safflower oil and coconut oil), resulting in control, high OA (high monounsaturated (MUFA)), and high LA (high polyunsaturated (PUFA)) meals, with all ingredients used commonly found in Western diets. The high fat meals had comparable ratios of all fats other than the one which had been elevated, with the amount of energy from the elevated fats in each high fat meal also being matched (for further details see Table 6.1). The two high fat meals were isoenergetic and had matched contributions from macronutrients to energy content. Each test meal contained 13.74 ± 0.658 kJ/g (as determined by nutritional analysis performed using Xyris FoodWorks (Xyris Pty. Ltd., Highgate Hill. QLD, Australia)) and provided 30 % of each participant's estimated daily energy intake calculated using the Mifflin-St Jeor equation (Mifflin et al., 1990) and estimates of usual daily activity levels.

Table 6.1: Nutritional Composition of Experimental Meals

	CONTROL	HIGH OA	HIGH LA
Carbohydrate (% E)	73.46	39.74	39.74
Protein (% E)	6.91	6.84	6.84
Fat (% E)	23.12	55.13	55.13
- Saturated (% Total Fat)	34.03	14.41	14.54
- MUFA (% Total Fat)	34.63	70.65	14.41
- Oleic Acid (% of MUFA)	95.05	97.88	94.94
- PUFA (% Total Fat)	31.32	14.93	70.74
- Linoleic Acid (% of PUFA)	94.97	94.08	98.68
Omega 6: Omega 3	16.7	13.09	77.20

% E = Percentage of total energy. OA; Oleic acid, LA; Linoleic acid

6.3.5 Body composition Assessment and Anthropometric Measurements

Body composition (fat mass % and lean mass %) was measured using dual energy x-ray absorptiometry (DXA) (Hologic Discovery QDR, Hologic (Australia) Pty Ltd, North Ryde, NSW, Australia) in the first week of the study. Anthropometric measurements were taken twice during the study and included height measurement, weight, waist circumference, hip circumference, blood pressure and heart rate.

6.3.6 Blood collection and Analysis

A total of five blood samples were collected over each 3 ¼ hour meal session (see Figure 6.1). Samples were analysed to measure blood glucose concentrations (all time points) and to quantify satiety and diabetes related markers, and cytokines (baseline, 1 hour post meal and 2 hours post meal) using multiplex assays and a Bioplex© 200 multiplex suspension array system (Biorad, California, USA), according to the manufacturer's instructions.

6.3.7 Appetite assessment

Participants completed an appetite questionnaire immediately before consumption of the meal and two hours after. This was in the form of a visual analogue scale (VAS) questionnaire (developed by Parker et al. (2004)) assessing appetite parameters (including nausea) and other sensations which may impact perceived appetite, such as anxiety and fatigue.

6.3.8 Statistical analysis

All statistical analysis was performed using Prism GraphPad. For VAS scores a 2-way ANOVA was utilised. Difference between blood glucose levels in response to test meal consumption at each time-point was determined via 2-way ANOVA with Tukeys multiple comparisons test, as was change between time-points within each test meal. For analysis of appetite regulating compounds and inflammatory markers, area under the curve was utilised. This was calculated as a change from baseline and also the net change from baseline to account for negative peaks, i.e. reductions in circulating concentrations over the period of sample collection. Analytes that did not have a full set of data inside the reporting range were excluded from analysis.

6.4 Results

6.4.1 Participants

Table 6.2: General Participant Characteristics

		Average ± SEM
Age (Years)		45.8 ± 3.6
Height (cm)		169.5 ± 2.2
Weight (Kg)		92.2 ± 4.5
BMI (Kg/ m^2)		32.0 ± 1.3
Waist Circumference (cm)	Female	102.4 ± 7.1
	Male	108.6 ± 7.9
Hip Circumference (cm)	Female	119.6 ± 4.1
	Male	111.3 ± 5.7
Waist to Hip Ratio	Female	0.83 ± 0.03
	Male	0.97 ± 0.01
Fat Mass (% of Total Mass)		37.4 ± 2.9
Lean Mass (% of Total Mass)		55.7 ± 2.8
Systolic Blood Pressure (mmHg)		128.4 ± 4.1
Diastolic Blood Pressure (mmHg)		82.3 ± 3.5
Heart Rate (bpm)		73 ± 4.4
Fasting Serum Glucose (mmol/L)		4.6 ± 0.1

Of the 14 who commenced the study, one participant was removed from analysis due to the final blood sample not being able to be collected for one meal session. Three participants left the study due to time constraints and two left the study due to discomfort during blood sampling. Eight participants completed the study, 5 females and 3 males. The average age was 45.8 ± 3.6 and average BMI 32.03 ± 1.3 kg/m^2. Further participant characteristics are shown in Table 6.2.

6.4.2 Appetite Measures

For the parameters related to appetite there was a significant reduction in feeling of hunger and a significant increase in feeling of fullness after consumption of all meals as shown in Figure 6.2. There was no significant change to perceived satisfaction and desire to eat following consumption of all three meals. In regards to prospective food intake amount (with 'none' being the bottom of the scale and 'A large amount' being the top of scale) there was a significant decrease following consumption of the control and high OA meals, though not following consumption of the high LA meal, as shown in Figure 6.2. VAS results showed no changes to perception of fatigue, nausea or anxiety as a result of the consumption of any of the test meals, as shown in Figure 6.3. This removes the influence of these variables on perceived hunger as a result of meal consumption. For further information regarding the scales used on the VAS questionnaire please see Appendix, Figure 9.3.

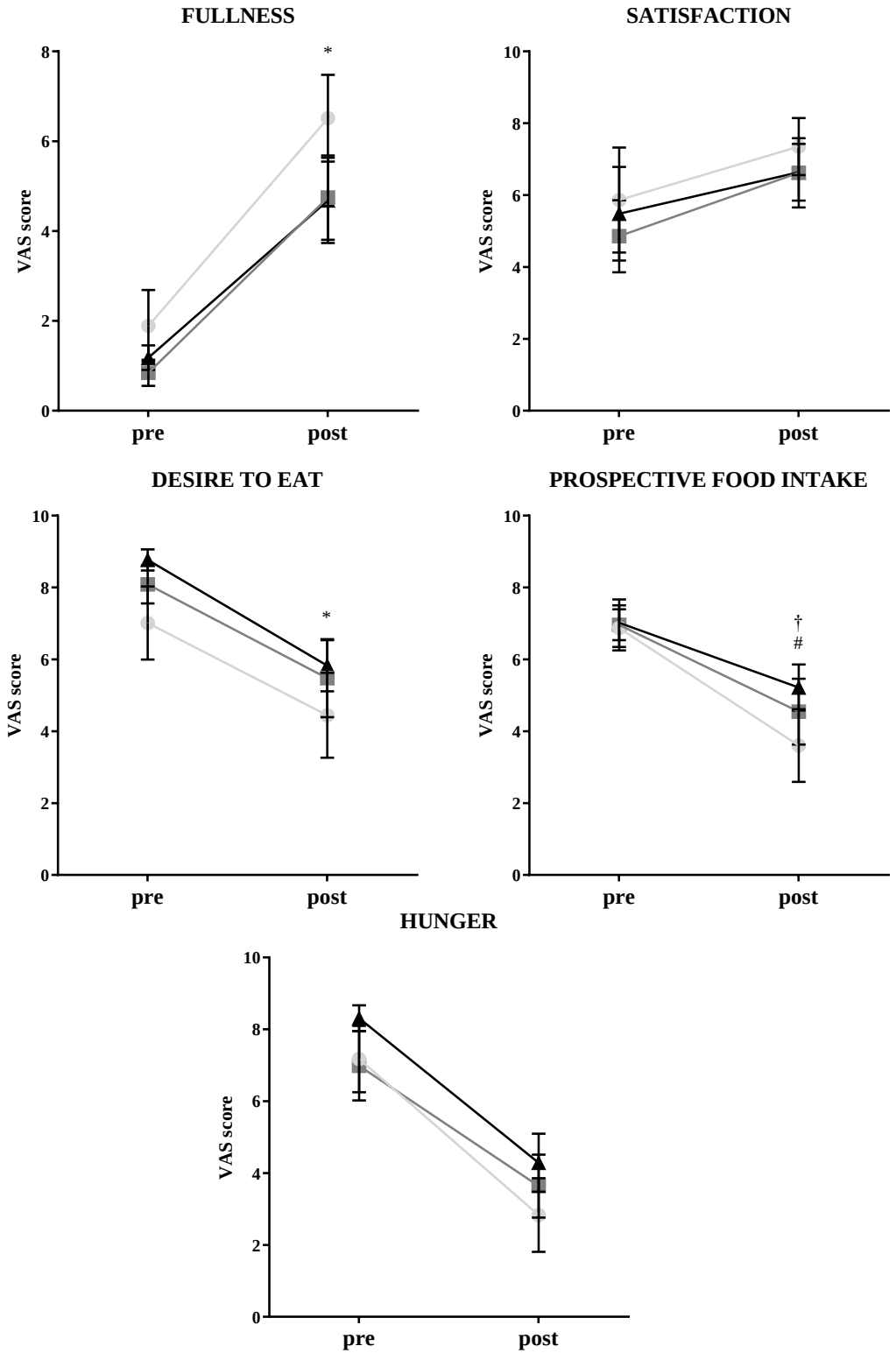

Figure 6.2: Participant Visual Analogue Scale appetite parameter responses, ● = control meal, ■ = high OA, ▲= high LA. Pre = VAS completed 15 minutes prior to consumption; Post = VAS completed 2 h post consumption. * = $p < 0.05$ all meals comparing pre-and post-consumption. † = $p < 0.05$ for control meal comparing pre-and post-consumption. # = $p < 0.05$ for high OA comparing pre-and post-consumption. n = 8 for each data set. All data shown as mean ± SEM.

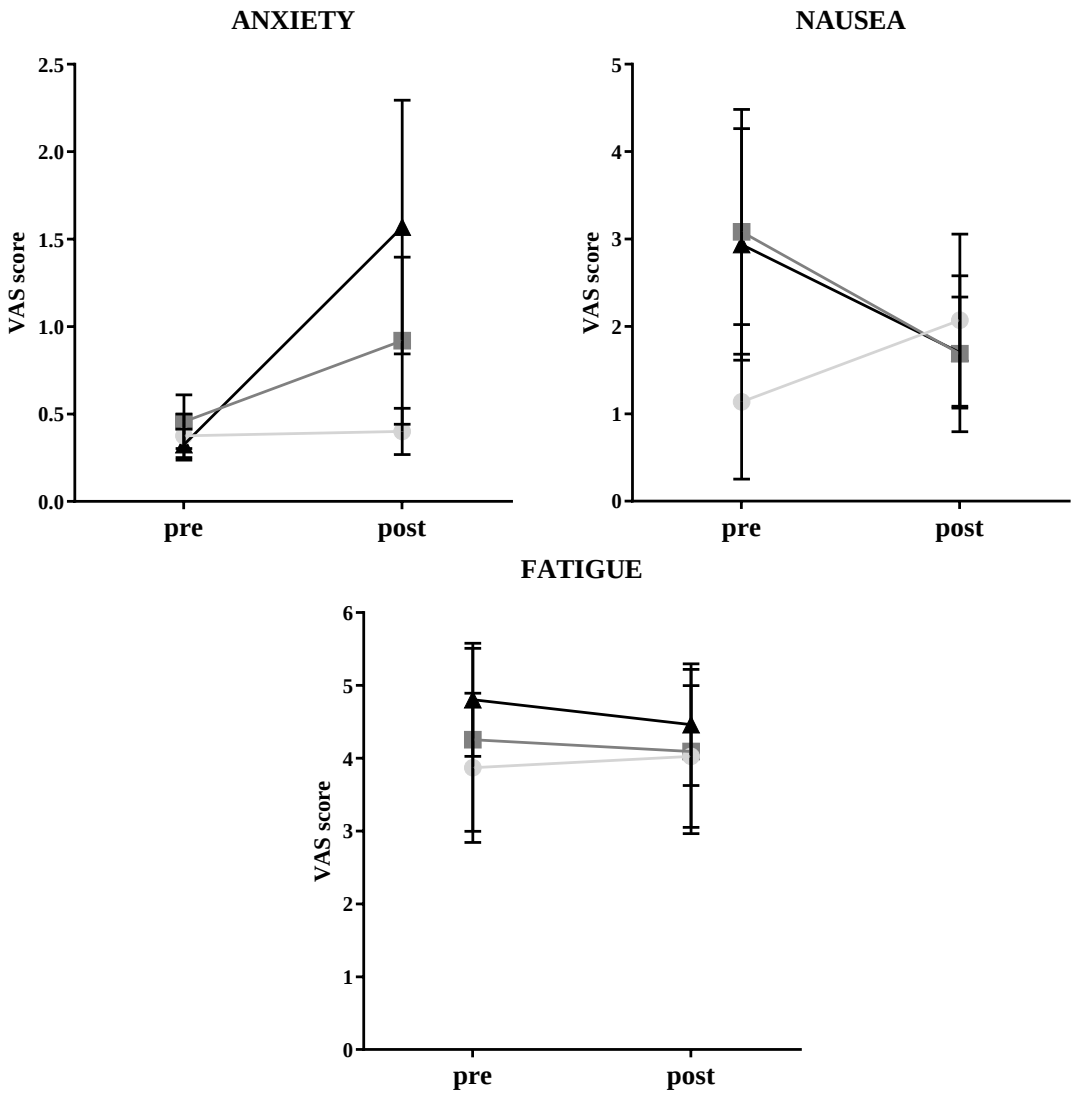

Figure 6.3: Participant Visual Analogue Scale other sensations responses, ● = control meal, ■ = high OA, ▲= high LA. Pre = VAS completed 15 minutes prior to consumption; Post = VAS completed 2 h post consumption. n = 8 for each data set. All data shown as mean ± SEM.

6.4.3 Biochemical Measures

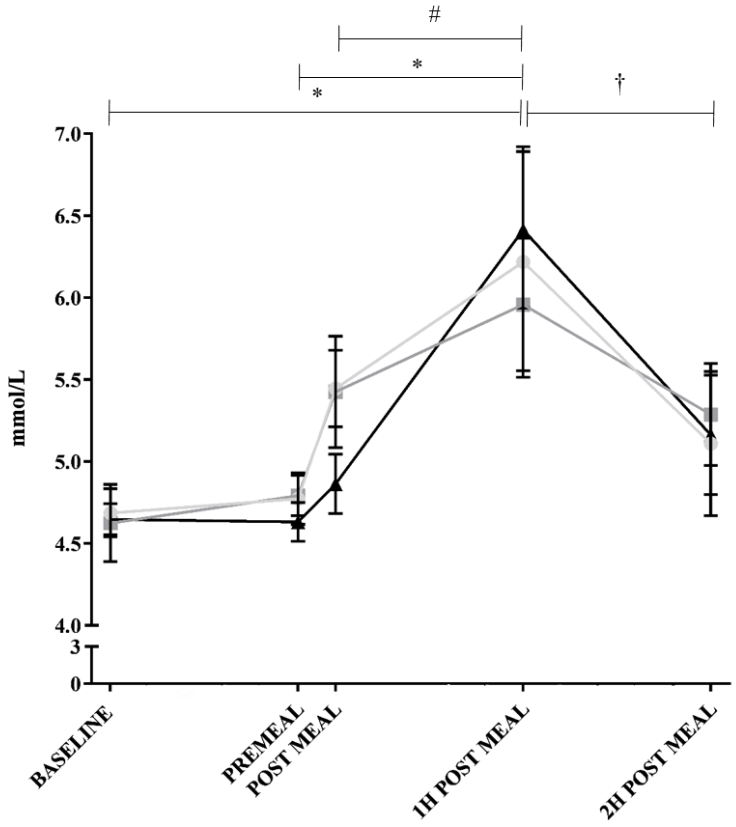

Figure 6.4: Blood glucose responses to ingestion of test meals, ● = control meal, ■ = high OA, ▲= high LA. * = p < 0.05 all meals comparing time-points. † = p <0.05 for control and high LA comparing time-points. # = p< 0.05 for high LA comparing time-points. All data shown as mean ±SEM, n = 8 for each data point.

Blood glucose measurement showed no significant difference between the test meals at any of the time-points. There was a significant increase in blood glucose concentrations following the consumption of all test meals between baseline and 1 hour post meal consumption and between pre-meal and 1 hour post consumption. When comparing time-points for the high LA meal, the blood glucose concentration at the 1 hour time point was significantly increased compared to immediately post consumption, however none of the other meals were increased from immediately post consumption to 1 hour post consumption. There was a significant decrease in blood glucose concentrations between 1 and 2 hours post consumption for the control and high LA meals, though not for the high OA meal.

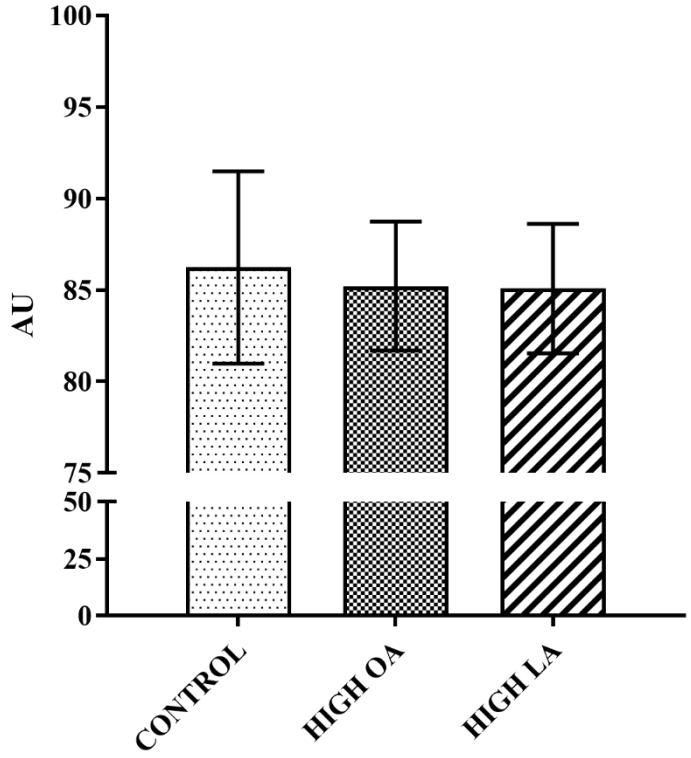

Figure 6.5: AUC of blood glucose responses to ingestion of test meals. Measured over the 2 hours post consumption. Displayed in arbitrary units (AU). n = 8 for each data set. Data shown as mean ± SEM.

Calculation of area under the curve of the blood glucose levels in the 2-hour post consumption of each test meal found no significant differences in blood glucose response between the different test meals as shown in Figure 6.5.

Consumption of all test meals resulted in a significant increase in insulin at the 1 hour post consumption time-point. In response to consumption of the control meal, insulin levels were still significantly elevated compared to baseline at the 2-hour post consumption time-point. In response to the consumption of the high LA test meal there was a significant decrease in insulin concentrations between the 1 and 2-hour post consumption time-points. Despite this, no significant differences were found between the test meals during subsequent AUC analysis as shown in Figure 6.6.

In response to the high LA test meal consumption there was a significant increase in Ghrelin production from baseline compared to the control meal when analysing the net AUC, additionally there was a significant reduction in Ghrelin concentrations following consumption of the control meal at both the 1 and 2-hour time-points when compared to baseline as shown in Figure 6.7.

In response to consumption of all test meals there was a significant increase in glucose-dependent insulinotropic peptide (GIP) at the 1 hour post consumption time-point, as shown in Figure 6.8. Consumption of the control and LA test meals resulted in a sustained significant difference when compared to baseline at the 2-hour post consumption time-point, though not for the high OA test meal. At the 2-hour time-point there was a significant decrease in GIP concentrations following consumption of the high OA test meal, though there were no significant differences for any of the AUC analyses between the different meals.

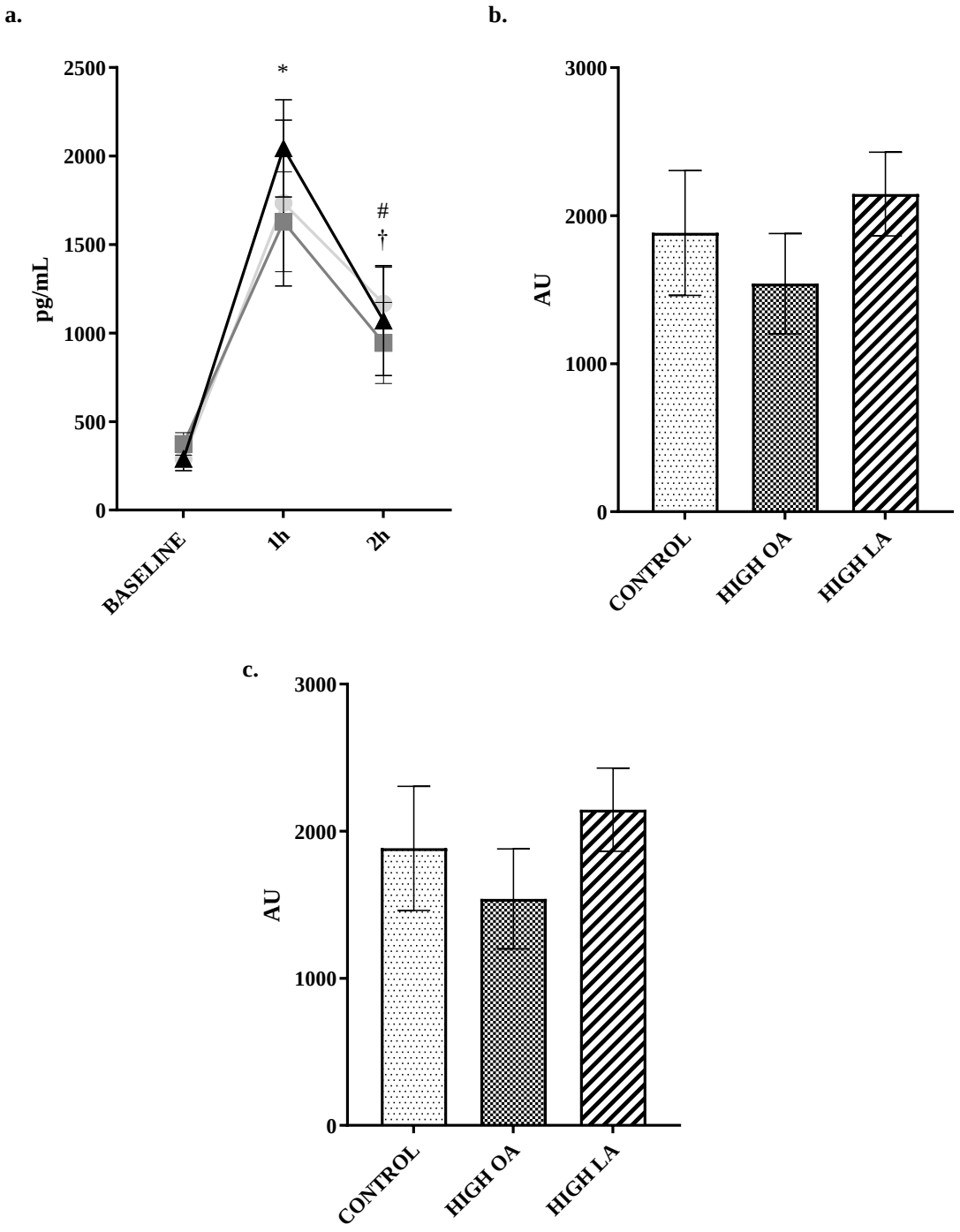

Figure 6.6: Change in Insulin over 2-hour postprandial period. a. plotted values. ● = control meal, ■ = high OA, ▲= high LA; b. Area under the curve (AUC) from baseline; c. Net AUC from baseline. All data displayed as Mean ± SEM, n = 8 for each data set. * = significantly different to baseline for all test meals. # = significantly different to baseline for control meal. † = significantly different to 1 h for the high LA test meal.

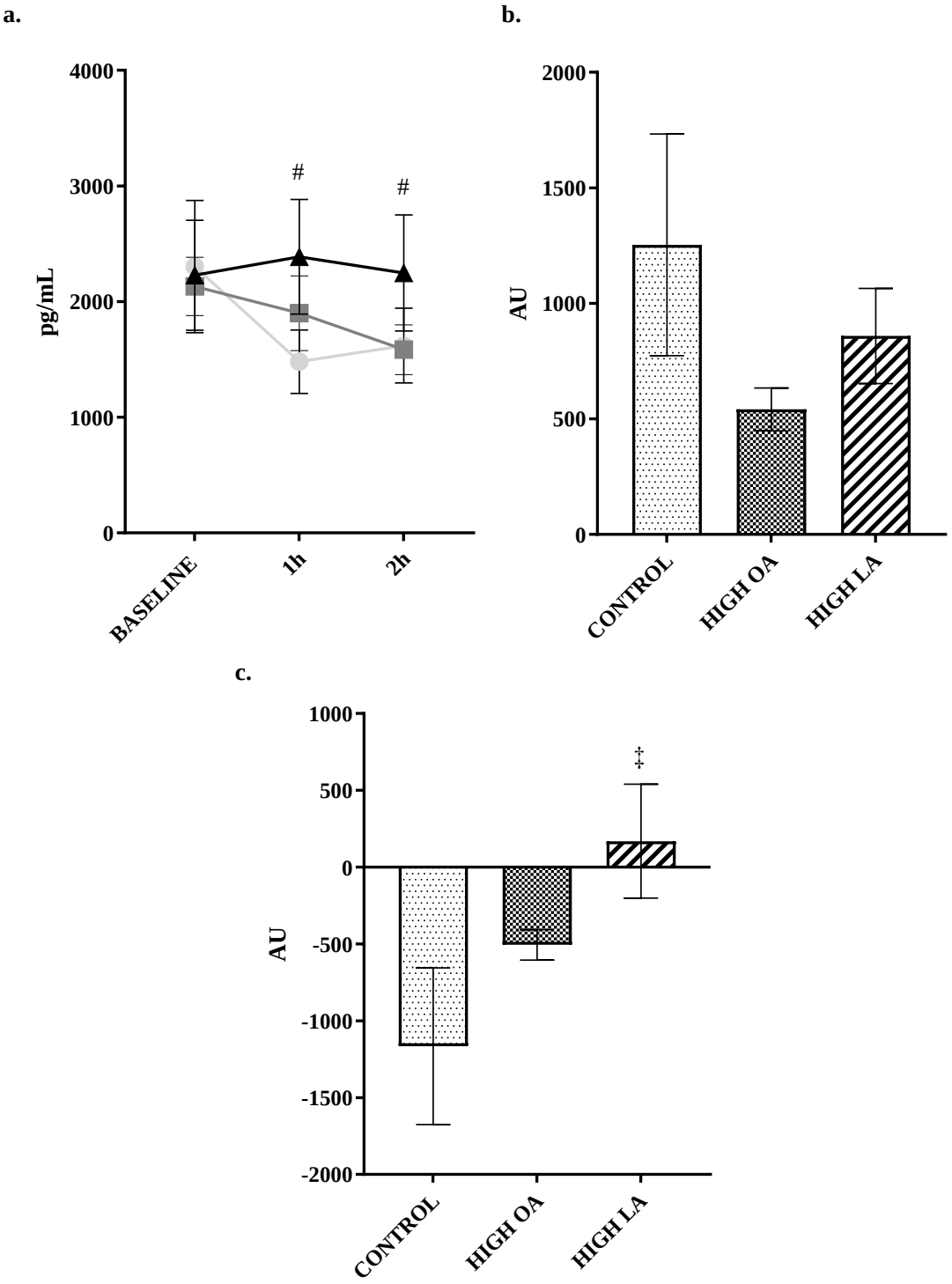

Figure 6.7: Change in Ghrelin over 2-hour postprandial period. a. plotted values., ● = control meal, ■ = high OA, ▲= high LA; b. Area under the curve (AUC) from baseline; c. Net AUC from baseline. All data displayed as Mean ± SEM, n = 8 for each data set. # = significantly different at this time-point compared to baseline for control meal. ‡ = significantly different to control meal.

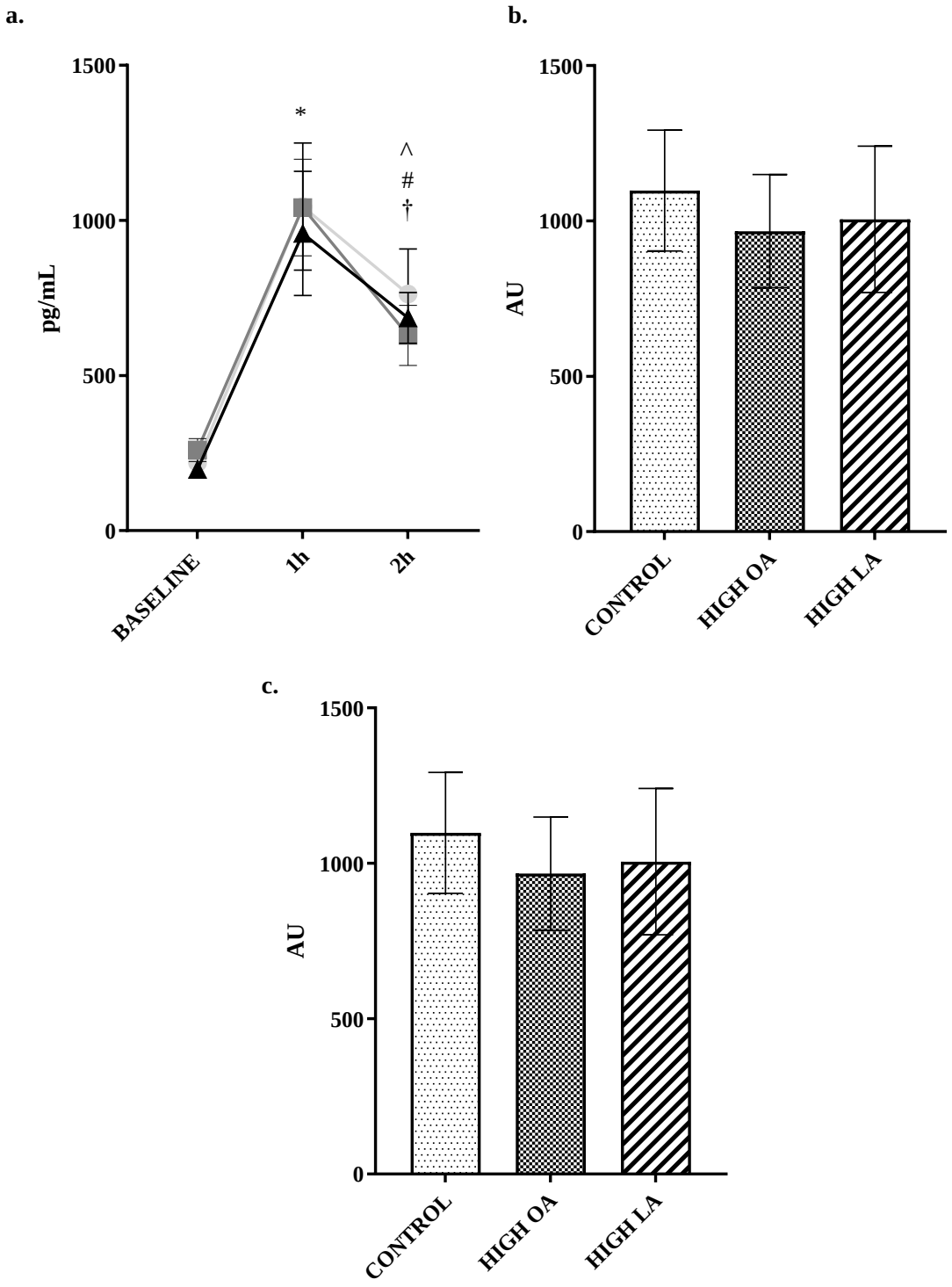

Figure 6.8: Change in glucose-dependent insulinotropic peptide (GIP) over 2-hour postprandial period. a. plotted values., ● = control meal, ■ = high OA, ▲= high LA b. AUC from baseline; d. Net AUC from baseline. All data displayed as Mean ± SEM, n = 8 for each data set. * = significantly different to baseline for all test meals. # = significantly different to baseline for control, † = significantly different to baseline for high LA test meal. ^ = significantly different to 1 h for the high OA test meal.

Consumption of test meals failed to elicit any significant differences in glucagon-like peptide 1 (GLP-1) or glucagon for all types of analysis performed as shown in Figures 6.9 and 6.10.

At 1 hour post meal consumption, there was a significant increase in resistin following consumption of the high LA test meal, though this failed to result in a significant difference in net AUC for the test period ($p = 0.13$ compared to control), as shown in Figure 6.11.

Consumption of test meals failed to elicit any significant differences in leptin for all types of analysis performed as shown in Figure 6.12.

As shown in Figure 6.13 net AUC indicates a trend towards reduced adiponectin following consumption of the control meal and an increase following consumption of the OA meal, though this did not reach statistical significance ($p = 0.12$).

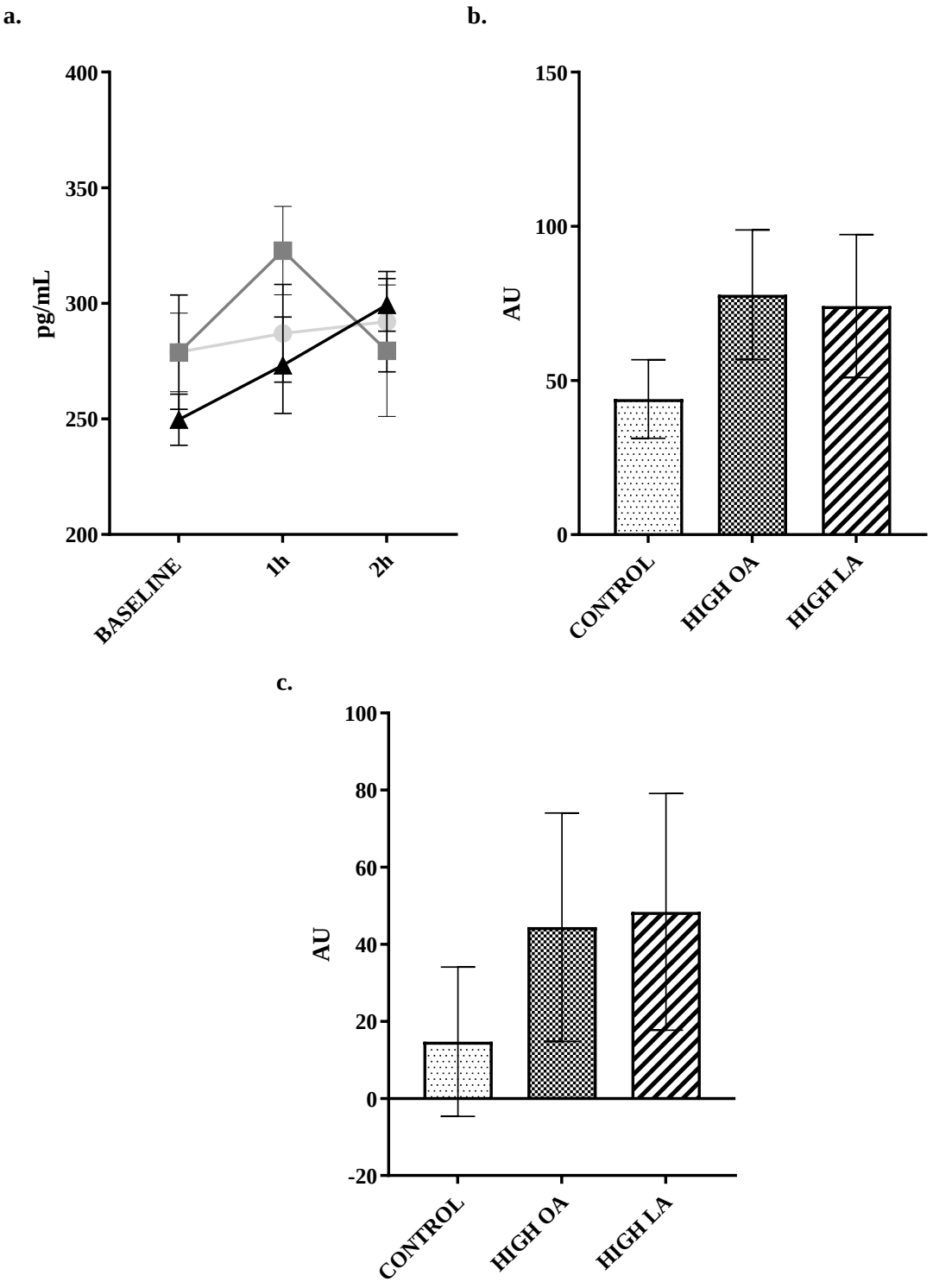

Figure 6.9: Change in glucagon-like peptide 1 (GLP-1) over 2-hour postprandial period. a. plotted values ● = control meal, ■ = high OA, ▲= high LA; b. Area under the curve (AUC) from baseline; c. Net AUC from baseline. All data displayed as Mean ± SEM, n = 8 for each data set.

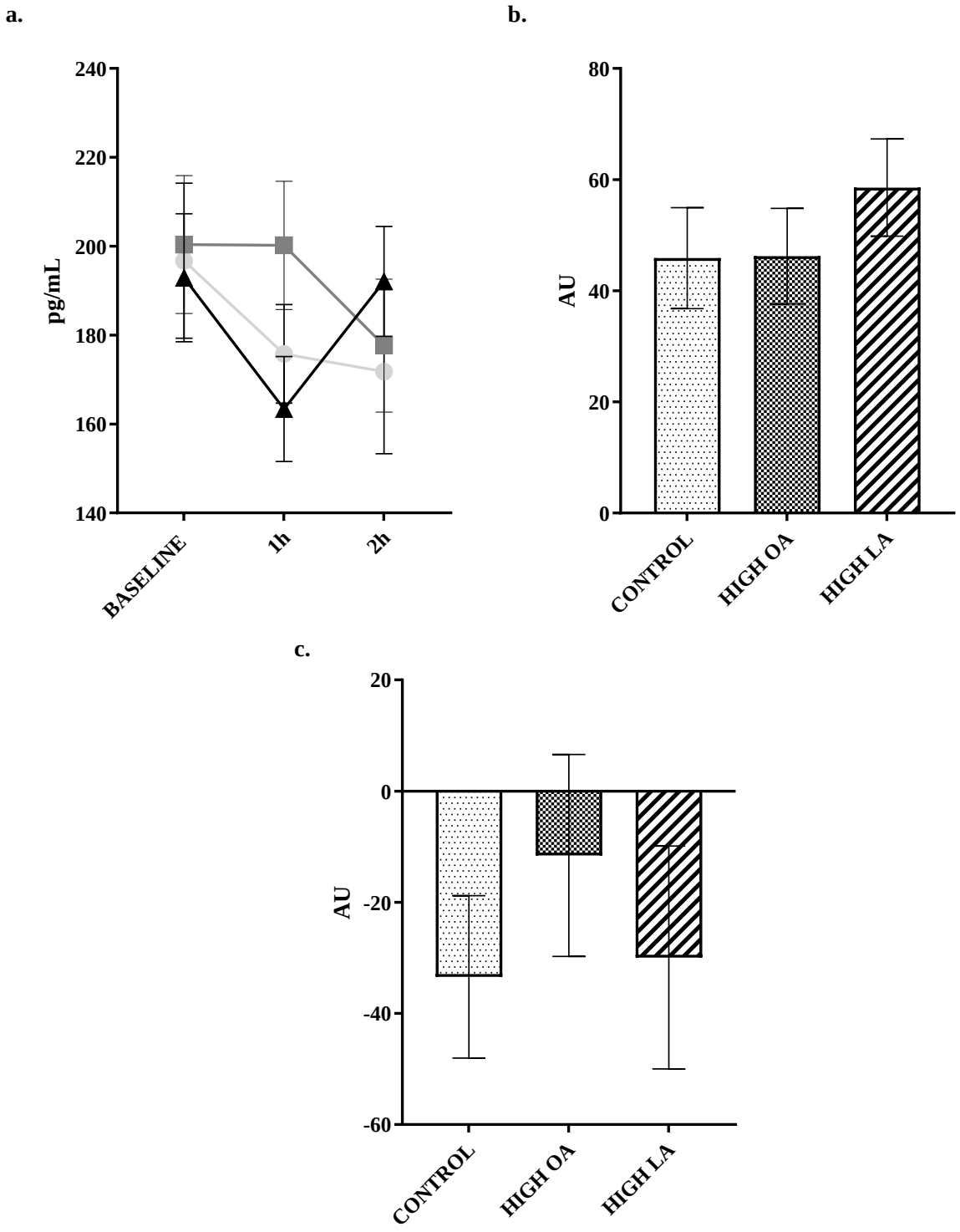

Figure 6.10: Change in Glucagon over 2-hour postprandial period. a. plotted values. ● = control meal, ■ = high OA, ▲= high LA; b. Area under the curve (AUC) from baseline; c. Net AUC from baseline. All data displayed as Mean ± SEM, n = 8 for each data set.

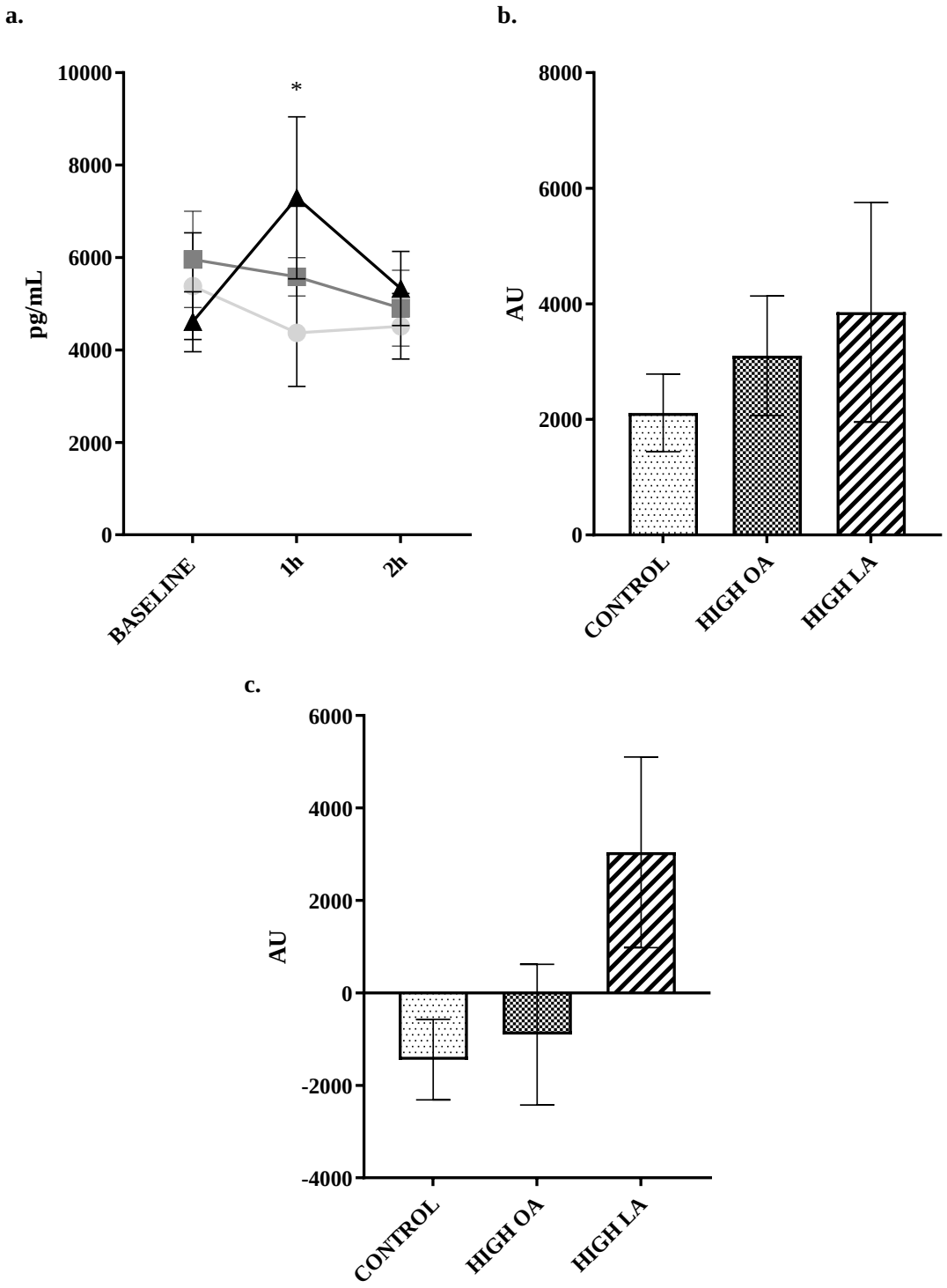

Figure 6.11: Change in Resistin over 2-hour postprandial period. a. plotted values., ● = control meal, ■ = high OA, ▲= high LA; b. Area under the curve (AUC) from baseline; c. Net AUC from baseline. All data displayed as Mean ± SEM, n = 8 for each data set. * = significantly different at this time-point to baseline for the LA meal.

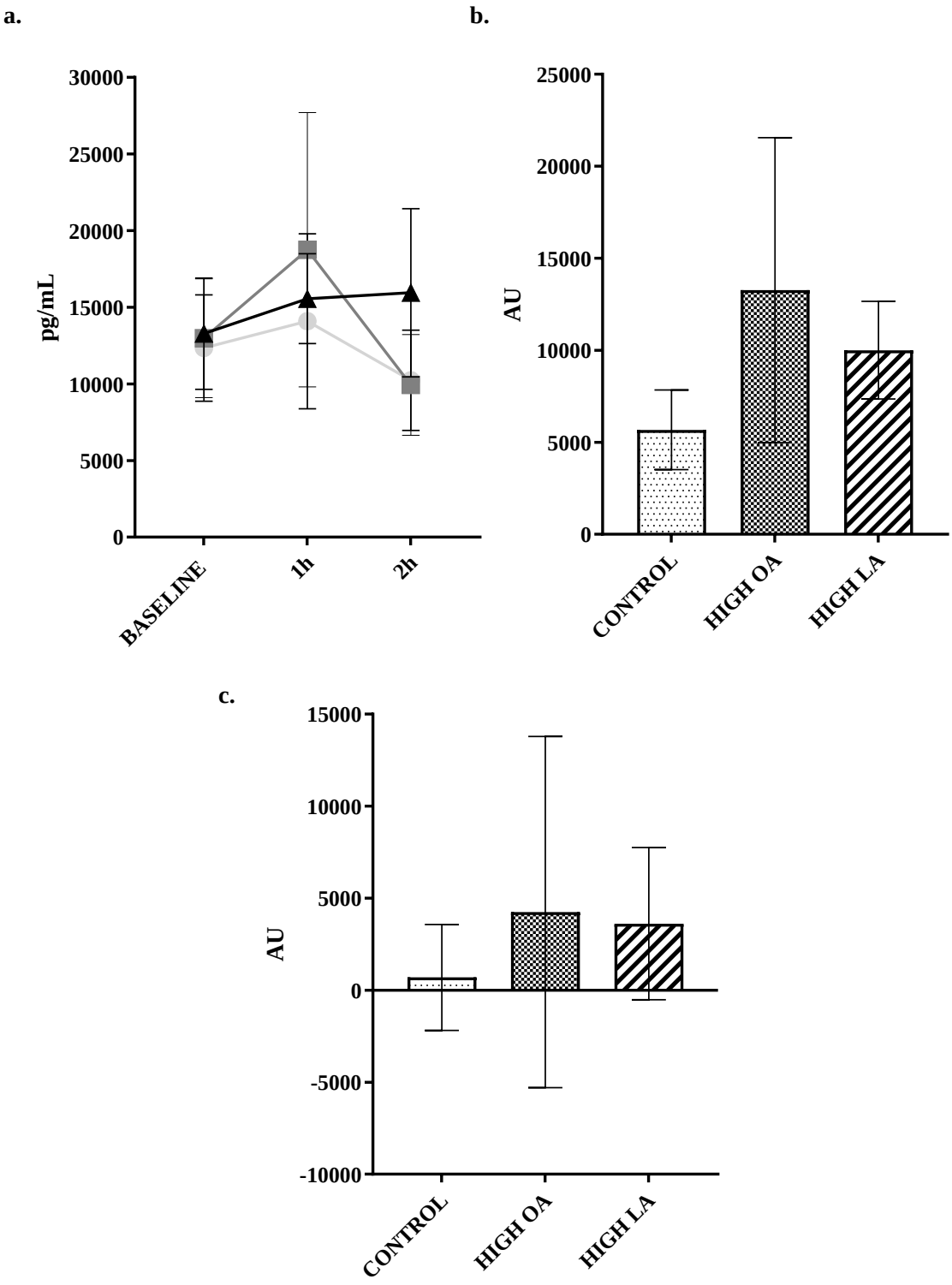

Figure 6.12: Change in Leptin over 2-hour postprandial period. a. plotted values., ● = control meal, ■ = high OA, ▲= high LA; b. Area under the curve (AUC) from baseline; c. Net AUC from baseline. All data displayed as Mean ± SEM, n = 8 for each data set.

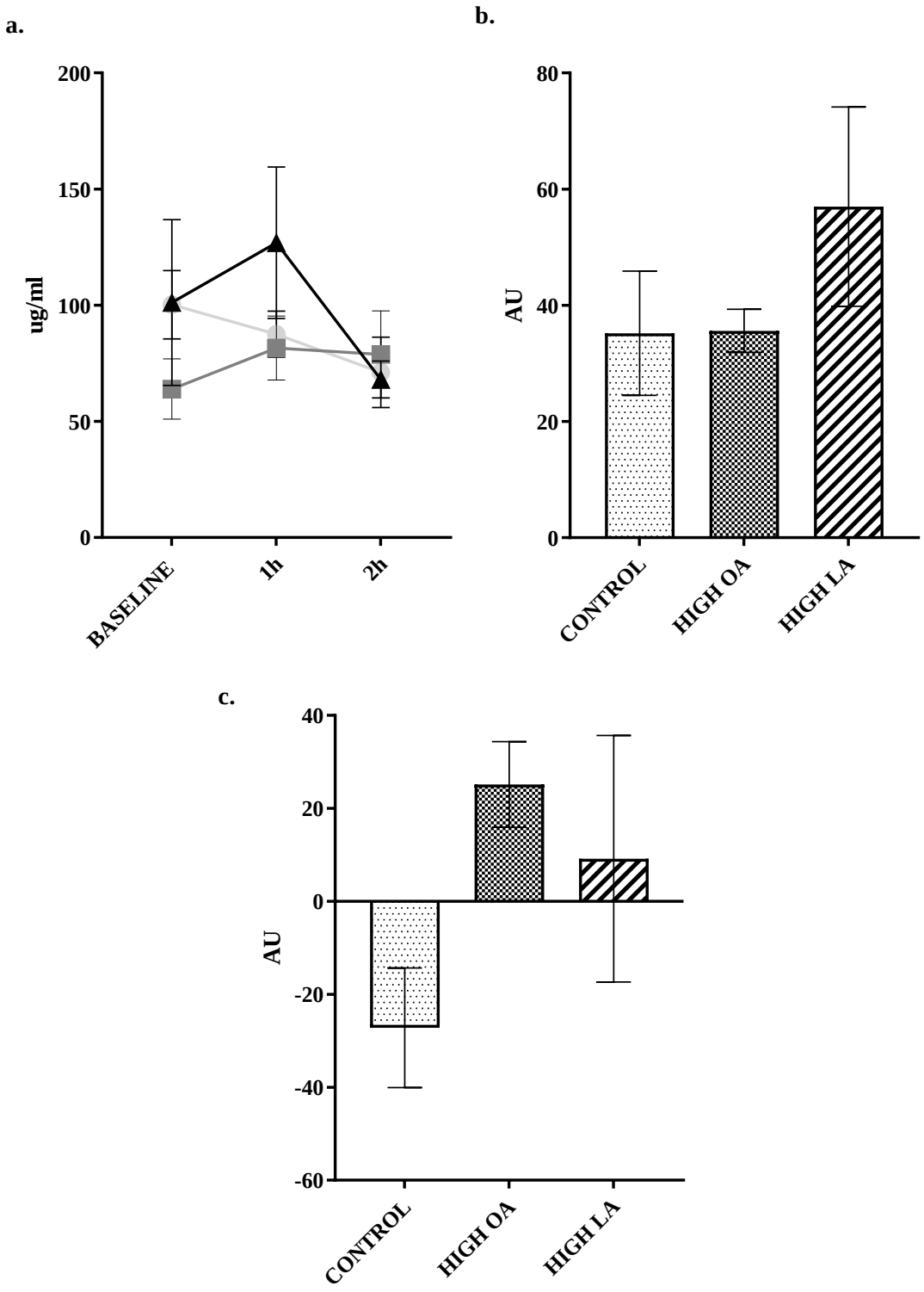

Figure 6.13: Change in Adiponectin over 2-hour postprandial period. a. plotted values. ● = control meal, ■ = high OA, ▲= high LA; b. Area under the curve (AUC) from baseline; c. Net AUC from baseline. All data displayed as Mean ± SEM, n = 8 for each data set.

There was no significant change to IL-1β, IL-6, IL-10, IL-13, or C-reactive protein (CRP) in response to test meal consumption as shown in Figures 6.4.14- 6.18. As shown in Figure 6.19 the response of tumour necrosis factor alpha (TNF-α) to meal consumption following the LA meal showed a trend towards a reduction, though this did not reach statistical significance ($p = 0.16$) when compared to the control meal.

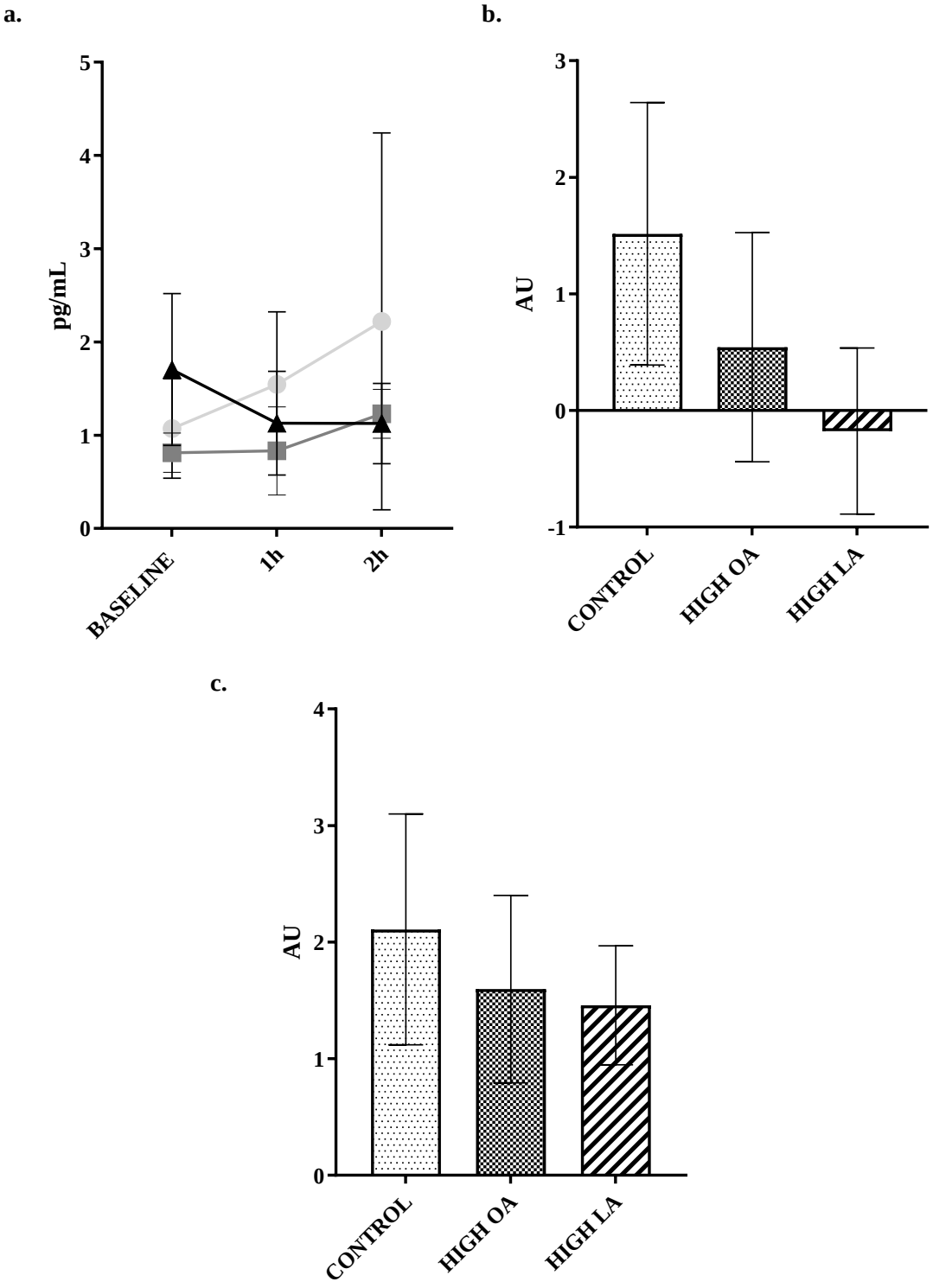

Figure 6.14: Change in IL-1β over 2-hour postprandial period. a. plotted values., ● = control meal, ■ = high OA, ▲= high LA; b. Area under the curve (AUC) from baseline; c. Net AUC from baseline. All data displayed as Mean ± SEM, n = 8 for each data set.

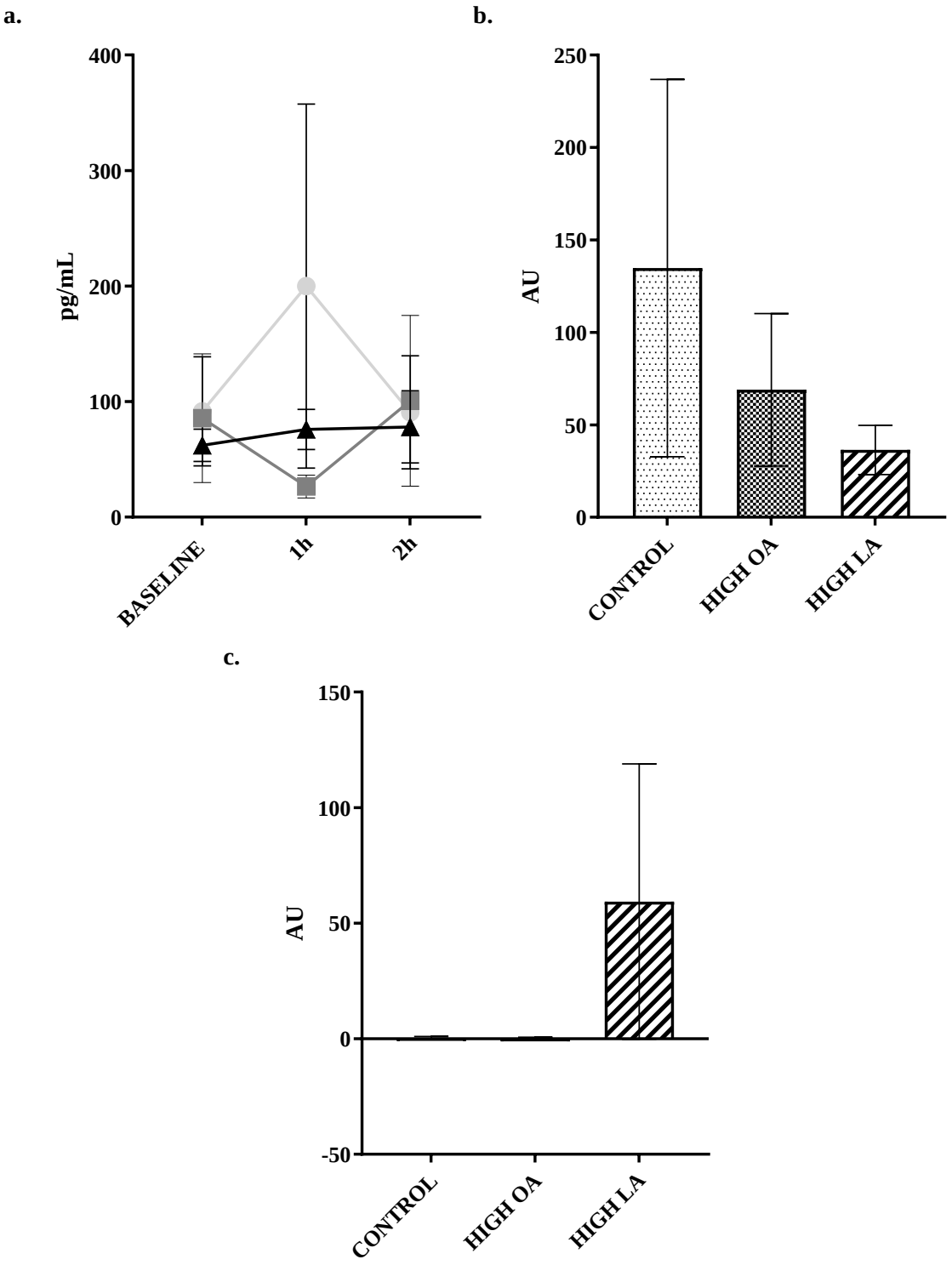

Figure 6.15: Change in IL-6 over 2-hour postprandial period. a. plotted values. ● = control meal, ■ = high OA, ▲= high LA; b. Area under the curve (AUC) from baseline; c. Net AUC from baseline. All data displayed as Mean ± SEM, n = 8 for each data set.

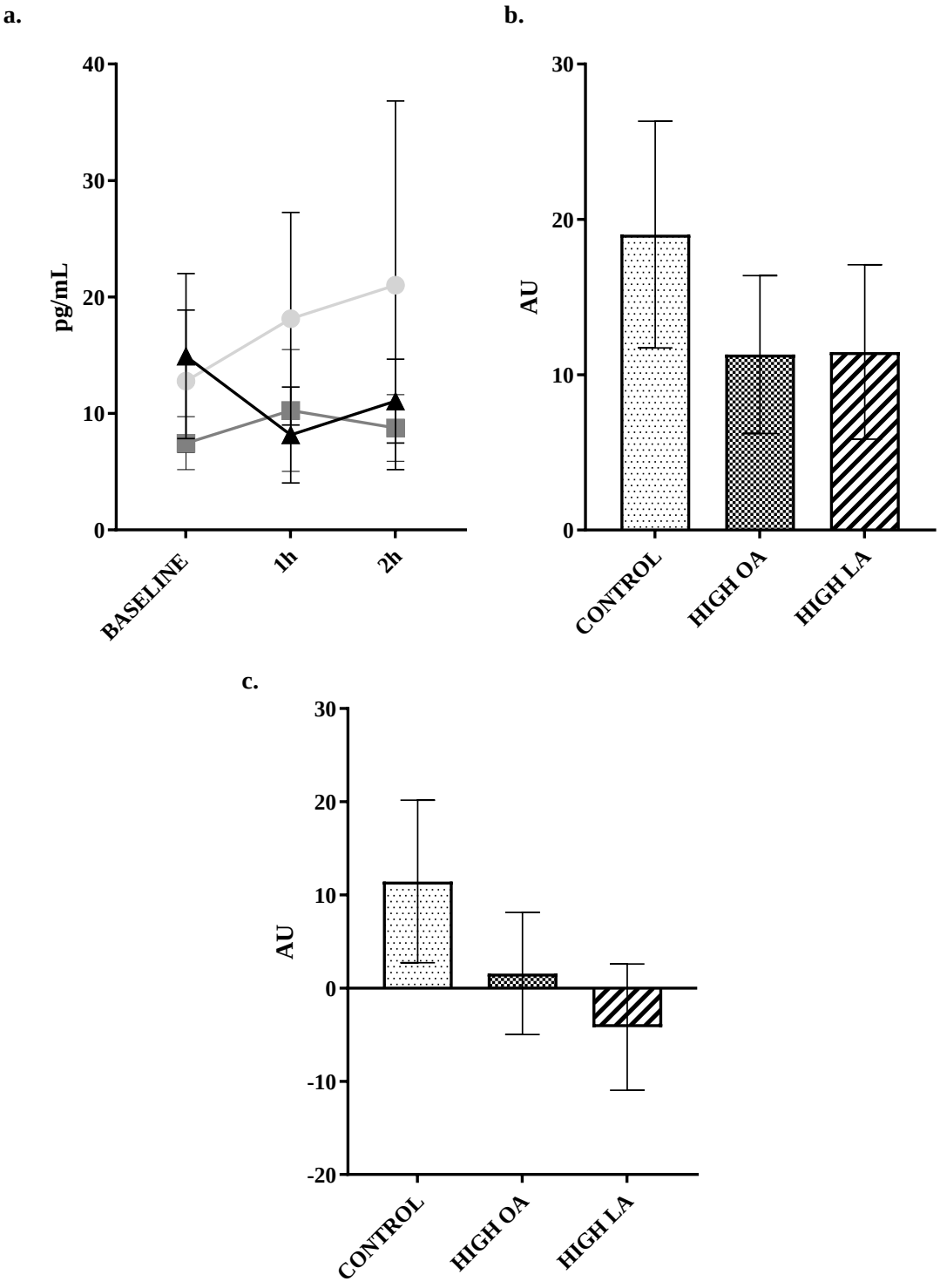

Figure 6.16: Change in IL-10 over 2-hour postprandial period. a. plotted values. ● = control meal, ■ = high OA, ▲= high LA; b. Area under the curve (AUC) from baseline; c. Net AUC from baseline. All data displayed as Mean ± SEM, n = 8 for each data set.

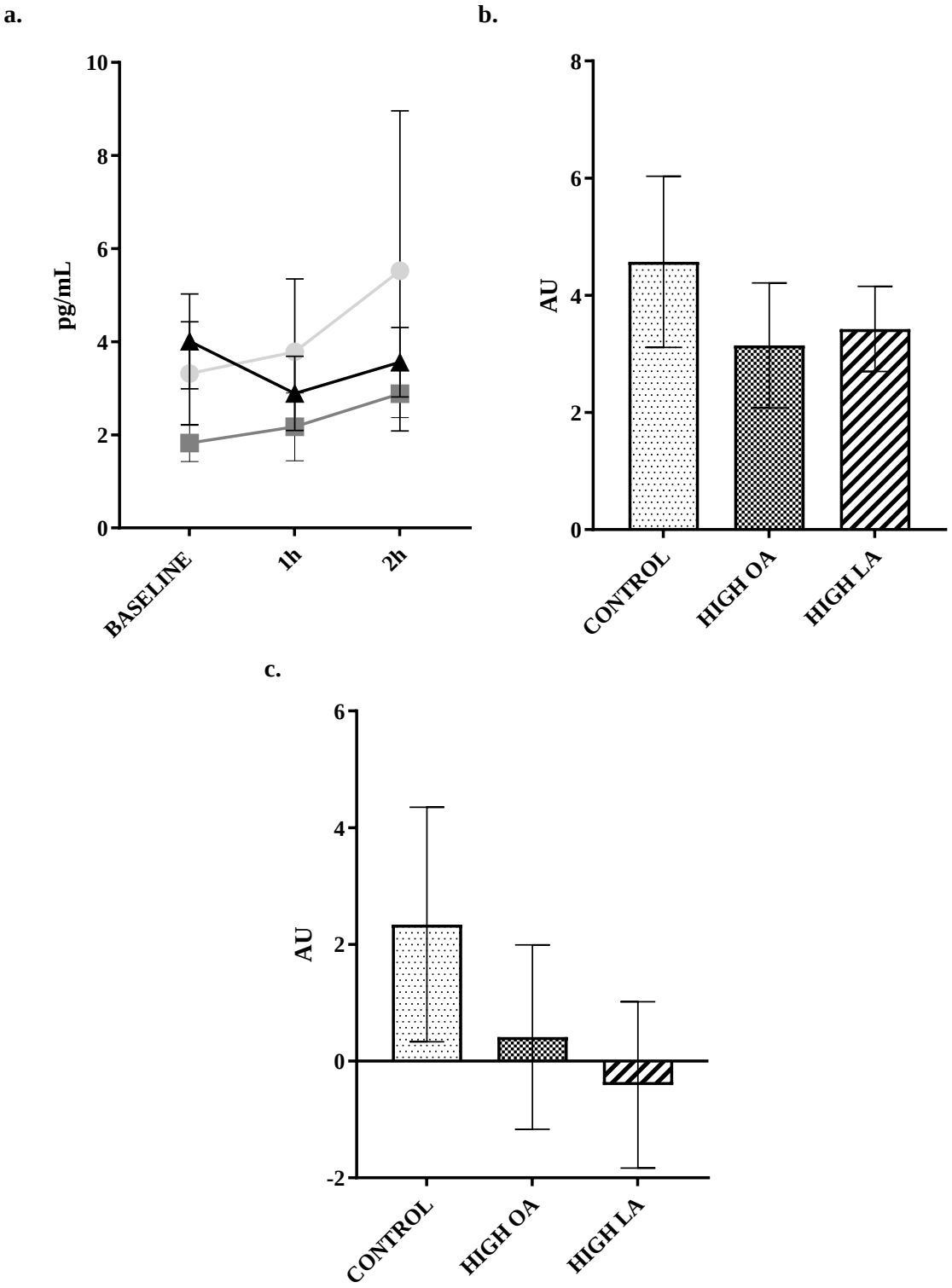

Figure 6.17: Change in IL-13 over 2-hour postprandial period. a. plotted values., ● = control meal, ■ = high OA, ▲= high LA; b. Area under the curve (AUC) from baseline; c. Net AUC from baseline. All data displayed as Mean ± SEM, n = 8 for each data set.

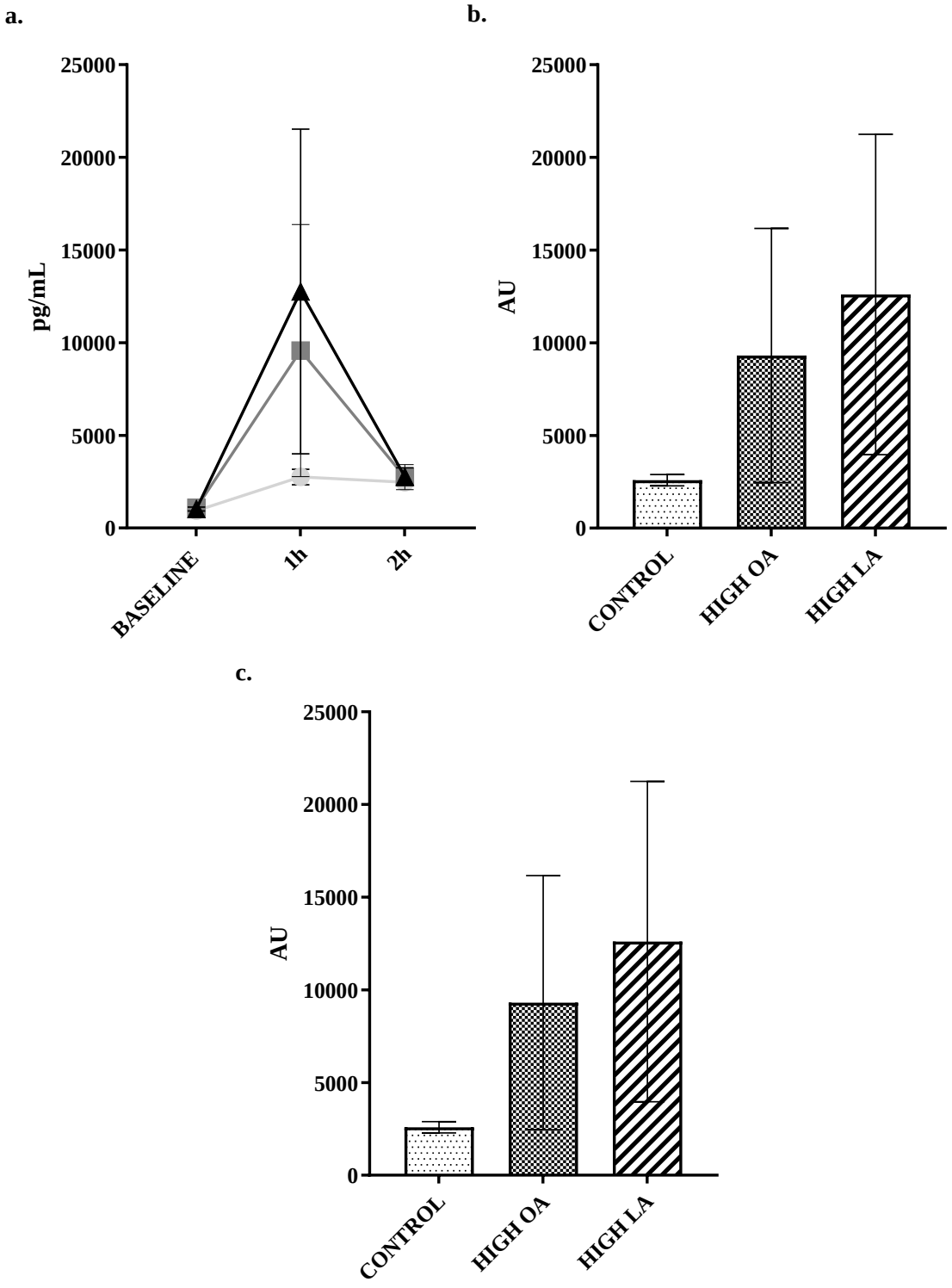

Figure 6.18: Change in C-reactive protein (CRP) over 2-hour postprandial period. a. plotted values., ● = control meal, ■ = high OA, ▲= high LA; b. Area under the curve (AUC) from baseline; c. Net AUC from baseline. All data displayed as Mean ± SEM, n = 8 for each data set.

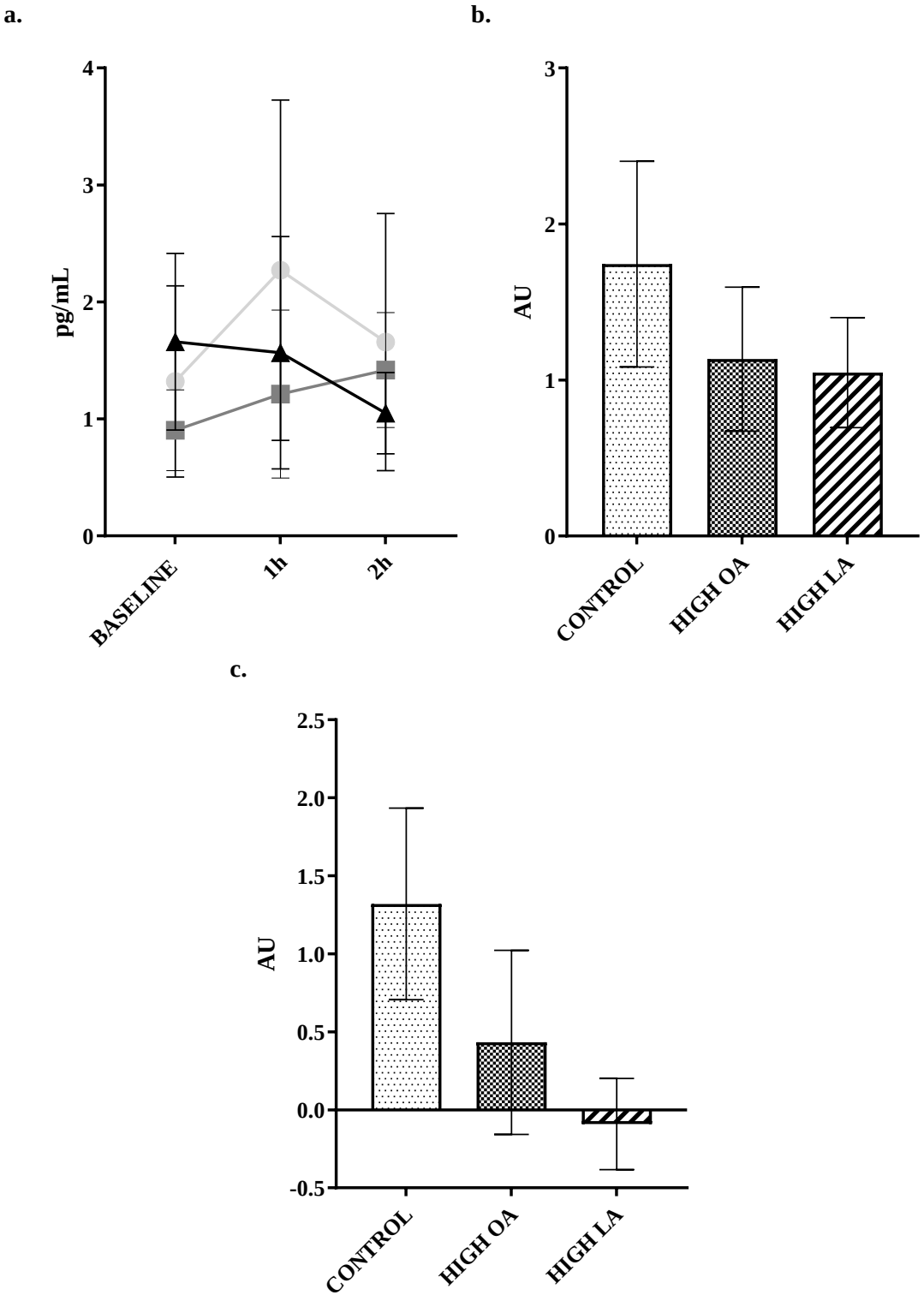

Figure 6.19: Change in tumor necrosis factor alpha (TNF-α) over 2-hour postprandial period. a. plotted values., ● = control meal, ■ = high OA, ▲= high LA, b. Area under the curve (AUC) from baseline; c. Net AUC from baseline. All data displayed as Mean ± SEM, n = 8 for each data set.

Measurement of Plasminogen activator inhibitor-1 (PAI-1), Visfatin, interferon gamma (IFN-γ), IL-2, IL-3, IL-4, IL-5 and IL-12p70 failed to yield complete data sets due to samples being outside the range of determination, and as such were removed from statistical analysis. There were no significant differences between meals observed between baseline measurements (pre-test meal consumption) for any of the analytes measured.

6.5 Discussion

The modern food environment has an abundance of cheap, highly palatable plant derived oils, yet the ability of FA to modulate appetite and food intake is currently unknown. In this study, using carefully designed meals with matched energy from macronutrients in the two high fat meals, and matched contents of the specifically elevated FA, we were able to show specific fatty acid regulation of postprandial appetite markers and perceived appetite.

The participant characteristics show that while they were all overweight or obese and had elevated central adiposity, their fasting blood glucose levels indicate they had no impairment to blood glucose management when compared to the national guidelines, and were normotensive (Colagiuri 2009; Stewart 2012). Though blood glucose responses showed no significant differences between test meals there was a significant increase in blood glucose concentrations between immediately post consumption and the 1 hour time point for the LA meal. Additionally, there was a significant decrease in blood glucose concentrations between the 1 and 2-hour post prandial time points in response to consumption of the control and LA test meals, which was not seen following consumption of the OA meal. Taken together this indicates that the LA meal resulted in a faster peak and then decline in blood glucose concentrations. As the two high fat test meals had matched fat content this difference between the two high fat meals cannot be attributed to post prandial lipaemia, additionally the two test fats were both 18 carbon chains, removing the influence of chain length, additionally the level of absorbance of the two FA is comparable (Jones et al., 1985), though differences in metabolism post absorption cannot be eliminated. A meta-analysis comparing low and high MUFA diets found that a high MUFA intake correlated with a decrease in HbA1c in diabetic individuals (Schwingshackl et al., 2011), with another study finding a high MUFA diet decreases fasting blood glucose when compared to high PUFA diets (Qian et al., 2016).

Additionally, studies have found that in the long term, high OA diets improve insulin sensitivity and are capable of lowering fasting insulin (Ryan et al., 2000), and decreasing the postprandial insulin response when compared to a high LA meal (Shah et al., 2007), though as shown here, there were no significant differences in postprandial insulin levels between the test meals. In regards to the control meal the insulin levels were still significantly elevated at the 2-hour time-point when compared to baseline, most likely due to the higher carbohydrate content of this meal.

Blood glucose responses in this study may have also been affected by resistin production, with a significant increase at the 1 hour time-point following consumption of the high LA test meal, though this was not translated to a significant difference in net AUC across the testing period ($p = 0.055$), most likely due to inter-subject variability. As resistin is an adipokine which has been shown to interfere with intracellular insulin signalling and also stimulates monocyte and macrophage inflammatory cytokine production, this may contribute to the systemic low-grade inflammation observed in obesity (Abate et al., 2014; Lee, S. et al., 2014). Treatment of cultured 3T3-L1 adipocytes with OA has found that when compared to the *trans* isomer of OA (elaidic acid) there is a significant reduction in resistin expression (Granados et al., 2011) though another group has found arachidonic acid (AA) is capable of decreasing resistin expression in the same cell type (Haugen et al., 2005). While human and mouse resistin only share a 59 % homology and it currently appears there are differences in regulation and function, human resistin is involved in inflammation-induced insulin resistance in the liver and skeletal muscle and may also be implicated in CVD (Park et al., 2017). Despite this evidence, there appears to be an absence of studies comparing the effect of dietary OA or LA on resistin expression, though one large scale study (~ 6600 participants) has found an inverse correlation between serum resistin levels and adherence to the Mediterranean diet and

MUFA intake (de León et al., 2014). Moreover, it appears the role of LA in modulating postprandial resistin levels in humans has not been sufficiently investigated to draw further inference.

Adiponectin is also an adipokine capable of influencing blood glucose levels, though working in opposition to resistin as it increase the sensitivity of tissues, such as skeletal muscle, to insulin, resulting in greater glucose uptake (Silha et al., 2003). Though this study did not find significant differences in adiponectin levels following meal consumption, net AUC data shows a trend ($p = 0.12$) of reduced adiponectin following consumption of the control meal and an increase following consumption of the OA meal. This is supported by studies showing an increase in circulating adiponectin in a subsection of the Nurse's Study cohort who habitually consumed a Mediterranean diet (Mantzoros et al., 2006), in a group of overweight men prescribed a Mediterranean style diet (Esposito, Di Palo, et al., 2010), and in a mixed gender study that aimed to increase monounsaturated fat to 10 % or more of energy consumed (Esposito & Giugliano, 2010). Furthermore, treatment of both cultured human and mouse adipocytes with OA has been found to reverse TNF-α induced depletion of adiponectin mRNA expression, on its own and in combination with the antioxidant phenol hydroxytyrosol, commonly found in olive oil (Scoditti et al., 2015).

This study also found that consumption of the high LA test meal resulted in an increase in ghrelin production when the net AUC was compared to the response to the control meal. Ghrelin is a neuropeptide which promotes food intake, with levels generally increasing prior to food intake and dropping in the postprandial period, with levels generally decreasing within 1 hour of food consumption (Callahan et al., 2004; Meier & Gressner, 2004). This indicates that LA may be able to modulate the normal ghrelin response to feeding. This response to ghrelin is in contrast to what was found by

Stevenson et al. (2017), in lean individuals following consumption of a 35 % fat meal (with 21 % from PUFA) which elicited no postprandial change to ghrelin when compared to a high carbohydrate control meal. This may indicate that the higher amount of fat (and load of PUFA) consumed in the current study (55 % of energy from fat, of which 70 % was LA) may have influenced the results found here. There is also the possibility that the increased adiposity of the subjects (which has been found to correlate with lower ghrelin production (Tschöp et al., 2001)) in the present study or the length of postprandial period (2 hours compared to the 4 hours in the published study) influenced the results. Adding to this increase in ghrelin, analysis of VAS responses to perceived prospective food intake found that the perceived amount of food which participants could comfortably consume decreased post consumption for both the control and high OA meals, though this did not decrease for the high LA meal. This indicates that though hunger had decreased and fullness increased there was not a decrease in how much they felt they could eat- possibly indicating promotion of hedonic food intake. Moreover, ghrelin is capable of stimulating hedonic food intake, an important factor when considering modern environments of abundant, high fat, palatable foods (Lindqvist et al., 2005; Monteleone et al., 2012).

GIP was increased at 1 hour compared to baseline following consumption of all meals, which was sustained at the 2-hour time-point for the control and LA meals, though there was a significant reduction at the 2-hour postprandial time-point following consumption of the OA meal. GIP secretion in response to both OA and LA consumption has been demonstrated previously, though over a considerably longer period of time (30 hours) with incremental fat consumption (Xiao et al., 2006). Despite this, other research comparing MUFA, PUFA and SFA containing meals in people with metabolic syndrome over a 6 hour period found no significant difference in AUC between the PUFA (LA) and MUFA (OA) meals when analysing AUC (Chang et al., 2016). In agreement with this,

earlier research has found no difference in 3 hour responses to ingestion of high LA and OA amounts at any measured time-points (Lardinois et al., 1988), indicating that the level of FA saturation may not effect GIP response. Changes to GLP-1 concentrations were not observed in the present study. This is not surprising due to the requirement of stimulation of L-type enterocytes in the ileum and colon to trigger secretion of GLP-1, with the time course of this study not allowing sufficient time for progression to this area of the GIT and differences between the test meal compositions (Gutierrez-Aguilar & Woods, 2011).

The finding of no significant difference in perceived appetite between the test meals following consumption is in line with a study conducted in lean healthy men consuming high OA and LA breakfasts (Strik et al., 2010). This study failed to elicit differences in perceived appetite following a mixed composition breakfast, nor was there a difference in energy intake at an *ad libitum* buffet style lunch following the meal intake, though this study did not look at hormonal responses (Strik et al., 2010). Interestingly, in a study using 15 healthy normal weight mixed gender participants, energy intake at a subsequent meal and over the following 24 hours was reduced following consumption of both extra virgin olive oil and high OA sunflower oil containing meals, compared to a high LA meal, with a corresponding increase in circulating OEA (Mennella, Ilario et al., 2015). Despite this, a number of other studies have also found no significant difference between test meals for any appetite parameters measured when comparing OA and LA (and in some instances SFA) meals in an acute timeframe (Casas-Agustench et al., 2009; Flint et al., 2003; MacIntosh et al., 2003) or when the fats are delivered via ileal infusion (Maljaars et al., 2009). This indicates that measurement of hormones involved in appetite modulation may be a better indicator of how appetite responses are affected by food intake than self-reporting.

The results of this study show that different plant derived fats have the potential to modulate postprandial hormonal responses, with a high LA meal leading to an increase in ghrelin, a key hormone in promoting food intake, potentially evidenced by this meal being the only one tested to not elicit a decrease in prospective food intake in the post prandial period. Consumption of the LA meal also caused a spike in the levels of resistin, an adipokine which decreases insulin sensitivity, 1 hour post meal consumption. Additionally, consumption of the high LA meal caused a significant peak in blood glucose levels 1 hour post consumption which was not observed following the same load of OA. Moreover, this is the first study of its kind (to the author's knowledge) to investigate such a wide range of appetite modulating hormones in response to an acute fatty acid intake with matched loads. Further investigation of the role of LA in modulating resistin and ghrelin levels may provide insight into how dietary fats are capable of affecting post prandial appetite and metabolism. As ghrelin has a role in hedonic food intake, the assessment of *ad libitum* energy intake at a subsequent meal may also be of interest. In line with other similar studies, a larger subject number may also be required to remove variables of gender, age and adiposity, which all have the potential to modulate appetite further.

Chapter 7: Discussion and Future directions

7.1 Overview of major findings

Over the time-period 1961-2009, Australia experienced a major dietary change, or nutrition transition, resulting in an increase in the contribution of lipids to total energy availability, and an increase in both LA availability and the contribution of plant based oils to LA availability, as had been hypothesised in section 1.27. This study is the first (to the authors' knowledge) to assess the changes in specific SFA, MUFA and PUFA and their sources over such a large time period. Results of this study show that over the time-period analysed there was a 16.7 % increase in energy availability from lipids, a similar trend to studies investigating cumulative change of energy from macronutrients in the USA, (Carden & Carr, 2013), Ireland (Sheehy & Sharma, 2011), Barbados (Sheehy & Sharma, 2010), Switzerland, France (Guerra et al., 2012) and Europe (Balanza et al., 2007), as well as China, which has recently gone through a nutrition transition (Popkin, 2001).

The increase in availability of energy from fat found in the current study is a result of an increase in the availability of the most common FA of the SFA, MUFA and PUFA classes, being PA, OA and LA. Furthermore, the finding of this study that OA is the most highly consumed FA of those measured is similar to American results which found OA to be the most highly consumed FA, providing 12 % of total energy in 2010 (Vannice & Rasmussen, 2014). Though OA accounted for the highest proportion of the FA surveyed here, the greatest cumulative change in FA energy availability was from LA, with a cumulative change of 120.5 % and a relative change of 176.8 % over the time-period analysed. A similar trend was found in the US by Blasbalg et al. (2011), who found an

increase in LA availability of 158 % over the time-period 1909-1999 when assessing relative change.

Comparing the average annual contribution of LA and AA to energy availability in the present study in the year 1995 results are comparable to those found by Hibbeln et al. (2006) in their assessment of Australian diets (LA (4.7 % as compared to the 5.3 % found here) and AA (0.07 % compared to 0.06 %)), with the slight differences possibly attributable to the use of different food composition databases in analysis. During the time-period 1961-2009 there was a cumulative change of 7.1 % and a relative change of 4.4 % in the contribution of LA to total PUFA availability. Interestingly, Meyer et al. (2003) when investigating PUFA intake using 24 hour recall data found that in 1995, LA contributed to approximately 88 % of total PUFA, around 8 % higher than found at this time point in the current study, possibly due to the intrinsic differences between the two dietary analysis methods. Further analysis of the commodities listed in the FBS found that the main source of the increased energy availability from LA was primarily plant oils, with cottonseed, palm and sunflower seed oils having the greatest increases in availability over the time-period analysed. The findings of this first study stress the need for further understanding of how particular FA contained within plant oils are capable of effecting health.

Chapters 4 and 5 of this thesis utilised a rodent model of obesity to investigate the effect of different dietary fats, being a predominately 'Western' style, a 'Mediterranean' style high OA and a high LA diet on body composition, metabolic health and tissue specific changes to expression of genes involved in FA metabolism. Additionally, changes to these parameters were also assessed following weight loss in DIO by feeding a lower energy chow diet. Though this study found no significant differences between body weights, food intake, blood pressure or percentage body fat for the three high fat fed

groups, which had been hypothesised, this study does show that there is a potential for the type of dietary fat to modulate food intake and energy efficiency, with reductions in both of these occurring for the high OA consuming animals towards the end of the study. Though the animals who were switched from the 'Western' diet to the chow at the start of the experimental period had a reduction in fat mass, and improvement in glucose handling, as had been hypothesised, there were no significant differences in adipose tissue gene expression. It had been hypothesised that the high LA consuming animals would have higher white adipose tissue PPARγ mRNA expression compared to the high OA consuming animals, as had been found in research performed by Ailhaud et al. (2006) though this may have been influenced by the duration of the study or the rodent strain used. Additionally, DIO appears to down regulate epidydimal adipose tissue PPARα mRNA expression regardless of dietary FA type.

Chapter 5 utilised the skeletal muscle from the animals discussed in Chapter 4, and aimed to investigate the ability of different types of dietary fat to modulate expression of genes related to FA transport and oxidation. This Chapter also investigated whether specific dietary FA were able to maintain adiponectin signalling or alter expression of genes related to oxidation or transport of FA, by incubating excised muscles with globular adiponectin at physiologically relevant levels. Expression of FAT/CD36 was higher in the soleus depot compared to EDL for the muscle incubated without adiponectin for the chow, and the 'Western' diet then chow group, as well as the high OA group. As the 'Western' diet then chow and the chow fed animals had comparable body fat percentages, this may indicate that high fat feeding with the majority of the fat being OA may preserve a lean phenotype of FAT/CD36 mRNA expression where there is adequate movement of FA into the cells for oxidation, both at rest and during contraction in this primarily oxidative muscle type (Holloway et al., 2008). Moreover, OA muscle content (a

representation of habitual FA intake profile), has a negative association with muscle FA deposition in unspecified abdominal muscles from male Sprague Dawley rats (Garcia-Escobar et al., 2008). This may indicate that a high OA diet prevents an influx of FA into the EDL for storage, as FAT/CD36 has been implicated in the initiation of high fat diet induced intramuscular lipid accumulation (Bonen, Arend, Parolin, et al., 2004; Mullen et al., 2007). Incubation of both soleus and EDL muscles from the high OA fed animals resulted in an increase in FAT/CD36, CPT1 and β-HAD expression, which was not seen in any of the other groups, indicating that a diet high in OA can help preserve adiponectin signalling in a rodent model of DIO, as has been hypothesised. This is important as adiponectin resistance has been found to precede intramyocellular lipid accumulation and the loss of insulin stimulated glucose uptake (Mullen, Smith, Junkin, & Dyck, 2007).

In an acute time-frame, the study in Chapter 6 was able to demonstrate that specific FA have the ability to modulate appetite parameters when consumed as part of a mixed composition meal. The use of a mixed composition meal, as opposed to a lipid emulsion, adds significance to the results as it replicates the ways in which nutrients are usually consumed (i.e. in combination as a meal) and provided the same percentage of energy that would normally come from a breakfast meal (Preziosi et al., 1999; Purslow et al., 2008; Winkler et al., 1999). As hypothesised, all meals resulted in a significant increase in fullness and a reduction in desire to eat two hours post consumption. There were no significant differences in postprandial insulin levels, disproving the hypothesis of there being a lower insulin response following the high OA meal when compared to the high LA meal as found in the study performed by Shah et al. (2007). Consumption of the control and high OA meals decreased prospective food intake, though this did not occur following the high LA meal. Additionally, which had not been hypothesised or previously found, following consumption of the LA meal there was a significant net increase in

ghrelin production, a hormone which encourages hedonic food intake and generally has a reduction in levels post-prandially (Cummings et al., 2001; Monteleone et al., 2012). This was coupled with an acute increase in resistin production at the one hour time point, indicating potential impairment of insulin signalling. Taken together this indicates that high LA meals may promote excess energy intake as a result of hedonic eating and impair glucose handling, though further investigation is required.

7.2 Future directions

In initial planning, one major focus of this thesis was to investigate the relationship between consumed FA and concentrations of specific endocannabinoids and ethanolamides in tissues and plasma from the animal study, and in plasma from the human study. To date this has not been performed, primarily due to the lack of a standard analytical technique or extraction protocol, the extremely volatile nature of the compounds, and the very low concentrations at which they are found (picomoles to fentomoles). Initial lipid extraction and comparison to standards performed at Victoria University indicated that levels could be quantified, with a further 6 weeks spent at the University of Texas at El Paso (UTEP), Biomolecule Analysis Core facility developing a protocol. Though a usable protocol was developed involving manual reverse solid phase column extraction there was not sufficient time to perform the laborious extractions (column extraction alone required more than 2 hours of constant attention and only one sample could be processed at a time). In the future, a return to UTEP or access to a triple quadrupole mass spectrometry system would allow for this novel research to be performed, with quantification of levels of OEA in plasma and skeletal muscle from the rodent study being of particular interest. Moreover, a separate protocol was developed during the visit to UTEP which allowed for the quantification of unique ceramide, diacylglyceride and triacylglyceride species (as opposed to the traditional method of

quantification of total levels) in muscle tissue. Progression of this would allow for the identification of specific derivatives of the precursor dietary FA and of specific molecules which are believed to have a role in the modulation of metabolism and insulin sensitivity.

Initial study design of Chapter 3 included quantification of n-3 FA and calculation of the n-6:n-3 over time, though due to the absence of complete profiles of the major n-3 FA for the majority of food commodities included, and the FAO categorisation of fish and seafood having divergent FA compositions, this was not able to be performed at this time. In the future, updating of Australian food composition databases by bodies such as FSANZ to include sufficient quantification of the n-3 class of FA would allow further extrapolation of data to determine the change in the n-6:n-3 of the Australian diet over the time measured, and would add significantly to the information relating to changes to our dietary intake. This would be an important step forward in determining the n-6:n-3 of Australian diets at a population level as currently the majority of information relating to this is generated from the Australian National Nutrition Surveys which use 24-hour dietary recall assessment, which may not capture the intake of high n-3 sources, such as fish (Meyer, 2016; Ridoutt et al., 2016; Sui et al., 2016). Moreover, the use of food disappearance data removes the variable of whether standard serving sizes are used (as set by the Australian dietary guidelines) or actual serving sizes, between which significant differences have been found in research conducted by Zheng et al. (2016), which may lead to intakes of particular FA being miscalculated.

In regards to the rodent study, the lack of hypothesised results found may have been influenced by the un-manipulated dietary components, namely the quantity and type of carbohydrate and the quantity of protein in the diet. Though there has been a large number of studies utilising rodent models to determine the most efficient method of inducing obesity and diabetes there is little consensus as to a dietary composition which is

considered best. This may be an important omission in our current knowledge and further research into optimal diet composition for inducing obesity and insulin resistance in rodent models would be of significant benefit to a large number of researchers worldwide. Similarly, the duration of the adiponectin incubation may have influenced the results, though the time used in this study has been found to elicit changes by other research groups previously (Mullen et al., 2010; Tishinsky et al., 2012; Tomas et al., 2002; Yoon et al., 2006), a longer duration may have resulted in different changes to gene expression than those found here.

In Chapter 4 a reduction in energy intake was found in the high OA fed animals when compared to the 'Western' diet group in the last two weeks of the experimental period, and a lower energy efficiency when compared to the high LA fed animals. This may indicate that the duration of the experimental period was too short and that sustained differences in energy intake in this group may have led to changes in adiposity and weight gain if the experimental period had been longer than 6 weeks, with other research finding 8 (Pranprawit et al., 2013) to 17 weeks (Marques et al., 2016) may be required. An ideal follow-on from this study would be to repeat the study with a longer experimental period to allow for the change in diet FA composition to fully influence tissue compositions and then other measured variables. Additionally, the use of the Sprague Dawley strain, chosen in this study due to it not having a specifically bred metabolic disease genotype, may have influenced the body weights and percentage adiposity of the animals found following the chronic consumption of specific FA elevated high fat diets, as well as their failure to develop insulin resistance or diabetes. The use of an *ob/ob* Zucker strain in further studies may show metabolic disturbances and differences in body weights and percentage adiposity between the specific diet treatments (Sato et al., 2007). Also, the use of a rodent respirometry system to measures energy expenditure and estimate substrate utilisation

would provide further data on the effect of different dietary fats on metabolism. Additionally, sampling of a subcutaneous white adipose tissue depot (such as the inguinal fat pad (Casteilla et al., 2001)) may provide further insight into the distribution of adipose tissue and also the modulation of metabolic genes by dietary FA in this compartment.

Though FAT/CD36 expression was measured in this study, the presence of the receptor and its ability to translocate to the cell membrane is yet to be measured. As obesity is capable of downregulating this translocation, measurement of this may show effects of FA on FAT/CD36 beyond mRNA expression (Han et al., 2007). Similarly, only expression of skeletal muscle metabolism related genes was performed and as such measurement of phosphorylated AMPKα2, the active form (Hawley et al., 1996), and deacetylate PGC1α (a more active form) (Draznin et al., 2012) via western blotting, may provide further insight into the modulation of skeletal muscle metabolism by dietary FA. A further limitation of this study may also be that the muscles were incubated whole. A standard operating procedure protocol produced to conduct similar experiments in excised rodent skeletal muscle suggests that muscles weighing more than 25 mg cannot maintain adequate perfusion for extended periods of time and may require stripping of the muscle to smaller portions (Barton et al., 2008). This may have influenced the results of this experiment due to two distinct factors- lack of adequate oxygen supply to the muscle fibres inside the muscle, and a lack of perfusion of the adiponectin containing medium to these fibres.

Measurement of deacetylated PGC1α is of particular interest in this area, as research has found that in C_2C_{12}, OA has been found to increase deacetylation and PGC1α activity, as well as complete FA oxidation, which did not occur when LA was added to the culture medium (Lim et al., 2013). Moreover, the concentration of OA used by Lim et al. (2013) correlates with human and mouse postprandial plasma levels, providing further promising

evidence for a benefit of OA in muscle metabolism. Finally, high OA diets have been found to increase rates of adipose tissue lipolysis, compared to LA (Soriguer et al., 2003) though the expression of lipolytic enzymes was not quantified in the present study.

In Chapter 5 the ability of different dietary FA was shown to be capable of modulating skeletal muscle gene expression and preserving adiponectin signalling, immunohistochemical analysis currently being performed on muscle sections in the UTEP College of Health Sciences, Skeletal Muscle Metabolism Laboratory, will provide further information regarding lipid, glycogen and mitochondrial content as well as GLUT4 translocation. As a large number of tissues during the rodent study were collected, though not analysed due to time constraints, this would be an ideal follow-on from the research conducted here. Of particular interest would be histological analysis of the livers (especially due to the increased weight of the livers from the high OA consuming animals) to determine fat deposition and structural changes, as well as expression of metabolism related genes and if they are modulated by the different diets. Additionally, analysis of the energy content of faeces (collected throughout the study) via bomb calorimetry would allow for assessment of FA malabsorption. Moreover, determination of tissue content, especially of the muscles, of the total amount of particular FA, and other lipid species such as ceramides and oxylipins, resulting from the different diets would provide further evidence of the role of diet in muscle metabolism.

Recent research has indicated the ability for cross-talk between adipose tissue and skeletal muscle, with secretion media from cultured primary subcutaneous and visceral adipocytes (using intact adipose tissue) from morbidly obese individuals (regardless of gender) being able to supress insulin signalling acutely in cultured myotubules through suppression of Akt phosphorylation (Sarr et al., 2017). The use of adipose tissue from habitual ‘Mediterranean’ and ‘Western’ diet consuming individuals could help to provide further

evidence as to the role of dietary FA, and the role of adipokines in muscle metabolism. This could be assessed through changes to gene expression and protein concentrations (measured via western blotting) and potentially changes to glucose uptake through the use of a cellular bioenergetics analytical instrument.

An ideal follow on from the acute FA study would be to investigate the long-term changes to appetite and regulating hormones by altering the dietary FA profile of an individual's habitual intake to have a low n-6:n-3, primarily through substitution of n-9 FA for n-6, which has been found to be achievable in long term studies in an Australian setting (Papamiltiadous et al., 2016; Wood et al., 2014; Wood et al., 2013). If the study was designed as a cross over study (e.g. 12 weeks on each diet) with a diet comprising the same foods though with a controlled n-6:n-3 of moderate range, the effect of LA intake on metabolic parameters such as appetite regulating hormones, cytokines and blood glucose regulation (using a hyperinsulinemic-euglycemic clamp) could be investigated. This could be done in two distinct sub-populations, obese insulin sensitive individuals and obese T2DM individuals to investigate the role of impaired glucose handling on appetite and metabolic regulation, with body composition assessed via DXA at the end of each diet period. Also, if participants had meal consumption sessions during which acute changes to appetite parameters and hormones could be measured, as was done here, it could remove the variable of the FA composition of the participants' background diets. A major limitation of dietary change studies is participant compliance to prescribed diets (Metz et al., 1997), one effective method of increasing this is providing the majority of recommended foods to the participant. This also allows for dietary compliance to be measured by returned food, and closer control of specific products being consumed, though this does greatly increase the cost and the time required to undertake the research. Additionally, blood glucose concentrations during the meal consumption sessions could

be greatly improved with the use of continuous blood glucose monitoring sensors as peaks may have been missed with the protocol used here. If muscle biopsies were collected at the end of each diet period the amount of muscle lipid deposition could be quantified, as could the proportion of specific ceramides and lipid oxidation intermediates and the association between these and other participant characteristics, such as glucose handling could be assessed. These samples could also be analysed for changes to the expression of genes related to FA metabolism and transport. Additionally, collection of muscle biopsies would allow for culturing of cells to determine glucose uptake and metabolism, and FA oxidation rates through analysis of oxygen consumption rate and extracellular acidification rate measured using a cellular bioenergetics analytical instrument (such as a Seahorse XF analyser). This would allow for investigation of dietary FA content influence on metabolism at a cellular level in both insulin sensitive and resistant states as the cells retain their donors' metabolic phenotypes (Ukropcova et al., 2005).

Though the study in Chapter 6 assessed perceived appetite, it could have been improved by measuring *ad libitum* intake at a subsequent meal, though this was deemed to be overly extending the time requirements of the participants when the study was designed. Adding this form of assessment to future, long term studies can help to determine if the perceived appetite or change to hormones actually results in an increase in energy intake (determined by weighing food before and after presentation to participants), and by providing foods of differing energy density this can also be used to determine if the intake of particularly energy dense foods is favoured (Monteleone et al., 2012; Votruba et al., 2009). Finally, in a larger study investigating the influence of different dietary FA on appetite, quantification of plasma FA and of their endocannabinoid and ethanolamide derivatives would provide novel information into dietary regulation of the endocannabinoid system, and associations between these and concentrations of hormones

and other circulating factors would provide valuable, novel information in the understanding of how appetite is regulated. If this was able to be done at intervals during the diet periods and on samples collected during meal challenge sessions it would provide an insight into both short and long-term appetite and endocannabinoid system regulation.

7.3 Summary of Findings

The research undertaken within this thesis indicates that over the time-period 1961-2009 the macronutrient and FA composition of the Australian food supply changed significantly, with an increase in the availability of plant derived oils resulting in a considerable increase in the LA content of the food supply. Additionally, this thesis has demonstrated that a diet high in OA in rodents with DIO is capable of preserving adiponectin signalling in both oxidative and glycolytic muscle types. In an acute timeframe, the ingestion of a high LA mixed composition breakfast meal in overweight or obese humans has also been shown to cause a sharp peak in blood glucose levels, an increase in the food intake promoting hormone, ghrelin, and a spike in the pro-inflammatory, insulin signalling interfering adipokine, resistin, one hour post consumption. Moreover, the high LA meal was the only meal consumed which did not decrease the perceived amount of food participants could comfortably consume in the post prandial period, indicating potential hedonic food intake stimuli in overweight and obese individuals. Taken together, the results of this thesis indicate that when comparing high OA and LA intakes, whether acute or in a habitual diet, promotion of a high OA intake may be beneficial for metabolic health and preservation of normal appetite regulation.

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Chapter 9: Appendices

VICTORIA UNIVERSITY
MELBOURNE AUSTRALIA

INFORMATION TO PARTICIPANTS INVOLVED IN RESEARCH

You are invited to participate

You are invited to participate in a research project entitled '**The effect of weight loss and meal composition on circulating endocannabinoid levels in overweight and obese humans**'.

This project is being conducted by a student researcher- Shaan Naughton as part of an Honours study at Victoria University, under the supervision of Dr. Andrew McAinch from the Faculty of Health, Engineering and Science and Dr. Michael Mathai from the Faculty of Health, Engineering and Science.

Project explanation

Obesity and overweight are two of the most important medical concerns of current day, due not only to the affect on general health and their influence on the development of other medical conditions such as type 2 diabetes and cardiovascular disease, but also due to the strain placed on the medical system. The last National Health Survey found that 68% of male and 55% of female adult Australians and are either overweight or obese, with the main influencing factors being a trend towards sedentary lifestyles and an increase in energy intake due to larger portion sizes and an increased intake of processed and convenience foods.

Appetite is a main regulator in food intake, with an increase being an influencing factor in the development of overweight and obesity. Appetite is influenced by numerous physiological and hormonal systems, one of which being the endocannabinoid system, with activation of this system resulting in an increase in hunger. Obese individuals often have a dysregulation of this system, which can affect energy metabolism. The effect of weight loss on this system has not been researched extensively, with very few human trials investigating how particular meal compositions can affect short term circulating levels of endocannabinoids.

This study aims to determine the effect of weight loss in overweight and obese individuals as a result of dietary modification on the levels of circulating endocannabinoids and other physical and biochemical parameters related to overweight and obesity. Assessing short term changes in circulating endocannabinoid levels after the consumption of a meal containing specific amounts of nutrients will result in a better knowledge of how food choices and dietary habits can affect short term appetite regulation.

Male and female participants between the ages of 18-60 who are overweight or obese (BMI >25 or waist circumference >94cm (male), >80cm female) and weight stable, who are not currently taking any weight loss medication/supplementation or fatty acid supplementation (e.g. Fish oils or omega 3) and are free from heart, liver or kidney disease, type 1 and type 2 diabetes or insulin resistance will be suitable for this study. Those who are pregnant, planning on becoming pregnant or breastfeeding are not suitable to be involved in this study.

What will I be asked to do?

- Attend an initial screening session (expected duration of ½ an hour) to determine eligibility to participate in the study, during this session a medical, diet and lifestyle history will also be taken, as well as initial anthropometric measures.
- attend a teaching clinic of either of the Flinders Lane, Footscray Park or St. Albans campus's weekly for nutrition consultation and anthropometric measurement (weight, waist and hip circumferences, blood pressure, and heart rate) on a weekly basis for a 10 week period (expected weekly duration of 45 minutes)

- complete a 3 day food diary (2 week days and one weekend day) every second week
- Present to the clinic after an overnight fast, approximately 12 hours after consumption of the last meal (water is permitted while fasting) for the food challenge sessions on eight occasions (once a week for the 4 weeks before and after the dietary intervention). On each occasion during each four week period participants will be required to consume a breakfast meal of toast and jam with different nutrient compositions in a 15 minute period. Participants will have 15ml venous blood taken by an experienced and qualified phlebotomist 1 hr before, immediately prior to the meal, immediately after consumption and one and two hours after consumption.
- Present to Footscray Park campus on a designated morning after an overnight fast at the beginning and end of the study for dual energy x-ray absorptiometry (DXA) scanning. A DXA scan is non-invasive, does not cause pain and does not require you to be in an enclosed space. You will be required to lie still on the scanning bed for 6 minutes while the scanner passes over the top you.

What will I gain from participating?

It is anticipated that some participants who undertake the treatment may experience some weight loss and learn about healthy nutrition and physical activity by the end of the study period. The DXA scan will provide accurate information to the participant in regards to their body composition and how this has changed during the weight loss period. In addition, participants will be contributing to the further understanding of the effect of weight loss on circulating endocannabinoid levels and how different meal compositions affect short term circulating endocannabinoid levels.

How will the information I give be used?

Blood samples collected in the study will be coded, so that individuals will not be identifiable to anyone other than the main investigator. All information provided by each participant during the study will be kept confidential at all times unless required by law to be disclosed. Any information that provides any form of personal identification will be kept in a secured locked filing cabinet and retained for 15 years from the end of the study (as per clinical trial guidelines). No information collected during the study will be disclosed to third party organisations. Participants have to right to access personal information collected at any time during study. As participation in the study is voluntary, provision of personal information is at the participants' discretion, but may influence the suitability of care provided and the validity of the study results. All results will be reported as part of an overall group.

What are the potential risks of participating in this project?

There are few potential risks of participating in this study, all of which have been minimised to ensure the safety of each participant. There is a very small risk of minor bruising or infection associated with blood sampling. To minimise the risk, each procedure will be performed by a qualified practitioner. Further, the area will be swabbed with disinfectant prior to sample collection to reduce the risk of infection. Compression will be applied to the area following the withdrawal of the catheter or needle to reduce risk of bleeding and haematoma. After use, the cannula or needle and syringe will be disposed of properly and safely to eliminate further risk of infection. In the case of an adverse reaction, infection or bruising associated with blood sampling; the subjects would be asked to contact one of the listed investigators and advised to see their own medical professional for assessment at our expense.

DXA scanning involves exposure to X-ray. The radiation exposure involved is less than the limit set by the Australian Radiation Protection and Nuclear Safety Agency

This research study involves exposure to a very small amount of radiation. As part of everyday living, everyone is exposed to naturally occurring background radiation and receives a dose of about 2 millisievert (mSv) each year. The effective dose from this study is about 0.01 mSv. At this dose level, no harmful effects of radiation have been demonstrated as any effect is too small to measure. The risk is believed to be minimal

How will this project be conducted?

The project will be conducted in the Nutritional Therapy teaching clinic, Victoria University, St Albans campus, Melbourne, Australia. If you are interested in this research please complete the attached consent form. You are encouraged to discuss this proposal with a medical practitioner, friend or family member. Please do not hesitate to contact the Chief Investigators or the Student Researcher to ask questions if you would like clarification about any topic. Once you have consented to be part of this research you will undergo an initial screening test to determine your medical suitability for this study.

Who is conducting the study?

Chief Investigators
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Any queries about your participation in this project may be directed to the Chief Investigator listed above.
If you have any queries or complaints about the way you have been treated, you may contact the Research Ethics and Biosafety Manager, Victoria University Human Research Ethics Committee, Victoria University, PO Box 14428, Melbourne, VIC, 8001 or phone (03) 9919 4148.

Figure 9.1: Plain Language Information for Participants

VICTORIA UNIVERSITY
MELBOURNE AUSTRALIA

CONSENT FORM FOR PARTICIPANTS INVOLVED IN RESEARCH

INFORMATION TO PARTICIPANTS:
We would like to invite you to be a part of a study entitled '**The effect of weight loss and meal composition on circulating endocannabinoid levels in overweight and obese humans**'

This study aims to determine the effect of weight loss in overweight and obese individuals as a result of dietary modification on the levels of circulating endocannabinoids and other physical and biochemical parameters related to obesity and overweight. Assessing acute changes in circulating endocannabinoid levels after the consumption of a meal containing specific amounts of nutrients will result in a better knowledge of how food choices and dietary habits can affect short term appetite regulation through modulation of the endocannabinoid system.

CERTIFICATION BY SUBJECT

I,
of

certify that I am at least 18 years old* and that I am voluntarily giving my consent to participate in the study: '**The effect of weight loss and meal composition on circulating endocannabinoid levels in overweight and obese humans**' being conducted at Victoria University by Dr Andrew McAinch

I certify that the objectives of the study, together with any risks and safeguards associated with the procedures listed hereunder to be carried out in the research, have been fully explained to me by:

Ms. Shaan Naughton

and that I freely consent to participation involving the below mentioned procedures:

- Attend the teaching clinics either of the Flinders Lane, Footscray Park or St. Albans campus's weekly for nutrition consultation and anthropometric measurement for a 10 week period
- Complete a 3 day food diary (2 week days and one weekend day) every second week
- Present to the clinic after an overnight fast, approximately 12 hours after consumption of the last meal (water is permitted while fasting) for the food challenge sessions on eight occasions (once a week for the 4 weeks before and after the dietary intervention). Participants will have venous blood taken by an experienced and qualified phlebotomist 1hr before, immediately prior to the meal, immediately after consumption and one and two hours after consumption. Completion of appetite surveys will also be required.
- Present to Footscray Park campus on a designated morning after an overnight fast at the beginning and end of the study for dual energy x-ray absorptiometry (DXA) scanning.

I certify that I have had the opportunity to have any questions answered and that I understand that I can withdraw from this study at any time and that this withdrawal will not jeopardise me in any way.

I have been informed that the information I provide will be kept confidential.

VICTORIA UNIVERSITY
MELBOURNE AUSTRALIA

Signed:

Date:

Any queries about your participation in this project may be directed to the researcher:
Dr. Andrew McAinch
(03) 9919 2019
Andrew.McAinch@vu.edu.au

If you have any queries or complaints about the way you have been treated, you may contact the Research Ethics and Biosafety Manager, Victoria University Human Research Ethics Committee, Victoria University, PO Box 14428, Melbourne, VIC, 8001 or phone (03) 9919 4148.

[*please note: Where the participant/s are aged under 18, separate parental consent is required; where the participant/s are unable to answer for themselves due to mental illness or disability, parental or guardian consent may be required.]

Figure 9.2: Form for Obtaining Informed Consent

Table 9.1: Test meal compositions per 100kJ

INGREDIENT	ENERGY (kJ)	AMOUNT (g)	CHO (g)	PRO (g)	LIPID (g)	LIPID COMPOSITION					FIBRE (g)
						SATURATED FATTY ACIDS (g)	OLEIC ACID(g)	OTHER MUFA (g)	LINOLEIC ACID (g)	OTHER PUFA (g)	
CONTROL											
high fibre white bread	41.9	4.3	1.9	0.394	0.112	0.017	0.043	0.051	0.034	0.043	0.27
reduced sugar strawberry jam	10.7	1.9	0.6	0.013	-	-	-	-	-	-	0.009
icing sugar	28.5	1.8	1.8	-	-	-	-	-	-	-	-
coconut oil	6.3	0.2	-	-	0.171	0.158	0.01	0.01	0.003	0.003	-
light extra virgin olive oil	6.3	0.2	-	-	0.171	0.026	0.132	0.134	0.011	0.012	-
safflower oil	6.3	0.2	-	-	0.171	0.012	0.021	0.021	0.137	0.138	-
TOTAL	**100.1**	**8.5**	**4.3**	**0.407**	**0.626**	**0.213**	**0.206**	**0.217**	**0.186**	**0.196**	**0.279**
HIGH OLEIC ACID											
high fibre white bread	41.9	4.3	1.9	0.394	0.111	0.017	0.043	0.051	0.034	0.043	0.27
reduced sugar strawberry jam	7.1	1.3	0.4	0.009	-	-	-	-	-	-	0.009
safflower oil	4.3	0.1	-	-	0.116	0.008	0.014	0.014	0.093	0.093	-
light extra virgin olive oil	46.7	1.3	-	-	1.263	0.189	0.973	0.987	0.082	0.086	-
TOTAL	**100.0**	**6.9**	**2.3**	**0.402**	**1.49**	**0.215**	**1.03**	**1.053**	**0.209**	**0.223**	**0.278**

INGREDIENT	ENERGY (kJ)	AMOUNT (g)	CHO (g)	PRO (g)	LIPID (g)	LIPID COMPOSITION					FIBRE (g)
						SATURATED FATTY ACIDS (g)	OLEIC ACID(g)	OTHER MUFA (g)	LINOLEIC ACID (g)	OTHER PUFA (g)	
HIGH LINOLEIC ACID											
high fibre white bread	41.9	4.3	1.9	0.393	0.111	0.013	0.0423	0.051	0.034	0.043	0.27
reduced sugar strawberry jam	7.1	1.3	0.4	0.008	-	-	-	-	-	-	0.009
coconut oil	4.7	0.1	-	-	0.128	0.119	0.007	0.007	0.002	0.002	-
light extra virgin olive oil		-	-	-	-	-	-	-	-	-	-
safflower oil	46.3	1.2	-	-	1.25	0.085	0.154	0.156	1.004	1.009	-
TOTAL	**100.0**	**6.9**	**2.3**	**0.401**	**1.49**	**0.217**	**0.204**	**0.215**	**1.04**	**1.054**	**0.278**

Name: _____ Date: _____ Time: _____

Questionnaire

Please answer each of the following eight questions according to how you are feeling *at this point in time* by crossing the horizontal line with a vertical mark.

Furthest to the *left* means that you *do not* have the feeling / symptom

Furthest to the *right* means that you *do* have this feeling / symptom

1. I feel hungry

   Not Hungry _____ Hungry

2. I feel full

   Not full _____ full

3. I feel Nauseas

   Not Nauseous _____ Nauseous

4. I feel drowsy

   Alert _____ drowsy

5. I feel anxious

   calm _____ anxious

6. I feel satisfied

   Not satisfied _____ Satisfied

7. How strong is your desire to eat?

   Weak _____ Strong

8. How much food do you think you could eat?

   None _____ A large amount

Adapted from: Parker et al., Appetite 43:227-233, 2004

Figure 9.3: Visual Analogue Scale Appetite Questionnaire